To George, for your patience, guide and friendship.

A Juan, Carmen, Vladimir y Antonio, que haría sin ustedes.

A Mario, mi compañero en todo, mi AMOR.

AGRADECIMIENTOS

Quiero agradecer a todas aquellas personas que han estado conmigo de manera personal y profesional en la elaboración de este trabajo.

Mis sinceros agradecimientos a:

Mis directoras Marisol Izquierdo y Lidia Robaina, por creer en mí y por su dirección, tanto en el ámbito científico, como personal.

Al Grupo de Investigación en Acuicultura, al Instituto Canario de Ciencias Marinas, a la Facultad de Ciencias del Mar y a la Facultad de Veterinaria, sus puertas siempre estuvieron abiertas.

A todas aquellas personas que no sólo me ayudaron en la realización de este documento, sino que también forjaron conmigo fuertes lazos de amistad, que perdurarán aún cerrado este capítulo de mi vida.

A TODOS, MILLONES DE GRACIAS

INDEX

1.- INTRODUCTION

1.1 COLOUR IN AQUACULTURE

Within the last few decades there has been a remarkable growth of the aquaculture industry (9.2% per year since 1970) due mainly to a plateau in wild captures and limited stock potential (FAO, 2006) (Figure 1), together with an increase in world population and global per capita fish consumption which has doubled over the past 50 years (Ahmed and Delgado 2000; Delgado *et al*., 2002, 2003). Besides these factors, aquaculture has also been promoted in view of the fact that it provides consumers with safe, nutritious and high quality food products.

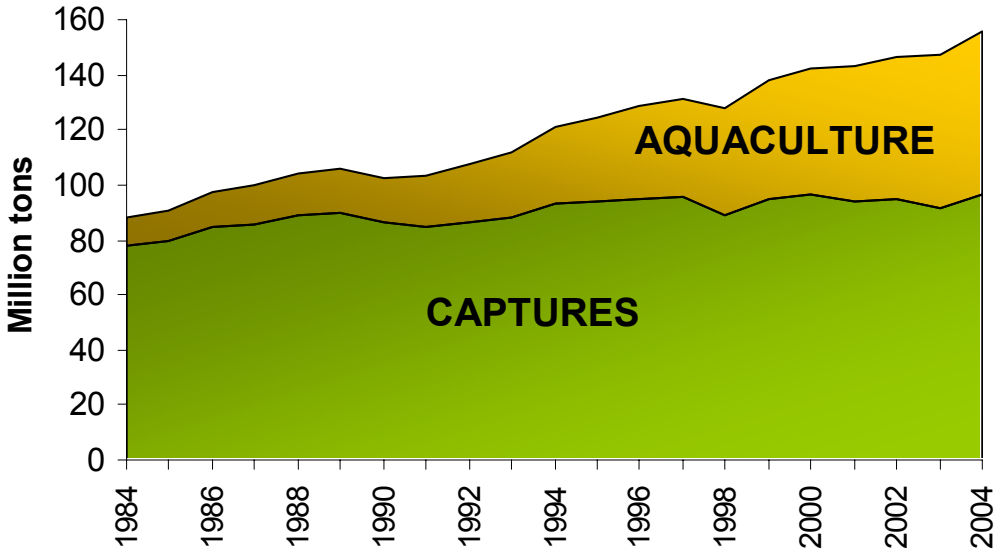


Figure 1. Global captures (T) and aquaculture production (T) including seaweed from 1984 to 2004 (FAO, 2006).

High quality aquaculture products must fulfil several requirements well appreciated by consumers, including the adequate colour, which is among the most important fish quality parameters in the market. Colour is the first characteristic perceived and is a determinant selection criterion, directly related to the subsequent acceptance or rejection (Shahidi *et al*., 1998). Traditionally, it has been held that coloration is associated to superior flavour, an opinion that still persists (Clydesdale, 1993; Sylvia *et al*., 1995; 1996). In fish, colour is much more than a cosmetic effect; consumers associate natural coloration with healthy and high quality products. For instance, muscle pigmentation in farmed salmonids is regarded as the most important quality parameter after freshness (Koteng, 1992). Aside from flesh pigmentation, vivid skin coloration of cultured red and yellow skinned fish such as red porgy, *Pagrus pagrus* (Basurco *et al*., 1999) Japanese red sea bream, *Pagrus major* (Lin *et al*., 1998) Australian snapper, *Pagrus auratus* (Booth *et al*., 2004; Doolan *et al*., 2005), and yellowtail, *Seriola quinqueradiata* (Miki *et al.,* 1985) are well appreciated, and lead to high market values. In ornamental fish, skin colour, is as well an important characteristic affecting market price and playing a major role in the overall appraisal (Gouveia and Rema, 2005). In crustaceans, such as shrimp, a bright and appropriate colour is also associated with freshness and quality, and the desired coloration must be preserved through storage, processing and cooking (Boonyaratpalin *et al*., 2001). In the sea urchin industry, based on the production of marketable gonads, the highest commercially valuable sea urchin gonads are bright yellow-orange (Shpigel *et al*., 2004).

Four main groups of pigments account for the coloration of mammals, birds, fish and invertebrates of economic importance. These are porphyrins, pteridines, melanins, and carotenoids (Hudon, 1994). Porphyrins are of primary importance mainly in the coloration of avian eggshell (Kennedy and Vevers, 1976; Lang and Wells, 1987). Pteridines are responsible for many of the bright yellows and reds in fish, amphibians and reptiles (Nixon, 1985; Ziegler, 1965); these pigments are water soluble and are produced endogenously (Hudon,

1994). Melanin gives all the blacks, greys and browns to vertebrates and many invertebrates, and also several of their reds and yellows. Melanins are heterogeneous polymers made up of metabolites of tyrosine (Hudon, 1994). Carotenoid pigments, obtained by animals from their diets, give most of the bright red, yellow, and orange colours well appreciated not only in aquaculture but also in the poultry industry (Toyomizu *et al*., 2001).

1.2 CAROTENOIDS

Carotenoids are among the most common natural pigments responsible for many of the hues found in nature as well as a variety of functions that will be discussed later on. More than 650 different naturally occurring carotenoids are known (Pfander, 1987). These pigments are found in bacteria, fungi, plants, and animals. Only plants, bacteria, fungi, and algae can synthesize carotenoids; animals cannot biosynthesize them thus, they must be obtained from the diet (Schiedt, 1998). In the animal kingdom, carotenoids are the most widely occurring pigments after melanin. Invertebrates and most vertebrates display great diversity and ability to modify the molecular structures of dietary carotenoids, whereas in mammals the occurrence and distribution of carotenoids is very limited (Schiedt, 1998).

1.2.1 CAROTENOID CHEMICAL STRUCTURE

Almost all carotenoids are tetraterpenoid compounds formed by eight isoprenoid units, being the skeleton of the molecule a central long system of alternating double and single bonds (Britton, 1995). The hydrocarbons corresponding to the empirical formula $C_{40}H_{56}$ are know as carotenes and the derivatives containing one or more oxygen functions (aldehyde, carboxylic acid, epoxy, hydroxy, keto, methoxy groups) are known as xanthophylls (Schmidt *et al*., 1994). Carotenoids may also be either acyclic, or possess a six member ring (occasionally five-member), at one or both ends of the molecule skeleton

(Schmidt et al., 1994). They can also occur in a free form, as well as in the forms of esters, glycosides, sulphates and carotenoproteins (Matsuno *and* Hirao, 1989).

1.2.2 CAROTENOID PHYSICAL AND CHEMICAL PROPERTIES

The physical and chemical properties of carotenoids are a consequence of their chemical structure. The conjugated double bond system, their most striking characteristic, is the light absorbing chromophore that confers these pigments their attractive colour. Most carotenoids absorb light in the range of 400-500 nm, having their maximum (λmax) at three wavelengths that result in a three peak spectra. The greater the number of conjugated double bonds, the higher the λmax values. Nevertheless, cyclization of the molecule results in: hypsochromic shift (displacement of λmax to lower wavelength), a hypochromic effect (decrease in absorbance), and a loss of fine structure (spectrum with less defined peaks). Lycopene, an unsaturated acyclic carotenoid, is red and has λmax at 444, 470, 502 nm; bicyclic β-carotene, although possessing the same number of conjugated double bonds as lycopene, is yellow orange and has a λmax at 450 and 477 nm and a mere inflection (shoulder) at 425 nm. Monocyclic γ-carotene is red orange and exhibits a λmax and spectrum, intermediate between those of lycopene and β-carotene. Other carotenoids instead of having a three peak spectra, have a spectrum that consists of a rounded almost symmetrical single maximum peak. This is the case for ketocarotenoids, such as astaxanthin, cantaxanthin and echinone. The wavelength of maximum absorption (λmax) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore of the molecule and provide valuable information for identifying carotenoids (Britton, 1995).

Aside from the light absorving properties, the polyene chain also gives carotenoids their distinctive chemical reactivity of being very prone to oxidation and isomerization. A great number of geometrical isomers can exist,

nevertheless carotenoids are naturally in the all-trans (all-E) form, although the presence of cis (Z) isomers, usually in small amounts, must always be considered (Schmidt *et al*., 1994). Instability of carotenoids towards oxidation is an important feature of the molecule in relation to free radical chemistry (Britton, 1995).

In respect to their solubility, carotenoids are like other groups of higher isoprenoids, being carotenes the typical non polar hydrocarbon, and xanthophylls more polar, yet still insoluble in water (Schimdt *et al*., 1994). Their lipophilic behaviour makes them prone to accumulate in lipophilic compartments like membranes or lipoproteins. The lipophilicity of these compounds also influences their absorption, transport and excretion in the organism (Stahl *et al*., 1993). Only in combination with proteins (carotenoproteins), carotenoids are soluble in an aqueous phase; the presence of carotenoproteins is observed in crustaceans such as lobster, crab and shrimp (Sahidi *et al*., 1998).

It is of great importance to understand the physical and chemical properties of carotenoids such as size, shape, polarity, since these are determinant in their ability to fit correctly into its molecular environment and allow them to function (Britton, 1995).

1.2.3 CAROTENOID BIOSYNTHESIS

Biosynthesis occurs through the isoprenoid or terpenoid pathway (Figure 2). The first committing step of carotenoid biosynthesis is a head to head coupling of two molecules of the C_{20} geranylgeranyl diphosphate (GGDP) to yield colourless phytoene. In the following steps, the introduction of double bonds creates the light absorbing properties that determine the colour of carotenoids. Finally, the plethora of subtle structural variations occurs, which account for the hundreds of carotenoids (Britton, 1998).

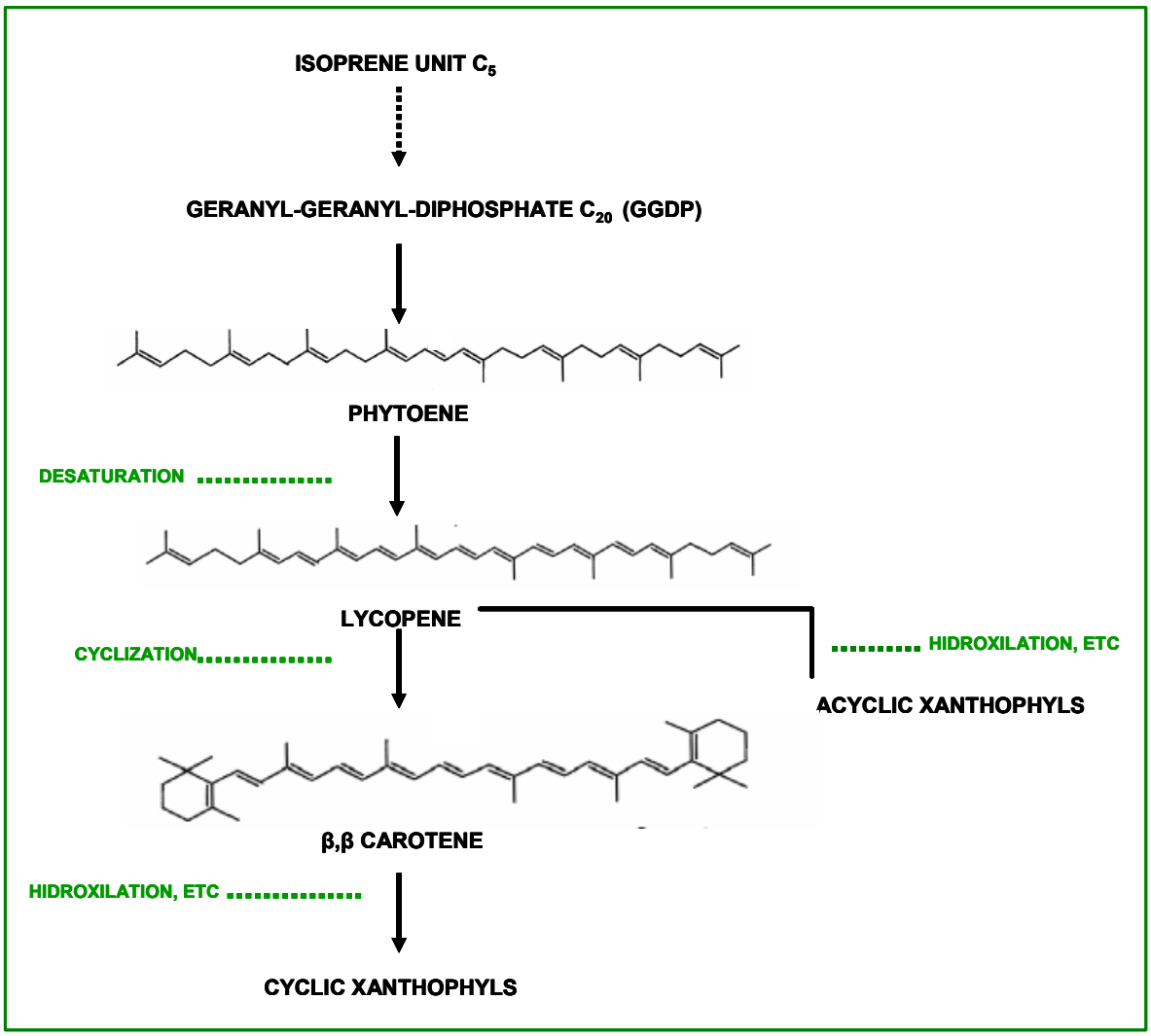


Figure 2. Summary of biosynthetic pathway for carotenoids

1.2.4 CAROTENOID FUNCTIONS

The functions of carotenoids can be divided into two groups: (1) phenological and, (2) physiological functions (Latscha, 1991). In nature, the most perceptible role of carotenoids is to confer the diversity of colour observed in plants, fruits, flowers, and in animal kingdom. Colour is not only a cosmetic effect, but also strongly influences communication signals such as luring,

warning, and camouflaging. In reproduction, carotenoids are often used by animals to produce sexual signals associated with mate choice (Kodric-Brown, 1985, 1987; Hill, 1991; Badyaev and Hill., 2000). In higher plants, carotenoids not only confer coloration but also flavour and fragrance characteristics (tobacco, flowers, and fruits), through carotenoids metabolites (Enzell, 1985).

The physiological functions of carotenoids in plants relate to their role in the photosynthetic process. In photo-protection, carotenoids dissipate the light energy used in photosynthesis and inhibit the formation of reactive oxygen species (ROS), both being defence mechanisms for excessive solar energy. Furthermore, carotenoids participate in light collection and the transfer of energy on to chlorophyll for photosynthesis (Deming-Adams *et al*., 1996).

In animals one of the most important physiological functions of carotenoids is their role as vitamin A precursors (Krinsky *et al*., 1993). This field has been extensively studied because of the physiological importance of vitamin A in growth, vision and reproduction. Not all carotenoids are vitamin A precursors; only 60 have been reported to be precursors of retinoids (Pfander, 1987). The main precursors are: α-carotene, γ-carotene, β-cryptoxanthin, echinone, β-apo-12´-carotenal, and of course β-carotene. From a structural point of view, retinoid precursors should at least have one unsubstituded β-ring. Nevertheless, certain xanthophylls such as canthaxanthin, astaxanthin, zeaxanthin, lutein and tunaxanthin, have been found to be also retinoids precursors in fish and rats (Matsuno, 1991).

Carotenoids are part of the oxidative defence mechanisms, scavenging two of the ROS molecules (Sies, 1986; Halliwell, 1996): (1) singlet molecular oxygen ($^1O_2$), an excited state of a partially reduced form of oxygen, unstable and highly reactive, and (2) peroxyl radicals, generated in the process of lipid oxidation (Stahl and Sies, 2003). Carotenoids react more efficiently with peroxyl radicals (Stahl and Sies, 2003). In general, carotenoids are considered

antioxidants, preventing diseases caused by oxidative stress (Chew and Park, 2004). Also, light exposure leads to the formation of ROS; carotenoids (with absorption maxima at around 450 nm) filter blue light, therefore they become a defensive barrier against the photooxidative damage of tissues (Stahl and Sies, 2003).

Carotenoids also participate in enhancing growth (Inborr and Lignell, 1997), immune function and disease resistance in higher animals and human (Bendich *et al*., 1986, Chew, 1992; Jyounchi *et al*., 1993; Chew and Park, 2004). In reproduction, aside from participating in sexual signals, they enhance reproductive performance (Lotthammer, 1978; Vassallo-Agius *et al*., 2001a, 2001b).

1.3 CAROTENOIDS IN AQUACULTURE

In the aquaculture industry, carotenoids have been included in diets of salmonids, crustaceans and other farmed fish, mainly as pigments to provide a desirable coloration to these cultured organisms. Consumers subconsciously relate product colour to nutritive value, healthiness, freshness and taste. Therefore, colour is a decisive quality criterion that has to be maintained and optimized. Carotenoids may not only contribute in improving quality by enhancing colour, but could also help to a better image in minds of consumers´ of aquacultural products, in view of increasing information available on carotenoids positive effect on human health. Aside from their quality enhancing properties, carotenoids seem to improve certain production parameters of farmed species. However, most of the research up to date has focused on their role as pigments.

Few studies have been conducted in carotenoids importance on molluscs, most of them dealing with their isolation and identification (Shahidi *et al*., 1998). In a study carried out by Vershinin (1996) with eight species of

freshwater and sea molluscs, only all-trans xanthophylls were found in the non-reproductive organs of all species, and carotenes were limited to hepatopancreas. In *Octopus vulgaris*, a well appreciated candidate in the aquaculture sector, three stereoisomers of astaxanthin were identified as main carotenoids (Moaka *et al*., 1989). Nevertheless there is no information on the possible role of astaxanthin in this species. The most probable function of carotenoids in mollusks could be the stabilisation of cell membranes fluidity (Vershinin, 1996).

In crustaceans, decapods such as lobster, shrimp, crayfish, and crab, the main carotenoids found are ketocarotenoids, astaxanthin being the most abundant (Schiedt, 1998). The formation of carotenoproteins complexes in crustaceans lead to brown, purple, green or blue colour of exoskeleton in live animals. Once carotenoproteins are exposed to temperatures higher than 60ºC, irreversible denaturalisation reactions occurs liberating astaxanthin and leading to the characteristic orange-red colour of astaxanthin that crustaceans show (Shahidi *et al*., 1998). *Penaeus species,* the most common farmed crustaceans, require the inclusion of a carotenoid source in their diet in order to achieve the desirable body coloration. Several studies carried out with *Penaeus monodon* and *Penaeus japonicus,* have demonstrated that these two species could attain the proper coloration by supplementing the diet with an astaxanthin source or by dietary supplementation of β-carotene, canthaxanthin, or zeaxanthin sources (Tanaka *et al*., 1976; Yamada *et al*., 1990; Chien and Jeng, 1992; Okada *et al*., 1994; Boonyaratpalin *et al*., 2001). Since crustaceans have the metabolic ability to introduce structural modifications into dietary carotenoids, in particular by introducing hydroxy groups at C(3) and C(3´) and keto groups at C(4) and C(4´), they can convert β-carotene, canthaxanthin or zeaxanthin into astaxanthin (Schiedt, 1998) (Figure 3). Dietary carotenoids converted into astaxanthin are deposited in the shrimp body in free form in association with proteins (carotenoproteins), and in esterified forms which are predominantly mono-ester and diester (Foss *et al*., 1987; Yamada *et al*., 1990). Though most research to

date has focus on the effect of carotenoid deficiency on *Penaeus species* coloration, there is growing evidence that suggests that carotenoids also influence growth (Dall, 1995; Petit *et al*., 1997), survival (Wyban *et al*., 1997), immune system (Liñán-Cabello *et al*., 2003; Supamattaya *et al*.,2005), reproduction (Liñán-Cabello *et al*., 2003), antioxidant activity (Meyers *et al*., 1997) and stress (Chien *et al*., 1999; Chien *et al*., 2003; Supamattaya *et al*.,2005).

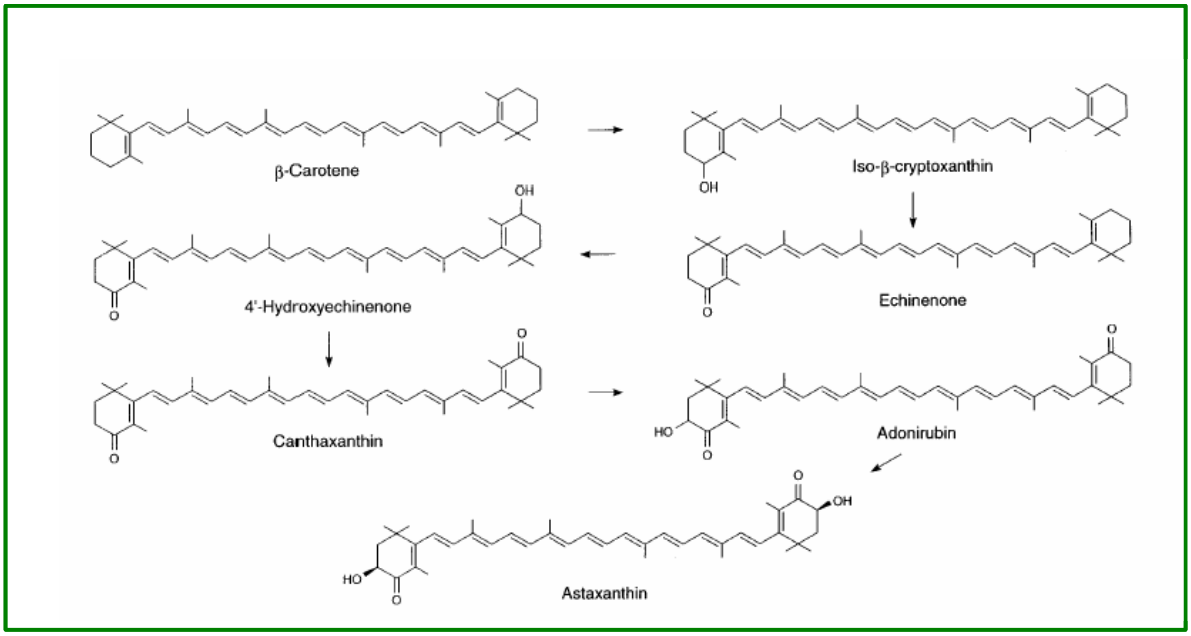


Figure 3. Astaxanthin formation from β-carotene in prawn *Penaeus japonicus* and lobster *Panulirus japonicus* (Bucheker, R., 1982).

Regarding equinoderms, sea urchins being the main cultured species, dietary carotenoids play an important role in the characteristic coloration of gonads. Echinone is the main carotenoid found in gonadal tissue, comprising as much as 83% of total carotenoids (Plank *et al*., 2002), which is synthesized from β-carotene (Shina *et al*., 1978; Goodwin, 1984; Matsuno and Tsushima, 2001). Carotenoid sources utilized in sea urchins are mainly natural, since synthetic sources such as β-carotene or astaxanthin do not improve gonad colour (Goebel and Barker, 1998; Havardsson and Imsland, 1999). Natural algal based diets or addition of natural β-carotene product derived from spray-dried algae, *Dunaliella salina*, results in the increase of gonad colour intensity (McBride *et al*., 1997; 1999; Robinson *et al*., 2002; Pearce *et al*., 2004). Aside from improving pigmentation, dietary carotenoids also have a biological role in sea urchins, participating in reproduction, early development, and biological defence (Tsushima *et al*., 1997; Kawakami *et al*., 1998; Shpigel *et al*., 2004).

1.4 CAROTENOIDS IN FISH

The main carotenoids present in fish are luteins, zeaxanthins, astaxanthins, and tunaxanthins (Matsuno, 2001) (Figure 4), being tunaxanthin the predominant carotenoid in marine fish (Sahidi, 1998). Since fish are not able of synthesizing carotenoids, these must be obtained from their feed. In farmed fish synthetic and natural carotenoid sources are thus supplemented to the diets.

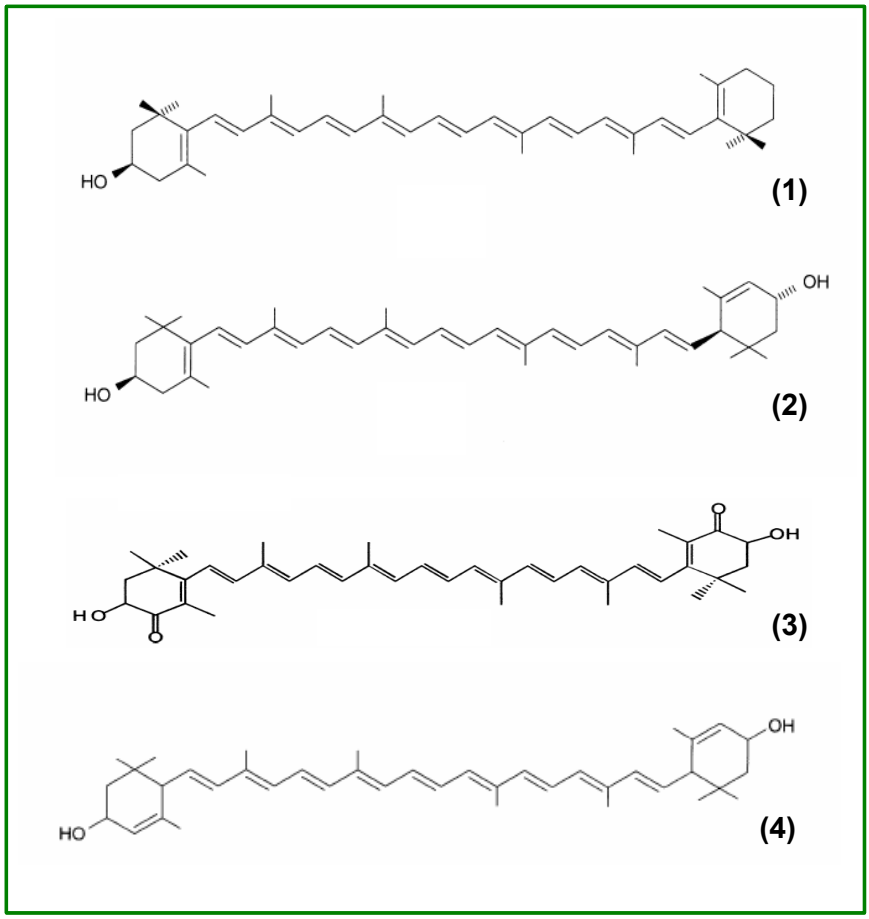


Figure 4. Chemical structure of the main carotenoids in fish: lutein (1), zeaxanthin (2), astaxanthin (3) and tunaxanthin (4).

1.4.1 CAROTENOID SOURCES

In the group of synthetic carotenoids, astaxanthin is the main pigment used world wide in the aquaculture industry (Higuera-Ciapara *et al*., 2006), and canthaxanthin (Figure 5) is the predominant source in salmonids (Torrissen, 1986; Choubert *et al*., 1994; Metusalach *et al*., 1996). However, as the naturally occurring carotenoid in salmonids flesh is astaxanthin, synthetic astaxanthin is the preferred source, as it produces the true colour; canthaxanthin gives the fillet a more yellow-orange coloration (Johnson, 1992). Astaxanthin industrially

produced is an identical molecule to that in living organisms, being a mixture of isomers (3S, 3S), (3R, 3S), and (3R, 3R) in the ratios of 1:2:1 (Figure 6).

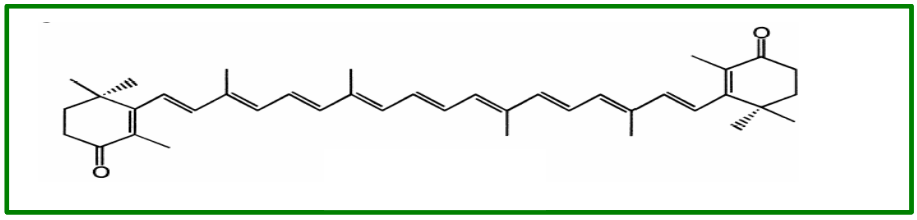


Figure 5. Chemical structure of canthaxanthin.

The high cost of synthetic pigments and the growing demand for natural pigmentation has stimulated the production of natural carotenoid sources with potential for industrialization. Nevertheless, these products so far have only taken up a small fraction of the market due to their limited production (McCoy, 1999). This is the case of some microorganisms such as microalgae, bacteria, and yeast species that have been reported to synthesize astaxanthin (Johnson and Schroeder, 1995; Armstrong, 1997) and other carotenoids of interest. The freshwater microalgae *Haematococcus pluvialis* has the ability to accumulate large amounts of astaxanthin: 1.5-5.0% w/w on a dry basis (Johnson and Schroeder, 1995; Krishna and Mohanty, 1998). *Haematococcus* primarily contains monoesters of astaxanthin linked to 16:0, 18:0, 18:2, and 18:1 (major) fatty acids (Restrøm *et al*., 1981), having the unesterified, mono and diesters an optically pure (3S, 3´S) chilarity (Grung *et al*., 1992). However, this alga exhibits some unfavourable characteristics like its slow growth rate and complex life cycle, when compared to other microalgae successfully cultivated at commercial scale like *Dunaliella spp*., and *Spirulina spp*. (Cifuentes *et al*., 2003). Feeding *Chlorella vulgaris* also produces positive pigmentation results in certain fish (Gouveia *et al*., 1996; 2002), and the microalgae *Chlorococcum sp* seems to be a promising source of astaxanthin, canthaxanthin and adonixanthin

(Higuera-Ciapara *et al*., 2006). It is worth noting that aside from giving an appropriate coloration, microalgae sources could provide better growth performance as it was shown in a study with *Penaeus monodon* larvae (Darachai *et al*., 1999).

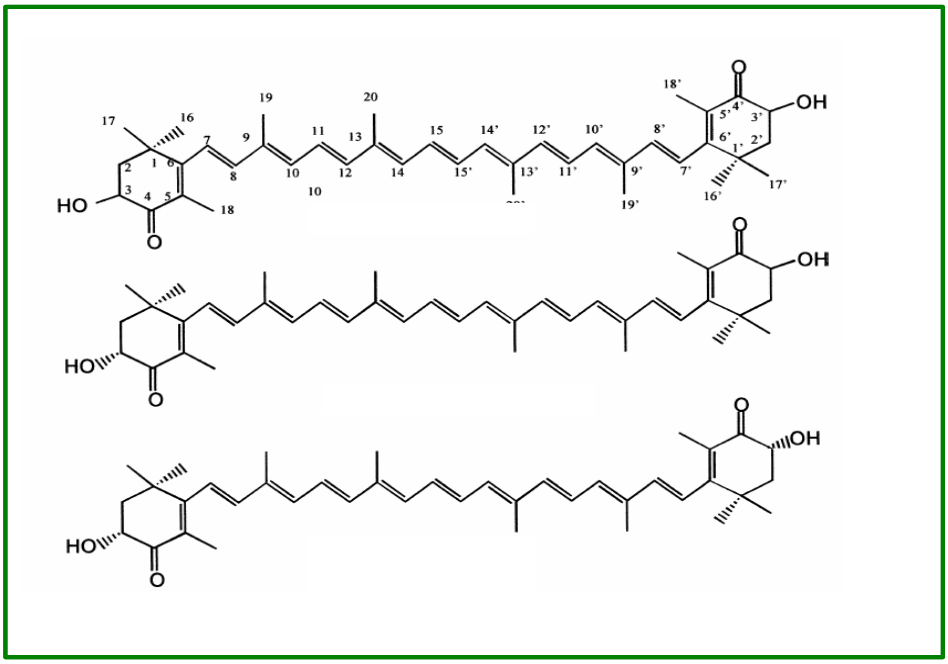


Figure 6. Astaxanthin configurational isomers (3S, 3´S) astaxanthin; (3R, 3´S) astaxanthin; (3R, 3´R) astaxanthin.

Among yeast, *Phaffia rhodozyma* is probably the most important as it contains astaxanthin as its main carotenoid (Andrewes and Starr, 1976), constituting approximately 85% of total pigments (Shahidi *et al*., 1998). The (3R, 3´R) optical isomer of astaxanthin predominates in red yeast, opposite to the normal (3S, 3´S) configuration found in other sources (Andrewes and Starr, 1976). The optical isomers are utilized to the same degree and the optical configuration is maintained after deposition in the flesh of rainbow trout (Foss *et*

al., 1984). Johnson *et al*., (1980) reported that *Phaffia rhodozyma* also serves as a good source of proteins and lipids. The inclusion of this carotenoid source, aside from its positive effect on fish pigmentation, enhances liver function and defensive potential against oxidative stress (Nakano *et al*., 1995; 1999).

Crustacean processing discards (shrimp, krill, and crab) are also potential carotenoid sources. Crustacean discards constitute an attractive ingredient for industrialization, since around 70% of the raw weights of the catch are processing discards (Wilkie, 1972; Simpson *et al*., 1985) with high carotenoid content and its use reduces the environmental problem caused by the large amounts of wastes (Torrisen *et al*., 1984; Sahidi *et al*., 1991; Sahidi, 1995). Astaxanthin is the predominant carotenoid (Sahidi *et al*., 1994; Higuera-Ciapara, 2006) in crustacean by-products that also include significant proportions of mineral salts (15-35%), proteins (25-50%) and chitin (25-35%) (Lee *et al*., 1982). Crustacean by-products have been successfully used for coloration of integument and flesh in feeds of fish with high economic importance (Satio *et al*., 1971; Spinelli *et al*., 1974; Torrissen *et al*., 1982; Meyers, 1977; Coral *et al*., 1997). However, certain disadvantages of this carotenoid source such as its variability in pigment concentration and its high ash and chitin content which reduces its digestibility for fish, severely limit the rate of inclusion in diet formulations.

Plants also have potential as carotenoid sources. Experiments with red pepper have given good results, although a lower efficacy was found in comparison to commercially available astaxanthin (Carter *et al*., 1994; Yanar *et al*., 1997). Furthermore, paprika oleoresin pigments confer a less desirable coloration in comparison to canthaxanthin in rainbow trout (Akhtar *et al*., 1999). In a study carried out with *Sparus aurata* fed a diet containing corn gluten meal (rich in zeaxanthin), the coloration of the front head and operculum achieved the characteristic yellow found in their wild counterparts (Robaina *et al*., 1997). It would be interesting to further investigate other probable plant-based carotenoid

sources for fish. Marigold, rich in lutein, might be an interesting dietary alternative given its efficacy with egg and skin coloration of poultry.

1.4.2 CAROTENOID ABSORPTION AND TRANSPORT

Carotenoids are hydrophobic compounds that are not easily solubilised in the aqueous environment of the gastrointestinal tract of fish; therefore, digestion, absorption and transport processes are associated to lipids (Castenmiller and West, 1998).

The intestinal absorption of carotenoids involves several steps, including disruption of the food matrix, dispersion in lipid emulsions and solubilisation into mixed bile salt micelles, before being carried to the enterocyte brush border were the absorption takes place (Furr and Clark, 1997; Tyssandier *et al*., 2001). In salmonids, approximately 35% of dietary astaxanthin is absorbed (Torrissen *et al*., 1989; Storebakken and No, 1992; Struksnaes *et al*., 2005) mainly along the proximal intestine (Torrissen, 1986; Al-Khalifa and Simpson, 1988; Torrissen, 1989; Hardy *et al*., 1990; White *et al*., 2002), taking approximately 18 to 30 hours (March *et al*., 1990; Choubert *et al*., 1994b). In comparison with other fish nutrients, absorption of carotenoids is considered slow. Furthermore, many authors suggest that the intestinal absorption from micelles is a passive diffusion process (Choubert *et al*., 1994b; Parker, 1996; Castenmiller and West, 1998; Van den Berg, 1999). Carotenoids are absorbed without prior metabolic conversion, except for xanthophylls esters, hydrolysed before absorption, by a non specific bile salt dependant lipase, since no esters are found in plasma or white muscle of salmonids (Schiedt, 1998; White *et al*., 2003b). Hence, astaxanthin esters found in the skin of salmonids are a result of re-esterification of free carotenoids with endogenous fatty acids (Foss *et al*., 1987). Studies in salmonids species regarding absorption have been assessed through monitoring carotenoid blood levels (Choubert *et al*., 1994b; Kiessling *et al*., 1995; Gobantes *et al*., 1997; White *et al*, 2002, 2003b). These levels are

affected by metabolism, absorption and excretion processes (Castenmiller and West, 1998). Therefore, this approach is informative but does not quantify carotenoid uptake by the intestine (White *et al.*, 2003a).

Salmonids preferentially absorb more polar carotenoids, particularly astaxanthin rather than canthaxanthin, zeaxanthin or carotenes (Foss *et al*., 1984; Guillou *et al*., 1992; Schiedt *et al*., 1985). The unesterified or esterified carotenoids forms also seem to influence absorption. Many studies have lead to contradictory results, with authors claiming that the free form is better absorbed than the ester form (Schiedt *et al*., 1985; Foss *et al*., 1987; Storebakken *et al*., 1987; Choubert and Heinrich, 1993), while others report that both forms are equally absorbed (Barbosa *et al*., 1999; Bowen *et al*., 2002). Japanese red sea bream seems to absorb synthetic astaxanthin dipalmitate more efficiently than unesterified astaxanthin, as reflected by skin pigmentation results (Ito *et al*., 1986).

In regard to transport, due to the hydrophobic nature of carotenoids these cannot circulate freely in plasma, and must be associated to plasma lipoproteins (Aas *et al*., 1999). Fish carotenoids are mostly transported to peripheral tissues by high density lipoproteins (HDL) (Nakamura *et al*., 1985) and to a limited extent (5-7%) by low density lipoproteins (LDL) (Ando *et al*., 1985). In rainbow trout and other *Oncorhynchus* species, astaxanthin and canthaxanthin were found to be present in all serum lipoprotein fractions (Choubert *et al*., 1992, 1994a; Choubert and Heinrich, 1993). In mature female fish, significant amounts of carotenoids also bind to vitellogenin, a female specific serum lipoprotein (Ando *et al*., 1986). During sexual maturation of *Oncorhynchus keta*, chum salmon, HDL and vitellogenin (VtG) were associated with carotenoid transport during re-distribution of carotenoids from muscle to the integument, and from muscle to ovaries, respectively (Ando and Hatano, 1988). Albumin, an abundant soluble protein in the body of all vertebrates and a major transport protein for fatty acids and hydrophobic compounds (Sheridan, 1988; Peters,

1996), is also suggested to play an important role in blood transport of carotenoids of Atlantic salmon (Aas *et al*., 1999).

1.4.3 CAROTENOID METABOLISM AND DEPOSITION

In fish, reductive and oxidative metabolic transformations play an important role (Schiedt, 1998). Carotenoid metabolism is suggested to take place in the organs where their metabolites are found (Storebakken *et al*., 1992), such as the liver (Hardy *et al*., 1990; Metusalach *et al*., 1996) or in the intestine (Aas *et al*., 1999). In salmonids, approximately 50% of dietary astaxanthin absorbed may be metabolised (Torrissen *et al*., 1989; Storebakken *et al*., 1992; Struksnaes *et al*., 2005). Early studies established a classification based on cartotenoids metabolic capacity of fish (Tanaka, 1978): A first type of fish cannot oxidize the ionone ring and, therefore, the specific oxygenated derivatives have to be included in their diet. A second type of fish, such as gold fish *Carassius auratus*, and the fancy red carp *Cyprinus carpio,* are able to oxidize 4 and 4´ positions of the ionone ring, hence being able to convert zeaxanthin and lutein to astaxanthin (Matsuno, 2001)

Salmonid species are capable of reducing, but not oxidizing dietary carotenoids to their own tissue-specific molecules. These reductive metabolic reactions involve the stepwise removal of the keto group at 4 and 4´ positions of the ionone ring (Matsuno and Tsushima 2001). The skin of this group of fish presents predominantly astaxanthin esters, when fed astaxanthin either free or esterified (Katsuyama *et al*., 1987; Bjerkeng *et al*., 2000). In a study carried out with Artic charr, aside from mono- and di-ester astaxanthin, small amounts of unesterified astaxanthin and yellow xanthophylls (iodaxanthin, tunaxanthin, lutein and zeaxanthin) were found, all of them expected metabolites of astaxanthin (Bjerkeng *et al*., 2000). When canthaxanthin was included in salmonid diets, β-carotene prevailed in the skin, followed by echinone and finally canthaxanthin; in Artic charr skin the presence of isocryptoxanthin was

also reported (Bjerkeng *et al*., 1990; Metusalach *et al*., 1995). All these carotenoids are reductive metabolites of canthaxanthin, β-carotene being the end product.

Yellowtail, an extensively cultured species in Japan that is characterized for exhibiting bright yellow bands near the lateral line, also seems to reduce ingested carotenoids (Fujita *et al*., 1983; Miki *et al*., 1985,). The yellow colour observed in the integuments is conformed predominantly by tunaxanthin (Hirao, 1967), although astaxanthin is the prevalent carotenoid in their natural diet. These observations suggest that astaxanthin must be reductively converted into tunaxanthin by the removal of the keto group at 4 and 4´ positions of the ionone ring, and the conversion of β-ring to ε-ring as shown in Figure 7 (Miki *et al*., 1985). When adult yellowtails were fed a lutein diet, an increase of skin tunaxanthin was also observed (Schiedt, 1998). Therefore, both astaxanthin and lutein could be tunaxanthin precursors, following the pathway shown in Figure 7, being 3´-epilutein the common carotenoid in both pathways. In black bass, *Micropterus salmoides*, tunaxanthin is also the predominant carotenoid in the integuments; no astaxanthin is found even though its diet is based on crustaceans, which is rich in astaxanthin (Yamashita and Matsuno, 1992). Striped jack (*Caranx delicatissimus*) presents more than 90% of tunaxanthin, lutein and zeaxanthin in the integuments, and when this fish is fed *Spirulina maxima* (rich in zeaxanthin) the content of these carotenoids increases (Shahidi *et al*., 1998). In both striped jack and black bass a reductive metabolic pathway from astaxanthin or zeaxanthin to tunaxanthin is suggested.

In relation to bright pink red skinned fish, such as Japanese red sea bream, astaxanthin esters are the predominant carotenoids deposited in their integuments, and in minor amounts, tunaxanthin (Tanaka *et al*., 1976; Allahpichay *et al*., 1984; Nakazoe *et al*., 1984). The inclusion of natural or synthetic dietary astaxanthin returned the characteristic skin colour, so well appreciated and lost under farmed conditions. Japanese red sea bream fed

diets supplemented with β-carotene and canthaxanthin, showed a decrease in skin carotenoid level, however when fed zeaxanthin or lutein a certain increase was shown, although not comparable to levels achieved when fed an esterified astaxanthin source (Nakazoe *et al*., 1984). Japanese red sea bream is capable of reducing but not oxidizing dietary carotenoids. The increase in skin carotenoid concentration observed with zeaxanthin and lutein supplementation could be due to the transfer of these carotenoids unchanged or perhaps due to a reductive metabolic process of both lutein and zeaxanthin to tunaxanthin; in yellowtail this transformation has also been suggested (Miki *et al*., 1985).

1.4.5 FUNCTIONS

In fish, carotenoids have similar functions as those previously found in other animal species: they are vitamin A precursors (Schiedt *et al*., 1985; Guillou *et al*., 1989; Christiansen *et al*., 1994; White *et al*., 2003a); markedly affect reproduction performance (Craik, 1985; Christiansen *et al*., 1996; Verakunpiriya *et al*., 1997; Chou *et al*., 2001; Vassallo-Agius *et al*., 2001a, b); are potent antioxidants (Bjerkeng *et al*., 1995; Shimidzu *et al*., 1996; Nakano *et al*., 1999; Bell, *et al*., 2000); enhance immune system (Nakano *et al*., 1995; Amar *et al*., 2003); and affect liver structure (Segner *et al*., 1989; Page *et al*., 2005). Although some authors claim that the biological functions of carotenoids in fish are still speculative (Choubert *et al*., 2005), other consider these compounds as important micronutrients that fish are not able to synthesize and, therefore, must be included in the diet (Baker *et al*., 2001).

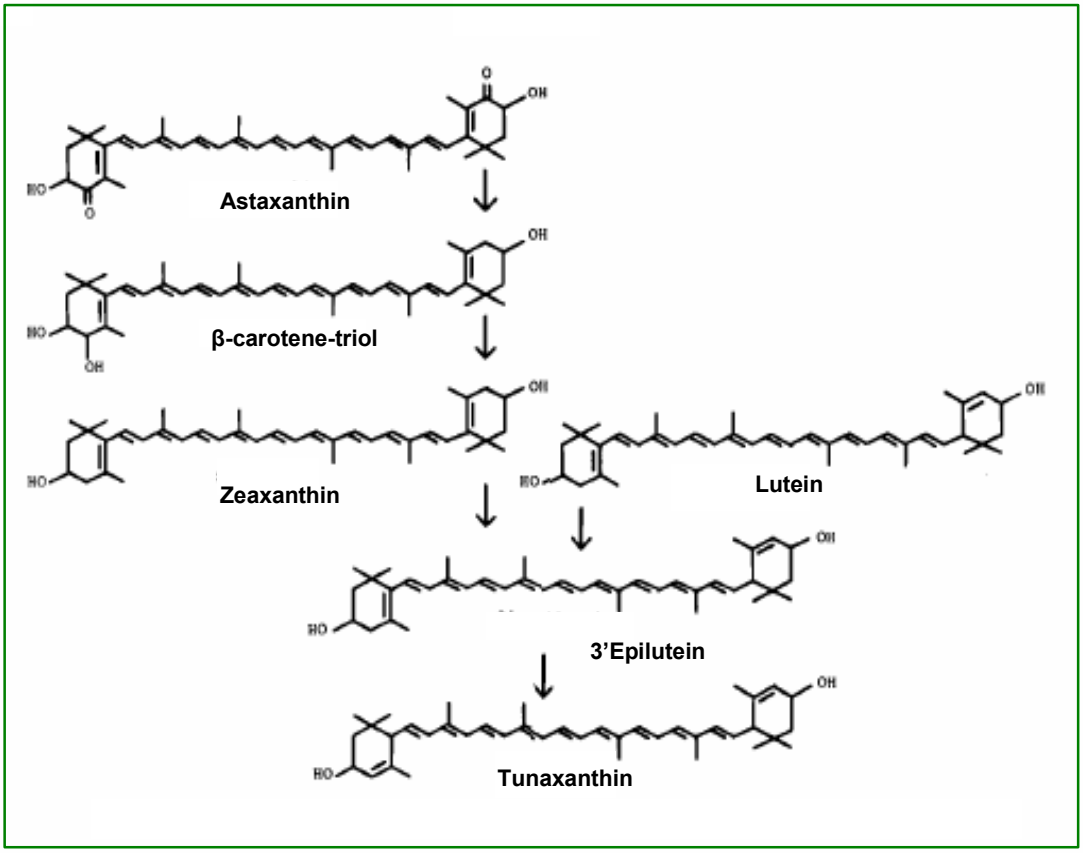


Figure 7. Presumed metabolic pathway of astaxanthin and lutein to tunaxanthin in yellowtail (Miki *et al*., 1985).

There is a controversy on the role of carotenoids in fish growth, several studies reporting a positive influence whereas others did not find any effect. In Atlantic salmon fry and juveniles the inclusion of synthetic astaxanthin and canthaxanthin not only enhanced growth but also survival (Torrissen, 1984; Christiansen *et al*., 1994, 1995; Christiansen and Torrissen, 1996). In Indian carp, the inclusion of β-carotene in the diet also promoted survival and growth (Goswami, 1993), and in red tilapia (*Oreochromis niloticus*), shrimp head meal, gave positive growth results when included in the diet. When using natural carotenoid sources it should be taken into consideration the presence of other components that could also enhance growth. Results attained with Atlantic salmon fry and juveniles, together with reports for shrimp and sea urchins (Petit

et al., 1997; Myuki *et al*., 1997) suggest that carotenoids may have a positive effect on growth, mainly during the first developmental stages. No clear effect of carotenoid supplementation time or carotenoid concentration in the diet has been reported on growth.

1.5 CAROTENOIDS IN RED PORGY

Red porgy is a member of the *Sparidae* family. It is geographically distributed along the British Isles to Senegal in the Eastern Atlantic, from North Carolina to Argentina in the Western Atlantic, and also in the Mediterranean and Adriatic seas (Kentouri *et al*., 1995; Pajuelo *et al*., 1996; Mihelakakis *et al*., 2001). Juveniles are found mainly around 20-50 m depth, on sandy bottoms, where smaller preys are plentiful; but adult specimens prefer deeper waters, around 250 m depth, where they have access to larger preys such as crustaceans, mollusc and small fish (Labropoulou *et al*., 1999). This species is highly appreciated in commercial fisheries for its appearance and meat quality, and for the growing concern about fishing declines (Vaughan *et al*., 2002). These facts, together with successful studies on red porgy development under aquaculture conditions (Kentouri *et al*., 1994, 1995; Hernandez-Cruz *et al*., 1999) and the comparable growth and survival to gilthead sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) (Divanach *et al*., 1993), makes this fish species a potential candidate for the aquaculture industry in the Mediterranean region. However, there are still certain limitations for the entrance of red porgy in aquaculture, the loss of the characteristic red-pink skin coloration and skin darkening (Kentouri *et al*., 1995). In turn, dark grey integuments are observed in farmed red porgy. These quality characteristics in cultured fish dramatically influence acceptability and market value.

1.5.1 RED PORGY SKIN COLOUR

Skin colour in fish results from the presence of different types of chromatophores, pigment cells, subdivided into at least six different types depending on the pigment present (Fujii, 2000): melanophores (black or brown), erythrophores (red), xanthophores (yellow), cyanophores (blue), leucophores (whitish) and iridophores (iridescent). Various combinations of these chromatophore species, in various proportions, determine coloration of fish integument (Fujii, 2000). In a study on red porgy skin pigment cells, predominantly xanthophores/erythrophores and melanophores were observed in the skin when this fish species was fed a diet with a carotenoid source; on the contrary, when a carotenoid depleted diet was supplied, very few xanthophores/erythrophores and mainly melanophores were found, and no red-pink coloration was observed (Chatzifotis *et al*., 2005). Similarly, in Japanese red sea bream, a close species to red porgy, erythrophores are assumed to be the predominant pigment cells in the skin, since the main pigment extracted from the vivid red-pink skin was astaxanthin diester (Tsukuda *et al*., 1966; Lin *et al*., 1997). Therefore, the inclusion of carotenoids in diets of cultured red porgy could be essential to provide the characteristic coloration of its integuments (Picture 1).

Encouraging results have being achieved by including dietary unesterified or esterified astaxanthin (Chebbaki, 2001; Cejas *et al*., 2003; Tejera *et al*., 2003, 2005; Chatzifotis *et al*., 2005; Kalinowski *et al*., 2005). However, studies on the appropriate dose and supplementation time with astaxanthin, and information on the possible carotenoids present in this species skin is yet unknown. Aside from astaxanthin, other dietary carotenoids such as β-carotene and lycopene have been used, however no desirable skin color has been attained (Chatzifotis *et al*., 2005). Fish developmental stage (Foss *et al*., 1984; Katsuyama *et al*., 1987; Bjerkeng *et al*., 1992), dietary components such as lipids and fatty acids (Torrissen *et al*., 1990; Bjerkeng *et al*., 1997; Bell *et al*., 1998; Nickell and Bomage, 1998a; Bjerkeng *et al*., 1999), genetic background

and fish metabolic turnover (March *et al*., 1990; Hudon, 1994; Bjerkeng *et al*., 1997) have been reported to influence carotenoid digestion, absorption, transport, and deposition; therefore, these factors must be taken into consideration when studying carotenoids.

Picture 1. Wild red porgy caught in the Coast of Fuerteventura.

Aside from the dietary component, fish can also change their skin coloration in response to factors such as environmental conditions, physiological status, and stressful stimuli (Szisch *et al*., 2002). These factors can generate two basic processes: physiological fast colour change and morphological colour change. The former is the aggregation or dispersion of pigmented granules, principally melanosomes and it is controlled by the sympathetic nervous and/or endocrine system. Morphological changes concern

changes in the type, number or density of melanophores and/or the net amount of melanin (Bagnara *et al*., 1973; Fujii, 1993; Sugimoto 1993, 2002; Szisch *et al*., 2002). Nevertheless, a prolonged physiological colour change may lead to a morphological one. Aquaculture conditions such as density, tank background, light spectrum, light intensity, and aquaculture related stressors, also seem to influence red porgy skin coloration. Most aquaculture conditions seem to cause physiological colour changes, although if the inappropriate conditions persist in time, morphological colour changes may occur, causing red porgy skin darkening, as melanin synthesis takes place.

2.- OBJECTIVES

The general objective of this thesis was to improve skin coloration of cultured red porgy, approaching it to their wild counterparts in view of the importance of this skin characteristic for its market value. To achieve this goal the following objectives were focused:

1.- Determine the effect of different dietary carotenoid sources on red porgy skin colour.

Studies were conducted evaluating red porgy skin colour sensibility to the dietary inclusion of shrimp shell meal, as a source of esterified astaxanthin, synthetic canthaxanthin and synthetic astaxanthin (Chapters 4 and 6). As well, various concentrations of the mentioned carotenoid sources were also tested on red porgy skin coloration by colorimetry, using the calculations provided by the International Lighting Standards Commission (CIE).

2.- Determine the effect of dietary carotenoids on red porgy culture performance.

Growth and feed utilization parameters were recorded from red porgy fed with synthetic canthaxanthin, shrimp shell meal and synthetic astaxanthin (Chapters 4, 5 and 6).

3.- Determine the adequate supplementation time with several types of carotenoid sources to give red porgy skin a coloration similar to wild specimens.

Red porgy were fed with shrimp shell meal or synthetic astaxanthin for different periods of time before harvesting and the skin colour achieved was compared to that of the wild specimens (Chapters 5 and 6).

4.- Analyse the effect of carotenoid feeding on red porgy skin carotenoid concentration.

Extraction and quantification of total skin carotenoid concentration of cultured red porgy fed different carotenoid sources for different lengths of supplementation time (Chapters 5 and 6). Establish the possible relationship between skin colour parameters and skin carotenoid concentration.

5.- Separate, quantify and tentatively identify red porgy skin carotenoids in cultured and wild red porgy.

Several TLC and HPLC methods were tested to separate, quantify and tentatively identify carotenoids present in red porgy skin (Chapters 6 and 7).

3.- MATERIAL AND METHODS

In the following chapter, material and methods utilized in the four experiments carried out in the present thesis, described in chapters 4, 5, 6 y 7 respectively, will be explained in general, giving more detail in each chapter.

3.1 ANIMALS

Cultured red porgies used in all of the experiments were produced at our research facilities in “Instituto Canario de Ciencias Marinas” Gran Canaria, Spain. At the beginning of each experience fish were individually weighted, measured and distributed according to the initial weight desired, trying to avoid significant differences in mean weight, among tanks. Aside from cultured red porgy, wild specimens captured near the Coast of Fuerteventura Island were also employed in experiments 2, 3 and 4.

3.2 EXPERIMENTAL CONDITIONS

All fish used in the different experiences were held indoors and with natural sea water continuously supplied under the experimental conditions described in Table 1 and the tanks utilized are shown in Pictures 1 and 2. Concerning wild specimens, these were distributed in four circular grey fibreglass tanks of 1 m^3 (Picture 3).

3.3 DIETS AND FEEDING

3.3.1 FORMULATION

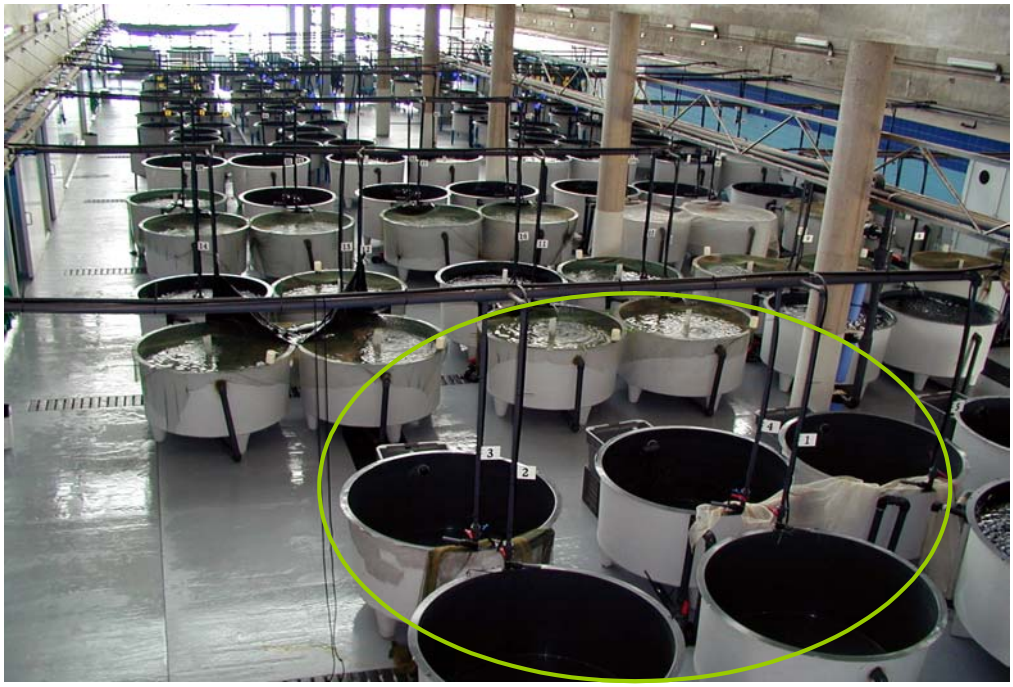
Most of the ingredients used for diet elaboration were obtained locally, fish meal and fish oil were provided by the company Proaqua-Spain, shrimp shell meal was supplied by Sopropeche – France, and synthetic carotenoids by DSM – Madrid, Spain. Diets were formulated after biochemical analysis of the used ingredients, if necessary. All experimental diets were formulated to have the same protein and lipid levels. Concerning vitamins and minerals premixes (Table 2) these were also the same for all the experimental diets.

Table 1. Experimental conditions utilized in all of the experiences

EXPERIMENTAL CONDITIONS	**EXP. I**	**EXP. II**	**EXP. III**	**EXP.IV**
Tank colour and shape	Grey-circular			
Tank material	Fibreglass			
Tank volume (l)	100	500	500	1000
Water flow (l/min)	3	6	6	12
Temperature (ºC)	17 – 19			
Oxigen (mg/l)	8-10			
Photoperiod	12 hours light:12 hours dark			

Picture 1. Experimental tanks of 100 l utilized in experiment 1.

Picture 2. Experimental tanks of 500 l utilized in experiments II and III.



Picture 3. Experimental tanks of 1000 l utilized with wild red porgy.

Table 2. Vitamin and mineral premixes used in all experimental diets.

VITAMINS (g/kg diet)		**MINERALS (g/kg diet)**	
Retinol acetate	0.025	$(H_2PO_4)Ca$	1.605
Menadione	0.02	$CaCO_3$	4.0
Cholecalciferol	0.005	$FeSO_4.7H_2O$	1.5
Cianocobalamine	0.5	$MgSO_4.7H_2O$	1.605
Biotin	0.001	K_2HPO_4	2.8
Folic acid	0.01	$Na_2PO_4H_2O$	1
Pyridoxine	0.04	$Al(SO_4)_3.6H_2O$	0.02
Riboflavine	0.05	$ZnSO_4.7H_2O$	0.24
Thiamine	0.04	$CuSO_4.5H_2O$	0.12
Choline	2.7	KI	0.02
Calcium pantothenate	0.12	$CoSO_4.7H_2O$	0.08
Niacin	0.2	$MnSO_4H_2O$	0.08
Ascorbic acid	2		
α-tocopherol	0.25		
Mio-inositol	2		

3.3.1 FEED PROCESSING

All the formulated diets were produced in our research facilities using a mixer (DANAMIX BM 330, Azpeitia, Gipuzcua, Spain) and a pelletizer (CPM, California Pellet Mill, USA).

3.3.2 FEEDING

Fish were fed until apparent satiation 2-3 times a day in all of the experiences. Before starting each experiment red porgy were fed a commercial

non pigmented diet for marine fish (Proaqua-Spain). In the case of wild red porgy these were fed to apparent satiation and with natural diet based on mussels.

3.4 BIOLOGICAL AND FEED UTILIZATION PARAMETERS

The following equations were used to study the effect of the experimental diets on growth and feed utilization parameters in red porgy.

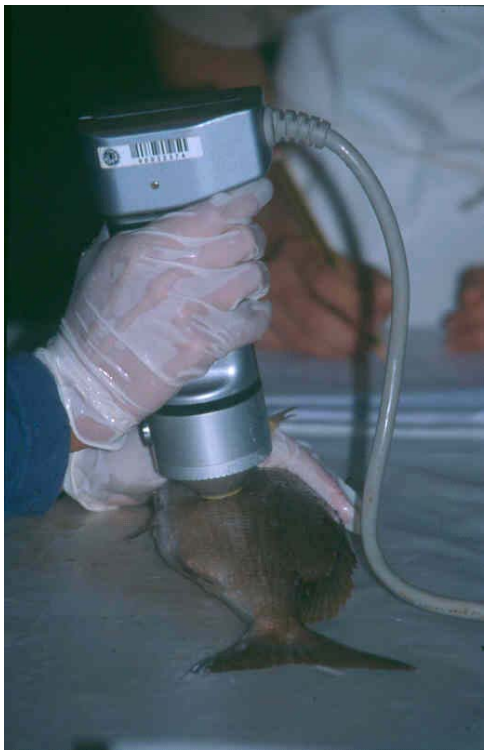
- Growth (%) = (Final mean weight – initial mean weight)*100
  Initial mean weight

- Specific growth rate (SGR) = (Ln final mean weight – Ln initial mean weight) *100
  Number of days

- Feed conversion ratio (FCR) = Feed intake (g)
  Weight gain (g)

3.5 SKIN COLOUR ANALYSIS

For skin colour measurements a portable colorimeter (Minolta Chroma Meter CR-200, Minolta, Osaka, Japan) was used in all of the experiences (Picture 4). This apparatus was utilized in the following way: triplicate measurements were taken at each sampling skin zone, the colour parameters measured were L*, lightness, which ranges from 0 for black and 100 for white; a* for red/green chromaticity; and b* for yellow and blue chromaticity, in accordance with the recommendations of the International Commission on Illumination, CIE (1976).



Picture 4. Colour measurements taken with the colorimeter.

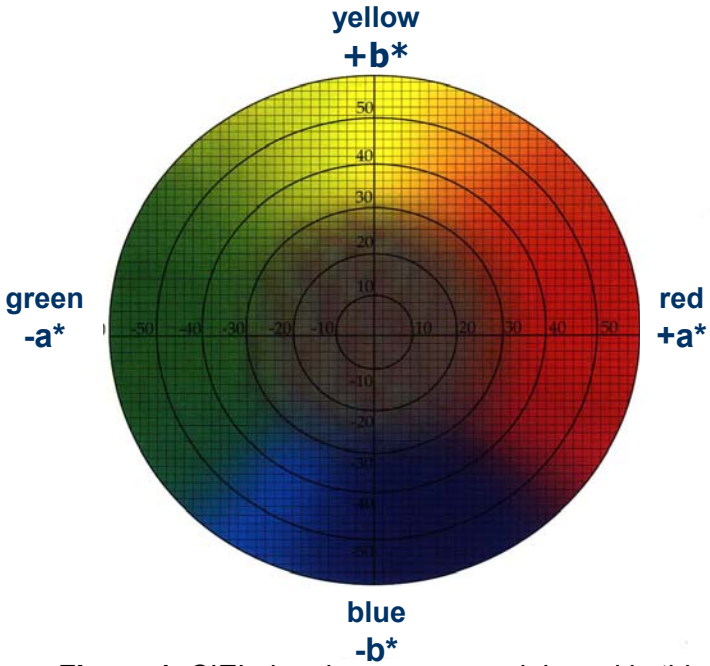


Figure 1. CIELab colour space model used in this study.

From a* and b* values, hue (H_{ab}) and chroma (C_{ab}) values were calculated. Hue, namely the observable colour (e.g. red, blue, yellow), is an angular measurement where 0º indicates a red hue, 90º denotes a yellow hue, 180º green and 270º blue one; and is calculated by the following equation:

$H_{ab}=\arctan(b^*/a^*)$ (Hunt, 1977)

Chroma is an expression of saturation or intensity of the colour attained and is expressed by the following equation:

$C_{ab}= (a^{*2}+ b^{*2})^{1/2}$ (Hunt, 1977)

3.6 BIOCHEMICAL ANALYSIS

Diets, whole fish and skin were kept at -80ºC, under nitrogen atmosphere until biochemical analyses were carried out. All the analyses were done in triplicate, except carotenoid concentration that was effectuated by duplicate (Barua *et al.,* 1993). Previous biochemical analysis, samples were properly homogenised.

3.6.1 PROXIMATE ANALYSES

For proximate composition of diets and fish the following methodologies were carried out:

· MOISTURE

Moisture was determined by drying the samples at 105º C until a constant weight was reached (AOAC, 1995).

· ASH

Ash content was determined by drying the samples in an oven at a temperature of 450º C until a constant weight was attained (AOAC, 1995).

· PROTEINS

Proteins were estimated from total nitrogen present in the sample, using Kjeldhal methodology (AOAC, 1995), after the digestion of the sample with concentrated sulphuric acid at a temperature of 420º C.

· LIPIDS

Total lipid was extracted from the diets by the method of Folch *et al*., (1957) using a mixture of chloroform:methanol (2:1, v/v) containing 0.01% of BHT. After lipid extraction, solvent is dried under nitrogen atmosphere and subsequently weighted.

3.6.2 FATTY ACID ANALYSES

Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipid following the method of Christie ESAS COMAS ANTES DE PARENTESIS!!! (1982). FAME were separated, identified and quantified by liquid gas chromatography under the conditions described by Izquierdo *et al*. (1990) and shown in Table 3. Individual methyl esters were identified by comparison with external standard EPA 28, Nippai, Ltd. Tokyo, Japan.

Table 3. Conditions of GC for fatty acid determination

Apparatus	Shimadzu GC-14-A (Shimadzu instrument division, Kyoto, Japón.
Integrator	Shimadzu C-R5A
Column	Capillary fused silica 30*0.32 mm D.I (Supelco, Inc., Bellefonte, EE.UU)
Transport gas	Helium
Pressure of gasses	He=1, H_2 =0.5, N_2= 0.5 y air 0.5 kg/cm^2
Detector	FID at 250 º C
Temperature of injector	250º C
Oven	Initial temperature - 180º C -10 minutes Increment of 2.5º C per minute Final temperature 215º C during12 minutes.

3.6.3 CAROTENOID ANALYSES

Carotenoid extraction, saponification, separation, relative quantification, and tentative identification were carried out using the following methodologies.

· CAROTENOID EXTRACTION FROM DIETS

Carotenoid extraction from the diets was conducted in duplicate according to Barua *et al*. (1993). Extraction is first carried out with ethyl acetate: methanol (1:1), followed by ethyl acetate, and finally hexane. Pooled supernatants were evaporated to dryness under nitrogen atmosphere, re-dissolving the carotenoid residue in a volume of hexane to have an absorbance between 0.2 and 0.8.

As synthetic carotenoids, included in some of the experimental diets, are in a starch matrix, extraction is done after an enzymatic digestion with Maxatase (International Biosynthetics, Rijswijk, Netherlands) as follows: Portions of

ground diet were mixed with 10 ml of water and 10 mg of Maxatase, followed by incubation in a water bath at 50ºC for 30 minutes (Weber, 1988).

For quantification of carotenoid concentration, absorbance of total carotenoids in hexane, previous filtration using a Pasteur pipette filled with cotton, was read at the λ_{max} in a spectrophotometer. Carotenoid concentration was estimated according to the following equation:

$$\mu g/g = 10000*V*A / W* E_{1\%,1cm}$$

V is total volume of the extract.
W is the weight of the sample.
A is the absorbance.
$E_{1\%, 1cm}$ is the absorbance of a 1% solution read in a 1cm cuvette

· CAROTENOID EXTRACTION FROM THE SKIN

Carotenoid extraction from skin samples was done according to Schiedt *et al*. (1995). Tissue was weighted and carotenoid extraction was carried out with acetone, until no more pigment was released. Partial evaporation of the combined acetone extracts is carried out to obtain approximately 5 ml. To remove water from the acetone extract, an equal volume of hexane is added, followed by 2 ml of water, the mixture is shaken cautiously, until two phases separate. The aqueous phase hypophase (lower) is run off and re-extracted with hexane. Solvent extraction is repeated until no more coloured material is extracted from the hypophase. The hexane layer (upper), containing carotenoids, is washed two to three times with water to remove traces of acetone. Afterwards, hexane is evaporated to dryness under nitrogen atmosphere. The extract was dissolved with hexane to attain an absorbance

between 0.2 and 0.8. Quantification of skin carotenoid concentration is done as described above.

· CAROTENOID SAPONIFICATION

For the break down of carotenoids esters, saponification was carried out according to Schmidt et al. 1994 with some modifications. A freshly prepared solution of NaOH in water (43% w/w) was added to the skin carotenoid extract dissolved in ethanol if the sample is not fully dissolved a few drops of diethyl ether are added. Approximately 0.5 ml of the NaOH is added every 5 ml of the carotenoid solution, to give an overall NaOH concentration of 7-8%. The solution is kept under nitrogen atmosphere overnight, at room temperature and wrapped with aluminium foil to protect it from light. On the following day, the saponification mixture is treated for the recovery of carotenoids. To recover carotenoids, the same volume of diethyl ether and water are added, and the solution is shaken. The upper phase with saponified carotenoids is collected and three additional water rinses are done. Afterwards, solvent is evaporated to dryness under nitrogen atmosphere, and the extract dissolved with a known volume of mobile phase and filtered through filter (PTFE 0.45 µm).

· CAROTENOID SEPARATION AND QUANTIFICATION BY THIN LAYER CHROMATOGRAPHY (TLC)

Carotenoid separation and relative quantification by TLC was done in the following way: Glass chamber were plates are developed is prepared by covering the inside with filter paper, the mobile phase is left for approximately 30 minutes, allowing chamber atmosphere saturation. Since light must be avoided when working with carotenoids, development chamber is also protected with tin foil paper. After development chamber is prepared, samples are applied in silica pre-coated G60 Merck glass plates (20x20cm) using glass capillaries. Application is done as a fine streak which contains a known amount of sample, approximately 3 µg. After sample application, the plate is placed in the development chamber and when solvent front has reached approximately 1 cm

above the plate, it is removed quickly and solvent position and separated carotenoids are marked with a pencil. Once the solvent from the plate has evaporated the Rf values are taken. The Rf value is defined as the distance moved by the solute divided by the distance moved by the mobile front.

For carotenoid relative quantification, each component was scrapped from the silica plate and washed several times with acetone until no colour is observed in the silica. There after, the acetone volume is filtered with a Pasteur pipette filled with cotton, evaporated to dryness and dissolved in a volume of hexane to give an absorbance between 0.2 and 0.8. Maximum absorbance was used for quantification, and as a know amount of sample was applied in the silica plate relative amounts of carotenoids may be estimated (%).

· CAROTENOID SEPARATION BY HIGH PRESSURE LIQUID CHROMTOGRAPHY (HPLC)

Conditions employed for skin carotenoid separation and identification using HPLC are shown in Table 4.

· CAROTENOID IDENTIFICATION

The following identification techniques were employed for tentative identification of the possible carotenoids present in the skin of cultured red porgy: spectral characteristics of the separated pigments, and comparison with published data, co-chromatographic comparisons with authentic carotenoids and a numerical notation (%III/II) which describes the ratio of the peak height of the longest wavelength absorption band (band III) to that of the middle absorption band (usually λ_{max}, band II) as a percentage.

Table 4. HPLC conditions employed for skin carotenoid separation.

Chromatograph	Thermo liquid chromatograph equipped with four pumps and a photodiode array detector (PDA)
Column	C_{18} Thermo column
Solvents	Dichloromethane-methanol-acetonitrile-water (HPLC grade, Sigma Chemical Co.)
Flor	1 ml/minute
Detection	PDA
Wavelength	480 nm and 440 nm
Temperature of column	Room temperature
Injection volume	100 µl

3.7 STATISTICAL ANALYSES

Means and standard deviations were calculated for each parameter measured. All data were tested for normality and homogeneity of variance. Differences among groups were determined by one-way ANOVA or non parametric statistics (Kruskal and Wallis). Afterwards an appropriate test was applied to identify groups that were significantly different. A minimum significance level of $P<0.05$ was used in all tests.

4.- EFFECT OF DIFFERENT CAROTENOID SOURCES AND THEIR DIETARY LEVELS ON RED PORGY (*Pagrus pagrus*) GROWTH AND SKIN COLOUR

Abstract

Whereas wild specimens exhibit a red pink silver colour, under captivity red porgy (*Pagrus pagrus*) skin turns dark grey. This characteristic is directly associated with acceptance by the consumers and market value. The present study was conducted to evaluate the effect of diet supplementation with two carotenoid sources, at two different concentrations, on growth and skin coloration. Fish of 44g initial mean weight were fed the following diets: control (with no added carotenoids), CTX 40 and CTX100 (diets with 40 and 100 mg of synthetic canthaxanthin/kg of diet respectively); SM20 and SM40 (diets with 20 and 40 mg of astaxanthin from shrimp shell meal/kg of diet respectively). Three samplings were carried out in this study, at the beginning, after 75 and 105 days of feeding. At each sampling, individual weight and colour measurements were performed. Colour was evaluated in three different body zones: front lateral (zone I), front dorsal (zone II) and caudal fin (zone III). Growth was not influenced by the inclusion of carotenoids in red porgy diet. Only astaxanthin from shrimp shell meal, particularly at 40 mg/kg diet, was able to give red porgy skin an overall reddish coloration, suggesting a better utilization of this source. Shrimp shell meal diets enhanced reddish hue and chroma values, nevertheless hue values at days 75 and 105 did not show a marked difference, and chroma after 75 days of feeding time tended to be reduced, suggesting skin colour saturation after a certain feeding time. Carotenoid supplementation did not influence positively on skin lightness. From the three evaluated zones, zone I of fish fed SM40 diet presented lower variation of hue, chroma and lightness; therefore, it could be proposed as a control zone for future studies. Results of this study suggests that the inclusion of astaxanthin, from shrimp shell meal, in

red porgy diets significantly improves skin coloration and markedly enhances the commercial value of this cultured species.

Keywords: Carotenoids; Chroma; Growth; Hue; Lightness; *Pagrus pagrus*; Skin

EFECTO DE DIFERENTES FUENTES DE CAROTENOIDES Y SU NIVEL EN LA DIETA SOBRE EL CRECIMIENTO Y COLORACIÓN DE LA PIEL DEL BOCINEGRO (*Pagrus pagrus*)

Resumen

En la naturaleza, el bocinegro (*Pagrus pagrus*) presenta una coloración rosada que se torna gris oscuro bajo condiciones de cultivo. Este fenómeno afecta de forma directa, tanto a la aceptación del producto por parte de los consumidores, como a su valor comercial. El presente estudio se diseñó con la finalidad de evaluar el efecto de la inclusión en la dieta de dos fuentes de carotenoides, a dos concentraciones diferentes, sobre el crecimiento y coloración de la piel de estos peces. Ejemplares con un peso medio inicial de 44g fueron alimentados con las siguientes dietas: control (sin inclusión de carotenoides); CTX 40 y CTX100 (suplementadas con 40 y 100 mg de cantaxantina sintética por kilogramo de dieta, respectivamente); SM20 y SM40 (suplementadas con 20 y 40 mg de astaxantina procedente de carcasa de langostino por kilogramo de dieta, respectivamente). Se realizaron tres muestreos a lo largo del estudio, al inicio, 75 y 105 días del mismo. En cada muestreo, se midió el color y se pesó los animales de forma individual. El color se evaluó en tres zonas corporales diferentes: anterior lateral (zona I), anterior dorsal (zona II) y aleta caudal (zona III). La inclusión de carotenoides en la dieta, no afectó al crecimiento de los bocinegros. Solamente la astaxantina de carcasa de langostino, cuando se incluyó a una concentración de 40 mg/kg de dieta, dio lugar a una coloración rojiza en la piel del bocinegro, lo que sugiere una mejor utilización de esta fuente. Ambas dietas con harina de carcasa de langostino promovieron los valores de croma y color, en este último caso a valores más rojizos. Sin embargo, a día 75 y 105, los valores de color no mostraron diferencias, y el croma obtenido a los 75 días del régimen alimenticio tendió a reducirse a día 105, sugiriendo una saturación del color en la piel tras un cierto tiempo de alimentación. En el presente experimento, la inclusión de

carotenoides en la dieta, no influyó en la luminosidad de la piel del bocinegro. De las tres zonas evaluadas, la zona I de los peces alimentados con la dieta SM40 presentaron una menor variación en los valores de color, croma y luminosidad; por lo que podría proponerse como zona de control para futuros estudios. Los resultados obtenidos en el presente estudio, sugieren que la inclusión de astaxantina de harina de carcasa de langostino en la dieta del bocinegro, mejora significativamente la coloración de la piel y aumenta de forma sustancial el valor comercial de esta especie de cultivo.

***Palabras clave**:* Carotenoides; Color; Crecimiento; Croma; Luminoidad; *Pagrus pagrus;* piel

4.1 INTRODUCTION

Despite the more optimistic projections still forecast a certain increase in European sea bass and gilthead sea bream markets during the next years, dependence of most Mediterranean marine fish farms on only two species markedly constrains the further development of aquaculture in this region. Hence, recent studies have being focusing along the last 10 years on diversification of cultured species, many of them belonging to the sparid family (Basurco and Abellan, 1999). Among them, red porgy, a species distributed along the Mediterranean and Atlantic coasts, has been found to be a suitable candidate for aquaculture diversification, for a high fillet quality and market price, among other considerations. Previous studies have shown that this species has a good adaptability to culture conditions, spontaneous spawning in captivity, and no serious problems of disease or mortalities (Kentouri *et al*., 1994; Kokokiris, 1998; Schuchardt *et al*., 2000). These characteristics together with the inability of the wild captures to match the market demands, makes this species a valuable candidate for finfish mariculture (Manooch and Hassler, 1978; Divanach *et al*., 1993). However, several authors as well as fish farmers have found that this species loses its natural skin coloration under culture conditions. Thus, whereas wild specimens exhibit a red pink silver colour, under captivity red porgy skin turn dark grey (Kentouri *et al*., 1995; Stephanou *et al*., 1995; TECAM, 1999; Cejas *et al*., 2003). Maintenance of the natural skin pigmentation is of great importance from a commercial point of view, being directly associated with acceptance or rejection by the consumers (Shahidi *et al*., 1998) and the product market price.

Skin and muscle pigmentation is frequently provided by carotenoid inclusion in fish diets. For instance, to preserve cultured salmon natural muscle coloration, diets for salmonids species include different types of carotenoid sources like synthetic astaxanthin and canthaxanthin (Torrissen, 1989; Bjerkeng, *et al*., 1992; Storebakken and No, 1992) carcass meal from red crustacean (Satio and Regier, 1971; Kotic *et al*., 1974) certain algae or red yeast (Johnson *et al*., 1980). Carotenoids are also included in commercial diets

for Japanese red sea beam to maintain good skin coloration (Katayama, 1972; Tanaka 1976; Nakazoe *et al*., 1984). Aside from colouring muscle and skin, there is evidence of the beneficial effects of dietary carotenoid supplementation, such as growth enhancement (Torrissen 1989; Storebakken and Goswami, 1996) or improvement of broodstock performance (Verakunpiriya *et al*., 1997; Watanabe and Vassallo-Agius, 2003).

4.2 MATERIALS AND METHODS

4.2.1 FISH

Hatchery reared red porgy, with an initial mean body weight of 44 g, were distributed in grey circular tanks of 100 l at an initial density of 5.3 kg/m^3.

4.2.2 DIETS

Five experimental dry pelleted diets containing 50% protein and 15% lipid were evaluated in triplicate groups for a period of 105 days. A control diet with no carotenoid source added. Two diets containing respectively 40 and 100 mg of synthetic canthaxanthin/kg diet, diets CTX40 and CTX100; and another pair containing two levels of shrimp shell meal to provide 20 and 40 mg totally of unesterified and esterified astaxanthin/kg diet, diets SM20 and SM40 respectively. Feed was supplied until apparent satiation, three times a day, and six days a week. The ingredient and biochemical composition of the diets are shown in Table 1, and the fatty acid profile in Table 2.

4.2.3 COLORIMETRIC ANALYSIS

Data on skin colour, whole body weight, and total length were recorded in anaesthetised fish, with 2-phenoxyethanol, at the beginning, after 75 and 105 days of feeding. Colour was determined in three zones of the left hand side of the fish: zone I, the front lateral, zone II the front dorsal, and zone III the caudal fin. Measurements were taken at each skin zone using a tristimulus colorimeter (Minolta Chroma Meter CR-200, Minolta, Osaka, Japan). The colour parameters

were L*, a* and b* in accordance with the recommendations of the International Commission on Illumination, CIE (1976). From a* and b* values, hue (H_{ab}) and chroma (C_{ab}) values were calculated, as specified in material and method section.

4.2.4 BIOCHEMICAL ANALYSIS

Biochemical analysis of feed was conducted in triplicate. Moisture, crude protein, and ash were determined according to AOAC (1995). Total lipid was extracted from the diets by the method of Folch *et al*., (1957). Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipid following the method of Christie, (1982). FAME were separated, identified and quantified by gas-liquid chromatography under the conditions described by Izquierdo *et al*., (1990), and individual methyl esters were identified by comparison with external standard EPA 28, Nippai, Ltd. Tokyo, Japan.

Carotenoid extraction from the diets was conducted in duplicate according to Barua *et al*., (1993). Quantification of carotenoid concentration in diets is described in material and methods. In both shrimp shell meal diets, were astaxanthin is the predominant carotenoid, the λ_{max} was 470 nm; and for canthaxanthin diets the λ_{max} was recorded at 466 nm. The extinction coefficient ($E_{1\%, 1cm}$) used for astaxanthin and canthaxanthin in hexane was 2100 and 2200 respectively (Britton, 1995).

4.2.5 STATISTICAL ANALYSIS

Means and standard deviations were calculated for each parameter measured. All data were tested for normality and homogeneity of variance. Differences among groups were determined by one-way ANOVA or non parametric statistics (Kruskal and Wallis). Duncan significant means test was applied to identify groups that were significantly different. A minimum significance level of $P<0.05$ was used in all tests. To evaluate variability of hue,

chroma and lightness, of coloured fish, between each zone, the coefficient of variation was estimated.

Table 1. Feed composition and proximate analyses of diets

Ingredient	**Control**	**CTX40**	**CTX100**	**SM20**	**SM40**
Fishmeal	70.9	70.9	70.9	66.3	61.7
Shrimp shell meal [a]				8.0	16.0
Gelatinized starch[b]	18.3	18.2	18.2	14.6	11.0
Vitamin premix	2.0	2.0	2.0	2.0	2.0
Mineral premix	2.0	2.0	2.0	2.0	2.0
CMC[c]	0.5	0.5	0.5	0.5	0.5
Fish oil	6.4	6.4	6.4	6.6	6.8
Canthaxanthin [d]		0.04	0.10		
Analysed composition (% dry wt)					
Protein	51.6	50.9	52.9	52.8	53.2
Lipids	13.3	15.2	13.9	14.1	14.3
Ash	10.8	9.2	10.4	12.4	13.7
Moisture	11.0	9.7	11.8	12.3	12.0
Astaxanthin (mg/kg)	9.3			21.1	38.0
Cantaxanthin (mg/kg)		38.3	100.6		

[a] Sopropeche shrimp shell meal, France

[b] Merigel 100 Amylum Group

[c] Carboxymethil cellulose Sigma

[d] CAROPHYLL red 10%, DSM.

Table 2. Fatty acid composition of the diets (g FA/100 g total FA).

Fatty acids	Control	CTX40	CTX100	SM20	SM40
14:00	5.56	5.59	5.39	5.36	5.24
15:00	0.46	0.26	0.26	0.48	0.47
16:00	18.63	18.33	17.73	18.28	18.30
17:00	0.46	0.48	0.48	0.48	0.48
18:00	2.41	2.34	2.30	2.38	2.33
20:00	0.12	0.12	0.11	0.12	0.12
22:0	0.02	0.13	0.11	0.12	0.05
Σ saturates	**27.6**	**27.25**	**26.38**	**27.22**	**26.99**
16:1n-7	5.36	5.79	5.43	5.66	5.76
16:1n-5	0.28	0.31	0.30	0.31	0.32
17:1	0.43	0.49	0.46	0.48	0.48
18:1n-9	19.48	19.43	19.31	18.77	19.91
18:1n-7	0.26	0.52	0.48	0.48	0.35
18:1n-5	0.01	0.02	0.02	0.02	0.02
20:1n-9+20:1n-7	8.49	7.63	7.70	7.39	7.04
20:1n-5	0.05	0.10	0.10	0.07	0.08
22:1n-11	10.64	9.04	11.79	8.93	8.37
22:1n-9	0.04	0.07	0.07	0.08	0.08
Σ monoenes	**45.04**	**43.37**	**45.66**	**42.19**	**42.41**
16:4n-3	0.21	0.16	0.24	0.24	0.22
18:3n-3	1.26	1.42	1.34	1.44	1.46
18.4n-3	2.04	2.29	2.12	2.29	2.22
20:3n-3	0.13	0.14	0.13	0.14	0.15
20:4n-3	0.45	0.49	0.36	0.5	0.51
20:5n-3	7.38	7.83	7.54	8.40	8.47
22:4n-3	0.06	0.05	0.06	0.05	0.05
22:5n-3	0.67	0.67	0.66	0.72	0.78
22:6n-3	10.98	11.49	11.08	12.23	12.08
Σ (n-3)	**23.18**	**24.54**	**23.53**	**26.01**	**25.94**
18:2n-6	1.94	2.23	2.12	2.19	2.17
18:3n-6	0.12	0.12	0.08	0.08	0.12
18:4n-6	0.11	0.05	0.07	0.06	0.12
20:2n-6	0.26	0.30	0.29	0.31	0.31
20:3n-6	0.04	0.05	0.05	0.05	0.05
20:4n-6	0.48	0.53	0.52	0.57	0.60
22:3n-6	0.21	0.22	0.22	0.23	0.21
22:4n-6	0.05	0.04	0.06	0.02	0.04
22:5n-6	0.18	0.18	0.19	0.19	0.19
Σ (n-6)	**3.39**	**3.72**	**3.60**	**3.70**	**3.81**
Σ (n-3) HUFA	**19.67**	**20.67**	**19.83**	**22.04**	**22.04**
Σ (n-6) HUFA	**1.22**	**1.32**	**1.33**	**1.37**	**1.4**
n-3/n-6	**6.84**	**6.60**	**6.54**	**7.03**	**6.81**

4.3 RESULTS

No significant differences were found in growth, SGR and FCR among fish from all dietary treatments (Table 3). Nevertheless, higher growth values were observed in fish feed SM40 diet and lower growth and SGR in fish fed CTX100.

Table 3. Effect of feeding carotenoids supplements on growth and feed utilization parameters after 105 days of experiment[1]

	Control	CTX40	CTX100	SM20	SM40
Initial weight (g)	43.9±3.7	43.2±4.2	45.1±3.4	44.5±3.6	43.4±3.3
Final weight (g)	113.3±13.0	108.8±15.8	105.7±12.0	113.2±12.1	113.6±14.9
Feed intake (g)	1024.2±95.4	999.9±13.3	1064.2±65.6	1079.3±99.5	1017.9±41.9
Growth[2] (%)	159.0±17.8	152.3±13.6	135.9±9.1	157.0±8.5	161.8±14.4
SGR[3]	0.91±0.07	0.88±0.05	0.82±0.04	0.90±0.03	0.91±0.06
FCR[4]	1.2±0.06	1.3±0.06	1.5±0.21	1.3±0.14	1.2±0.1

Values are mean ± S.D. of three groups per treatment

[1] No significantly differences (P>0.05) were observed among treatments means

[2] Growth (%) = 100x (final weight – initial weight)/initial weight

[3] SGR: specific growth rate = 100x (ln final weight – ln initial weight)/ nº days

[4] FCR: feed conversion ratio= feed intake (g) / weight gain (g)

Skin hue values attained in zone I are shown in Figure 1. Fish feed SM20 and SM40 diets had a significantly (P<0.05) more reddish hue at day 75. After 105 days of feeding, the tendency of all dietary treatments was to increase skin hue values, in other words to give a more yellowish coloration. Only fish fed SM40 diet maintained the reddish hue, differing significantly (P<0.05) from the rest. Chroma values of fish feed SM40 diet were significantly (P<0.05) higher than in the other treatment fish at both sampling points, although a lowering of skin chroma was observed at day 105 (Figure 2). Skin lightness (L^*) results show no significantly differences among treatments, and final values are slightly higher than the initial (Figure 3). Coefficient of variation of hue, chroma and lightness of fish fed SM40 diet, in this zone, was 6.9, 17.9 and 9.1% respectively.

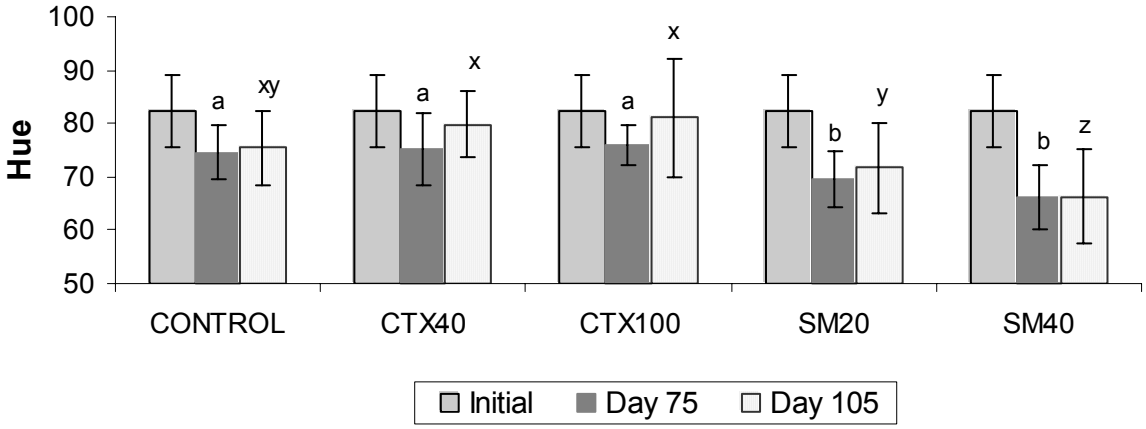


Figure 1. Hue values (º) at day 0, 75 and 105 in zone I. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

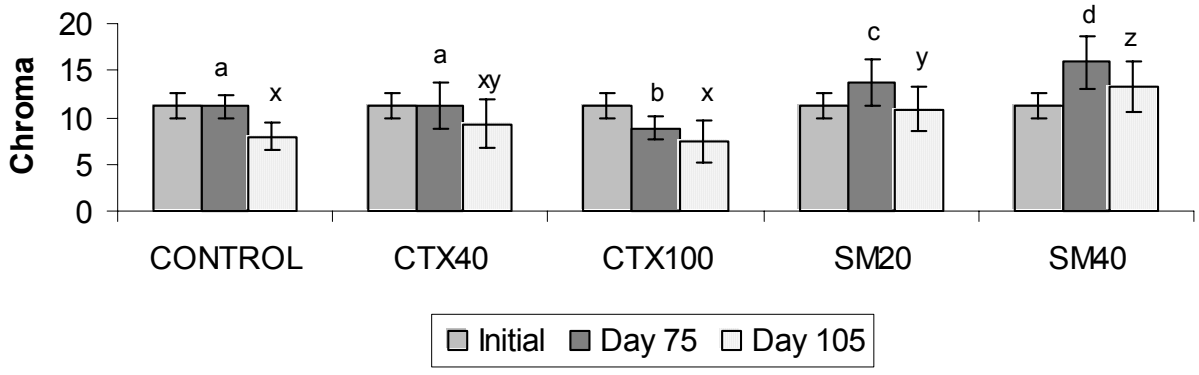


Figure 2. Chroma values at day 0, 75 and 105 in zone I. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

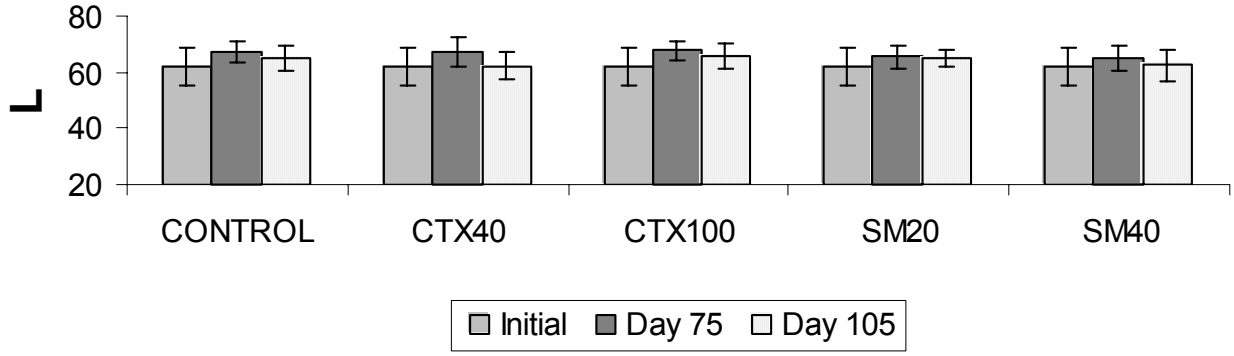


Figure 3. Lightness values at day 0, 75 and 105 in zone I. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

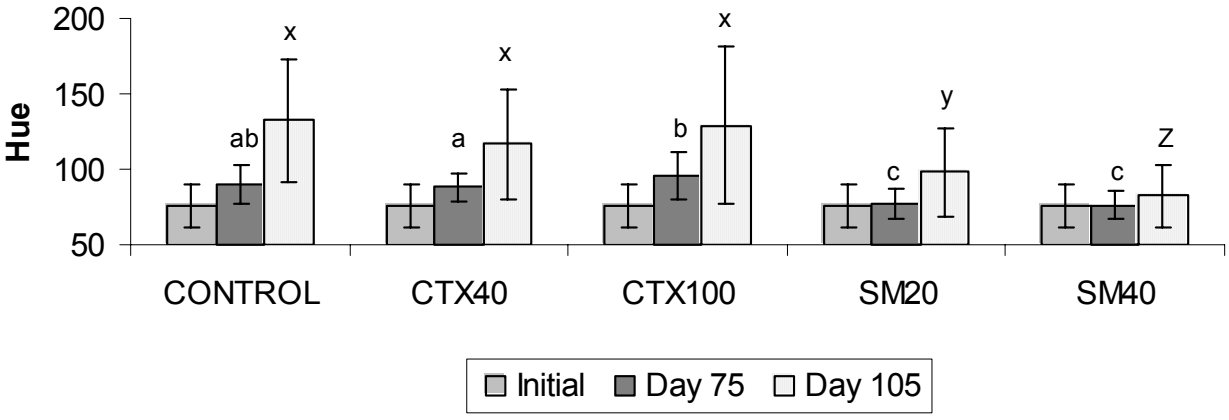


Figure 4. Hue values (º) at day 0, 75 and 105 in zone II. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

In zone II, skin from fish fed control, CTX40 and CTX100 diets showed significantly (P<0.05) higher hue values, exhibiting a yellowish hue (Figure 4). The inclusion of shrimp shell meal in the diet did not alter skin hue values in this zone. Regarding skin chroma, it was reduced in all fish, being results of SM40 animals significantly (P<0.05) higher than the rest (Figure 5). Skin lightness in this evaluated zone decreased along the experiment in all experimental fish (Figure 6). Hue, chroma and lightness of fish fed SM40 diet presented coefficient of variation of 25.1, 42.4 and 13.1% respectively.

Hue results in zone III are shown in Figure 7, fish feed SM20 and SM40 diets were significantly (P<0.05) more reddish throughout the experiment; although, redness from day 75 to 105 tended to be reduced in fish fed both shrimp shell meal diets. Skin chroma values from SM20 and SM40 fish were significantly (P<0.05) higher than the rest of treatment groups, even at the final sampling when all values tended, once again, to be reduced (Figure 8). Lightness results showed no statistical differences among treatments (Figure 9). Coefficient of variation of hue, chroma and lightness of SM40 diet fish was 7.4, 27.6 and 11.5% respectively.

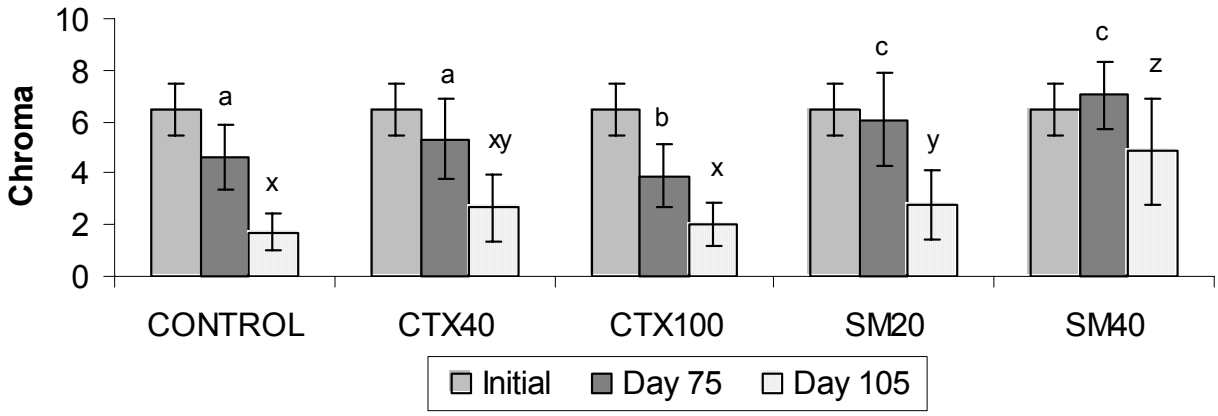


Figure 5. Chroma values at day 0, 75 and 105 in zone II. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

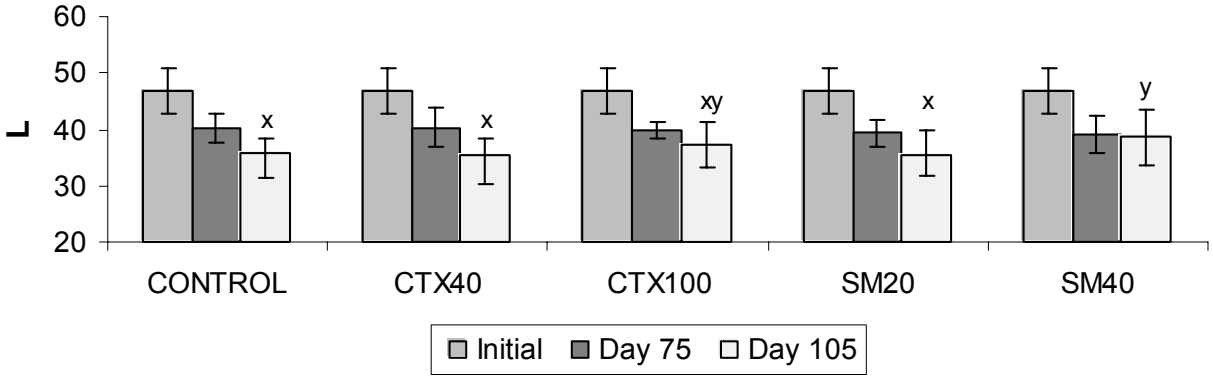


Figure 6. Lightness values at day 0, 75 and 105 in zone II. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

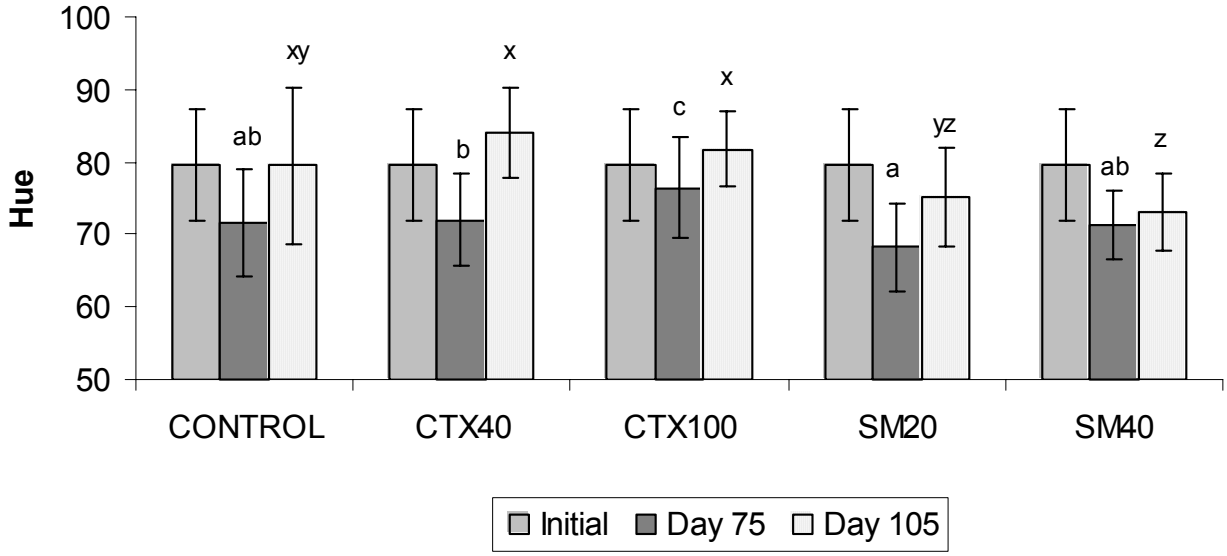


Figure 7. Hue values (º) at day 0, 75 and 105 in zone III. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

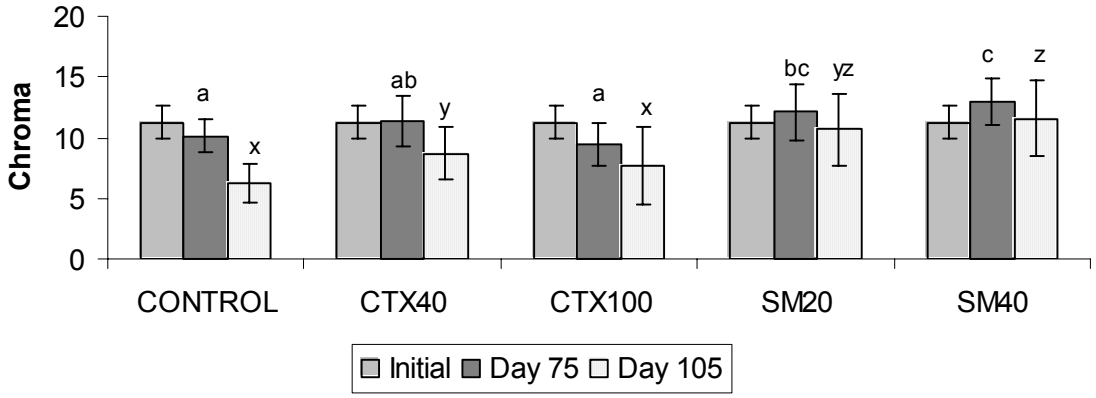


Figure 8. Chroma values at day 0, 75 and 105 in zone III. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

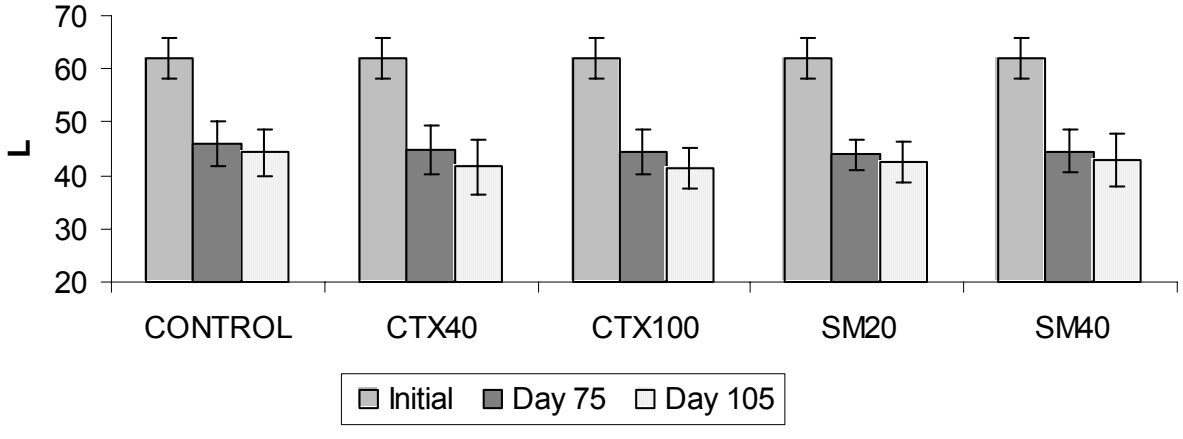


Figure 9. Lightness values at day 0, 75 and 105 in zone III. Bars represent mean ± S.D. Means with no letters or with common letters denote no significant differences (P>0.05).

4.4 DISCUSSION

Carotenoids are known to have a positive role in the intermediary metabolism of fish (Tacon, 1981; Segner *et al*. 1989) that could enhance nutrient utilisation and may ultimately result in improved growth (Amar *et al*., 2001). In the present study, fish feed a carotenoid source did not differ from control fish in growth and FCR. These results are in accordance with another study carried out with red porgy juveniles fed krill meal, for 75 days, as a source of astaxanthin (Chebakki, 2001) and agree well with that of Gomes *et al*., (2002) who did not find growth or feed efficiency enhancement when gilthead sea bream were fed different carotenoids for 9 weeks. Also, a study with rainbow trout found no significant differences (Nickell and Bromage, 1998). Nevertheless, this study with rainbow trout found that fish fed an astaxanthin supplemented diet from 6.5g and 25.5g until 400g exhibited higher wet weight than fish fed astaxanthin from 120.5g up to 400g. These results suggest that supplementation for longer periods are needed to evaluate a possible role on growth.

From the two carotenoid sources and dietary levels used in the present study, only the inclusion of 40 mg of astaxanthin from shrimp shell meal/kg diet, which is mainly in esterified form, was able to give red porgy skin an overall reddish coloration. This result suggests that this species utilizes well esterified astaxanthin. Astaxanthin esters are also efficiently used for deposition and coloration of the skin in red sea bream (Nakazoe, 1984; Ito, 1986; Lorenz, 1998) and Australian snapper, another close species to red porgy (Booth *et al*., 2004). Also, in gilthead sea bream pigments from algae, mainly esterified, were efficiently absorbed, as shown in plasma carotenoid composition (Gomes *et al*., 2002). Nevertheless, in salmonids esterified astaxanthin is not that well utilized as unesterified astaxanthin (Torrissen *et al*., 1979; Schiedt *et al*., 1985; Foss *et al*., 1987), although there is some controversy in this field.

In concern to canthaxanthin supplemented diets, the effect on skin was to give a more yellowish hue and atypical chroma values. Usually, an increase

of dietary carotenoids levels raises chroma, however fish fed CTX100 diet attained lower chroma values than fish fed CTX40 diet. Japanese red sea bream, fed β-carotene or canthaxanthin showed a decrease in the carotenoid level in the integuments (Lorenz, 1998). Decrease in deposition can be explained by a limitation in the rate of absorption (Torrissen *et al*., 1990). In rainbow trout it is stated that astaxanthin is a better pigment source than canthaxanthin; also, it has been found to be better absorbed through the digestive tract and a preferred deposition of astaxanthin in the flesh (Torrisen, 1989).

There is an uneven distribution of colour in red porgy skin, the front lateral zone exhibiting a more reddish hue and higher chroma, followed by the caudal and finally dorsal zone. These results suggested a progressive deposition of astaxanthin in these three body zones, since coloration and the concentration of carotenoids are significantly related (Bjerkeng, 2000). Uneven colour distribution was also found in salmonids muscle, where a longitudinal variation in carotenoid content and red colour was found, with more astaxanthin deposited in the caudal than in the anterior part (Bjerkeng, 2000). A part from an uneven distribution, the variability of hue, chroma and lightness of pigmented fish from SM40 diet was lower in zone I than in the other two zones.

Enhancement of reddish hue and increment in chroma values with the inclusion of shrimp shell meal in the diet tended to be reduced after 75 days of feeding. This behaviour of both hue and chroma is due to a* (redness) and b* (yellowness) colour variables that increased until day 75 and there on a decreased suggesting a skin colour saturation could be taking place. In agreement, an apparent colour saturation point was found in red sea bream after two months feeding on 100 mg/kg diet of esterified astaxanthin (Ito et al., 1986). This saturation plateau found in fish fed carotenoids seems to depend on genetical factors (Torrissen *et al*., 1984) nutritional (Torrissen, 1985) and biological factors such as fish size and specie. Juvenile salmonids (< 200 g) do not efficiently deposit carotenoid in the muscle (Torrissen *et al*., 1989; Hatlen *et*

al., 1995), fish must reach a minimum body weight before pigmentation of the muscle can occur (Yarzhombeck, 1970; Spinelli and Mahnken, 1978; McCallum *et al*., 1987; Torrissen, 1989).

In concern to skin lightness, carotenoid supplementation did not seem to influence positively on this colour variable. Skin pigmentation in vertebrates is modified by hormonal stimulation, background colour and the illumination (Sugimoto, 1993; Duray *et al*., 1996; Crook, 1997; Healey, 1999; Popoutsoglou *et al*., 2000; Rotlland *et al*., 2003). For instance, in Australian red snapper, a close species to red porgy, skin darkness seems to be a response to the proximity of sea cages to the surface of the water and the subsequent effect of sunlight exposure on melanin concentration in the skin (Booth *et al*., 2004). Handling stress and sacrifice methods are also among factors that negatively influence fish skin lightness (Lin *et al*., 1998).

The present work suggests that *Pagrus pagrus* skin pigmentation can be modified from a dark grey to a red pink silver colour, supplementing the diet with 40 mg of astaxanthin/kg diet from shrimp shell meal. From the three evaluated zones, the front lateral zone seems accumulate more astaxanthin, showing a more reddish coloration than the other two zones and also less variability. In further studies, this zone can be used as a control for evaluating the overall redness attained in this species. Nevertheless, since diet is not the only parameter that modifies skin pigmentation, its relation with environmental factors and aquaculture related stressors should be considered for the adequate red porgy skin coloration.

5.- TIME EFFECT OF SHRIMP SHELL MEAL DIETARY SUPPLEMENTATION ON RED PORGY *Pagrus pagrus* SKIN COLORATION AND GROWTH

Abstract

Red porgy, *Pagrus pagrus*, is a candidate for aquaculture diversification in the Mediterranean area. The main restriction for the development of this species, under cultured conditions, is the loss of their natural red-pink coloured skin. Several studies, using different dietary astaxanthin sources have given positive results on red porgy skin coloration. Nevertheless, there is scarce information on the more adequate supplementation time with astaxanthin for cultured red porgy to acquire a skin colour similar to wild specimens. This study evaluated the effect of supplementing red porgy diet with shrimp shell meal, containing esterified astaxanthin, during different periods prior to harvest on skin colour, skin carotenoid concentration and growth. The experiment was carried out for 180 days, testing a control diet with no carotenoids and an SM diet with inclusion of 16 % shrimp shell meal in substitution of fish meal. Four treatments were assayed. Control treatment, fish were fed throughout the experiment on control diet; SM60, SM120 and SM180 treatments were fish were fed first the control diet and thereafter for 60, 120 and 180 days before harvesting on the SM diet. Fish growth was significantly improved by increased feeding period with SM diet. Skin a*(redness) parameter increased with supplementation time, whereas b*(yellowness) increased until approximately 120 days supplementation. Skin hue values lowered, skin turning more reddish, with increasing supplementation time. Carotenoid concentration in the skin also increased with increasing supplementation time. Overall results showed that a period of 120 to 180 days feeding 16 % shrimp shell meal in substitution of fish meal on a *Pagrus pagrus* diet could provide this species skin with an adequate red-pink coloration similar to wild specimens.

Keywords**:** Carotenoids; Colour*;* Growth; Red porgy; Shrimp shell meal; Skin; Supplementation time.

5.- EFECTO DEL TIEMPO DE SUPLEMENTACIÓN CON HARINA DE CARCASA DE LANGOSTINO SOBRE EL CRECIMIENTO Y COLORACIÓN DE LA PIEL DEL BOCINEGRO *Pagrus pagrus*

Resumen

El bocinegro, *Pagrus pagrus* es una de las especies candidatas para la diversificación de la acuicultura en el Mediterráneo, siendo la limitación principal para el desarrollo del cultivo de esta especie, la pérdida de su coloración natural rosa-rojiza bajo condiciones de cautividad. La inclusión de diferentes fuentes de astaxantina en la dieta ha dado resultados positivos en la coloración del bocinegro en diversos estudios. Sin embargo, la información acerca del tiempo más adecuado para que los bocinegros de cultivo adquieran un color similar a los ejemplares salvajes, cuando son alimentados con una dieta con astaxantina, es escasa. Este estudio evaluó el efecto sobre el crecimiento, color y concentración de carotenoides en la piel, de la inclusión en la dieta de astaxantina esterificada proveniente de la harina de carcasa de langostino, a lo largo de diferentes períodos antes del sacrificio. Durante el experimento, de 180 días de duración, se evaluó una dieta control sin carotenoides y una dieta SM con inclusión del 16% de harina de carcasa de langostino en sustitución de la harina de pescado. Se ensayaron cuatro tratamientos. El control consistió en peces alimentados a lo largo de todo el período experimental con la dieta control; y los tratamientos SM60, SM120 y SM180, todos los peces se alimentaron inicialmente con la dieta control, y posteriormente durante los 60, 120 y 180 días previos al sacrificio, con la dieta SM. El crecimiento mejoró de forma significativa al aumentar el tiempo de alimentación con la dieta SM. El valor del parámetro a*(rojo) en la piel aumentó con el tiempo de alimentación con la dieta SM, mientras que b*(amarillo), lo hizo hasta aproximadamente 120 días de inclusión. El color en la piel mostró una tendencia rojiza cuando se aumentó el tiempo de inclusión. Del mismo modo, la concentración de carotenoides en la piel, aumentó con el tiempo de

inclusión. De forma general, los resultados mostraron que los bocinegros alimentados durante 120 a 180 días con una dieta con una sustitución del 16% de la harina de pescado por harina de carcasa de de langostino, adquirieron una coloración rosa-rojiza similar a la de los individuos del medio natural.

Palabras claves: Bocinegro; carotenoides; color; crecimiento; harina de carcasa de langostino; piel; tiempo inclusión.

5.1 INTRODUCTION

Whereas in some fish species such as salmonids, fillet colour constitutes an important quality parameter (Sigurgisladottir *et al.,* 1997), in others, is adequate skin pigmentation what greatly improves fish appearance, being associated by the consumer with product freshness and differentiation. Such is the case of red porgy, a high value species, of increasing interest for Mediterranean aquaculture, which despite being characterized by a natural red-pink skin coloration, acquires a grey colour under culture conditions (Kentouri *et al.,* 1995; Stephanou *et al.,* 1995; TECAM, 1999; Cejas *et al.,* 2003).

Since fish, like other vertebrates, are unable to synthesize carotenoids the novo (Goodwin, 1984) skin coloration is highly dependent on the carotenoids present in the diet. Previous studies with cultured *Pagrus pagrus* have shown an improvement in skin coloration by the inclusion of different astaxanthin sources such as krill meal (Chebakki, 2001), shrimp (*Pleisonika sp*.) (Cejas *et al.,* 2003), synthetic astaxanthin (Tejera *et al*., 2003), shrimp shell meal (Chapter 4) or *Haematococcus pluvialis* (Chatzifotis *et al*., 2005). However, the optimum duration of carotenoid supplementation time for red porgy to achieve skin coloration similar to wild specimens has not been yet established, despite their important economical and practical consequences. Determining the proper supplementation time is not only of consideration for attaining a desirable pigmentation, but also to reduce feed and production costs. For instance, in salmonids muscle pigmentation is responsible for 15-20% of the total feed cost, or 6-8% of the total production cost (Torrissen, 1995). Although, pigments cost have been lately reduced due to market competition and the increase in the price of other dietary components such as fish meal and fish oil.

Aside from providing the adequate coloration in different tissues, in certain studies dietary carotenoids have shown to enhance fish growth (Sommer *et al*., 1992; Christiansen *et al.,* 1994; Christiansen *et al*., 1995;

Christiansen *et al.*, 1996; Izquierdo *et al*., 2005); whether in others such positive effect has not been found (Bell *et al*., 2000; Gomes *et al*., 2002). This influence on growth has also been observed in other cultured animals such as shrimp, *Penaeus japonicus* and *Penaeus monodon* (Petit *et al*., 1997; Supamattaya *et al.*, 2005) and in sea urchin *Pseudocentrotus depressus* (Tsushima *et al*., 1997). Previous studies supplementing red porgy diet with an esterifed astaxanthin source also suggested an enhancement of growth, although no significant differences were obtained among treatments (Chapter 4).

5.2 MATERIALS AND METHODS

5.2.1 FISH

One hundred and sixty eight hatchery reared red porgy, with an initial mean weight of 185 g were randomly distributed in 12 grey circular tanks, of 500 l, at an initial density of 5.2 kg/m^3.

5.2.2 DIETS

During a 180 days period, two isocaloric (15%) and isoproteic (50%) diets were tested. A control diet without an added carotenoid source and shrimp shell meal diet (SM) with 16 % substitution of fish meal by shrimp shell meal (Sopropeche, France). Four different treatment groups were established, depending on the feeding period of each diet. Control treatment: control diet fed for 180 days. SM60 treatment: control diet fed for the first 120 days followed by SM diet fed for 60 days before harvest. SM120 treatment: control diet fed for the first 60 days followed by SM diet fed for 120 days before harvest. SM180 treatment: fed for 180 days on SM diet. Each treatment was tested in triplicate. Manual feeding was continued until apparent satiation, two times a day, and six days a week. Ingredient content, proximate and astaxanthin composition of the diets are shown in Table 1, and fatty acid profile in Table 2.

Table1. Feed composition and proximate analyses of diets

Ingredient	**Control**	**SM**
Fishmeal	75.3	65.6
Shrimp shell meal [a]		16.0
Fish oil	7.8	8.0
Gelatinized starch [b]	12.4	5.9
Vitamin premix	2.0	2.0
Mineral premix	2.0	2.0
CMC [c]	0.5	0,5
Analysed composition (% dry matter)		
Protein	51.6	53.2
Lipids	13.3	14.3
Ash	10.8	13.7
Moisture	11.0	12.0
Astaxanthin (mg/kg)		21.2

[a] Sopropeche shrimp shell meal

[b] Merigel 100 Amylum Group

[c] Carboxymethil cellulose Sigma

5.2.3 SAMPLING AND COLORIMETRIC ANALYSIS

Colour and weight were determined in all fish, at the beginning of the experiment and at days 60, 120 and 180. Sampling for colour and skin carotenoid concentration was carried out in the left hand side of the fish and only in the front lateral zone as suggested in Chapter 4. Colorimetric analysis was done using a tristimulus colorimeter (Minolta Chroma Meter CR-200, Minolta, Osaka, Japan) as described in material and methods. For skin carotenoid determination, 9 fish per treatment were sampled and for whole fish analysis 6 fish per treatment were sampled at the end of the study.

5.2.4 WILD RED PORGY

Wild fish with a mean weight of approximately 104 g, captured in the Coast of Fuerteventura Islands, were randomly distributed in four grey circular tanks of $1m^3$ at a density of 4.5 kg/m^3. Fish were fed to apparent satiation, two times a day, with natural diet based on mussels. After one week acclimation period, skin colour

sampling was carried out in all fish, without using 2-phenoxy ethanol, and as specified with cultured fish lines above. Only one week of acclimation was necessary since after that time good animal behaviour and feed intake was observed.

5.2.5 BIOCHEMICAL ANALYSIS

Feed samples and whole fish were analysed in triplicate. Moisture, crude protein, and ash were carried out according to AOAC, (1995). Total lipid was done by Folch *et al*., (1957). Fatty acid methyl esters in the diet were obtained by transmethylation as described by Christie (1982). FAME were separated, identified and quantified by gas-liquid chromatography under the conditions described by Izquierdo *et al*., (1990), and individual methyl esters were identified as described in material and methods.

Carotenoid extraction from the diets was conducted according to Barua *et al*., (1993). Carotenoid extraction from skin samples was done according to Schiedt *et al*., (1995). Quantification of carotenoid concentration in diets and skin tissue is described in material and methods.

5.2.6 STATISTICAL ANALYSIS

All data were tested for normality and homogeneity of variance. Differences among groups were determined by one-way ANOVA or non parametric statistics (Kruskal Wallis). Duncan significant means test was applied to identify groups that were significantly different. Differences were considered significant when $P<0.05$. Relationship between lightness (L*) colour variable and skin carotenoid concentration was determined by means of correlation coefficient (r) and the type of relation by simple regression analysis. To calculate the significance of correlation an ANOVA was carried out, considering significant when $P<0.01$.

Table 2. Fatty acid composition of the experimental diets (g FA/100 g total FA)

Fatty acids	Control	SM40
14:00	7.65	7.70
15:00	0.52	0.52
16:00	19.40	19.31
17:00	0.22	0.22
18:00	2.48	2.48
20:00	0.12	0.12
22:0	0.02	0.02
Σ saturates	**30.41**	**30.37**
16:1n-7	7.34	7.24
16:1n-5	0.46	0.44
17:1	0.86	0.87
18:1n-9	12.34	12.13
18:1n-7	3.10	3.02
18:1n-5	0.50	0.51
20:1n-9+20:1n-7	9.60	9.73
20:1n-5	n.d	n.d
22:1n-11	12.89	12,98
22:1n-9	n.d	n.d
24:1	1.51	1.51
Σ monoenes	**48.60**	**48.43**
16:4n-3	0.04	0.05
18:3n-3	0.86	0.86
18.4n-3	1.94	1.98
20:3n-3	0.10	0.10
20:4n-3	0.45	0.46
20:5n-3	6.51	6.48
22:4n-3	0.05	0.05
22:5n-3	0.55	0.56
22:6n-3	6.33	6.42
Σ (n-3)	**16.28**	**16.96**
18:2n-6	2.02	2.06
18:3n-6	0.15	0.15
18:4n-6	0.13	0.13
20:2n-6	0.18	0.18
20:3n-6	0.04	0.06
20:4n-6	0.48	0.45
22:4n-6	0.04	0.07
22:5n-6	0.12	0.12
Σ (n-6)	**3.16**	**3.22**
Σ (n-3) HUFA	**13.44**	**14.07**
Σ (n-6) HUFA	**0.86**	**0.88**
n-3/n-6	**5.15**	**5.27**

5.3 RESULTS

Increasing feeding time with SM diet resulted in an improvement in growth in terms of final body weight, percentage gain weight and SGR (Table 3). Hence, growth in SM180 fish, fed on SM diet for the entire experiment, was significantly higher ($P<0.05$) than in control fish (Table 3). Feed conversion ratios were also slightly better, although no significant differences ($P>0.05$) with control fish were found. In agreement with growth improvement, whole body protein content was increased by increasing feeding time with SM diet (Table 4). Red porgy from SM180, SM120 and SM60 treatments achieved slightly higher protein content, although not differing significantly ($P>0.05$) from the control treatment group. On the contrary, whole body total lipids were reduced by the inclusion of SM in the diet, particularly with shorter feeding periods, being significantly lower ($P<0.05$) in fish from SM60 and SM120 than in control fish.

Table 3. Growth and feed utilization (mean±SD) in red porgy fed carotenoid supplementation for different periods of time before harvest.

	Control	**SM60**	**SM120**	**SM180**
IBW (g)	187.3± 16.4	187.5± 18.5	186.3± 16.8	184.9± 14.7
FBW (g)	375.6± 51.3a	399.2± 59.5ab	407.6± 37.9ab	437.4± 58.3b
*__Growth (%)__	100.3± 6.5a	113.1± 3.9ab	118.4± 4.5ab	136.9± 17.8b
**__SGR__	0.39± 0.02a	0.42± 0.01ab	0.44± 0.01ab	0.48± 0.04b
***__FCR__	2.5± 0.3	2.1± 0.1	2.2± 0.3	2.1± 0.2

*Growth (%) = ((Final weight (g) – Initial weight (g))/Initial weight)*100

**SGR =((Ln Final weight – Ln Initial weight)/ nº days) x 100

***FCR = Feed intake (g) / Weight increase (g)

Table 4. Proximate composition (mean ± SD) of whole body (dry basis) from red porgy fed carotenoid supplementation for different periods of time before harvest.

	Control	SM60	SM120	SM180
Protein (%)	49.99±2.40	51.76±1.66	50.95±5.63	52.30±1.61
Lipids (%)	34.68±1.21b	31.03±3.06a	31.70±2.21a	33.83±3.40ab
Ash (%)	12.39±3.23	12.88±1.48	14.88±2.15	10.61±2.57
Moisture (%)	63.14±2.24	65.19±0.55	63.63±1.78	65.50±0.59

5.3.1 SKIN COLOUR

After 60 days of experiment, a*, b* and chroma values in the skin of SM180 fish, which at the moment had been fed on SM diet for only 60 days, were significantly ($P<0.05$) higher than in the other fish, which have only received the control diet for such period (Table 5). Hue values were also influenced positively, SM180 treatment group showing significantly ($P<0.05$) lower values. Skin lightness did not seem to be influenced as the other colour parameters (Table 5).

Table 5. Skin coloration in red porgy after 60 days of experiment (Values are mean ± S.D.; different letters in a column denote significant differences ($P<0.05$))

	a*	b*	Hue	Chroma	L
Control	2.79±0.99a	6.10±2.02a	67.55±6.89b	6.83±1.85a	66.25±7.7b
SM60	2.60±0.86a	6.81±2.10a	65.83±5.33b	7.39±2.44a	65.92±4.39b
SM120	3.28±1.05a	6.93±1.63a	66.03±8.12b	7.65±1.36a	56.11±5.39a
SM180	5.21±1.30b	9.15±2.05b	60.18±5.25a	10.57±2.27b	57.66±6.06a

After 120 days of experiment, colour variables a*, b* and chroma were the highest (P<0.05) in SM180 fish fed for a longer period of time (120 days at the moment) on SM diet, followed by SM120 fish fed the SM diet for 60 days. Hue values were significantly (P<0.05) lower in fish fed SM diet with increasing supplementation time, showing a more reddish colour SM180 and SM120 fish, but without a significant (P>0.05) difference between them (Table 6). Regarding lightness, values were significantly (P<0.05) reduced in fish fed SM diet at this moment (SM120 and SM180) although in this case fish fed SM diet for 60 days (SM120) showed the lowest value (Table 6).

Table 6. Skin colouration in red porgy after 120 days of experiment (Values are mean ± S.D.; different letters in a column denote significant differences (P<0.05))

	a*	b*	Hue	Chroma	L
Control	3.93±1.15a	7.51±1.98a	61.92±6.81b	8.52±2.10a	59.30±4.79c
SM60	3.53±1.06a	7.30±1.27a	64.38±6.41b	8.15±1.40a	60.14±5.83c
SM120	6.53±1.05b	10.84±1.96b	58.62±4.99a	12.70±1.94b	53.01±5.41a
SM180	7.50±1.46c	12.68±3.28c	58.53±6.82a	14.83±3.18c	55.96±4.65b

At the end of the trial, a* (redness) was significantly higher (P<0.05) in specimens from SM180 group, fed throughout the entire experiment on SM diet, followed by SM120 and SM60 groups, fed on shrimp shell meal diet for 120 and 60 days respectively (Table 7). Comparing final results with wild specimens, similar a* values are achieved when cultured red porgy are fed on SM diet for a period of 120 to 180 days (Table 7).

Regarding b* (yellowness) the increment with increasing supplementation time is only observed until approximately 120 days of feeding, whereas there on values decrease (Table 7). Therefore, by the end of the trial SM180 group does not differ ($P>0.05$) from SM120 and SM60 animals, but all of them are higher than control fish. Yellowness values achieved by cultured red porgy fed for 60, 120 and 180 prior to harvest are still low in comparison with wild fish (Table 7).

Table 7. Skin colour and carotenoid concentration (wet basis) in red porgy after 180 days of experiment (Values are mean ± S.D.; different letters in a column denote significant differences ($P<0.05$)). Comparison with wild red porgy colour parameters.

	a*	b*	Hue	Chroma	L	Carotenoids (μg/g)
Control	4.00±1.69a	8.87±2.16a	66.51±7.17b	9.79±2.49a	59.08±3.79c	4.68±0.58a
SM60	6.24±1.47b	12.27±2.42b	62.77±5.35b	13.82±2.53b	56.17±5.69bc	8.35±1.56b
SM120	7.84±0.98c	12.22±2.70b	56.50±7.24a	14.62±2.25b	51.59±3.39a	10.35±1.29bc
SM180	9.04±1.10d	11.19±2.38b	50.47±5.77a	14.45±2.25b	55.08±4.32b	13.99±3.05c
WILD	**8.7**	**13.1**	**56.7**	**15.9**	**69.4**	

Since hue is determined by the b*/a* ratio, lower ratios giving lower hue values. Fish from SM180 group, with the highest a* value, showed a more reddish overall coloration, although not differing ($P>0.05$) from SM120 group, only from control and SM60 fish ($P<0.05$) (Table 7). Comparing hue of wild red porgy with experimental fish, a similar value is reached when cultured red porgy are fed for a period of 120 to 180 days on SM diet (Table 7). Chroma, also determined by a* and b* behaviour, was statistically similar in fish fed on the SM diet; since b* values decreased from day 120 of supplementation, the only

significant differences (P<0.05) observed was with the control group (Table 7). Final chroma values achieved by cultured red porgy in the present study are lower than chroma values observed in wild specimens (Table 7). Picture 1 shows clearly the skin colour results attained by both SM120 and SM180 treatment groups and the difference with control fish.

Picture 1. The first red porgy fish, starting from the top, was fed on the control diet, the second was fed on SM diet for 120 days and the last two red porgy were fed on SM diet for 180 days.

Skin lightness from red porgy of control and SM60 treatment groups attained significantly higher (P<0.05) values than the rest of experimental fish (Table 7), also a negative correlation (P<0.05) between this colour parameter

and skin carotenoid concentration was found (Figure 1). Lightness is the only skin colour parameter in cultured red porgy that differs greatly from their wild counterparts (Table 7).

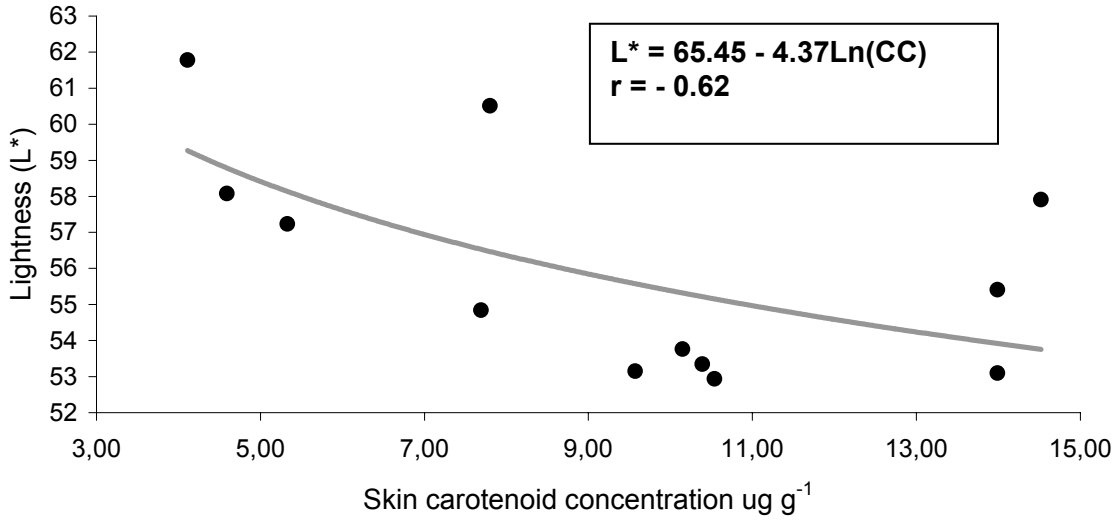


Figure 1. Relationship between skin carotenoid concentration (CC) and skin lightness (L*).

In concern to skin carotenoid concentration in red porgy skin, an increment with supplementation time was observed, although no significant differences ($P>0.05$) were found between fish fed for 180 or 120 days on SM diet, only with SM60 and control fish (Table 7).

5.4 DISCUSSION

Carotenoid pigmentation in fish is not only affected by the type of dietary carotenoid supplemented, but also by other factors such as pigment source, concentration, length of carotenoid feeding time and by other dietary ingredients composition (Bjerkeng, 2000). Pigmentation studies on red porgy skin have been focused mainly on the inclusion of the appropriate type of carotenoid in the diet for this tissue to acquire the natural red-pink coloration, characteristic of this species (Chatzifotis *et al*., 2005; Tejera *et al*., 2005; Chapter 4). A previous study with juvenile red porgy found that diet supplementation with esterified astaxanthin, from shrimp shell meal, provides this fish with a favourable skin colour (Chapter 4). The present study not only corroborates the previous observation, but also finds an effect of supplementation time. Feeding for 120 days, before harvesting, on SM diet enhanced both skin redness (a*) and yellowness (b*). However, longer feeding periods only increased redness but not skin yellowness.

Changes in skin colour variables a* and b* are most probably reflecting the behaviour of astaxanthin esters and tunaxanthin esters in red porgy skin. Both xanthophylls have been reported as the main carotenoids in red porgy integuments (Tejera *et al*., 2005) and in *Pagrus major*, a close species to *Pagrus pagrus* (Allahpichay *et al*., 1984); also, as reported by Bjerkeng and Berge, (2000), esterified forms are found to predominate in fish skin. Astaxanthin with a longer chromophore exhibits a red coloration and tunaxanthin with a shorter chromophore exhibits a yellow colour. Therefore, the increase of skin redness with increasing supplementation time could be due to the direct skin deposition of esterified astaxanthin, the main carotenoid found in shrimp shell meal (Choubert and Luquet, 1983, Shahidi and Synowiecki, 1991). Nevertheless, as shrimp shell meal does not contain tunaxanthin or its main precursor lutein (Miki *et al*., 1985) the increase in yellowness (b*) could be related with a tunaxanthin synthesis from astaxanthin. Despite tunaxanthin synthesis has not been yet study in red porgy, production of this xanthophyll

from dietary astaxanthin has been found to occur in another Pagrus species, Japanese red sea bream (Allahpichay *et al*., 1984). Therefore, skin yellowness decrease after 120 days of supplementation with SM diet, could be due to a limitation in pigment synthesis. Similarly, in a study carried out with red porgy juveniles fed a diet with also a substitution of 16% of fish meal by shrimp shell meal, an increase of b* value was observed in the first 75 days of experiment, afterwards a decrease of this colour variable took place (Chapter 4). The start of the decrease of b* value in the previous study was observed earlier, day 75, in comparison with the present study, day 120. This is probably due to the size of fish in both experiments; in salmonids it has been reported that fish below 200 g do not efficiently deposit carotenoids (Torrissen *et al*., 1989; Hatlen *et al*., 1995).

Skin lightness was not positively affected by shrimp shell meal feeding time, since the highest values observed were in fish fed on the control diet, without carotenoid supplementation. To support this finding, a negative correlation was found between this colour variable and skin carotenoid concentration. In salmonids such as Atlantic salmon and Artic charr a negative correlation was also reported between muscle carotenoid concentration and muscle lightness (L*) (Bjerkeng *et al.,* 1997; Hatlen, 1998). Besides dietary carotenoid content, husbandry conditions, such as background colour, light spectrum, and density, affect skin lightness. A white background, blue spectrum and densities below 25 kg/m^3 for adult red porgy can give skin lightness values close to wild specimens (Van der Salm, *et al*., 2004). Also in a study carried out with *Pagrus auratus*, a close species to *Pagrus pagrus*, white tanks seem to be important to attain an optimum skin colour (Doolan *et al*., 2004). In our trial, the grey background and the full spectrum illumination used, together with the shrimp shell meal, could have negatively affected this colour variable.

In concern to growth, the inclusion of shrimp shell meal on red porgy diet, as a source of esterified astaxanthin, enhanced growth performance with

increasing supplementation time, confirming our previous study which suggested a significant growth improvement by shrimp shell meal supplementation if feeding periods were longer than 105 days (Chapter 4). Effects of carotenoid supplementation on fish growth are although contradictory (Izquierdo *et al*., 2005). For instance, better growth has been found when rainbow trout (Sommer *et al*., 1992) or Atlantic salmon (Christiansen *et al*., 1994, 1995; Christiansen and Torrissen, 1996) diets were supplemented with carotenoids. Whereas other authors have not been able to find such positive effect in these same fish (Amar *et al*., 2003; Bell *et al*., 2000) or other ones (Gomes *et al*., 2002). These differences could be related to the different carotenoid sources used by those authors, and the inclusion of other nutrients. Therefore, we could not discard that in the present experiment growth enhancement could be related to other components of shrimp shell meal such as certain amino acids, fatty acids or phospholipids and instead of the esterified astaxanthin present in this raw material.

The present study suggests that a*, b*, hue and chroma colour variables attained by cultured red porgy fed a diet supplemented with 16% shrimp shell meal in substitution of fish meal for a period of 120 to 180 days before harvest, provides this species skin with a coloration close to wild specimens. The only colour parameter that differed greatly from wild red porgy was lightness, results from this study suggests that a dietary control with carotenoids is not what influences most on this colour parameter.

6.- EFFECT OF SUPPLEMENTATION TIME WITH UNESTERIFIED ASTAXANTHIN ON COLOUR AND CAROTENOIDS IN RED PORGY SKIN

Abstract

Synthetic astaxanthin is widely used in the aquaculture sector, being principally included in the diet of farmed species with a pigmentation purpose. The objective of this study was to investigate the effect of incorporating synthetic astaxanthin, as a source of unesterified astaxanthin, in red porgy diet for different lengths of time, on skin colour, skin carotenoid concentration and composition. Also, the present work aims to find the possible relationship between skin colour parameters (a*, b*, hue, chroma and L*) and skin total carotenoid concentration. To achieve this information an experiment was conducted for 90 days, with fish of initial mean weight of 250g. Three treatment groups were established, each one tested in triplicate. Control group: fed on a control diet (with no carotenoid inclusion) during the entire experiment. ASTX60 group: fed for the first 30 days of the trial on the control diet and 60 days before harvest on a diet with 100 mg of unesterified astaxanthin/ kg of diet (ASTX100). ASTX90 group: fed on ASTX100 diet 90 days before harvesting. Skin colour parameters (a*, b*, hue and chroma) after 30 to 60 days of feeding with ASTX100 diet were similar to values found in wild red porgy. However, by the end of the trial, ASTX60 and ASTX90 treatment groups fed ASTX100 diet for 60 and 90 days respectively, presented significantly lower skin carotenoid concentration in comparison to values found in wild specimens' skin. This contradiction observed between skin colour variables and skin carotenoid concentration could be explained by the relationship observed between skin colour variables and skin total carotenoid concentration, which shows a logarithmic pattern. Thin layer chromatography analysis of red porgy skin revealed the presence of a red and a yellow fraction were astaxanthin and

tunaxanthin seem to be the predominant carotenoids respectively. This finding is observed in both wild and experimental fish. Relative amounts of the red fraction are found to be higher in cultured specimens from both ASTX90 and ASTX60 treatment groups in comparison to wild red porgy. On the other hand, the yellow fraction was found to be higher in wild animals than in cultured red porgy.

Key words**:** carotenoids; red porgy; skin; thin layer chromatography; unesterified astaxanthin

6.- EFECTO DEL TIEMPO DE SUPLEMENTACIÓN CON ASTAXANTINA NO ESTERIFICADA EN LA COLORACIÓN Y CAROTENOIDES EN LA PIEL DEL BOCINEGRO

Resumen

La astaxantina sintética es ampliamente utilizada en el sector acuícola, y su inclusión en la dieta, de diversas especies, tiene principalmente fines pigmentantes. El objetivo del presente estudio es evaluar el efecto de la inclusión, durante diferentes períodos de tiempo, de astaxantina sintética, como fuente de astaxantina no esterificada, en la dieta del bocinegro, sobre el color de la piel, concentración de carotenoides en la piel y la composición de carotenoides en la misma. Además, tiene como finalidad encontrar una posible relación entre los parámetros de coloración de la piel (a*, b*, L*, color e intensidad del color) y la concentración total de carotenoides en la misma. Para todo ello, se llevó a cabo un experimento de 90 días de duración, con peces de un peso medio inicial de 250 g. Se establecieron tres tratamientos, cada uno de ellos por triplicado. El grupo control: se alimentó con la dieta control (sin inclusión de carotenoides) durante todo el período experimental. El grupo ASTX60: alimentado durante los primeros 30 días con la dieta control, y 60 días previos al sacrificio con una dieta con 100 mg de astaxantina no esterificada por kilogramo de dieta (ASTX100). El grupo ASTX90: alimentado con la dieta ASTX100 noventa días previos al sacrificio. Los resultados de los parámetros de coloración de la piel, menos la luminosidad, mostraron que alimentar el bocinegro durante 30 a 60 días con la dieta ASTX100, fue suficiente para alcanzar valores similares a los de los peces salvajes. Sin embargo, al final del experimento, los peces de los tratamientos ASTX60 y ASTX90, alimentados por 60 y 90 días con la dieta de ASTX100 respectivamente, presentaron una concentración total de carotenoides en la piel significativamente inferior a los de los peces salvajes. Esta contradicción podría deberse a la relación observada

entre las variables de coloración de la piel y la concentración total de carotenoides de la misma que sigue un patrón logarítmico. El análisis de cromatografía de capa fina de la piel del bocinegro, mostró una banda roja y otra amarilla, donde la astaxantina y tunaxantina respectivamente parecen ser los carotenoides predominantes. Este resultado se observó tanto en los peces salvajes como en los experimentales. Se encontró también que las cantidades relativas de la fracción roja son mayores en los peces correspondientes a los tratamientos ASTX60 y ASTX90 que en los individuos salvajes. Por otro lado, la fracción amarilla fue mayor en los bocinegros salvajes, comparado con los peces de cultivo.

***Palabras clave*:** astaxantina no esterificada; bocinegro; carotenoides; cromatografía en capa fina, piel

6.1 INTRODUCTION

Colour perceived in wild red porgy skin constitutes an appreciated and determinant market characteristic. The loss of this attribute in farmed red porgy is one of the barriers to move onwards to grand scale production. Several studies have been carried out focusing on dietary control to manage this skin problem and the addition of different sources of dietary astaxanthin have shown promising results (Chebbaki, 2001; Cejas *et al*., 2003; Tejera *et al*., 2003, 2005; Chatzifotis *et al*., 2005; Chapters 4 and 5).

One of the astaxanthin sources tested with red porgy was synthetic astaxanthin, returning the red-pink coloration to integuments (Tejera *et al*., 2003, 2005). This astaxanthin source is the main carotenoid used world wide in the aquaculture industry, being primarily used as an animal feed additive to provide coloration (Higuera-Ciapara *et al*., 2006). However, it has been suggested that skin coloration of red porgy fed synthetic astaxanthin is not so close to that of wild individuals as that obtained by feeding esterified astaxanthin (Tejera *et al*., 2005). In Japanese red sea bream, *Pagrus major*, esterified forms of astaxanthin seem to be better utilized than unesterified forms, since a colour saturation level is achieved earlier with unesterified astaxanthin (Ito *et al*., 1986; Guerin and Hosokawa, 2001).

Several methods have been used to determine and evaluate fish skin and fillet colour results. Visual grading is perhaps one of the oldest and easiest techniques, aided by the use of colour charts or fans (Bolten *et al*., 1966; Francis and Clydesdale, 1975). However, the degree of precision of this method is relatively low since it is dependant in several environmental factors, such as light conditions, and the individual perception ability of different graders (King, 1996). Portable instruments that register reflectance measure colour in a more convenient, precise and objective way, being CIE 1976 L*a*b* system the most frequently used. Due to the fact that colour in fish tissues is mostly caused by deposition of carotenoids, a relationship between these two variables must

exist. For instance, in salmonids, muscle coloration and carotenoid concentration are significantly related, different mathematical models describing the relationship between instrumentally assessed muscle coloration and total carotenoid concentration (Christiansen *et al*., 1995; Hatlen *et al*., 1998; Bjerkeng *et al*., 1997; Bjerkeng, 2000). Therefore, a colorimeter in conjunction with a predicted equation can be easily converted to an indicative carotenoid concentration value.

Aside from evaluating the inclusion of synthetic astaxanthin in skin colour and skin carotenoid concentration a tentative identification and quantification will be carried out using thin layer chromatography which is a simple and useful technique for carotenoid studies.

6.2 MATERIALS AND METHODS

6.2.1 FISH

Hatchery reared red porgy, of initial mean weight of 250 g were placed in grey circular tanks of 0.5 m^3 provided with a flow through water system, at an initial density of 7.0 kg/m^3.

6.2.2 DIETS

Two isoproteic and isocaloric diets were formulated: a control diet with no carotenoid supplementation and the ASTX100 diet with 100 mg/kg of Carophyll Pink from DSM (8% of unesterified astaxanthin). Three treatment groups, evaluated in triplicate for 90 days, were established. Control group: fed only on the control diet. ASTX60 group: fed during the first 30 days of the trial on the control diet and 60 days before harvest on ASTX100 diet. ASTX90 group: received only ASTX100 diet. The ingredients and biochemical composition of the diets are shown in Table 1.

6.2.3 SAMPLING

Animals were weighted at the beginning and at the end of the study. Colour data was recorded at the left hand side, on the front lateral zone of all fish at the beginning and every 30 days, following the methodology described in Material and Methods section. Skin samples, of the same area were colour was measured, were taken for carotenoid analysis at the end of trial from 9 fish per treatment. Skin samples were immediately stored at –80ºC until analysis. Fish were anaesthetised with 2-phenoxyethanol.

Table 1. Feed composition and proximate analyses of diets

Ingredient	**Control**	**ASTX100**
Fishmeal	68.8	68.8
Gelatinized starch [a]	18.0	18.0
Vitamin premix	2.0	2.0
Mineral premix	2.0	2.0
CMC [b]	0.5	0.5
Fish oil	8.7	8.7
Astaxanthin[c]		0.125
Analysed composition (% dry matter)		
Protein	51.6	52.4
Lipids	13.3	14.9
Ash	10.8	9.8
Moisture	11.0	10.1
Astaxanthin (mg/kg)	9.33	92.60

[a] Merigel 100 Amylum Group

[b] Carboxymethil cellulose Sigma

[c]CAROPHYLL Pink (8% astaxanthin) DSM

6.2.4 WILD RED PORGY

Wild fish with a mean weight of approximately 104 g were captured in the Coast of Fuerteventura Island and maintained as specified in chapter 5. Skin

colour was measured in all fish as described also in chapter 5, and for skin carotenoid analysis, 10 fish were taken randomly from the four tanks established.

6.2.5 BIOCHEMICAL ANALYSIS

All analyses were conducted in triplicate. Moisture, crude protein and ash from diets were carried out according to AOAC (1995), whereas total lipids were extracted by chloroform/methanol (Folch *et al*., 1957).

Total carotenoid extraction from the diets was carried out according to Barua *et al*. (1993) as described in Material and Methods. Total carotenoid extraction from red porgy skin was done by the methodology of Schiedt *et al*. (1995). Quantification of total carotenoid concentration in diets and skin tissue is described in Material and Methods section.

6.2.6 THIN LAYER CHROMATOGRAPHY

Hexane/acetone (4:1) and hexane/diethyl ether (20:1) were used as developing systems for red porgy skin carotenoids separation, tentative identification and for relative quantification. Detail on TLC procedure is described in Material and Methods.

6.2.7 STATISTICAL ANALYSIS

All data were tested for normality and homogeneity of variance. Detection of differences among groups was determined by one-way ANOVA or non parametric test (Kruskal-Wallis). Duncan test for multiple comparisons of means was applied to identify groups that were significantly different. Differences were considered significant when $P<0.05$. The relationship between colour parameters and skin carotenoid concentration were determined by means of

correlation coefficient (r) and the type of relation by simple regression analysis. To calculate the significance of correlation an ANOVA was carried out ($P<0.01$).

6.3 RESULTS

Growth was positively affected by the inclusion of 100 mg/kg of unesterified astaxanthin in red porgy diet, regardless the supplementation time used. Fish from ASTX90 and AST60 treatments fed with astaxanthin showed higher ($P<0.05$) final weights when compared to those of control treatment (Table 2). However, no significant differences were obtained in growth rates or feed utilization despite the slightly better results for ASTX90 and ASTX60 fish.

Table 2. Growth parameters and feed conversion rate of control, ASTX90 and ASTX60 by the end of the trial. Values are mean ± S.D ($P>0.05$)

	Control	**ASTX60**	**ASTX90**
IBW (g)	247.7±24.5	250.1±27.9	254.3±22.3
FBW (g)	378.2±49.9a	402.7±52.2b	404.4±41.7b
***Growth (%)**	49.0±6.7	61.4±11.4	58.8±7.3
****SGR**	0.44±0.05	0.53±0.08	0.51±0.05
*****FCR**	2.5±0.3	2.2±0.2	2.2±0.2

6.3.1 SKIN COLOUR AND CAROTENOIDS

Skin redness (a*) increased with increasing feeding time with ASTX100 diet (Figure 1). By the end of the trial, fish from ASTX90 treatment group, fed on ASTX100 diet for 90 days attained the highest values ($P<0.05$). The lowest skin redness was observed in control fish which did not receive any carotenoid supplementation in the diet (Figure 1).

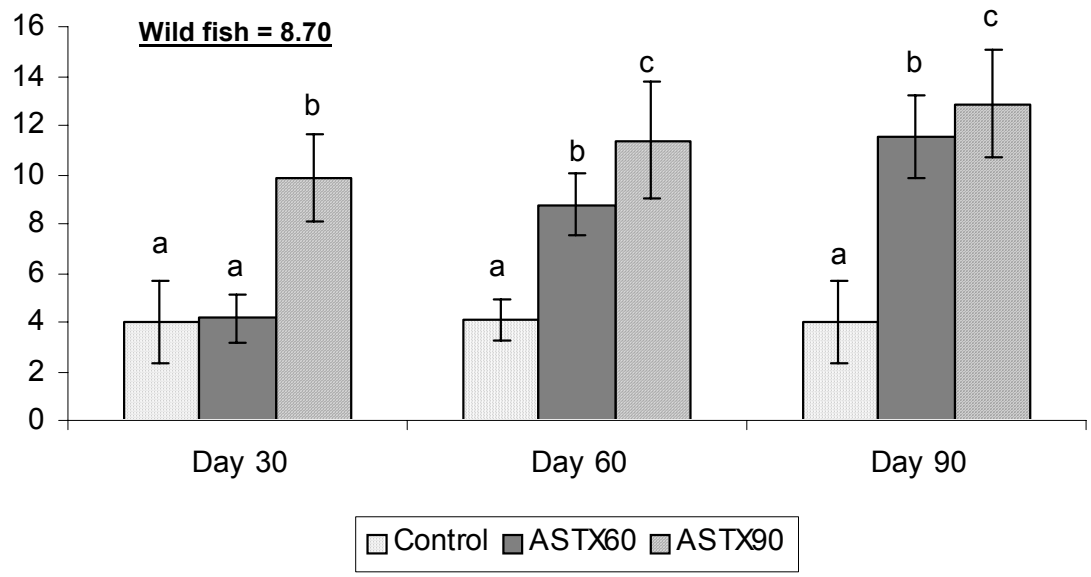


Figure 1. Redness (a*) values at day 30, 60 and 90 of feeding the experimental diets. Bars represent mean ± S.D. Different letters for a given time denote significant differences (P>0.05).

Skin yellowness (b*) increased after 60 days of feeding with ASTX100 diet, maintaining similar values for the rest of the experiment (Figure 2). By the end of the trial b* values from both ASTX60 and ASTX90, fed for 60 and 90 days respectively on ASTX100 diet, showed similar results, being significantly higher ($P<0.05$) than those from control fish (Figure 2). Skin hue decreased with increasing supplementation time with ASTX100 diet, acquiring a more reddish colour (Figure 3). At the end of the experiment ASTX90 fish showed significantly lower hue values ($P<0.05$) than the rest of treatment groups. Skin chroma presented a similar behaviour than skin yellowness, therefore by the end of the study both fish from ASTX60 and ASTX90 did not differ from each other ($P>0.05$), only from control fish (Figure 4). Regarding lightness, this was the only skin colour parameter that was not positively influenced by astaxanthin inclusion and supplementation time. Fish fed on the control diet were significantly ($P<0.05$) lighter, throughout the trail, than the other two treatment groups (Figure 5). Comparing skin colour results with wild red porgy, fish fed on

ASTX100 diet for a period between 30 to 60 days acquired skin coloration close to wild specimens (Picture 1). Only skin lightness achieved was lower than that of wild red porgy.

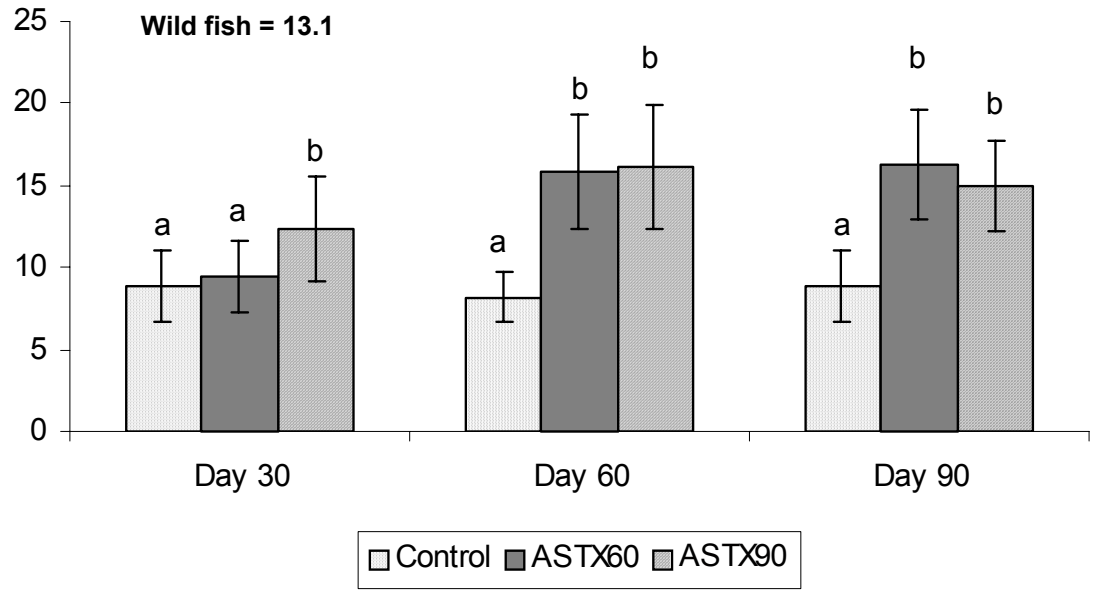


Figure 2. Yellowness (b*) at day 30, 60 and 90 of feeding the experimental diets. Bars represent mean ± S.D. Different letters for a given time denote significant differences (P>0.05).

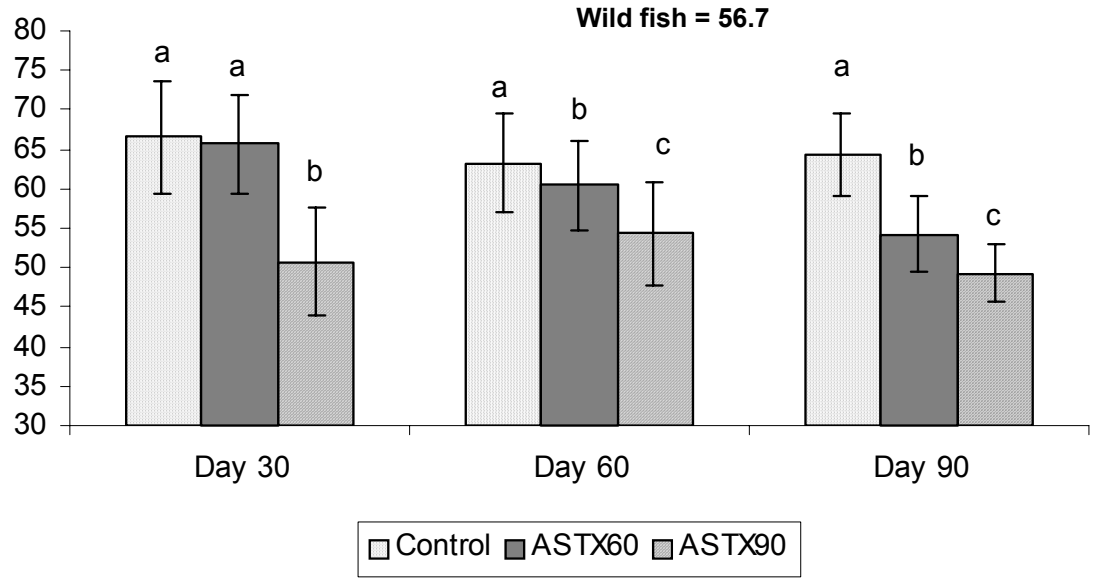


Figure 3. Hue at day 30, 60 and 90 of feeding the experimental diets. Bars represent mean ± S.D. Different letters for a given time denote significant differences (P>0.05).

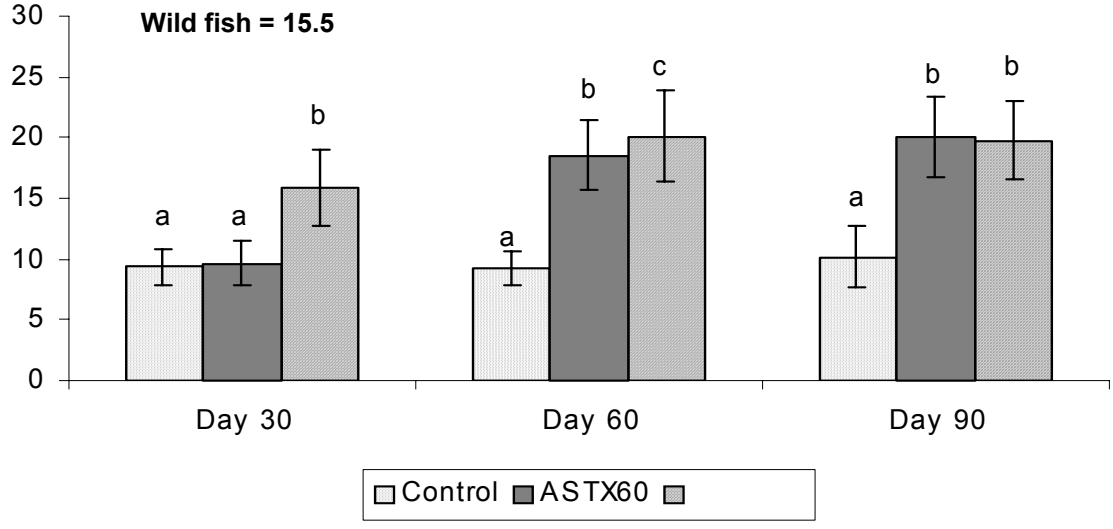


Figure 4. Chroma values at day 30, 60 and 90 of feeding the experimental diets. Bars represent mean ± S.D. Different letters for a given time denote significant differences (P>0.05).

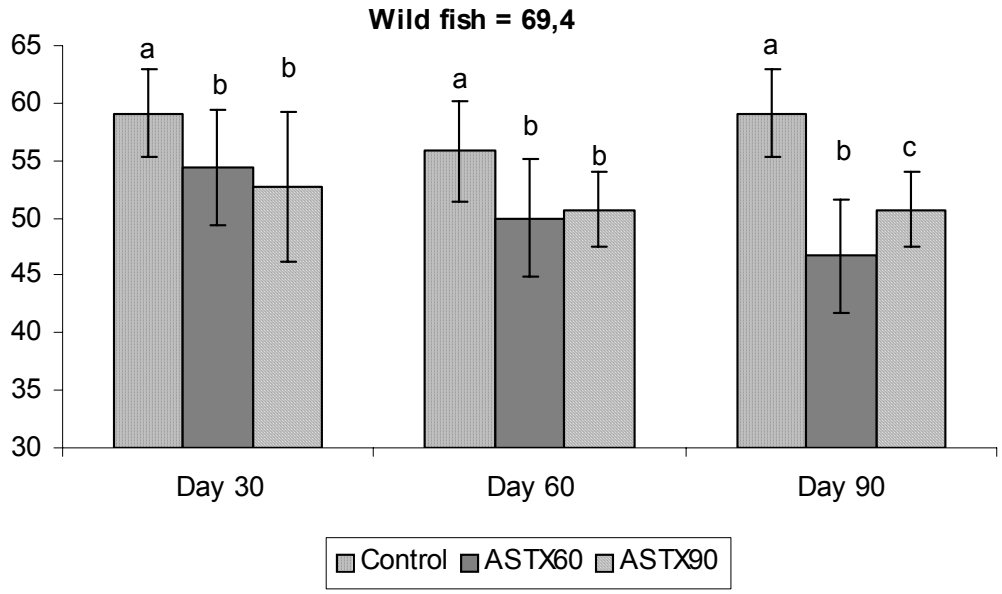


Figure 5. Lightness at day 30, 60 and 90 of feeding the experimental diets. Bars represent mean ± S.D. Different letters for a given time denote significant differences (P>0.05).

Picture 1. Red porgy on the left hand side were fed on the control diet and fish on the right hand side were fed on ASTX100 diet.

Skin carotenoid concentration was also enhanced by the addition of dietary astaxanthin and increased with the supplementation time. At the end of the trial the highest skin carotenoid concentration was obtained in ASTX90 fish differing significantly ($P<0.05$) from the rest of treatment groups (Table 3), whereas the lowest was found in control fish ($P<0.05$). In comparison to wild red porgy, skin carotenoid concentrations were found to be considerably higher than in cultured animals.

Table 3. Skin carotenoid concentration (μg/g) at the end of the trial

	Total carotenoids (μg/g)
Control	4.8±0.58a
ASTX60	17.44±1.38b
ASTX90	22.61±1.73c
WILD	53.99±10.8

All colour parameters were significantly correlated ($P<0.01$) with skin carotenoid concentration. Positive relationships were found between carotenoid concentration and redness (a*), yellowness (b*) and chroma (Figures 4, 5, 6); whereas lightness (L*) and hue were negatively correlated with skin carotenoid concentration (Figure 7 and 8). The colour parameter which gave the highest correlation with carotenoid concentration was redness. The regression model that fits best with the relationship between colorimetric variables and total skin carotenoid concentration shows a logarithmic pattern (Figures 4, 5, 6, 7, 8).

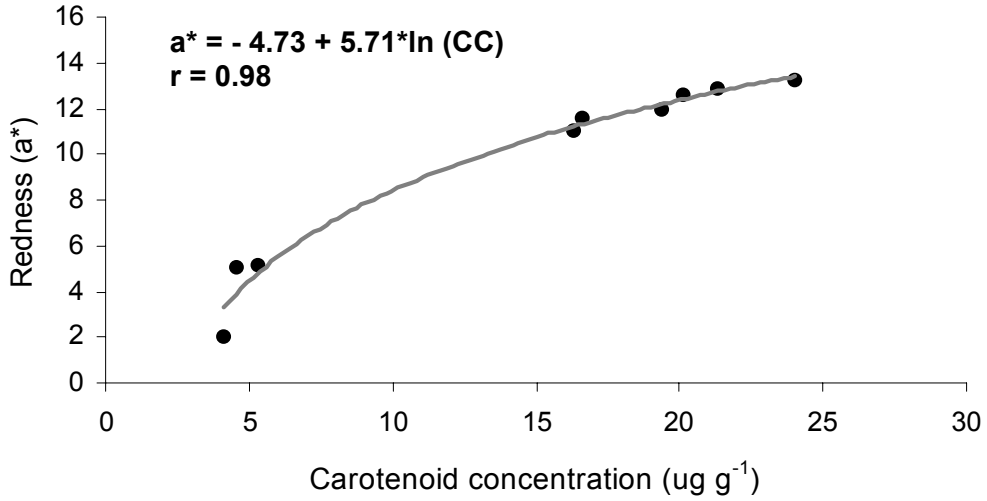


Figure 4. Relationship between skin carotenoid concentration (CC) and skin redness.

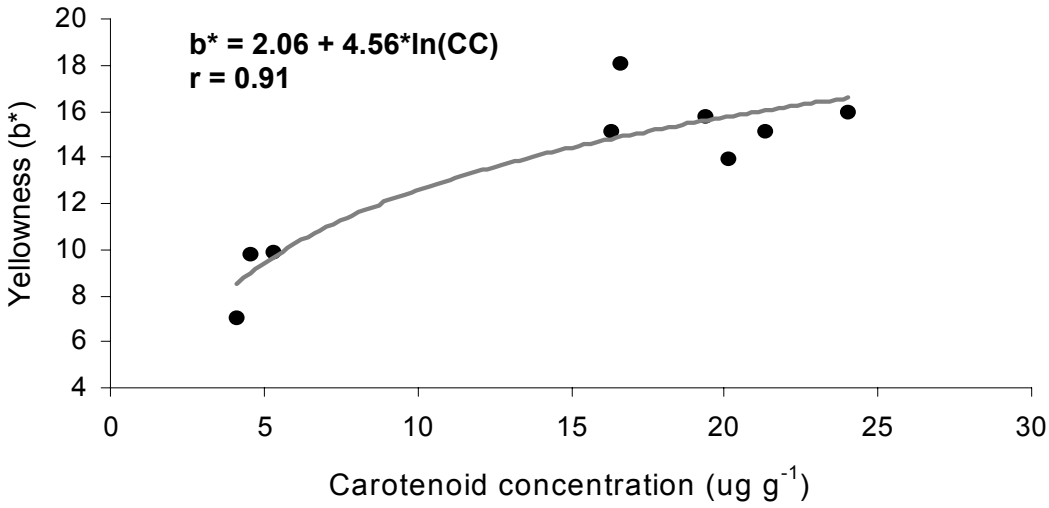


Figure 5. Relationship between skin carotenoid concentration (CC) and skin yellowness.

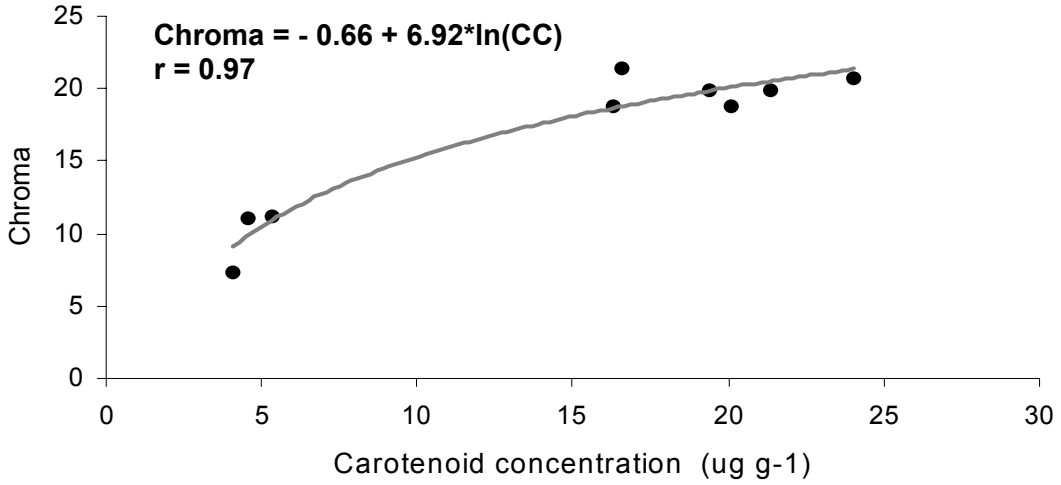


Figure 6. Relationship between skin carotenoid concentration (CC) and skin chroma.

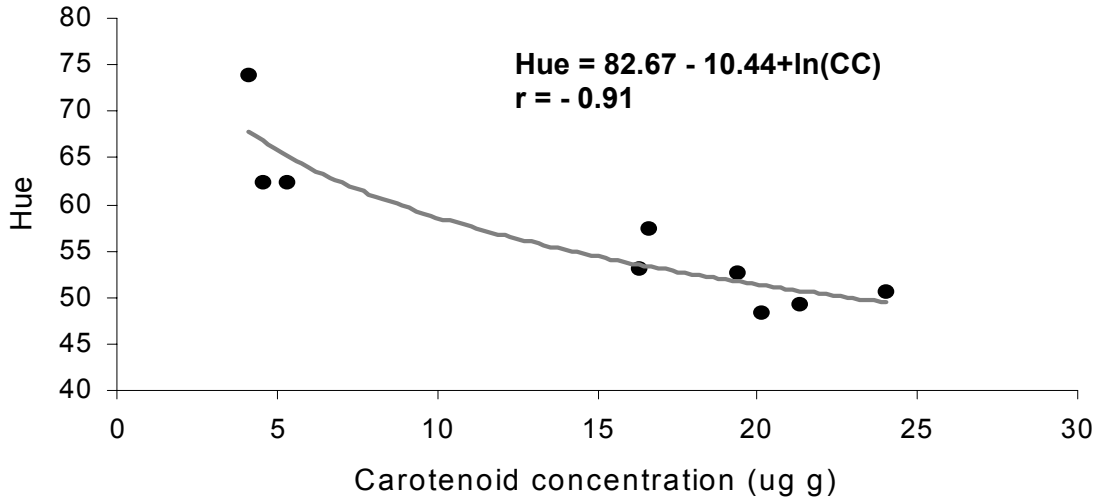


Figure 7. Relationship between skin carotenoid concentration (CC) and skin hue.

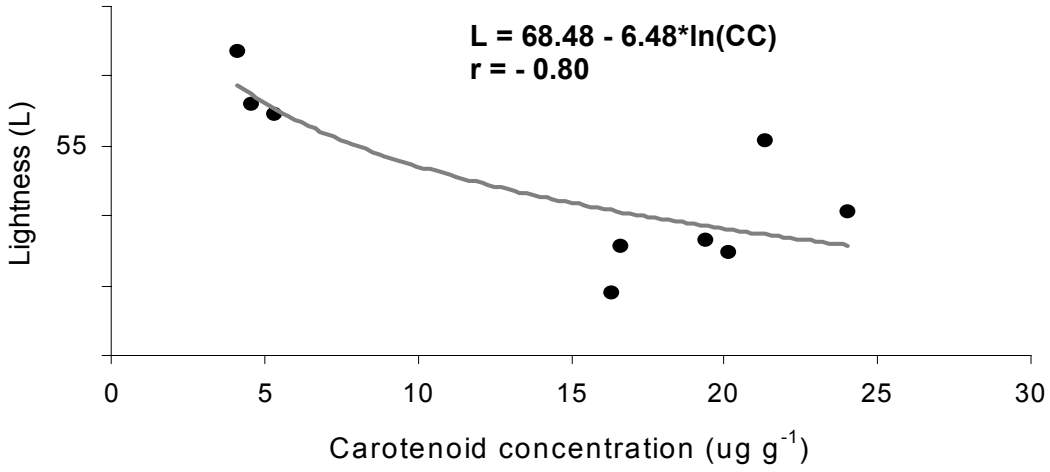
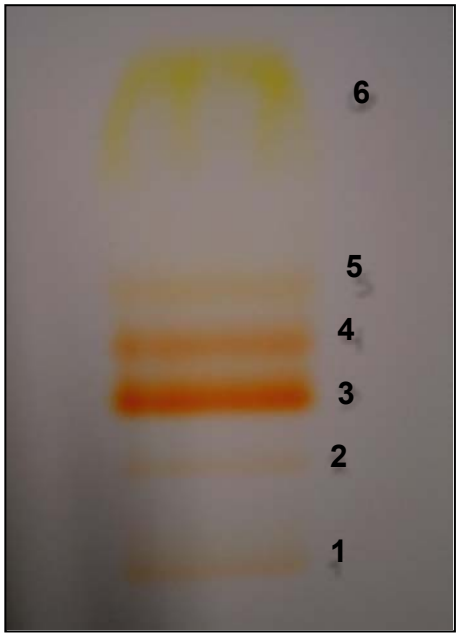


Figure 8. Relationship between skin carotenoid concentration (CC) and skin lightness.

Separation of carotenoids from red porgy skin by thin layer chromatography revealed with the hexane/acetone mobile phase six fractions (1-6) in order of increasing polarity, in both astaxanthin treatment groups (ASTX60 and ASTX90) and wild fish (Picture 2). The first five fractions showed a reddish coloration and the last fraction a yellow colour. Skin from control animals only showed the most predominant fractions 3, 4 and 6. Co-chromatographic comparisons with authentic carotenoids show the absence of unesterified astaxanthin (Rf = 0.17), canthaxanthin (Rf = 0.33), and β-carotene (Rf = 0.74). With the hexane/diethyl ether mobile phase two fractions, a red (Rf=0) and a yellow fraction (Rf=0.21), were separated also in both cultured and wild specimens. Since quantification was carried out using a spectrophotometer as specified in material and methods, the hexane/diethyl ether mobile phase was more suitable, since bands achieved were easier to measure with this methodology. ASTX90 group showed the highest relative amount of the red

fraction (86.4%). Wild red porgy showed the lowest and highest relative amounts of the red and yellow fractions respectively, with values of 64.1% and 35.9% respectively. The ratios of the red and yellow fraction in ASTX90, ASTX60 and wild red porgy were of 6.4, 5.2 and 1.8 respectively. Concerning the spectral characteristics, the red fraction presented a round symmetric band as a spectrum with a λ_{max} at 470 nm, while the yellow fraction presents a three bands spectral shape with a λ_{max} at 440nm.



Picture 2. Skin carotenoid fractions obtained by TLC (mobile phase hexane:acetone 4:1) from red porgy fed ASTX100 diet.

6.4 DISCUSSION

The inclusion of 100 mg of unesterified astaxanthin/ kg of diet seems to positively influence red porgy growth, although results visualized were not as noticeable as when longer supplementation periods, with a carotenoid diet, were tested on this species (Chapter 5). Therefore, it seems that prolonged supplementation time, with an astaxanthin source, seems to have a stronger influence on red porgy growth, than it does a high dietary carotenoid dose, as employed in the present study.

The logarithmic correlations observed between the different skin colour variables (a*, b*, hue, chroma, L*) and skin carotenoid concentration, denote the response of colour parameters diminishing at increasing carotenoid levels, therefore becoming increasingly difficult to predict carotenoid concentrations. Therefore, the logarithmic pattern found between skin colour parameters and skin carotenoid concentration, could explain the great differences observed between skin carotenoid concentration of cultured fish in comparison to wild animals and the similarity in a*, b*, hue and chroma of cultured red porgy to their wild counterparts by day 30 of feeding ASTX100 diet. Consequently, colour parameters have a limited use for prediction of carotenoid concentration, when carotenoid concentrations are high, as values diminish at increasing carotenoid levels (Bjerkeng, 2000). Similarly, in salmonids such as Artic charr, *Salvelinus alpinus*, Atlantic salmon, *Salmo salar*, Coho salmon, *Oncorhynchus kisutch*, and rainbow trout, *Oncorhynchus mykiss*, carotenoid concentration in fillet and a*, b*, hue, chroma, and L* variables show a non-linear correlation (Skrede *et al*., 1989; Smith *el al*., 1992; Christiansen *et al*., 1995; Hatlen *et al*., 1998; Nickell and Bromage, 1998). As well, redness is the colour parameter more highly correlated with muscle carotenoid concentration (Hatlen *et al*., 1998; Bjerkeng, 2000) as occurred in red porgy skin, in the present experiment.

The inclusion of unesterified astaxanthin in cultured red porgy diet has not only returned the red-pink colour to this species but also to other cultured

red skinned sparids such as *Pagrus auratus* and Japanese red sea bream, *Pagrus major* (Ito *et al*., 1986; Guerin and Hosokawa, 2001; Booth *et al*., 2004; Doolan *et al*., 2004). However, in Japanese red sea bream unesterified astaxanthin is not as effective as its esterified forms. Ito *et al*. (1986) reported a saturation point, with no further increase in skin carotenoid concentrations, after only a month of feeding an experimental diet supplemented with 100 mg of unesterified astaxanthin/ kg of diet, whereas the saturation point was found after two months when the feed contained esterified astaxanthin from *Haematococcus pluvialis* (Guerin and Hosokawa, 2001). The saturation point was not reached in the present study, since skin carotenoid concentrations at the end of the experiment differed among treatments. However, if considering colour variables behaviour, throughout the experiment, skin yellowness (b*) and chroma did show a plateau. These results are in agreement with another study carried out with red porgy using shrimp shell meal, as an astaxanthin source, where skin redness increased throughout the experimental period, while yellowness only increased up to day 120 and there on a decrease started (Chapter 5). Therefore, when red porgy is only provided with astaxanthin in the diet, b* colour variable increases up to a certain level and there on decreases. Results from the present work and from Chapter 4 and 5, point out that the increase of yellowness and the time it takes until it reaches a plateau could be related with astaxanthin concentration, source, and physiological status of the fish.

By the end of the study, relative amounts of skin carotenoids were in accordance to the colorimetric results. Feeding astaxanthin for 90 days gave the highest skin redness (a*) and the highest content of the red fraction carotenoids, whereas feeding astaxanthin for 60 days gave the highest skin yellowness (b*) and the highest amount of yellow carotenoids. The spectral characteristic of both red and yellow fractions suggests the presence of astaxanthin and tunaxanthin. Both xanthophylls have been found in this (Tejera *et al*., 2005; Pavlidis *et al*., 2006) and other species (Allahpichay *et al.,* 1984).

Factors affecting coloration include: the relative proportions of different carotenoids deposited, the relative abundances of cell-constituents interacting with carotenoids and the presence of other colour-masking compounds (Hatlen and Bjerkeng, 1998). In the present work, the proportions of red to yellow xanthophylls in cultured and wild fish skin, revealed great differences, that could affect the overall skin coloration. In red porgy from ASTX60 and ASTX90 treatment groups, the astaxanthin/tunaxanthin proportions are significantly higher in comparison to wild specimens, giving a more reddish skin coloration to cultured fish. This finding could be related to an excess of dietary astaxanthin in cultured red porgy diet.

The negative correlation found between skin lightness (L*) and skin carotenoid concentration point out a certain negative influence of dietary carotenoid on this colour variable. Thus, control fish showed the highest skin lightness values in comparison to fish fed ASTX100 diet, despite being still lower than in wild red porgy. Nevertheless, the reduction in skin lightness by dietary astaxanthin is not as marked, as that produced by others factors such as husbandry conditions. For instance, red porgy exposed to white tanks, blue spectrum and under low light intensity (Van der Salm et al., 2004) attained skin lightness values of 65, whereas red colour tanks gave skin lightness of 55. Inadequate aquaculture conditions causes red porgy skin darkening by affecting melanin synthesis. This pigment has been reported to have photo protective properties (Amstrong et al., 2000), therefore when red porgy is reared in cages, shows a darker skin, than fish cultured in tanks, due to the different light conditions (Sarmiento, 2006). But melanin synthesis is not only induced by adverse culture conditions, but also by protein rich diets high in tyrosine, since this amino acid is a melanin precursor (Chatzifotis et al., 2005).

In summary, feeding cultured red porgy 30 days before harvest with ASTX100 diet gives a skin coloration close to wild red porgy. Red porgy skin colour is determined by deposition of both red and yellow xanthophylls and

hence, supplementation with only astaxanthin, at such high concentration, produced fish with a more reddish skin than wild ones. Further studies are still needed on skin carotenoid identification, quantification and metabolism taking place in this tissue, in order to attain cultured specimens similar to wild red porgy, acceptable to the market requirements.

7.- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR TENTATIVE IDENTIFICATION OF CAROTENOIDS IN WILD RED PORGY SKIN

Abstract

In order to provide farmed red porgy with a skin colour similar to their wild counterparts, information on carotenoid profile and metabolic transformations of dietary carotenoids, by this fish species, in the skin are needed. To attain this data, a study was conducted with wild specimens captured from the Coast of Fuerteventura Island. Fish were maintained until acclimation and then skin samples were taken. Preparation and identification of wild red porgy skin carotenoids were carried out by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with photodiode array detection (PDA). TLC allowed a first separation of two main fractions, red and yellow, from the complex carotenoid content of wild red porgy skin. Afterwards, carotenoids from the red fraction were separated using a quaternary gradient and elution on a C_{18} reversed-phase column. Eleven carotenoids were achieved within 50 minutes, and based on the on the on-line spectral data obtained by PDA and comparison with reported values, astaxanthin mono and diesters were tentatively identified. In relation to the yellow fraction, carotenoids were separated, after saponification, with an isocratic system and elution on a C_{18} reversed-phase column. Eight carotenoids were separated within 15 minutes, and identification carried out in the same way as with carotenoids from the red fraction. Seven carotenoids were tentatively identified in the yellow fraction: tunaxanthins A, B, C, zeaxanthin, 3'epillutein, β-carotene triol and β-carotene. Results observed suggest that astaxanthin esters could be directly obtained through the diet and deposited in the skin without metabolic transformation, whereas the other identified skin carotenoids could be derived from astaxanthin.

Keywords: Astaxanthin esters; β-carotene; β-carotene triol; 3'epilutein; HPLC; skin; TLC; Tunaxanthin A, B, C; Wild red porgy; Zeaxanthin.

7.- CARACTERIZACIÓN DE LOS CAROTENOIDES EN LA PIEL DE BOCINEGRO SILVESTRE MEDIANTE CROMATOGRAFÍA LÍQUIDA DE ALTA RESOLUCIÓN

Resumen

Con la finalidad de obtener ejemplares de bocinegro de cultivos con una coloración natural lo más cercana posible a los ejemplares silvestres, es necesario caracterizar el perfil de carotenoides en la piel de éstos ejemplares silvestres y la utilización metabólica de los carotenoides que ingieren en su dieta. El presente estudio fue realizado utilizando piel de bocinegros silvestres desarrollando una metodología adecuada para identificar los carotenoides de la piel de los mismos. Cromatografía de capa fina y cromatografía líquida de alta resolución junto con un detector "diode array" fueron utilizados para la separación e identificación de los carotenoides presentes en la piel de los bocinegros silvestres. La cromatografía en capa fina fue utilizada en primer lugar debido a la complejidad de los carotenoides en la piel de esta especie y no siendo posible la separación de los mismos en un solo cromatograma. Haciendo uso de la cromatografía en capa fina se obtuvieron dos bandas, una de color rojo y otra amarilla. La separación de los carotenoides de la banda roja fue llevada acabo con un sistema de gradiente cuaternario y con una columna de fase reversa C_{18}. Una vez separados los carotenoides en un periodo de tiempo de 50 minutos, basándonos en la información del espectro dada por el detector en línea "diode array" y comparando con valores reportados en la literatura, mono y diesteres de astaxantina fueron identificados. En lo concerniente a la banda amarilla, los carotenoides fueron separados, previa saponificación, haciendo uso de un sistema isocrático y con una columna de fase reversa C_{18}. Ocho fueron los carotenoides separados en un periodo de tiempo de 15 minutos, y la identificación fue realizada de la misma manera que con los carotenoides encontrados en la banda roja. Se identificaron siete carotenoides en la banda amarilla: tunaxantina A, B, C, zeaxantina,

3’epiluteína, β-caroteno triol y β-caroteno. Los resultados observados sugieren que los ésteres de astaxantina encontrados fueron obtenidos a través del alimento y depositados en la piel sin ser transformados, mientras que el resto de pigmentos parecen ser metabolitos de astaxantina.

Palabras claves: β-caroteno; β-caroteno triol; 3’epiluteína; HPLC; piel; TLC; tunaxantina A, B, C; bocinegro silvestre; zeaxantina.

7.1 INTRODUCTION

Fish cannot synthesize carotenoids *de novo* being only able to convert them into different molecular species or tissue specific compounds. Hence, fish obtain carotenoids from the diet, assimilating and storing them as such, or converting to other metabolic forms or catabolising them completely (Hard, 1992). Differences exist among fish species in their ability to metabolize carotenoids (March and MacMillan 1996; Bjerkeng, 2000). According to their metabolic faculty two types of fish could be found, the "goldfish-type" and the "seabream-type" (Tanaka, 1978). The former involve most fresh water red fish, capable of oxidizing carotenoids that contain 3-hydroxy-β rings at the C(4) position, converting zeaxanthin into astaxanthin, and as a minor process, the oxidation of 4-keto-β rings at the C(3) position, converting canthaxanthin and β,β-carotene into astaxanthin (Schiedt, 1998). While the "seabream-type", mostly red marine fish, have neither of these oxidative capabilities (Schiedt, 1998).

Red porgy, characterized and well appreciated by its red-pink skin, fits in the "seabream-type" kind of fish. In a study carried out with this fish species, the inclusion of dietary β-carotene (Chatzifotis *et al.,* 2005) in the diet of farmed red porgy did not return the red-pink skin coloration, lost under aquaculture conditions (Kentouri *et al*., 1995; Stephanou *et al*., 1995; TECAM, 1999). Also, no improvement in farmed red porgy skin coloration was observed when canthaxanthin was included in the diet (Chapter 4). Hence, red porgy does not seem to be able to oxidise the ionone ring to astaxanthin, one of the carotenoids that confer the characteristic integument coloration to red porgy (Tejera *et al.,* 2005; Pavlidis *et al.,* 2006). Therefore, the inclusion of astaxanthin in the diet is needed in order to return the characteristic red-pink skin coloration (Chapters 2, 3; Chebakki, 2001; Cejas *et al.,* 2003; Tejera *et al*., 2003; Chatzifotis *et al*., 2005).

Although red porgy does not seem capable of oxidizing dietary carotenoids, it seems able to reduce these compounds (Chapter 6). When this

fish species is fed a diet with only astaxanthin, both astaxanthin and tunaxanthin esters were found in the integuments (Chapter 6; Tejera *et al.,* 2005). This finding not only suggests the role of astaxanthin as a tunaxanthin precursor, but also the capacity of this fish species to reduce carotenoids. This ability has also been observed in a closely related species, Japanese red sea bream, *Pagrus major* (Allahpichay *et al.,* 1984), the proposed transformation of astaxanthin to tunaxanthin being through "β-carotene-triol" metabolic pathway (Fujita *et al.,* 1983).

The present study was conducted to tentatively identify carotenoids present in wild red porgy skin, to try and understand the carotenoid metabolism in this marine species skin, in order to define other adequate dietary carotenoids for cultured red porgy, aside from astaxanthin.

7.2 MATERIALS AND METHODS

7.2.1 SAMPLES

Wild red porgy with a mean weight of approximately 104 g were caught in the Coast of Fuerteventura Island and maintained in our research group facilities until fish were acclimatized, as specified in material and methods. Skin samples were taken from the left hand side of the fish, in the front lateral zone, as suggested in Chapter 4. Skin was dissected from 10 fish randomly taken from the experimental tanks and samples were stored at -80ºC until carotenoid analyses.

7.2.2 CAROTENOID EXTRACTION AND PREPARATIVE TLC

Carotenoid extraction from red porgy skin was carried out according to Schiedt *et al*. (1995) as described in material and methods. For separation of red and yellow skin carotenoids, silica gel thin-layer chromatography (TLC) was utilized, using hexane/ diethyl ether (20:1) as a solvent system. The two bands obtained (red and yellow) were scrapped from the silica plate and washed several times with acetone until no colour was observed. Relative quantification

of the red and yellow band was done as described in material and methods section. After quantification, the acetone volume was evaporated to dryness, under nitrogen atmosphere, and the extract dissolved in the HPLC mobile phase. Before injecting each skin colour band, each one was filtered through a 0.45 µm PTFE. In order to break down carotenoids esters, samples were saponified as described in material and methods using Schmidt *et al.* (1994) procedure.

7.2.4 HPLC

Carotenoid pigments were analyzed by RP-HPLC under the conditions described in material and methods. For separation and identification of carotenoids from both of the TLC fractions, RP-HPLC was utilized; however, a different methodology was employed for each fraction. Red xanthophylls from red porgy skin were separated according to Yuan and Chen (1998) methodology, using a gradient system with the following mobile phase:

A: dichloromethane, **B:** methanol, **C:** acetonitrile and **D:** water.

· From 0 to 8 minutes: isocratic

A: B: C: D = 5: 85: 5.5: 4.5.

· From 8 to 14 minutes: gradient

A: B: C: D = 5: 85: 5.5: 4.5 to A: B: C: D = 22: 28: 45: 4.5.

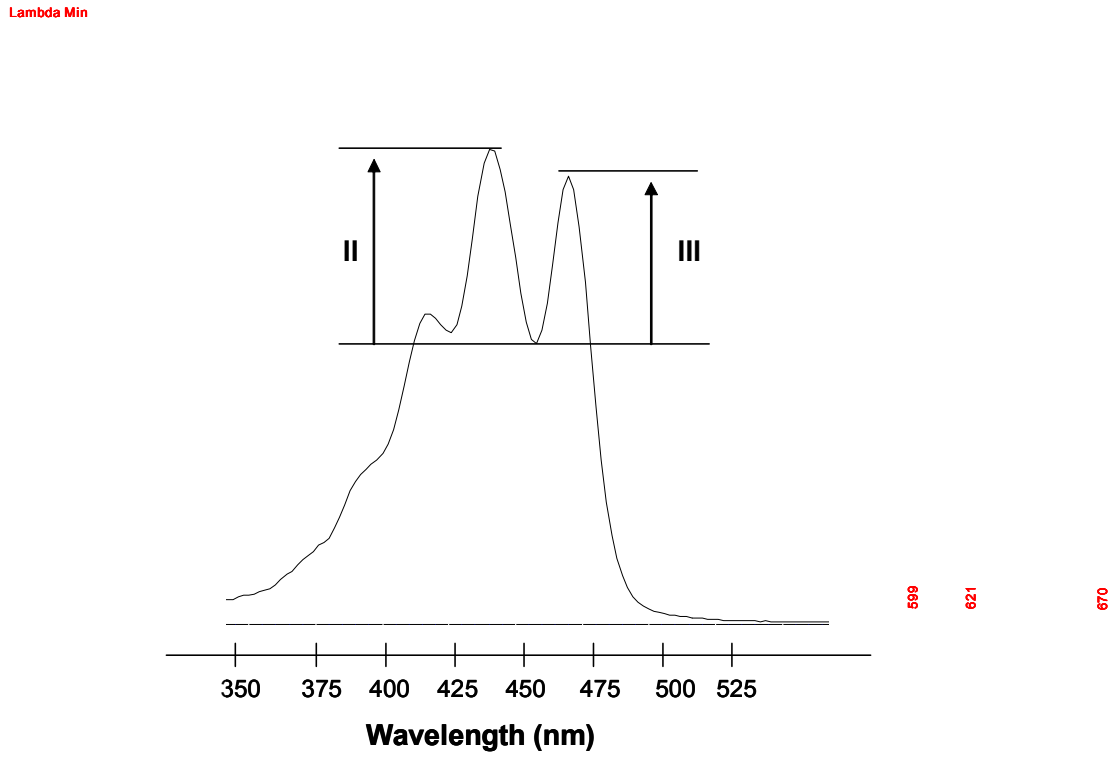
· From minute 14 to 50: isocratic

A: B: C: D = 22: 28: 45: 4.5.

The total running time was 50 minutes and the absorption spectra of carotenoids were displayed between 250 and 700 nm. Peaks were measured at a wavelength of 480 nm to facilitate detection of astaxanthin.

Saponified yellow carotenoids from red porgy skin were separated with an isocratic system with dichloromethane: methanol: acetonitrile: water (5: 70: 20.5: 4.5) as a mobile phase. Total running time was 15 minutes and the absorption spectra of carotenoids were displayed between 250 and 700 nm. Peaks were measured at a wavelength of 440 nm to facilitate detection of tunaxanthin.

Due to the lack of commercial standards for each of the carotenoids studied the identification techniques used were the following: comparison of spectra (by PDA) with published data and a numerical notation (%III/II) which describes the ratio of the peak height of the longest wavelength absorption band (band III) to that of the middle absorption band (usually λ_{max}, band II) as a percentage (Picture 1). The ratio of peak heights has proven useful to compare



Picture 1. Spectral fine structure (as % III/II) of tunaxanthin.

7.3 RESULTS AND DISCUSSION

The complexity of red porgy skin carotenoid extract, made it difficult to separate all compounds in a single chromatographic run. Separation in TLC plates allowed the isolation of red (65%) and yellow carotenoids (35%) from wild red porgy skin carotenoid extract.

7.3.1 CAROTENOIDS IN THE TLC RED FRACTION

Carotenoids present in the skin red fraction, separated with the methodology of Yuan and Chen (1998) gave eleven peaks. The typical chromatogram and chromatographic data obtained are shown in Figure 1 and Table 1 respectively. Using the photodiode array detector the spectrum of each peak was recorded during its elution, showing all of them a round shape with a λ_{max} of approximately 480 nm, except for peak 6 that showed a λ_{max} of 472 nm (Table 1). As one of the main carotenoids in red porgy skin have been suggested to be astaxanthin esters (Chapter 5; Tejera *et al.,* 2005; Pavlidis *et al.,* 2006), the use of the algae *Haematococcus pluvialis* and *Chlorococcum*, both with significant amounts of astaxanthin esters (Droop, 1955; Donkin, 1976; Johnson and An., 1991; Zhang et al., 1997; Yuan and Chen, 1998; Liu and Lee, 1999; Yuan et al., 2002), were used for comparing the spectrum and λ_{max}. In two studies carried out by Yuan and Chen (1998) and Yuan *et al.,* (2002) with *Haematococcus pluvialis* and with *Chlorococcum* respectively, astaxanthin esters showed a λ_{max} of 480 and 482 nm; therefore the λ_{max} of the peaks from the present work could possibly be astaxanthin esters. Slightly differences in λ_{max} is understandable considering that reproducibility of recording spectrophotometer in the 400-500 nm region is about ± 1-2 nm. Analyzing retention times, tentatively the first three peaks (1-3) are likely to be monoesters, and approximately five minutes after diesters (peaks 4-11).

Concerning peak 6, this could be a cis isomer due to the shift in the maximum absorbance to 472 nm (Nyambaka and Ryley, 1996). All-trans

astaxanthin, the more common isomer found in nature, is readily isomerized to cis forms specially 9-cis and 13-cis isomers, for steric reasons, by the action of light or heat (Quackenbush, 1987; Johnson and An, 1991). Cis-isomers of carotenoids may have essential metabolic functions and are naturally formed in certain organisms (Johnson and An, 1991). Therefore, it is likely that astaxanthin esters in red porgy skin could also exist as cis-isomers other than only all-trans isomers.

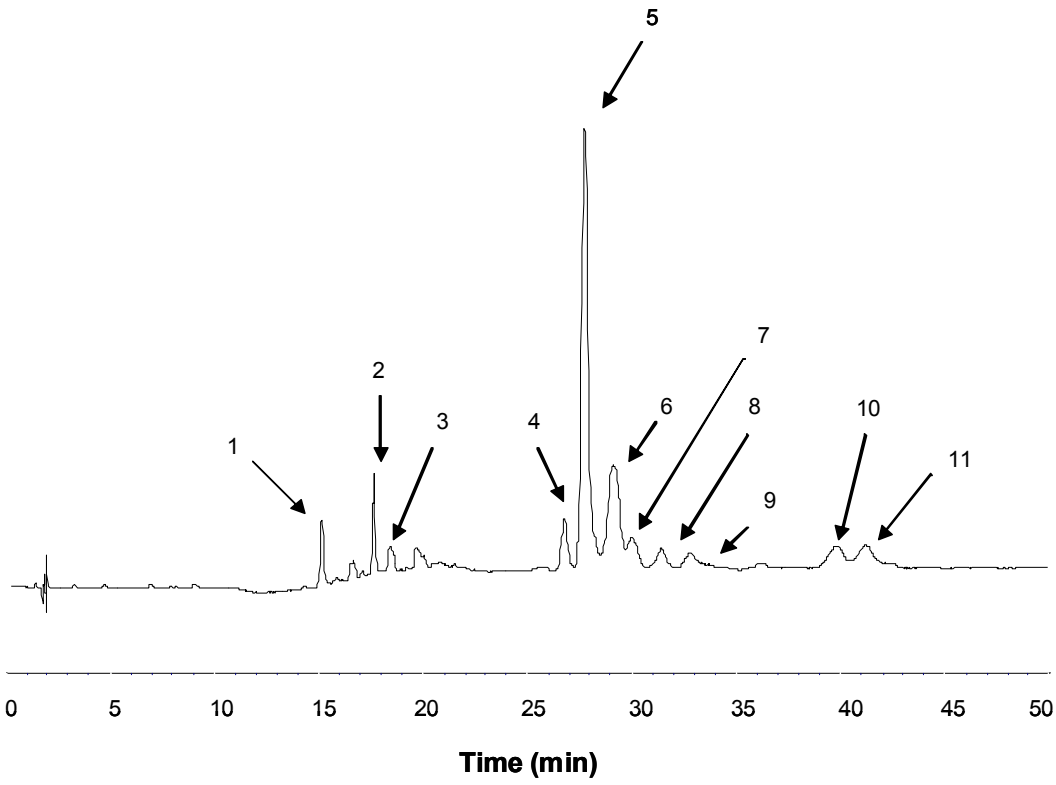


Figure 1. RP- HPLC chromatogram of TLC red fraction obtained from wild red porgy skin. C_{18} column (150x4.6 mm, 5µm). Mobile phase presents dichloromethane, methanol, acetonitrile and water. Detection at 480 nm.

Table 1. Retention time, maximum absorbance (λ_{max}) and tentative identification of carotenoids present in the skin red fraction.

Peak	Retention time	λ_{max} (nm)	Tentative identification
1	15.10	480	Astaxanthin monoester
2	17.6	480	Astaxanthin monoester
3	19.6	482	Astaxanthin monoester
4	26.9	480	Astaxanthin diester
5	27.9	482	Astaxanthin diester
6	29.2	472	Cis-astaxanthin diester
7	30.1	480	Astaxanthin diester
8	31.6	480	Astaxanthin diester
9	33.0	482	Astaxanthin diester
10	40.1	482	Astaxanthin diester
11	41.5	480	Astaxanthin diester

7.3.2 CAROTENOIDS IN THE TLC YELLOW FRACTION

Carotenoids from the skin yellow fraction were separated and tentatively determined, previous saponification, following an isocratic RP-HPLC procedure. Saponification had to be carried out due to the complexity of carotenoids present in this fraction. Eight peaks were obtained and the typical chromatogram and chromatographic data are shown in Figure 2 and Table 2 respectively. The spectrum of each eluted peak was detected using the photodiode array detector, showing a clear three peak spectrum, and in other cases two peaks with a mere shoulder (Table 2). Since no standards were available for identification, III/II% values were compared to literature values. The presumed metabolic route from dietary astaxanthin or lutein to tunaxanthin, proposed by Miki et al. 1985 (Figure 3), has also been utilized for tentative identification of the carotenoids found in red porgy skin yellow fraction. The eight peaks obtained from the skin yellow fraction were identified as follows:

β-catotene triol **(1)**: the more polar carotenoid, eluting first in the chromatograph. In literature this carotenoid presents a similar spectrum to β-carotene (Miki *et al.,* 1985), results in this study agree well, and the %III/II also coincides with β-carotene.

Zeaxanthin **(2)**: in literature %III/II values fluctuate from 25 to 36 (Khachik et al., 1987; Britton, 1995; Mouly et al., 1999; Lee et al., 2001), therefore peak 2 could be identified as zeaxanthin.

3'Epilutein **(3)**: detected as one of the major carotenoids, its spectrum has been reported to be similar to α-carotene (Miki *et al.,* 1985). Results from this work also show similarity to α-carotene.

Tunaxanthin **(4, 5, 6)**: in a study carried out with Artic charr, this carotenoid was found in the skin of this fish species and the III/II% was around 87 (Bjerkeng *et al.,* 2000). Hence, values from peaks 4 and 5 correspond. In marine teleosts a mixture of tunaxanthins A, B and C, which are stereoisomers of each other, have been reported by Rønenberg *et al.,* (1978) and Matsuno *et al.,* (1980). Therefore, peaks 4, 5 and 6, in order of decreasing polarity (Miki *et al.,* 1985) could be tunaxanthins A, B and C. Peak 6, presenting an additional peak at 325 nm, could be a cis isomer due to the shift in the maximum absorbance (Nyambaka and Ryley, 1996), and also for the presence of a peak in the UV region.

β-carotene **(7)**: the %III/II corresponds to values reported in literature (Britton, 1995) and in a study done with Japanese red sea bream, a close species to red porgy, β-carotene was found in the skin (Ha *et al.,* 1993), therefore it could also be present in red porgy skin .

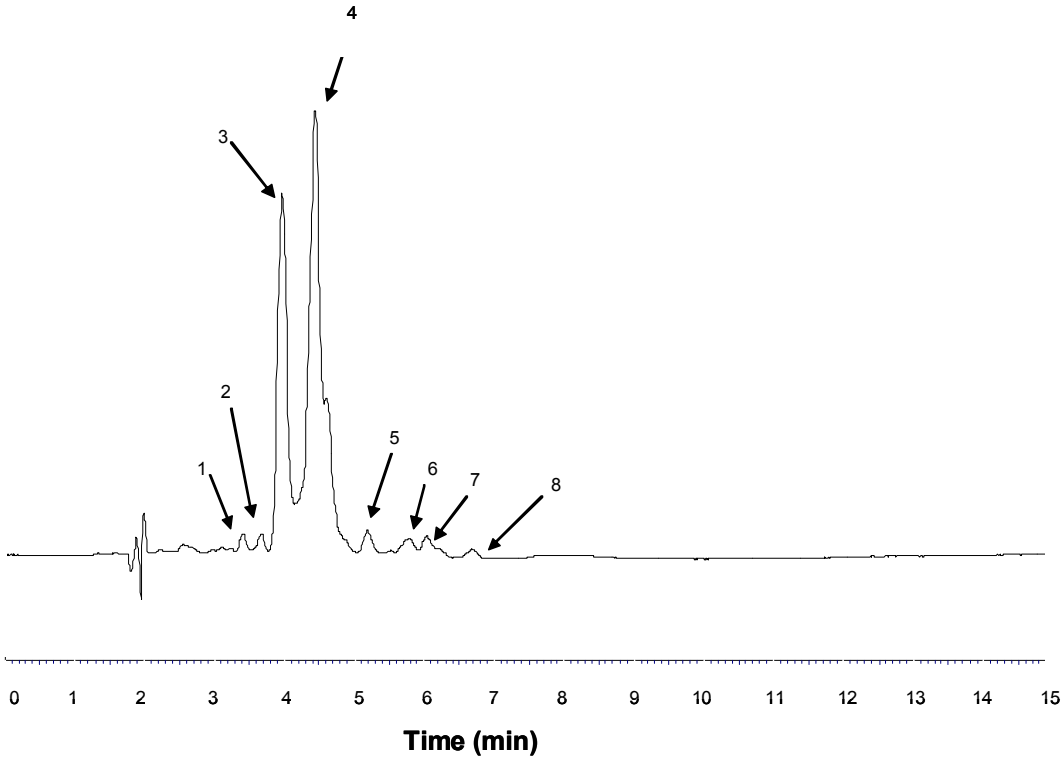


Figure 2. RP- HPLC chromatogram of TLC yellow fraction obtained from wild red porgy skin. C18 column (150x4.6 mm, 5µm). Mobile phase presents dichloromethane, methanol, acetonitrile and water. Detection at 440 nm.

Table 2. Retention time (min), maximum absorbance (λ_{max}), % III / II and tentative identification of carotenoids present in the skin yellow fraction.

Peak	Retention Time (min)	λ_{max} (nm)	% III / II	Tentative Identification
1	3.36	425^{sh}, 452, 478	25.8	β-carotene triol
2	3.62	425^{sh}, 450, 476	34.2	Zeaxanthin
3	3.92	415^{sh}, 441, 465	57.4	3´epilutein
4	4.38	419, 441, 470	88.9	Tunaxanthin
5	5.11	415, 437, 465	87.9	Tunaxanthin
6	5.69	325^{+}, 412, 434, 462	73.7	Tunaxanthin
7	5.93	425^{sh}, 447, 473	68.3	Unidentified
8	6.58	420^{sh}, 446, 471	25	β -carotene

(sh) shoulder. (+) cis peak

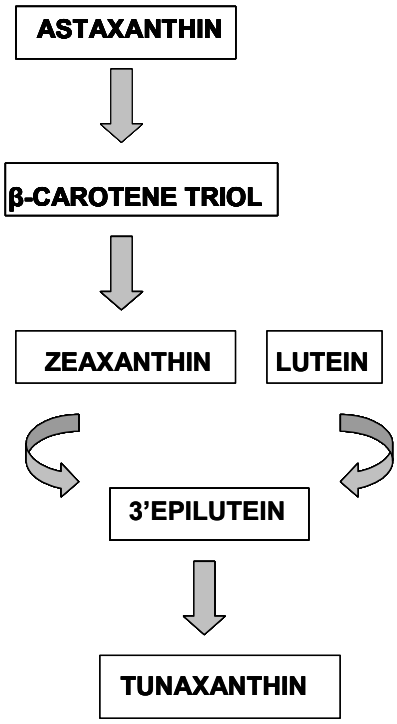


Figure 3. Presumed metabolic pathway from astaxanthin and lutein to tunaxanthin in yellowtail (Miki *et al.,* 1985)

7.3.3 RED PORGY SKIN CAROTENOID METABOLISM

Red porgy diet is mainly based on crustaceans (Labropoulou *et al.,* 1999) rich in astaxanthin mono and diesters (Shahidi *et al*., 1994; Higuera-Ciapara *et al.,* 2006). Based on this, we can suggest the presence of three types of carotenoids in this species skin. Carotenoids obtained from the diet and deposited as such in this tissue, intermediate metabolites and final metabolites of the ingested dietary carotenoids.

Astaxanthin esters found in the integuments of wild red porgy were probably obtained from the diet and deposited in the skin with no previous metabolic transformation. Astaxanthin di-esters (40%) and both mono-ester and unesterified astaxanthin, making up 15%, were also found in wild red porgy

from the Coasts of Crete (Pavlidis *et al.,* 2006). Nevertheless, in the present work unesterfied astaxanthin was not detected in the skin. The prevalence of astaxanthin diesters has been also found in cultured specimens fed a diet with the inclusion of an astaxanthin source (Tejera *et al.,* 2005). The above hypothesis suggesting that dietary astaxanthin is directly deposited in red porgy integuments, could be supported by the absence of possible compounds indicating a metabolic transformation from a dietary carotenoid to astaxanthin.

In concern to skin tunaxanthin, this carotenoid is suggested to be a final metabolite of the absorbed dietary astaxanthin. The transformation of dietary astaxanthin to tunaxanthin appears to be a common aspect of carotenoid metabolism among fish species, occurring in both freshwater and marine fish integuments such as: red sea bream, *Pagrus major*, yellowtail, *Seriola quinqueradiata*, black bass, *Micropterus salmoides*, tilapia and in Artic charr, *Salvelinus alpinus* (Fujita *et al.,* 1983a; Fujita *et al.,* 1983b; Miki *et al.,* 1985; Yamashita and Matsuno, 1992; Bjerkeng *et al.,* 2000). Regarding β-carotene triol, zeaxanthin, 3'epilutein, these are suggested to be intermediate metabolites of the metabolic route from astaxanthin to tunaxanthin. In a study carried out by Miki *et al.,* (1985) with yellow tail the "β-carotene-triol" metabolic pathway from astaxanthin to tunaxanthin showed the same metabolic intermediates.

In yellowtail not only astaxanthin has been reported as a tunaxanthin precursor, also zeaxanthin and lutein (Miki *et al.,* 1985), both abundant xanthophylls in the marine environment. When lutein is metabolised to tunaxanthin, the conversion takes place through 3'epilutein pathway. It would be interesting, in future studies, to evaluate in cultured red porgy the inclusion of lutein or zeaxanthin and analyze if tunaxanthin is synthesized. In a study carried out by Olsen and Baker (2006), with Atlantic salmon, the inclusion of synthetic lutein does not seem to interfere with astaxanthin absorption; therefore, it would be interesting to try and include lutein in red porgy diet with the aim of not utilizing astaxanthin as a tunaxanthin precursor and instead lutein that is a cheaper carotenoid. However, it should be taken into consideration the above

observations pointing out the need of astaxanthin in this fish diet since apparently is not capable of transforming other dietary carotenoids into astaxanthin.

CONCLUSIONS

Effect of different dietary carotenoid sources on red porgy skin colour

1. Synthetic canthaxanthin included up to 100 mg/kg of diet did not improve red porgy skin coloration.

2. Shrimp shell meal was found a good esterified astaxanthin source giving red porgy skin a colour close to the wild specimens, when it replaces 16% of fish meal.

3. Synthetic astaxanthin improves skin coloration of cultured red porgy at an inclusion level of 100 mg/kg of diet.

4. Skin colour parameters such as redness, yellowness, hue and chroma are improved by the inclusion of an astaxanthin source in red porgy diet. However, skin lightness is negatively affected by the inclusion of an astaxanthin source in the diet.

5. The front lateral zone of red porgy skin was found to be the best indicator of the adequate red porgy skin coloration, therefore proposed for future studies.

Effect of different carotenoid sources on red porgy culture performance

6. Both synthetic canthaxanthin and astaxanthin fed for about 100 days to red porgy in up to 100mg/kg inclusion levels did not affected fish growth or feed utilization.

7. Shrimp shell meal included in 16% of replacement of fish meal in red porgy diets for more than 120 days, markedly enhanced growth parameters.

Effect of carotenoid supplementation time with different carotenoid sources on skin colour

8. A feeding period of 120 to 180 days, before harvesting, is necessary to provide cultured red porgy with skin coloration similar to their wild counterparts, if shrimp shell meal is used as a carotenoid source in replacement of 16% of fish meal.

9. A feeding period of 30 days, before harvesting, is necessary to achieve cultured red porgy with skin colour close to wild animals, when synthetic astaxanthin is included at 100mg/kg diet.

Effect of carotenoid feeding on total skin carotenoid concentration

10. Carotenoid concentration in red porgy skin increments when supplementation time with shrimp shell meal or synthetic astaxanthin increases.

11. A positive correlation, following a logarithmic pattern, was found between skin colour parameters, a*, b* and chroma and skin carotenoid concentration.

12. A negative correlation, following a logarithmic pattern, was observed between the skin colour parameters L* and hue, and skin carotenoid concentration.

Separation, quantification and tentative identification of cultured and wild red porgy skin carotenoids

13. Red porgy skin colour is determined by the relative amount of red and yellow carotenoids. An over supplementation of astaxanthin in cultured red porgy diet gives a more reddish skin coloration than desired, comparing with wild fish.

14. The main carotenoids found in cultured red porgy fed on an astaxanthin supplemented diet and wild specimens were esters of astaxanthin and esters of tunaxanthin.

15. In order to maintain the characteristic reddish skin coloration, astaxanthin must be an essential constituent of red porgy diet, in view of the inability of this fish species to oxidize dietary carotenoids. As red porgy shows the ability to reduce dietary astaxanthin to tunaxanthin, most probably by the β-catotene triol metabolic route, other tunaxanthin precursors rather than astaxanthin could be evaluated in future studies.

REFERENCES

Aanesen-Berit, A., Breivik, H., Sanna Lola, I., 2000. Pigment. UK Patent Nº WO0062625.

Aas, G.H., Bjerkeng, B., Storebakken, T. & Ruyter, B., 1999. Blood appearance, metabolic transformation and plasma transport proteins of C-astaxanthin in Atlantic salmon (*Salmo salar* L.). Fish Physiology and Biochemistry, 21: 325-334.

Ahmed, M. & Delgado, C., 2000. Introduction to the issues and context of rapid changes in world demand of fish. Paper presented in the Bien. Meeting Int. Inst. for Fish Econ. and Trade (II FET), 10-14 July, 2000, Corvallis, Oregon, USA. Reprinted in fish to 2020: implications of global fish outlook for developing countries. Penag, Malaysia: World Fish Center.

Ako, H., Tamaru, C.S., Asano, L., Yuen, B. & Yamamoto, M., 2000. Achieving natural coloration in fish under culture. Page 1-4 in C.C.-T. Tamaru, C.S. Tamaru, J.P. McVey, and K. Ikuta, editors. Spawning and maturation of aquatic species, UJNR Technical Report No. 28. University of Hawaii Sea Grant College Program, Honolulu, Hawaii.

Akhtar, P., Gray, J. I., Cooper, T.H., Graling, D. L. & Booren, A. M., 1999. Dietary pigmentation and deposition of α-tocopherol and carotenoids in rainbow trout muscle and liver tissue. J. Food Sci., 64: 234-239.

Alaluf, S., Heinrich, U., Stahl, W., Tronnier, H. & Wiseman, S., 2002. Dietary carotenoids contribute to normal human skin colour and UV photosensitivity. J. Nutr., 132: 399-403.

Al-Khalifa, A.S. & Simpson, K.L., 1988. Metabolism of astaxanthin in the rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol., B 91: 563-568.

Allahpichay, I., Shimizu, C. & Kono, M., 1984. Pigmentation of cultured red sea bream, *Chrysophrys major,* using astaxanthin from Antarctic krill, *Euphausia superba*, and a mysid, *Neomysis* SP. Aquaculture, 38: 45-57.

Amar, E.C., Kiron, V., Satoh, S. & Watanabe, T., 2001. Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Research, 32 (1): 162-163.

Amar, E.C., Kiron, V., Satoh, S. & Watanabe, T., 2003. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish. Shellfish. Immunol., 16: 527-537.

Amstrong, G.A., 1997. Genetics of eubacterial carotenoid biosynthesis: a colourful tale. Annu. Rev. Microbiol., 51: 629-659.

Amstrong, T. N., Cronin, T. W. & Bradley, B. P., 2000. Microspectrophotometric analysis of intact chromatophores of the Japanese Medaka, *Orysias latipes*. Pigm. Cell Res., 13: 116-119.

Ando, S. & Hatano, M., 1988. Bilirubin-binding protein in the serun of spawning migrating chum salmon, *Oncorhynchus keta*: Its identity with carotenoid carrying lipoprotein. Fish Physiol. Biochem., 5: 69-78.

Ando, S., Takeyama, T. & Hatano, M., 1986. Transport associated with serum vitellogenin of carotenoid in chum salmon (*Oncorhynchus keta*). Agric. Biol. Chem., 50: 557-563.

Ando, S., Takeyama, T., Hatano, M. & Zama, K., 1985. Carotenoid carrying lipoproteins in the serum of chum salmon (*Oncorhynchus keta*) associated with migration. Agric. Biol. Chem., 49: 2185-2187.

Andrewes, A. G. & Starr, M.P., 1976. (3R, 3´R)-astaxanthin from the yeast *Phaffia rhodozyma*. Phytochemistry, 15: 1009-1011.

AOAC, 1995. Official Methods of Analysis of the Association of Analytical Chemistry. Washington, DC, 1018 pp.

Badyaev, A. V. & Hill, G. E., 2000. Evolution of sexual dichromatism: Contribution of carotenoid versus melanin based coloration. Biol. J. Linn. Soc., 69: 153-172.

Bagnara, J.T. & Hadley, M.E., 1978. Chromatophores and colour change – The comparative physiology of animal pigmentation. Prentice-Hall, Inc., New Jersey, pp 202.

Bagnara, J.T., Taylor, J.D. & Prota, G. 1973. Colour changes, unusual melonosomes and new pigment from leaf frogs, 1034-1035. www.sciencemag.org

Baker, R.T.M., 2001. Canthaxanthin in aquafeed applications: Is there any risk? Trends in Food Science and Technology, 7 (4): 240-243.

Baker, R.T.M., Pfeiffer, A.M., Schöner, F.J. & Smith-Lemmon, L., 2002. Pigmentation efficacy of astaxanthin and canthaxanthin in fresh-water reared Atlantic salmon, *Salmo salar.* Animal Feed Science & Technology, 99: 97-106.

Barbosa, M.J., Morais, R. & Choubert, G., 1999. Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 176: 331-341.

Barua, A.B., Kostic, D. & Olson, J.A., 1993. New simplified procedures for the extraction and simultaneous high performance liquid chromatographic analysis of retinol, tocopherols, and carotenoids in human serum. J. Chromatogr., 617: 257-264.

Basurco, B. & Abellán, E., 1999. Finfish species diversification in the context of the Mediterranean marine fish farming development. Cahiers Options Méditerranéennes, Ser. B: Etudes et Recherches, 24: 9-25.

Bell, J.G, McEvoy, J., Webster, J.L., McGhee, F., Millar, R.M. & Sergeants, J.R., 1998. Flesh lipid and carotenoid composition of Scottish farmed Atlantic salmon (*Salmo salar*). J. Agric. Food. Chem., 46: 119-127.

Bell, J.G, McEvoy, J., Tocher D.R. & Sargent, J.R., 2000. Depletion of α-Tocopherol and Astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defense and fatty acid metabolism. American Society for Nutritional Sciences, 1800-1807.

Bendich, A. & Shapiro, S.S., 1986. Effect of β-carotene and canthaxanthin on the immune responses of the rat. J. Nutr., 116 (11): 2254-2262.

Berticat, O., Nègre-Sardargues, G. & Castillo, R., 2000. The metabolism of astaxanthin during the embryonic development of the crayfish *Astacus leptodactylus* Eschscholtz (Crustacean, Astacidea). Comparative Biochemistry and Physiology, B 127: 309-318.

Birkeland, S. & Bjerkeng, B., 2004. Extractabilities of astaxanthin and protein from muscle tissue of Atlantic salmon (*Salmo salar*) as affected by brine concentration and pH. Food Chemistry, 85: 559-568.

Bjerkeng, B., 2000. Carotenoid pigmentation of salmonid fishes - recent progress. In: Cruz-Suárez, L. E., Rique-Marie, D., Tapia-Salazar, M., Overa-Novoa, M.A. y Civera-Cerecedo, R., (Eds.). Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola., 19-22 Noviembre, 2000. Mérida, Yucatán.

Bjerkeng, B. & Johnsen, G., 1995. Frozen storage of rainbow trout (*Oncorhynchus mykiss*) as affected by oxygen, illumination and fillet pigment. Journal of Food Science, 60 (2): 284-288.

Bjerkeng, B. & Berge, G.M., 2000. Apparent digestibility coefficients and accumulation of astaxanthin *E/Z* isomers in Atlantic salmon (*Salmo salar* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). Comparative Biochemistry and Physiology, B 127: 423-432.

Bjerkeng, B., Storebakken, T. & Liaaen-Jensen, S., 1990. Response to carotenoids by rainbow trout in the sea: resorption and metabolism of dietary astaxanthin and canthaxanthin. Aquaculture, 91: 153-162.

Bjerkeng, B., Storebakken, T. & Liaaen-Jensen, S., 1992. Pigmentation of rainbow trout from start feeding to sexual maturation. Aquaculture, 108: 333-346.

Bjerkeng, B., Hatlen, B. & Wathne, E., 1999. Deposition of astaxanthin in fillets of Atlantic salmon (*Salmo salar*) fed diets with herring, capelin, sand eel, or Peruvian high PUFA oils. Aquaculture, 180: 307-319.

Bjerkeng, B., Hatlen, B. & Jobling, M., 2000. Astaxanthin and its metabolites idoxanthin and crustaxanthin in flesh, skin and gonads of sexually immature and maturing Arctic charr (*Salvelinus alpinus* (L.)) Comparative Biochemistry and Physiology, B 125: 395-404.

Bjerkeng, B., Refstie, S., Fjalestad, K. T., Storebakken, T., Rodbotten, M. & Roem, A., 1997. Quality parameters of the flesh of Atlantic salmon (*Salmo salar*) as affected by dietary fat content and full soybean meal as a partial substitute for fish meal in the diet. Aquaculture, 157: 297-309.

Botlen, R. S., Mann, J. H. & Gushue, W., 1966. Use of standardized colour surfaces in the grading of canned salmon for colour. Journal of the Fisheries Research Board of Canada, 24: 1613-1622.

Boonyaratpalin, M., Thongrod, S., Supamattaya, K., Britton, G. & Schlipalius, L.E., 2001. Effect of β-carotene source, *Dunaliella salina*, and astaxanthin on pigmentation, growth, survival and health of *Penaeus monodon*. Aquaculture Research, 32 (1): 182-190.

Booth, M., Warner-Smith, R., Allan, G. & Glencross, B., 2004. Effects of dietary astaxanthin source and light manipulation on the skin colour of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801). Aquaculture Research, 35: 458-464.

Bortolotti, G., Negro, J.J., Tella, J.l., Marchant, T.A. & Bird, D.M., 1996. Sexual dichromatism in birds independent of diet, parasites and androgens. Proceeding of the Royal Society of London, B 263: 1171-1176.

Bowen, J., Soutar, C., Serwata, R.D., Lagocki, S., White, D.A., Davies, S.J. & Young, A.J., 2002. Utilization of (3S, 3'S)-astaxanthin acyl esters in pigmentation of rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition, 8: 59-68.

Bragadóttir, M., 1998. Redfish colour – Processing improvement on board freezing trawlers. Icelandic Fisheries Laboratories Report summary, 11: 2-13.

Bragadóttir, M., 2001. Endogenous antioxidants in fish. Thesis of Master, department of Food Science, University of Iceland.

Brawner, W.R. III, Hill, G.E. & Sundermann, C.A., 2000. The effects of coccidaial and mycoplasmal infections on plumage pigmentation in male house finches (*Carpodacus mexicanus*). Auk., 117: 952-963.

Britton, G., 1995. Structure and properties of carotenoids in relation to function. FASEB J., 9:1551-1558.

Britton, G., 1995. UV/Visible spectroscopy. In Carotenoids: Spectroscopy (Britton, G., Liaaen-Jensen, S., Pfander, H., Eds). Vol. 1B. Birkäusen, Basel. 1998.

Britton, G., 1998. Overview of carotenoid biosynthesis. In: Britton, G., Liaaen-Jensen, S., Pfander, H., (Eds). Carotenoids, vol. 3. Birkäusen, Basel., 14-15.

Brush, A.H., 1990. Metabolism of carotenoid pigments in birds. FASEB Journal, 4: 2969-2977.

Buchecker, R., 1982. A chemist's view of animal carotenoids. Carotenoid Chemistry and Biochemistry, (G. Britton & T.W. Goodwin Eds).

Carter, J.V., Palafox, J.T.P. & Islas, R.P., 1994. Bioensayo de pigmentación de trucha arcoiris (*Oncorhynchus mykiss*) con extractos de chili ancho (*Capsicum annuum*). Arch. Latinoam. Nutr., 44: 252-255.

Castañeda, M.P., Hirschler, E.M. & Sams, A.R., 2005. Skin pigmentation evaluation in broilers fed natural and synthetic pigments. Poultry Science, 84 (1): 143-147.

Castenmiller, J.J.M. & West, C.E., 1998. Bioavailability and bioconversion of carotenoids. Annu. Rev. Nutr., 18: 19-38.

Cejas, J., Almansa, E., Tejera, N., Jerez, S., Bolaños, A. & Lorenzo, A., 2003. Effect of dietary supplementation with shrimp on skin pigmentation and lipid composition of red porgy (*Pagrus pagrus*) alevins. Aquaculture, 218: 457-469.

Chan, H.W.S., 1987. The mechanism of autoxidation, In: Autoxidation of unsaturated lipids (Chan, H.W.S., Eds). Academic Press, London, UK, 1-16.

Chatzifotis, S., Pavlidis, M., Doñate Jimeno, C., Vardanis, G., Sterioti, A. & Divanach, P., 2005. The effect of different carotenoid sources on skin coloration of cultured red porgy (*Pagrus pagrus*). Aquaculture Research, 36 (15): 1517-1525.

Chebbaki, K., 2001. Efecto de la nutrición sobre la coloración de la piel y la calidad del filete en bocinegro (*Pagrus pagrus*). Tesis de Master, Universidad de Las Palmas de Gran Canaria.

Chew, B. P., 1992. Role of carotenoids in the immune response. J. Dairy Sci., 75: 265.

Chew, B. P. & Park, J. S., 2004. Functions and actions of retinoids and carotenoids: building on the vision of James Allen Olson. American Society of Nutritional Sciences, 257S-261S.

Chien, Y.H. & Jeng, S.C., 1992. Pigmentation of kuruwa prawn (*Penaeus japonicus* Bate) by various pigment sources and levels and feeding regimes. Aquaculture, 102: 333-346.

Chien, Y., Pan, C. & Hunter, B., 2003. The resistance to physical stresses by *Penaeus monodon* juveniles fed diets supplemented with astaxanthin. Aquaculture, 216 (1-4): 177-191.

Chien, Y.H., Chen, I.M., Pan, C.H. & Kurmaly, K., 1999. Oxygen depletion stress on mortality and lethal course of juvenile tiger prawn *Penaeus monodon* fed high level of dietary astaxanthin. J. Fish. Soc. Taiwan, 26: 85–93.

Chou, Y.H. & Chien, Y.H., 2001. Effects of astaxanthin and vitamin E supplement in Japanese sea bass *Lateolabrax japonicus* brood stock diet on their fecundity and egg quality. 6th Asian Fisheries Forum, Book of Abstracts, p 60.

Choubert, G. & Luquet, P., 1983. Utilization of shrimp meal for rainbow trout (*Salmo gairdneri* Rich.) pigmentation. Influence of fat content of the diet. Aquaculture, 32: 19-26.

Choubert, G. & Heinrich, O., 1993. Carotenoid pigments of the green alga *Haematococcus pluvialis*: assay on rainbow trout, *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin. Aquaculture, 112: 217-226.

Choubert, G., Milicua, J.C. & Gomez, R., 1994a. The transport of astaxanthin in immature rainbow trout *Oncorhynchus mykiss* serum. Comp. Biochem. Physiol., 108 A: 245-248.

Choubert, G., Cravedi, J-P. & Laurentie, M., 2005. Pharmacokinetics and bioavailabilities of ^{14}C-keto-carotenoids, astaxanthin and canthaxanthin, in rainbow trout, *Oncorhynchus mykiss*. Aquaculture Research, 15 (9): 1526-1534.

Choubert, G., Gomez, R. & Milicua, J.C.G., 1994b. Response of serum carotenoid level to dietary astaxanthin and canthaxanthin in immature rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol., 109 A: 1001-1006.

Choubert, G., Milicua, J.C., Martinez, R.G., Sance, S., Petit, H., Nègre-Sandargues, G., Castillo, R. & Trilles, J.P., 1992. Distribution of canthaxanthin in immature rainbow trout (*Oncorhynchus mykiss)*. Comp. Biochem. Physiol., 103 A: 403-405.

Christiansen, R. & Torrissen, O.J., 1996. Growth and survival of Atlantic salmon, *Salmo salar* L. fed different dietary levels of astaxanthin. Juveniles. Aquaculture Nutrition, 2 (1): 55-62.

Christiansen, R. & Torrissen, O.J., 1997. Effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (*Salmo salar L*.) Aquaculture, 153: 51-62.

Christiansen, R., Lie, Ø. & Torrissen, O.J., 1994. Effect of astaxanthin and vitamin A on growth and survival during feeding of Atlantic salmon, *Salmo salar* L. Aquaculture Research, 25 (9): 903-914.

Christiansen, R., Lie, Ø. & Torrissen, O.J., 1995. Growth and survival of Atlantic salmon, *Salmo salar* L., fed different levels of astaxanthin. First-feeding fry. Aquaculture Nutrition, 1 (3): 189-198.

Christiansen, R., Glette, J., Lie, Ø., Torrissen, O.J. & Waagbø, R., 1995. Antioxidant status and immunity in Atlantic salmon, *Salmo salar*, L., fed semi-purified diets with and without astaxanthin supplementation. J. Fish Dis., 18: 317-328.

Christie, W.W., 1982. Lipid analysis. Pergamon Press, Oxford, UK, 207pp.

CIE, 1976. Official recommendations on uniform colour space, colour difference equations and metric colour terms. Suppl. Nº 2 to CIE Publication Nº 15, Colorimetry. Commission International de l´Éclairage, Paris.

Cifuentes, A.S., González, M.A., Vargas, S., Hoeneisen, M. & González N., 2003. Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions. Bio. Res., 36: 343-357.

Clydesdale, F.M., 1993. Colour as a factor in food choice. Crit. Rev. Fd. Sci. Nut., 33: 83-101.

Coral-Hinostroza, G.N. & Bjerkeng, B., 2002. Astaxanthin from the red crab langostilla (*Pleuroncodes planipes*): optical *R/S* isomers and fatty acid moieties of astaxanthin esters. Comparative Biochemistry and Physiology, B 133 (3): 437-444.

Coral, H. G., Hubermann, A., De la Lanza, G. & Monroy-Ruiz, J., 1997. Pigmentation of the rainbow trout (*Oncorhynchus mykiss*) with oil extracted astaxanthin from the langostilla (*Pleurocondes planipes*). Archivos Latino-americanos de Nutrición, 47: 237-241.

Cortés, C., Esteve, M.J., Frígola, A. & Torregrosa, F., 2004. Identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with ultraviolet-diode array detection. J. Agric. Food Chem., 52: 2203-2212.

Craik, I.C.A., 1985. Egg quality and egg pigment content in salmonids fishes. Aquaculture, 47 (1): 61-88.

Crook, A.C., 1997. Colour patterns in a coral reef fish. Is background complexity important? J. Exp. Mar. Biol. Ecol., 217: 237-252.

Czeczuga, B. & Czeczuga-Semeniuk, E., 1998. Carotenoids in the Norway haddock *Sebastes marinus* L. Bull. Sea Fish. Inst. Gdynia, 143 (1): 3-9.

Dall, W., 1995. Carotenoids versus retinoids (vitamin A) as essential growth factors in penaeid prawns (*Penaeus semisulcatus*). Mar. Biol., 124: 209-213.

Darachai, J., Pitatiratitivorakul, S. & Menasveta, P., 1999. Effect of astaxanthin on growth and survival of *Penaeus monodon* larvae. In: Proceedings of the 37th Kasetsart University Annual Conference, 36-41. Oates, C.G., Eds.

Davies, M.L., 2003. Assessment of the south Atlantic red porgy (*Pagrus pagrus*) population under a moratorium. Thesis of Master of Science, Fisheries and Wildlife Sciences. Virginia Polytechnic Institute, Blacksburg, Virginia, USA.

Delacour, J., 1928. Food and colour retention. Avicultural Magazine, 4: 167.

Delgado, C.L., Rosegrant, M.W., Meijer, S., Wada, N. & Ahmed, M., 2002. Fish as food: projections to 2020. Reprinted in fish to 2020: implications of global fish outlook for developing countries. Penag, Malaysia: World Fish Center.

Delgado, C.L., Wada, N., Rosegrant, M.W., Meijer, S. & Ahmed, M., 2003. Fish to 2020. Supply and Demand in Changing Global Market. International Food Policy Research Institute, Washington, DC, USA, 226pp.

Deming-Adams, B., Gilmore, A. M. & Adams III, W. W., 1996. In vivo functions of carotenoids in higher plants. FASEB J., 10: 403-412.

Divanach, P., Kentouri, M., Charalambakis, G., Pouget, F., & Sterioti, A., 1993. Comparison of growth performance of six Mediterranean fish species reared under intensive conditions in Crete (Greece), in raceways with the use of self-feeders. In: Barnabe, G., Kestemont, P. (Eds.) Production, Environment and Quality. Bordeaux Aquaculture 92. Special Publication Nº 1, European Aquaculture Society, Ghent, 285-297.

Donkin, P., 1976. Ketocarotenoid biosynthesis by *Haematococcuss lacustrus*. Phytochemistry, 15: 711-715.

Doolan, B.J., Allan, G.L., Booth, M.A. & Jones, P.L., 2004. Improving skin colour in farmed snapper (red sea bream, *Pagrus auratus*).School of Ecology and Environment, Deakin University, Warrnambool, Victoria 3280, Australia. www.was.org/documents.

Droop, M.R., 1955. Carotenogenesis in *Haematococcuss pluvialis*. Nature, 175: 42.

Dufva, R. & Allander, K., 1995. Intra specific variation in plumage coloration reflects immune response in great tit (*Pagrus major*) males. Functional Ecology, 9: 785-789.

Duray, M.N., Estudillo, C.B. & Alpasan, L.G., 1996. The effect of background colour and rotifer density on rotifer intake, growth and survival of grouper. (*Epinephelus suillus*) larvae. Aquaculture, 146: 217-224.

Enzell, C., 1985. Biodegradation of carotenoids an important route to aroma compounds. Pure & Appl. Chem., 57 (5): 693-700.

Erdman, J.W.Jr., Bierer, T.L. & Gugger, E.T., 1993. Absorption and transport of carotenoids. Annuals of the New York Academy of Sciences, 691: 76-85.

FAO, 1990. Food and Agriculture Organization of the United Nations (FAO-UN) CWP Handbook of Fishery Statistical Standards - Section J: Aquaculture. FIGIS Ontology Sheets.

FAO, 2002. The State of World Fisheries and Aquaculture. FAO, Rome Fisheries Department. Fishery Information. Data and Statistics Unit. Fishstat plus: Universal software for fishery statistical times series. Version 2.3.

Folch, J., Lees, M.S. & Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem., 226: 479-509.

Foscarini, R., 1988. A Review: Intensive Farming Procedure for Red Sea Bream (*Pagrus major*) in Japan. Aquaculture, 72: 191-246.

Foss, P., Storebakken, T., Schiedt, K., Liaaen-Jensen, S., Austreng, E. & Streiff, K., 1984. Carotenoids in diets for salmonids. I. Pigmentation of rainbow trout with the individual optical isomers of astaxanthin in comparison with canthaxanthin. Aquaculture, 41: 213-226.

Foss, P., Storebakken, T., Austreng, E. & Liaaen-Jensen, S., 1987. Carotenoids in diets for salmonids. V. Pigmentation of rainbow trout and sea trout with astaxanthin and astaxanthin dipalmitate in comparison with canthaxanthin. Aquaculture, 65: 293-305.

Fox, D.L. & McBeth, J.W., 1970. Some dietary carotenoids and blood-carotenoids levels in flamingos. Comparative Biochemistry and Physiology, 34: 707-713.

Francis, F.J. & Clydesdale, F.M., 1975. Food colorimetry: theory and applications. AVI. Westport, Connecticut.

Fujii, R., 1993. Cytophysiology of fish chromatophores. International review of Cytology, 143: 191-255.

Fujii, R., 2000. The regulation of motile activity in fish chromatophores. Pigment Cell Research, 13: 300-319.

Fujita, T., Satake, M., Hikichi, S., Takeda, M., Shimeno, S., Kuwabara, H., Miki, W., Yamaguchi, K. & Konosu, S., 1983. Pigmentation of cultured yellowtail with krill oil. Bull, Jpn. Soc Sci. Fish., 49: 1595-1600.

Furr, H.C. & Clark, R.M., 1997. Intestinal absorption and tissue distribution of carotenoids. Nutritional Biochemistry, 8 : 364-377.

Gaillard, M., Juillet, C., Cézilly, F. & Perrot-Minnot, M.J., 2004. Carotenoids of two freshwater amphipod species (*Gammarus pulex* and *G. roeseli*) and their common acanthocephalan parasite *Polymorphus minutes*. Comparative Biochemistry and Physiology, B 139: 129-136.

Gobantes, I., Choubert, G., Laurentie, M., Milicua, J-C. G. & Gomez, R., 1997. Astaxanthin and canthaxanthin kinetics after ingestion of individual doses by immature rainbow trout *Oncorhynchus mykiss*. J. Agric. Food Chem., 45: 454-458.

Goda, M. & Fujii, R., 1995. Blue chromatophores in two species of callionymid fish. Zool. Sci., 12: 811-813.

Goebel, N. & Barker, M.F., 1998. Artificial diets supplemented with carotenoid pigments as feeds for sea urchin. In: Mooi, R., Telford, M. (Eds.), Echinoderms. Balkeman, San Francisco, 667-672.

Gomes, E., Dias, J., Silva, P., Valente, L., Empis, J., Gouveia, J.B. & Young, A., 2002. Utilization of natural and synthetic sources of carotenoids in the skin pigmentation of gilthead Sea bream (*Sparus aurata*). European Food Research and Technology, 214: 287-293.

Goodwin, T.W., 1951. Carotenoids in fish. Department of Biochemistry, the University of Liverpool, UK. Bioch. Soc. Symp., 6: 63-82.

Goodwin, T.W., 1984. The Biochemistry of Carotenoids: Animals, Vol. II. Chapman and Hall, London.

Goto, S., Kogure, K., Abe, K., Kimata, Y., Kitahama, K., Yamashita, E. & Terada, H., 2001. Efficient radical trapping at the surface and inside the phospholipids membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. Biochimica et Biophysica Acta, 15112: 251-258.

Gouveia, L. & Rema, P., 2005. Effect of microalgal biomass concentration and temperature on ornamental goldfish (*Carassius auratus*) skin pigmentation. Aquaculture Nutrition, 11: 19-23.

Gouveia, L., Gomes, E. & Empis, J., 1996. Potential use of microalga (*Chlorella vulgaris*) in the pigmentation of rainbow trout (*Oncorhynchus mykiss*) muscle. Zertschrift fur Lebensmittel Untersuchung und-Forschung, 202: 75-79.

Gouveia, L., Choubert, G., Gomes, E., Pereira, N., Santinha, J. & Empis, J., 2002. Pigmentation of gilthead sea bream, *Sparus aurata* (L. 1875), using *Chlorella vulgaris* (Chlorophyta, Volvocales) micro alga. Aquaculture Research, 33 (12): 987-993.

Gouveia, L., Rema, L., Pereira, O. & Empis, J., 2003. Coloring ornamental fish (*Cyprinus Carpio* and *Carassius auratus*) with microalgal biomass. Aquaculture Nutrition, 9: 123-129.

Granado, F., Olmedilla, B., Gil-Martínez, E & Blanco, I., 1998. Lutein ester in serum after lutein supplementation in human subjects. British Journal of Nutrition, 80: 445-449.

Grether, G.F., Hudon, J. & Endler, J.A., 2001. Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulate*). The Royal Society, London, UK, 268: 1245-1253.

Grung, M., D´Souza, M.L., Borowitzka, M. & Liaaen-Jensen, S., 1992. Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3´S)-astaxanthin esters. J. Appl. Phycol., 4: 165-171.

Guerin, M. & Hosokawa, H., 2001. Pigmentation of red sea bream with natural astaxanthin derived from the alga *Haematococcus pluvialis* comparison with synthetic astaxanthin. Aquaculture, book of abstracts, 263.

Guillou, A., Choubert, G. & de la Noüe, J., 1992. Absorption and blood clearance of labelled carotenoids (^{14}C-astaxanthin, ^{3}H-cantaxanthin and ^{3}H-zeaxanthin) in mature female rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol., 103 A: 301-306.

Guillou, A., Choubert, G., Storebakken, T., de la Noüe, J. & Kaushik, S., 1989. Bioconversion pathway of astaxanthin into retinol$_2$ in mature rainbow trout (*Salmo gairdnieri* Rich). Comp. Biochem. Physiol., 94 B: 481-485.

Ha, B.S., Kang, D.S., Kim, J.H., Choi, O.S. & Ryu, H.Y., 1993. Metabolism of dietary carotenoids and effects to improve the body colour of cultured flounder and red sea bream *Pagrus major*. Bulletin of the Korean Fisheries Society, 26: 91-101.

Halliwell, B., 1996. Antioxidants in human health and disease. Annu. Rev. Nutr., 16: 33-50.

Hambre, K., Christiansen, R., Waagbø, R., Maage, A., Torstensen, B.E., Lygren, B., Lie, Ø., Wathne, E. & Albrektsen, S., 2004. Antioxidant vitamins, minerals and lipid levels in diets for Atlantic salmon (*Salmo salar*, L.): effects on growth performance and fillet quality. Aquaculture Nutrition, 10: 113-123.

Hardy, R.W., Torrissen, O.J. & Scott, T.M., 1990. Absorption and distribution of C-labelled canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 87: 331-340.

Hatlen, B., Jobling, M. & Bjerkeng, B., 1998. Relationship between carotenoid concentration and colour of fillets of Artic Charr, *Salvelinus alpinus* (L.), fed astaxanthin. Aquaculture Research, 29: 191-202.

Hatlen, B., Aas, G. H., Jørgensen, E. H., Storebakken, T. & Goswami, U. C., 1995. Pigmentation of 1, 2, and 3 years old Arctic charr, *Salvelinus alpinus* (L.), fed two different dietary astaxanthin concentrations. Aquaculture, 138: 303-312.

Hatlen, B., Arnesen, A.M., Jobling, M., Siikavuopio, S. & Bjerkeng, B., 1997. Carotenoid pigmentation in relation to feed intake, growth and social interactions in Arctic charr, *Salvelinus alpinus* (L.), from two anadromous strains. Aquaculture Nutrition, 3 (3): 189-199.

Havardsson, B. & Imsland, A., 1999. The effects of astaxanthin in feeds and environmental temperature on carotenoid concentration in the gonads of the green sea urchin *Strongylocentrotus droebachiensis.* Muller. J. World Aquac. Soc., 30 (2): 208-218.

Healey, E.G., 1999. The skin pattern of young plaice and its rapid modification in response to graded changes in background tint and pattern. J. Fish Biol., 55: 937-971.

Hernández-Cruz, C.M., Salhi, M., Bessonart, M., Izquierdo, M.S., Gonzalez, M.M. & Fernández-Palacios, H., 1999. Rearing techniques for red porgy (*Pagrus pagrus*) during larval development. Aquaculture, 179 (1-4): 489-497.

Higuera-Ciapara, I., Felix-Valenzuela, L. & Goycoolea, F.M., 2006. Astaxanthin: A review of its chemistry and applications. Critical Reviews in Food Science and Nutrition, 46:186-196.

Hill, G.E., 1991. Plumage coloration is a sexually selected indicator of male quality. Nature, 350: 337-339.

Hill, G.E., 1998. An easy, inexpensive method to quantify plumage coloration. Journal of Field Ornithology, 69: 353-363.

Hirao, S. 1967. Carotenoid pigments in fish. A review of carotenoids in fish. Bull. Jap. Soc. Sci. Fish., 33 (9): 866-871.

Hirschberg, J., 1999. Production of high-value compounds: carotenoids and vitamin E. Current Option in Biotechnology, 10: 186-191.

Houde, A.E. & Torio, A.J., 1992. Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology, 3: 346-351.

Hudon, J., 1994. Showiness, carotenoids and captivity: a comment on Hill (1992). Auk., III: 218-221.

Hudon, J., Grether, G.F. & Millie, D.F., 2003. Marginal differentiation between the sexual and general carotenoid pigmentation of guppies (*Poecilia reticulate*) and a possible visual explanation. Physiological and Biochemical Zoology, 76 (6): 776-790.

Hunt, R.W.G., 1977. The specification of colour appearance: I. Concepts and terms. Colour Res. Appl., 2: 55-68.

Inborr, J. & Lignell, Å., 1997. Effect of feeding astaxanthin-rich algae meal (*Haematococcus pluvialis*) on performance and carotenoid concentration of different tissues of broiler chickens. In: Proceedings of the XIII WPSA Conference on Poultry Meat Quality in Poznan, Poland, September 22–25, Session M1, 39-43.

Ingle de la Mora, G., 2002. Incremento al valor agregado de salmónidos a través de su pigmentación con colorantes naturales. In: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Gaxiola-Cortés, M.G., Simoes, N. (Eds). Avances en nutrición acuícola VI. Memorias del VI Simposium Internacional de Nutrición Acuícola 3 al 6 septiembre 2002. Cancún, México.

Ito, Y., Kamata, T. & Sameshima, M., 1986. Studies on the improvement of body colour of red sea bream *Pagrus major* by astaxanthin and astaxanthin dipalmitate. Suisanzoshoku, 34: 77-80.

Iwamoto, R.N., Myers, J.M. & Hershberger, W.K., 1990. Heritability and genetic correlations for flesh coloration in pen reared coho salmon. Aquacuture, 86: 181-190.

Izquierdo, M. S., Watanabe, T., Takeuchi, T., Arakawa, T. & Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red sea bream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), the Current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, 221 -232.

Izquierdo, M.S., Kalinowski, C.T., Thongrod, S.Y. & Robaina, L.E., 2005. Nutritional needs for correct pigmentation in European red porgy *Pagrus pagrus In:* Nutritional Biotechnology in the Feed and Food Industries. T.P. Lyons y K.A. Jacques Eds. Nottingham Univ. Press, 307-323.

Johnson, E.A. & An, G.H., 1991. Astaxanthin from microbial sources. Critical reviews of Biotechnology, 11: 297-326.

Johnson, E. A. & Schroeder, W. A., 1995. Microbial carotenoids. In: Fiechter (Eds). Advances in Biochemical Engineering. Berlin: Springer, 119-178.

Johnson, E.A., Villa, T.G. & Lewis, M., 1980. *Phaffia rhodozyna* as an astaxanthin source in salmonids diets. Aquaculture, 20: 123-134.

Johnson, L., 1992. Recovery of pigments an chitin from pink shrimp peeling wastes. In Advances in seafood biochemistry: Composition and quality, Flick, G.J. and Martin, R.E., Eds., Technomic Publishing Co. Inc., Lancaster and Basel, 123.

Jyounchi, H., Zhang, L., Gross, M. & Tomita, Y., 1993. Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances *in vivo* antibody production to T-dependant antigens without facilitating polyclonal B-cell activation. Nutr. Cancer, 19: 269-280.

Kalinowski, C.T., Robaina, L.E., Fernández-Palacios, H., Schuchardt, D.H. & Izquierdo, M.S., 2005. Effect of different carotenoid sources and their dietary levels on the red porgy (*Pagrus pagrus*) growth and skin colour. Aquaculture, 244 (1-4): 223-231.

Katayama, T., Ikeda, N. & Harada, K., 1965. Carotenoids in sea bream, *Chrysophrys major* Temmick and schlegel. Bull. Jpn. Soc. Sci. Fish., 31: 947-952.

Katayama, T., Yokoyama, H. & Chichester C.O., 1970. The biosynthesis of astaxanthin 1. The structure of α-doradexanthin and β-doradexanthin. Int. J. Biochem., 1: 438-444.

Katayama, T., Shintani, K. & Chichester, C.O., 1973. The biosynthesis of astaxanthin – VII. The carotenoids in sea bream, *Chrysophrys major* Temmick and Schlegel. Comparative biochemistry and physiology part B: Biochemistry and Molecular Biology, 44 (1): 253-257.

Katsuyama, M., Komori, T. & Matsuno, T., 1987. Metabolism of three steroisomers of astaxanthin in fish, rainbow trout and tilapia. Comp. Biochem. Physiol., 86 B: 1-5.

Kawakami, T., Tsushima, M., Katabami, Y., Mine, M., Ishida, A. & Matsuno, T., 1998. Effect of beta, beta-carotene, beta-echinenone, astaxanthin, fucoxanthin, vitamin A and vitamin E on the biological defense of the sea urchin *Pseudocentrotus depressus.* J. Exp. Mar. Biol. Ecol., 226 (2): 165-174.

Kennedy, G. Y. & Vevers, H. G., 1976. A survey of avian eggshell pigments. Comp. Biochem. Physiol., 55 B: 117-123.

Kentouri, M., O'Neil, D., Divanach, P. & Charalambakis, G., 1994. A study of the quantitative water requirements of red porgies *Pagrus pagrus* L. (Pisces: Sparidae), during early on growing under self-feeding conditions. Aquacult. Fish. Manage., 25: 741-752.

Kentouri, M., Pavlidis, M., Papandroulakis, N. & Divanach, P., 1995. Culture of red porgy, *Pagrus pagrus,* in Crete. Present knowledge, problems and perspectives. Cahiers Options Méditerranéennes, 16: 65-78.

Khachick, F. & Beecher, G.R., 1987. Application of a C-45-β-Carotene as an Internal Standard for the quantification of Carotenoids in Yellow/Orange vegetables by Liquid chromatography. J. Agric. Food Chem., 35: 732-738.

Kiessling, A., Dosanjh, B., Higgs, D., Deacon, G. & Rowshandeli, N., 1995. Dorsal aorta cannulation: a method to monitor changes in blood levels of astaxanthin in voluntarily feeding Atlantic salmon, *Salmo salar* L. Aquac. Nutr., 1: 43-50.

Kiessling, A., Espe, M., Ruohonen, K. & Mørkøre., 2004. Texture, gaping and colour of fresh and frozen Atlantic salmon flesh as affected by pre-slaughter iso-eugenol or CO_2 anaesthesia. Aquaculture, 236: 645-657.

King, T.L., 1996. Use of colorimetric analysis to estimate salmonids flesh carotenoid content. The progressive Fish-Culturist, 58: 215-218.

Kobayashi, M. & Sakamoto, Y., 1999. Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. Biotechnology Letters, 21 (4): 265-269.

Kodric-Brown, A., 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology, 17: 199-206.

Kodric-Brown, A., 1989. Dietary carotenoids and male mating sucess in the guppy: an environmental component to female choice. Am. Nat., 124: 309-323.

Kokokiris, L., 1998. The reproductive cycle and hermaphrodite pattern in *Pagrus pagrus.* Ph.D. Thesis, University of Crete, pp. 219.

Koteng, D. F., 1992. Markedsundersøkelse Norsk Laks. FNL Bergen, Norway.

Kotic, I.V., Tolokonnikov, G.Y. & Dubrovin, V., 1974. The effect of krill meal additions to feeds on muscle pigmentation in the rainbow trout, *Salmo gairdneri*. J. Ichtyol., 19: 114-123.

Krinsky, N., Wang, X. D., Tang, G. & Russell, R. M., 1993. Mechanisms of carotenoids cleavage to retinoids. Ann. N.Y. Acad. Sci., 691: 167-176.

Krishna, K. B. & Mohanty, P., 1998. Secondary carotenoid production in green algae. J. Sci. Industr. Res., 57: 51-63.

Labropoulou, M., Machias, A. & Tsimenides T., 1999. Habitat selection and diet of juvenile red porgy, *Pagrus pagrus* (Linnaeus, 1758). Fisheries Bulletin, 97: 495-507.

Lang, M.R. & Wells, J. W., 1987. A review of eggshell pigmentation. Worlds Poultry Sci. J., 43: 238-246.

Latscha, T., 1991. Carotenoids in aquatic animal nutrition. In D.M. Akiyama and R.K.H. Tan, Eds. Proceeding of the Aquaculture Feed Processing, 68-79.

Lee, H.S., Castle, W.S. & Coates, G.A., 2001. High-performance liquid chromatography for the characterization of carotenoids in the new sweet orange (Earlygold) grown in Florida, USA. Journal of Chromatography, A 913: 371-377.

Lee, J.E. & Peniston, Q., 1982. Utilization of shellfish waste for chitin and chitosan production. In: Chemistry and Biochemistry of Marine Food Products: 415. Martin, R., Flick, G., & Hebard, C., (Eds.). Avi Wesport., CT. USA.

Liaaen-Jensen, S., 1978. Marine Carotenoids. In: Scheuer, P. J., (Eds). Marine Natural Products. Chemical and Biological Perspectives, Vol. 2. Academic Press, 1.

Lin, M.Q., Ushio, H., Ohshima, T., Yamanaka, H. & Koizumi, C., 1998. Skin color control of the red sea bream (*Pagrus major*). Lebensm.-Wiss.u.-Technol., 31: 27-32.

Liñán-Cabello, M.A., Paniagua-Michel, J. & Zenteno-Savin, T., 2003. Carotenoids and retinal levels in captive and wild shrimp, *Litopenaeus vannamei.* Aquaculture Nutrition, 9: 383-389.

Liu, B.H. & Lee, Y.K., 1999. Composition and biosynthetic pathways of carotenoids in the astaxanthin-producing green alga *Chlorococcum* sp. Biotechnology Letters, 21: 1007-1010.

Lorenz, T.R., 1998. A review of astaxanthin as a carotenoid and vitamin source for sea bream. Naturerose Technical Bulletin Nº 052. Cyanotech Corporation, Hawaii, USA.

Lorenz, T.R. & Cysewski, G.R., 2000. Commercial potential for *Haematococcus* micro algae as a natural source of astaxanthin. Trends Biotechnol., 18 (4): 160-167.

Lotocka, M. & Styczyńska-Jurewics, E., 2001. Astaxanthin, canthaxanthin and astaxanthin esters in the copepod *Arcartia bifilosa* (Copepoda, Calanoida) during ontogenetic development. Oceanologia, 43 (4): 487-497.

Lotthammer, K.H., 1978. Original researches. In: Importance of Beta-carotene for Bovine Fertility.Proc. Roche Symposium London. Hoffmann-La Roche, Basel Switzerland.

Louis, E.D., Jurewicz, E.C., Watner, D. & Factor-Litvak, P., 2002. Skin color and the risk and severity of essential tremor: a reflectance spectroscopy study. Parkinsonism and related disorders, 9: 239-241.

Lowe, G.M., Vlismas, K. & Young, A.J., 2003. Carotenoids as prooxidants? Molecular Aspect of Medicine.

Lubzens, E., Lissauer, L., Levavi-Sivan, B., Avarre, J-C. & Sammar, M., 2003. Carotenoid and retinoid transport to fish ocytes and eggs: what is the role of retinol binding protein? Molecular Aspects of Medicine, 24: 441-457.

Manooch, C.S. & Hassler, W.W., 1978. Synopsis of biological data on the red porgy, *Pagrus pagrus* (Linnaeus). NOAA Technical Report NMFS Circular, 412: 1-18.

Mantiri, D.M.H., Nègre-Sardargues, G., Charmantier, G., Trilles, J-P., Milicua, J-C.G. & Castillo, R., 1996. Nature and metabolism of carotenoid pigments during the embryogenesis of the European lobster *Homarus gammarus* (Linné, 1758). Comparative Biochemistry and Physiology, 115A (3): 237-241.

March, B.E., Hajen, W.E., Deacon, G., MacMillan, C. & Walsh, M.G., 1990. Intestinal absorption of astaxanthin, plasma astaxanthin concentration, body weight, and metabolic rate as determination of flesh pigmentation in salmonids fish. Aquaculture, 90: 313-322.

Matsui, K., Marunouchi, J. & Nakamura, M., 2002. An ultra structural and carotenoid analysis of the red ventrum of the Japanese newt, *Cynops pyrrhogaster*. Pigment Cell Res., 15: 265-272.

Matsui, K., Takaichi, S. & Nakamura, M., 2003. Morphological and biochemical changes in carotenoid granules in the ventral skin during growth of the Japanese newt *Cynops pyrrhogaster*. Zoological Science, 20: 435-440.

Matsuno, T., 1991. Xanthophylls as precursors of retinoids. Pure & Appl. Chem., 63 (1): 81-88.

Matsuno, T. & Hirao, S., 1989. Marine carotenoids. In: Marine Biogenic Lipids, Fats and Oils. Vol. 1, R. G. Ackman (Eds), CRC press, Boca Raton, Florida, 251-388.

Matsuno, T. & Tsushima, M., 1995. Comparative biochemical of carotenoids in sea cucumbers. Comparative Biochemistry and Physiology, III B (4): 597-605.

Matsuno, T. & Tsushima, T., 2001. Carotenoids in the sea urchins. In: Lawrence, J.M. (Eds), Edible sea urchins: Biology and Ecology. Amsterdam, Elsevier, 115-138.

Matsuno, T., Ohkubo, M., Toriiminami, Y., Tsushima, M., Sakaguchi, S., Minami, T. & Maoka, T., 1999. Carotenoids in food Chain between freshwater fish and aquatic insects. Comparative Biochemistry and Physiology, B 124: 341-345.

McBride, S.C., Pinnix, W.D., Lawrence, J.M., Lawrence, A.L. & Mulligan, T.M., 1997. The effect of temperature on production of gonads by the sea urchin *Strongylocentrotus franciscanus* fed natural and prepared diets. Muller. J. World Aquac. Soc., 28: 357-365.

McCoy, M., 1999. Astaxanthin market a hard one to crack. Chemical Engineering News, April, 5: 15-17.

McGraw, K.J. & Hill, G.E., 2000. Differential effects of parasitism on the expression of carotenoid and melanin based ornamental coloration. Proceeding of the Royal Society of London, B 267: 1525-1531.

McGraw, K.J. & Hill, G.E., 2001. Carotenoids access and intraspecific variation in plumage pigmentation in male American goldfinches (*Carduelis tristis*) and Northern Cardinals (*Cardinalis cardinalis*). Functional Ecology, 15 (6): 732-739.

McLean, J.A., Karadas, F., Surai, P.F., McDevitt, R.U.M. & Speake, B.K., 2005. Lipid-soluble and water-soluble antioxidant activities of the avian intestinal mucosa at different sites along the intestinal tract. Comparative Biochemistry and Physiology, B 141: 366-372.

Mendes-Pinto, M.M., Choubert, G. & Morais, R., 2004. Effect of dietary bile extracts on serum response of astaxanthin in rainbow trout (*Oncorhynchus mykiss*): a preliminary study. Aquaculture Nutrition, 10: 353-357.

Metusalach, Synowiecki, J. & Brown, J., 1995. A review of carotenoid of pigmentation additives in fish feeds. J. Agric. Food Chem., 43: 1570-1573.

Metusalach, Brown, J.A. & Shahidi, F., 1997. Effects of stocking density on colour characteristics and deposition of carotenoids in cultured Arctic charr (*Salvelinus alpinus*). Food Chemistry, 59 (1): 107-114.

Metusalach, Synowiecki, J., Brown, J. & Shahidi, F., 1996. Deposition and metabolism of dietary canthaxanthin in different organs of Arctic charr (*Salvelinus alpinus* L.). Aquaculture, 142: 99-106.

Meyers, S. P., 1977. Using crustacean meals and carotenoid fortified diets. Feedstuffs, 43: 26-28.

Meyers, S.M. & Latscha, T., 1997. Carotenoids. In: Crustacean Nutrition Advances in World Aquaculture, (Abramo, L.R.D., Conkli, D.E., Akiyama, D.M., Eds.). World Aquaculture Society, Baton Rouge, LA, 6: 164-193.

Meyer, P., Riesen, R. & Pfander, H., 1995. Example 10: Carotenoid Glycosides and Glycosyl Esters. In: Carotenoids Isolation and Analysis, Birkäuser Verlag, Basel, Vol 1 A.

Miki, W., 1991. Biological functions and activities of animal carotenoids. Pure & Appl. Chem., 63 (1): 141-146.

Miki, W., Yamaguchi, K., Konosu, S., Takane, T., Satake, M., Fujita, T., Kuwabara, H., Shimeno, S. & Takeda, M., 1985. Origino of tunaxanthin in the integument of yellowtail (*Seriola quinqueradiata*). Biochemistry and Molecular Biology, 80 (2): 195-201.

Mihelakakis, A., Yoshimatsu, T. & Tsolkas, C., 2001. Spawning in captivity and early life history of cultured red porgy, *Pagrus pagrus*. Aquaculture, 199 (3-4): 333-352.

Moaka, T., Yokoi, S. & Matsuno, T., 1989. Comparative Biochemical studies of carotenoids of nine species of Cephalopoda. Comp. Biochem. Physiol., 92 B: 247-250.

Morrisey, P.A., Sheehy, P.J.A., Galvin, K., Kerry, J.P. & Buckley, D.J., 1998. Lipid stability in meat and meat products. Meat Science, 49 (1): 573-586.

Mouly, P.P., Gaydou, E.M. & Corsetti, J., 1999. Characterization of paprika (*Capsicum annuum*) extract in orange juices by liquid chromatography of carotenoid profile. J. Agric. Food Chem., 47: 968-976.

Nakamura, K.M., Hata, M. & Hata, M., 1985. A study on astaxanthin in salmon *Oncorhynchus keta* serum. Bull. Jap. Soc. Sci. Fish., 51: 979-983.

Nakano, T., Tosa, M. & Takeuchi, M., 1995. Improvement of biochemical features in fish health by red yeast and synthetic astaxanthin. J. Agric. Food Chem., 43: 1570-1573.

Nakano, T., Kanmuri, T., Sato, M. & Takeuchi, M., 1999. Effect of astaxanthin rich red yeast (*Phaffia rhodozyma*) on oxidative stress in rainbow trout. Biochimica et Biophysica Acta, 1426: 119-125.

Nakazoe, J., Ishii, S., Kamimoto, H. & Takeuchi, M., 1984. Effects of supplemental carotenoid pigments on the carotenoid accumulation in young red sea bream *Chrysophrys major.* Bull. Tokai Regional Fish. Res. Lab., 113: 29-41.

Nègre-Sadargues, G., Castillo, R., Petit, S., Gomez, R., Milicua, J-C.G. & Choubert, G., 1992. Utilization of synthetic carotenoids by the prawn *Penaeus japonicus* (Crustacean Decapoda) reared under laboratory conditions. MNHN, Paris, France, p. 107.

Negro, J.J. & Garrido-Fernández, J., 2000. Astaxanthin is the major carotenoid in tissues of white storks (*Ciconia ciconia*) feeding on introduced crayfish (*Procambarus clarkia*). Comparative Biochemistry and Physiology, B 126: 347-352.

Nelis, H.J.C.F., Lavens, P., Van Steenberge, M.M.Z., Sorgeloos, P., Criel, G.R. & De Leenheer, A.P., 1988. Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia.* Journal of lipid Research, 29.

Nickell, D.C. & Bromage, N.R., 1998a. The effect of dietary lipid level on variation of flesh pigmentation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 161: 237-251.

Nickell, D.C. & Bromage, N.R., 1998b. The effect of timing and duration of feeding astaxanthin on the development and variation of fillet colour and efficiency of pigmentation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 169: 233-246.

Nishigaki, I., Dmitrovski, A.A., Miki, W. & Yagi, K., 1994. Suppressive effect of astaxanthin on lipid peroxidation induced in rats. J. Clin. Biochem. Nutr., 16: 161-166.

Nixon, J. C., 1985. Naturally occuring pterins. In: Folates and pterins, Blaxley and Benkovic (Eds.), John Wiley & Sons, 2: 1.

Nyambaka, H. & Ryley, J., 1996. An asocratic recersed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids (α- and β-carotene) in dark green vegetables. Food Chem., 55: 63-72.

Ohkubo, M., Tsushima, M., Maoka, T. & Matsuno, T., 1999. Carotenoids and their metabolism in the goldfish *Carassius aurata* (Hibuna). Comparative Biochemistry and Physiology, B 124: 333-340.

Okada, S., Nur-E-Borhan, S. A., Watanabe, S. & Yamaguchi, K., 1994. Pigmentation of cultured black tiger prawn by feeding a Spirulina supplemented diet. 3. International Marine Biotechnology Conference, Tromsoe, Norway, Tromsoe University, Tromsoe, p. 61.

Olsen, R.E. & Baker, R.T.M., 2006. Lutein does not influence flesh astaxanthin pigmentation in the Atlantic salmon (*Salmo salar* L.). Aquaculture, 258 (1-4): 558-564.

Olson, V.A. & Owen, I.P.F., 1998. Costly sexual signals: are carotenoids rare, risky or required? Trend in Ecology and Evolution, 13: 510-514.

Østerlie, M., Bjerkeng, B. & Liaaen-Jensen, S., 1999. Accumulation of astaxanthin all-*E*, 9*Z* and 13*Z* geometrical isomers and 3 and 3'*RS* optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. American Society for Nutritional Sciences, 391-398.

Page, G.I. & Davies, S.J., 2002. Astaxanthin and canthaxanthin do not induce liver or kidney xenobiotic-metabolizing enzymes in rainbow trout (*Oncorhynchus mykiss* Walbaum). Comparative Biochemistry and Physiology, C 133: 443-451.

Page, G.I., Russell, P.M. & Davies, S.J., 2005. Dietary carotenoid pigment supplementation influences hepatic lipid and mucopolysaccharide levels in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology, 142 B: 398-402.

Pajuelo, J.G. & Lorenzo, J.M., 1996. Life history of the red porgy *Pagrus pagrus* (Teleostei: Sparidae) of the Canary Islands, Central East Atlantic. Fisheries Research, 28: 163-177.

Palace, V.P., Khaper, N., Quin, Q. & Singal, P.K., 1999. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Free radical & Medicine, 26 (5-6): 746-761.

Palozza, P. & Krinsky, N. I., 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Archives of Biochemistry and Biophysics, 297: 291-295.

Papoutsoglou, S.E., Mylonakis, G., Miliou, H., Karakatsouli, N.P. & Chadio, S., 2000. Effects of background colour on growth performances and physiological responses of scaled carp (*Cyprinus carpio* L.) reared in a close circulating system. Aquacult. Eng., 22: 300-318.

Parker, R.S., 1996. Absorption, metabolism and transport of carotenoids. FASEB Journal, 10: 542-551.

Pavlidis, M., Papandroulakis, N. & Divanach, P., 2006. Coloration pattern of red skin *Sparidae* (red porgies): A method for the comparison of chromaticity parameters. Aquaculture, 258 (1-4): 211-219.

Pearce, C.M., Daggett, T.L. & Robinson, S.M.C., 2004. Effect of urchin size and diet on gonad yield and quality in the green sea urchin (*Strongylocentrotus droebachiensis*). Aquaculture, 233: 337-367.

Peters, T., 1996. All about albumin: Biochemistry, Genetics, and Medical Applications. Academic Press, San Diego.

Petit, H., Negre-Sadargues, G., Castillo, R. & Trilles, J-P., 1997. The effects of dietary astaxanthin on growth and moulting cycle of post larval stages of the prawn, *Penaeus japonicus* (Crustacean, Decapoda). Comp. Biochem. Physiol., 117 A (4): 539-544.

Pfander, H., 1987. Key to carotenoids. Birkhäuser, Basel.

Plank, L.R., Lawrence, J.M., Lawrance, A.L. & Olvera, R., 2002. The effect of dietary carotenoids on gonad production and carotenoid profiles in the sea urchin *Lytechinus variegates*. J. World Aquac. Soc., 33 (2): 127-137.

Quackenbush, J., 1987. Reverse phase HPLC separation of cis- and trans-carotenoids and its application to β-carotenes in food materials. Journal of liquid chromatography, 10 (4): 643-653.

Raila, J., Mathews, U. & Schweigert, F.J., 2001. Plasma transport and tissue distribution of β-carotene, vitamin A and retinol-binding protein in domestic cats. Comparative Biochemistry and Physiology, A 130: 849-856.

Raila, J., Schuhmacher, A., Gropp, J. & Schweigert, F.J., 2002. Selective absorption of carotenoids in the common green iguana (*Iguana iguana*). Comparative Biochemistry and Physiology, A 132: 513-518.

Řehulka, J., 2000. Influence of astaxanthin on growth rate, condition, and some blood indices of rainbow trout, *Oncorhynchus mykiss.* Aquaculture, 190: 27-47.

Restrøm, B., Borch, G., Skulberg, O.M. & Liaaen-Jensen, S., 1981a. Optical purity of (3S, 3´S)-astaxanthin from *Haematococcus pluvialis*. Phytochemistry, 20 (11): 2561-2564.

Restrøm, B., Borch, G. & Liaaen-Jensen, S., 1981b. Fatty acid composition of some esterified carotenols. Comp. Biochem. Physiol., 69 B: 625-627.

Robaina, L., Moyano, F.J., Izquierdo, M.S., Socorro, J., Vergara, J.M. & Montero, D., 1997. Corn gluten, meat and bone meals as protein sources in diets for gilthead sea bream (*Sparus aurata*): nutritional and histological implications. Aquaculture, 157: 347-359.

Robinson, S.M.C., Castell, J.S. & Kennedy, E.J., 2002. Developing suitable colour in the gonads of cultured green sea urchins (*Strongylocentrotus droebachiensis*). Aquaculture, 206: 289-303.

Rønnestad, I., Helland, S. & Lie, Ø., 1998. Feeding *Artemia* to larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) results in lower larval vitamin A content compared with feeding copepods. Aquaculture, 164: 159-164.

Rotlant, J., Tort, L., Montero, D., Pavlidis, M., Martinez, M., Wenderlaar Bonga, S.E. & Balm, P.H.M., 2003. Background colour influence on the stress response in cultured red porgy *Pagrus pagrus.* Aquaculture, 223: 129-139.

Salguero, A., de la Morena, B., Vigara, J., Vega, J.M., Vilchez, C. & Leon, R., 2003. Carotenoids as protective response against oxidative damage in *Dunaliella bardawil*. Biomolecular Engineering, 20: 249-253.

Satio, A. & Regier, L.W., 1971. Pigmentation on brook trout (*Salvelinus fontalis*) by feeding dry crustacean waste. J. Fish. Res. Board Can., 26: 357-360.

Schiedt, K., 1998. Absorption and metabolism of carotenoids in birds, fish and crustacean. In: Britton, G., Liaaen-Jensen, S., Pfander, H., (Eds). Carotenoids, vol. 3. Birkäusen, Basel, pp 285.

Schiedt, K. & Liaaen-Jensen, K., 1995. Isolation and analysis. In: G. Britton, S. Liaaen-Jensen and H. Pfander, Editors, Carotenoids, Isolation and Analysis**,** Birkhäuser, Basel, 1 A: 81-108.

Schiedt, K., Bischof, S. & Glinz, E., 1991. Recent progress on carotenoid metabolism in animals. Pure & Appl. Chem., 63 (1): 89-100.

Schiedt, K., Leuenberger, F.J., Vecchi, M. & Glinz, E., 1985. Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. Pure Appl. Chem., 57: 685-692.

Schierle, J., Ardí, W., Faccin, N., Bühler, I. & Schüep, W., 1995. Example 8: Geometrical Isomer and β,β-Carotene. A rapid routine method for quantitative determination. In: Carotenoids Isolation and Analysis, Birkäuser Verlag, Basel, Vol 1 A.

Schmidt, K., Connor, A. & Britton, G., 1994. Analysis of pigments: carotenoids and related polyenes. In: Goodfellow, M., O´Donell, A.G., (Eds). Chemical methods in procaryotic systematics, pp 403.

Schuchardt, D., Vergara, J.M., Robaina, L. & Montero, D., 2000. The effects of varying dietary protein and lipid levels on growth, feed efficiency, protein utilization and body composition of red porgy fingerlings. The Ninth Intern. Symp. on Nutrition and Feeding in Fish 2000, Miyasaki, Japan, p. 91.

Schüep, W. & Schierle, J., 1995. Example 9: Astaxanthin. Determination of stabilized, added astaxanthin in fish feed and pre-mixes. In: Carotenoids Isolation and Analysis, Birkäuser Verlag, Basel, 1 A: 273-276.

Schüep, W., Hess, D. & Schierle, J., 1995. Example 7: Human Plasma. Simultaneous determination of carotenoids, retinol and tocopherols. In: Carotenoids Isolation and Analysis, Birkäuser Verlag, Basel, 1 A.

Segner, H., Arend, P., Von Poeppinghaussen, K. & Schmidt, H., 1989. The effect of feeding astaxanthin to *Oreochromis niloticus* and *Colisa labiosa* on the histology of the liver. Aquaculture, 79: 381-390.

Shahidi, F., 1995. Role of chemistry and biotechnology in value-added utilization of shellfish processing discards. Can. Chem. News, Sept., 25-29.

Shahidi, F. & Synowiecki, J., 1991. Isolation and characterization of nutrients and value-added produces from snow crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. J. Agric. Food Chem., 39: 1527-1532.

Shahidi, F., Synowiecki, J. & Penney, R.W., 1994. Chemical nature of xanthophylls in flesh and skin of cultured arctic char (*Salvelinus alpinus* L.). Food Chemistry, 51: 1-4.

Shahidi, F., Metusalach, A. & Brown, J.A., 1998. Carotenoid pigments in seafood and aquaculture. Crit. Rev. Food Sci. Nutr., 38: 1-67.

Sheehan, E.M., O'Connor, T.P., Sheehy, P.J.A., Buckley, D.J. & FitzGerald, R., 1998. Stability of astaxanthin and canthaxanthin in raw and smoked Atlantic salmon (*Salmo salar*) during frozen storage. J. Agric. Food Chem., 63 (3): 313-317.

Sheridan, M.A., 1988. Lipid dynamics in fish: absorption, transport, deposition and mobilization. Comp. Biochem. Physiol., 90 B: 679-690.

Shimidzu, N., Goto, M. & Miki, W., 1996. Carotenoids as singlet oxygen quenchers in marine organisms. Fisheries Science, 62 (1): 134-137.

Shina, A., Gross, J. & Lifshitz, A., 1978. Carotenoids of the invertebrates of the Red Sea (Eilat Shore): II. Carotenoid pigments in the gonad of the sea urchin *Tripneustes gratilla* (Echinodermata). Comp. Biochem. Physiol., 61 B: 123-128.

Shpigel, M., McBride, S.C., Marciano, S. & Lipatch, I., 2004. The effect of photoperiod and temperature on the reproduction of European sea urchin, *Paracentrotus lividus.* Aquaculture, 232: 1-4.

Shpigel, M., McBride, S.C., Marciano, S., Ron, S. & Ben-Amotz, A., 2005. Improving gonad colour and somatic index in the European sea urchin *Paracentrotus lividus*. Aquaculture, 245: 101-109.

Sies, H., 1986. Biochemistry of oxidative stress. Angew. Chem. Int. Eds. Engl., 25: 1058-1071.

Sigurgisladottir, S., Torrissen, O., Lie, Ø., Thomassen, M. & Hafsteinsson, H., 1997. Salmon quality: Methods to determine the quality parameters. Reviews in Fisheries Sciences, 5 (3): 223-252.

Simpson, B.K. & Haard, N.F., 1985. The use of proteolyc enzymes to extract carotenoproteins from shrimp processing wastes. J. Appl. Biochem., 7: 212-222.

Skrede, G., Storebakken, T. & Næs, T., 1989. Color evaluation in raw, baked and smoked flesh of rainbow trout (*Oncorhynchus mykiss*) fed astaxanthin or canthaxanthin. J. Food Sci., 55: 1574-1578.

Smith, B.E., Hardy, R.W. & Torrissen, O.J., 1992. Synthetic astaxanthin deposition in pan-size coho salmon (*Oncorhynchus kisutch*). Aquaculture, 104: 105-119.

Sommer, T.R., Potts, W.T. & Morrissy, N.M., 1991. Utilization of micro algal astaxanthin by rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 94: 79-88.

Sommer, T.R., D´Souza, F.M.L. & Morrissy, N.M., 1992. Pigmentation of adult rainbow trout, *Oncorhynchus mykiss*, using the green alga *Haematococcus pluvialis.* Aquaculture, 106: 63-74.

Spinelli, J. & Mahnken, C., 1978. Carotenoid deposition in pen-reared salmonids fed diets containing oil extracts of red crab (*Pleurocodes planipes*). Aquaculture, 13: 213 -223.

Spinelli, J., Lehman, L. & Wieg, D., 1974. Composition, processing and utilization of red crab (*Pleurocondes planipes*) as an aquacultural feed ingredients. J. Fish Res. Bd. Can., 31: 1025-1029.

Stahl, W. & Sies, H., 2003. Antioxidant activity of carotenoids. Institut für Biochemie und Molekularbiologie I, Heinrich-Heine Universität Düsseldorf, P.O. Box 101007, D-40001 Dusserldorf, Germany. Molecular Aspect of Medicine, 24: 345-351.

Stahl, W., Schwarz, W. & Sies, H., 1993. Human serum concentrations of all-trans-beta- carotene and alpha-carotene but not 9-cis-beta-carotene increase upon ingestion of a natural isomer mixture obtained from *Dunaliella salina* (Betatene). J. Nutr., 123: 847-851.

Stephanou, D., Georgiou, G. & Shourkri, E., 1995. Reproduction and larval rearing of the common sea bream (*Pagrus pagrus*), an experimental culture. Cahiers Options Méditerranéennes, 16: 79-87.

Storebakken, T. & No, H.K., 1992. Pigmentation of rainbow trout. Aquaculture, 100: 209-229.

Storebakken, T. & Goswami, U.C., 1996. Plasma carotenoid concentration indicates the availability of dietary astaxanthin for Atlantic salmon, *Salmo salar.* Aquaculture, 146: 147-153.

Storebakken, T., Foss, P., Schiedt, K., Austreng, E., Jensen, S.L. & Manz, U., 1987. Carotenoids in the diets for salmonids IV. Pigmentation of Atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. Aquaculture, 65: 279-292.

Storebakken, T., Sørensen, M., Bjerkeng, B. & Hiu, S., 2004. Utilization of astaxanthin from red yeast, *Xanthophyllomyces dendrorhous*, in rainbow trout *Oncorhynchus mykiss*: effects of enzymatic cell wall disruption and feed extrusion temperature. Aquaculture, 236: 391-403.

Sugimoto, M., 1993. Morphological colour changes in the medaka *Oryzias latipes*, after prolonged background adaptation-I. Changes in the population and morphology of the melanophores. Comp. Biochem. Physiol., A 104: 513-518.

Sugimoto, M., 2002. Morphological colour changes in fish: regulation of pigment cell density and morphology. Microscopy Research and Technique, 58: 496-503.

Supamattaya, K., Kiriratnikom, S., Boonyaratpalin, M. & Borowitzka, L., 2005. Effect of a *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp (*Penaus monodon*). Aquaculture, 248: 207-216.

Sylvia, G., Morrisey, M.T., Graham, T. & Garcia, S., 1995. Organoleptic qualities of farmed and wild salmon. J. Aquat. Food Prod. Technol., 4: 51-64.

Sylvia, G., Morrisey, M.T., Graham, T. & Garcia, S., 1996. Changing trends in seafood markets: the case of farmed and wild salmon. J. Food Prod. Market, 3: 49-63.

Szisch, V., van der Salm, A.L. & Wendelaar Bonga, S.E., 2002. Physiological colour changes in the red porgy, *Pagrus pagrus*, following adaptation to blue lighting spectrum. Fish physiology & Biochemistry, 27: 1-8.

Tacon, A.G.J., 1981. Speculative review of possible carotenoid functions in fish. Prog. Fish-Cult., 43: 205-208.

Tanaka, Y., 1978. Comparative biochemical studies on carotenoids in aquatic animals. Mem. Fac. Fish. Kagoshima Univ., 27: 355-422.

Tanaka, Y., Katayama, K.L., Simpson, K. & Chichester, C.O., 1976. The carotenoids in marine red fish and the metabolism of the carotenoids in sea bream, *Chrysophrys major* Temminch and Schegel. Bull. Jpn. Soc. Sci. Fish., 42: 1177-1182.

TECAM, 1999. Survey on Mediterranean Marine Finfish Species Diversification. Marine finfish species diversification: current situation and prospects in Mediterranean aquaculture. Cahiers Options Méditerranéennes, Ser. B: Études et Recherches, 24: 169.

Tejera, N., Cejas, J.R., Jerez, S., Rodríguez, C., Bolaños, A., Santamaría, F.J. & Lorenzo, A., 2003. Efecto de diferentes fuentes de astaxanthina en la dieta sobre la pigmentación de alevines de bocinegros (*Pagrus pagrus*). IX Congreso Nacional de Acuicultura, Cádiz, Libro de actas, 468-469.

Tejera, N., Cejas, J.R., Bolaños, A., Rodríguez, C., Jerez, S., Villamandos, J.E. & Lorenzo, A., 2005. Los carotenoides en la pigmentación del bocinegro (*Pagrus pagrus*). X Congreso nacional de Acuicultura Gandia, (Valencia), libro de actas, 1: 42-43.

Tomita, H., 1961. Melanin formation in the medaka. http://biol1.bio.nagoya-u.ac.jp:8000/melanin.html

Thompson, I., Choubert, G., Houlihan, D.F. & Secombes, C.J., 1995. The effects of dietary vitamin A and astaxanthin on the immunocompetence of rainbow trout. Aquaculture, 133: 91-102.

Torrissen, O.J., 1984. Pigmentation of salmonids. Effects of carotenoids in eggs and start feeding diet on survival and growth rate. Aquaculture, 43: 185-193.

Torrissen, O.J., 1985. Pigmentation of salmonids: factors affecting carotenoid deposition in rainbow trout (*Salmo gairdneri*). Aquaculture, 46: 133-142.

Torrissen, O.J., 1986. Pigmentation of salmonids – a comparison of astaxanthin and canthaxanthin as pigment sources for rainbow trout. Aquaculture, 53: 271-278.

Torrissen, O.J., 1989. Pigmentation of salmonids: Interaction of astaxanthin and canthaxanthin on pigment deposition in rainbow trout. Aquaculture, 79: 363-374.

Torrissen, O.J. & Braekken, O.R., 1979. The utilisation of astaxanthin forms by rainbow trout (*Salmo gairdneri*). In Halver, J.E., Tiews, K. (Eds.), Finfish Nutrition and Fish feed Technology, Vol. II Heenemann, Berlin, 377-382.

Torrissen, O.J. & Naevdal, G., 1984. Pigmentation of salmonids-genetical variation in carotenoid deposition in rainbow trout. Aquaculture, 38: 59-66.

Torrissen, O.J. & Naevdal, G., 1988. Pigmentation of salmonids-variations in flesh carotenoid of Atlantic salmon. Aquaculture, 68: 305-310.

Torrissen, O. J., Hardy, R. W. & Shearer, K. D., 1989. Pigmentation of salmonids – carotenoids deposition and metabolism, CRC Crit. Rev. Aquat. Sci., 1: 209-225.

Torrissen, O.J., Hardy, R.W., Shearer, K.D., Scott, T.M. & Stone, F.E., 1990. Effects of dietary canthaxanthin level and lipid level on apparent digestibility coefficients for canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 88: 351-362.

Toyomizu, M., Sato, K., Taroda, H., Kato, T. & Akiba, Y., 2001. Effects of dietary *Spirulina* on meat colour in muscle of broiler chickens. British Poultry Science, 42: 197-202.

Trams, E.G., 1969. Carotenoids transport in the plasma of the scarlet ibis (*Eudocimus rubber*). Comparative Biochemistry and Physiology, 28: 1177-1184.

Tsavalos, A.J., Harper, M., Daniels, M. & Young, A.J., 1992. Secondary carotenoid synthesis in micro algae. N. Murata (Eds) Research in Photosynthesis, Vol III: 47-50.

Tsushima, M., Kawakami, T., Mine, M. & Matsuno, T., 1997. The role of carotenoids in the development of the sea urchin *Pseudocentrotus depressus*. Invertebr. Reprod. Dev., 32 (2): 149-153.

Tsushima, M., Yamashita, E., Katsuyama, M. & Matsuno, T., 1998. Relationship of carotenoid composition between flounder *Paralichthys olivaceus* and its feed. Fish. Sci., 64 (3): 501-502.

Tsushima, M., Ikuno, Y., Nagata, S., Kodama, K. & Matsuno, T., 2002. Comparative biochemical studies of carotenoids in catfishes. Comparative Biochemistry and Physiology, B 133: 331-336.

Tyczkowski, J.K. & Hamilton, P.B., 1986. Evidence for differential absorption of zeacarotene, cryptoxanthin and lutein in young broiler chickens. Poultry Science, 65: 1137-1144.

Tyssandier, V., Lyan, B. & Borel, P., 2001. Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles. Biochimica et Biophysica Acta, 1533: 285-292.

Undeland, I., Hall, G. & Lingnert, H., 1999. Lipid oxidation in fillets of herring (*Clupea harengus*) during ice storage. J. Agric. Food Chem., 47: 524-532.

Unuma, T., 2002. Gonadal growth and its relationship to aquaculture in sea urchins. In: Yokota, Y., Matranga, V., Smolenicka, Z. (Eds.), the sea urchin: From Basic Biology to Aquaculture. A. A. Balkema Publishers, The Netherlands, 115-128.

Van den Berg, H., 1999. Carotenoid interactions. Nutr. Rev., 57: 1-10.

Van der Salm, A.L., 2005. Ph.D. Thesis, αMSH in fish. Functions in stress responses and skin colour change.

Van der Salm, A.L., Martinez, M., Flik, G. & Vandelaar Bonga, S.E., 2004. Effects of husbandry conditions on the skin colour and stress response of red porgy *Pagrus pagrus*. Aquaculture, 241 (1-4): 371-386.

Vassallo-Agius, R., Imaizumi, H., Watanabe, T., Yamazaki, T., Satoh, S. & Kiron, V., 2001. The influence of astaxanthin supplemented dry pellets on spawning of striped jack. Fisheries Sciences, 67: 260-270.

Vassallo-Agius, R., Watanabe, T., Imaizumi, H., Yamazaki, T., Satoh, S. & Kiron, V., 2001. Effect of dry pellets containing astaxanthin and squid meal on the spawning performance of striped jack *Pseudocaranx dentex*. Fisheries Sciences, 67: 667-674.

Vaughan, D.S. & Prager, H., 2002. Severe decline in abundance of the red porgy (*Pagrus pagrus*) population of the South-Eastern United States. Fisheries Bulletin, 100 (2): 351-375.

Verakunpiriya, V., Watanabe, K., Mushiake, K., Kawano, K., Kobayashi, T., Hasegawa, I., Kiron, V., Satoh, S. & Watanabe, T., 1997. Effect of krill meal supplementation in soft dry pellets on spawning and quality of egg of yellowtail. Fisheries Sci., 63: 433-439.

Vershinin, A., 1996. Carotenoids in mollusca: approaching the functions. Comp. Biochem. Physiol., 11 B (3): 63-71.

Wallat, G.K., Lazur, A.M. & Chapman, F.A., 2005. Carotenoids of different types and concentration in commercial formulated fish diets affect color and its development in the skin red oranda variety of goldfish. N. Am. J. Aquacult., 67 (1): 42-51.

Wallat, G.K., Luzuriaga, D.A., Balaban, M.O. & Chapman, F.A., 2002. Analysis of skin color development in live goldfish using a color machine vision system. N. Am. J. Aquacult., 64: 79-84.

Watanabe, T. & Vassallo-Agius, R., 2003. Broodstock nutrition research on marine finfish in Japan. Aquaculture, 227: 35-61.

Weber, S., 1988. Determination of stabilised, added astaxanthin in fish feeds and premixes with HPLC. In: Keller, H.E. (Eds), Analytical methods for vitamins and carotenoids in feeds. Roche Publication Nº 2264, Basel, Switzerland, 59-61.

Wilkie, D., 1972. The carotenoid pigmentation of *Pleuroncodes planipes* Stimpson (Crustacea, Decapoda, Galatheidae). Comm. Biochem. Physiol., 423: 731-734.

Winklhofer-Roob, B.M., Rock, E., Ribalta, J., Shmerling, D.H. & Roob, J.M., 2003. Effect of vitamin E and carotenoid status on oxidative stress in health and disease. Evidence obtained from human intervention studies. Molecular Aspects of Medicine, 24 (6): 391-402.

White, D.A., Ørnsrud, R. & Davies, S.J., 2003. Determination of carotenoid and vitamin A concentrations in everted salmonids intestine following exposure to solutions of carotenoid in vitro. Comparative Biochemistry and Physiology, 136 (3): 683-692.

White, D.A., Page, G.I., Swaile, J., Moody, A.J. & Davies, S.J., 2002. Effect of esterification on the absorption of astaxanthin in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Res., 33: 343-350.

White, D.A., Moody, A.J., Serwata, R.D., Bowen, J., Soutar, C., Young, A.J. & Davies, S.J., 2003. The degree of carotenoid esterification influences the absorption of astaxanthin in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Nutrition, 9: 247-251.

Woodall, A.A., Britton, G. & Jackson, M.M., 1996. Dietary supplementation with carotenoids: effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. British Journal of Nutrition, 76: 307-317.

Woźniak, M., 1996. The role of carotenoids in fish. Acta Acad. Agricult. Tech. Olst. Protectio Aquarum et Piscatoria, 22.

Wyban, J., Martinez, G. & Sweeney, J., 1997. Adding paprika to *Penaeus vannamei* maturation diet improves nauplii quality. World Aquacult., 28: 59-62.

Xu, Y. & Ding, Z., 2004. Traced studies on metabolism of astaxanthin in Atlantic salmon (*Salmo salar*). Journal of Experimental Zoology, 301 A: 317-323.

Yamada, S., Tanaka, Y., Sameshima, M. & Ito, Y., 1990. Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. I. Effects of dietary Astanxanthin, β-Carotene and Canthaxanthin on Pigmentation. Aquaculture, 87: 323-330.

Yamashita, E. & Matsuno, T., 1992. A series of new apocarotenoids from the black bass. Nippon Suisan Gakkaishi, 58: 2277-2282.

Yanar, M., Kumlu, M., Celik, M., Yanar, Y. & Tekelioglu, N., 1997. Pigmentation of rainbow trout (*Oncorhynchus mykiss*) with carotenoid from red pepper. Israeli J. Aquacult. Badmidgeh, 49: 193-198.

Yarzhombeck, A. A., 1970. Carotenoids in salmonidae and their relation to reproduction in these fishes. In: Problems in Fish Biology. Fish Res. Board Canada, Translation Series Nº 1641.

Yokoyama, A. & Miki, W., 1995. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin producing bacterium *Agrobacterium aurantiacum*. FEMS Microbiology Letters, 128: 139-144.

Yousry, M. & Naguib, A., 2000. Antioxidant activities of astanxanthin and related carotenoids. J. Agric. Food Chem., 48: 89-98.

Yuan, J.P. & Chen, F., 1997. Identification of astaxanthin isomers in *Heamatococcus lacustris* by HPLC-photodiode array detection. Biotechnology Techniques, 11 (7): 455-459.

Yuan, J.P. & Chen, F., 1998. Chromatographic Separation and Purification of *trans*-Astaxanthin from the Extracts of *Haematococcus pluvialis.* J. Agric. Food Chem., 46: 3371-3375.

Yuan, J.P. & Chen, F., 1999. Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. J. Agric. Food Chem., 47: 31-35.

Yuan, J.P., Gong, X.D. & Chen, F., 1996. Separation and identification of astaxanthin esters and chlorophylls in *Haematococcus lacustris* by HPLC. Biotechnol. Tech., 10 (9): 655-660.

Yuan, J.P., Gong, X.D. & Chen, F., 1997. Separation and analysis of carotenoids and chlorophylls in *Haematococcus lacustris* by high-performance liquid chromatography photodiode array detection. J. Agric. Food Chem., 45: 1952-1956.

Yuan, J.P., Chen, F., Liu, X. & Li, X-Z., 2002. Carotenoid composition in the green microalga *Chlorococcum*. Food Chemistry, 76: 319-325.

Zhang, D.H., Lee, Y.K., Ng, M,L. & Phang, S.M., 1997. Composition and accumulation of secondary carotenoids in *Chorococcum sp*. Journal of Applied Phycology, 9: 147-155.

Ziegler, I., 1965. Pterine als wirkstoffe und pigmente. Ergebn. Physiol., 56: 1-66.

INTRODUCCIÓN

A.- COLOR EN ACUICULTURA

Durante las últimas décadas se ha presenciado un proceso de extraordinario crecimiento de la industria acuícola, 9.2 % anual desde 1970, debido principalmente a un estancamiento de los volúmenes de pesca (Figura 1) y limitaciones del stock potencial (FAO, 2002), así como al incremento del consumo per capita global de pescado, el cual se ha duplicado en los últimos cincuenta años (Ahmed y Delgado, 2000; Delgado *et al*., 2002, 2003). Aparte del descenso de la captura y del incremento del consumo de pescado, la expansión de la acuicultura se ha producido en vista del hecho que ésta provee a los consumidores alimento sano, nutritivo y de alta calidad.

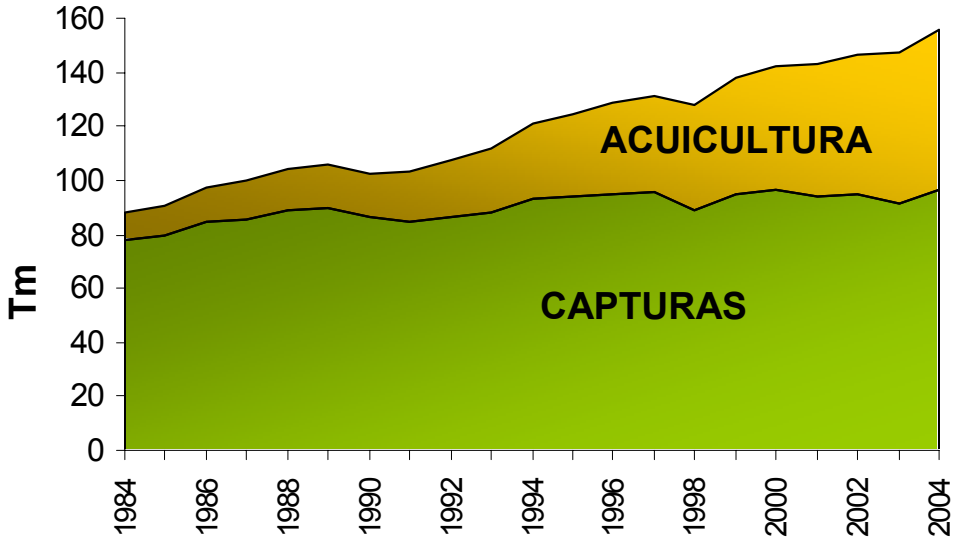


Figura 1. Capturas globales (Tm) y producción por acuicultura (Tm) incluyendo las algas desde 1984 hasta el 2004.

Los productos de acuicultura de alta calidad comprenden varias características muy apreciadas por los consumidores, siendo el color uno de los más importantes. El color es la primera característica percibida y es un criterio determinante de selección, directamente relacionado con la subsiguiente aceptación o rechazo (Shahidi *et al.,* 1968). Tradicionalmente, se ha relacionado la coloración con un sabor superior, una opinión que todavía persiste (Clydesdale, 1993; Sylvia *et al*., 1995; 1996). En los peces, el color es mucho más que un simple efecto cosmético; los consumidores asocian el color natural con salud y producto de alta calidad. La pigmentación del músculo en salmónidos cultivados se considera como el más importante parámetro de calidad, después de la frescura (Koteng, 1992). No sólo la coloración de músculo cuenta, también es altamente apreciada y materia de altos precios de mercado los vivos colores rojos y amarillos de la piel de especies cultivadas como *Pagrus pagrus*, bocinegro (Basurco y Abellan*.,* 1999), *Pagrus major*, dorada japonesa (Lin *et al*., 1998), *Pagrus auratus*, snapper australiano (Booth *et al*., 2004; Doolan *et al*., 2004) y *Seriola quinqueradiata*, seriola (Miki *et al*., 1985). En peces ornamentales, el color de la piel es también una importante característica que afecta el precio de mercado y juega un rol importante en la aceptabilidad general de estas especies (Gouveia y Rema, 2005). En crustáceos tales como el langostino, un brillante y apropiado color se asocia con frescura y calidad, debiendo preservarse esta coloración durante el almacenamiento, procesamiento y cocinado (Boonyaratpalin *et al*., 2001). En el cultivo de erizos, basado en la producción de gónadas, aquellas que presentan una coloración amarillo-naranja brillante son las de mayor valor comercial (Shpigel *et al*., 2004).

Cuatro grupos principales de pigmentos son responsables de la coloración en mamíferos, aves, peces e invertebrados de importancia económica. Estos son las porfirinas, pteridinas, melaninas y carotenoides (Hudon, 1994). Las porfirinas son de importancia primordial en la coloración de la cáscara de huevos de especies aviares (Kennedy y Vevers, 1976; Lang *et al*., 1987). Pteridinas son responsables por muchos de los amarillos y rojos brillantes en

peces, anfibios y reptiles (Nixon, 1985; Ziegler, 1965); estos pigmentos son solubles en agua y son de producción endógena (Hudon, 1994). Las melaninas dan lugar a los negros, grises y marrones de vertebrados y muchos invertebrados, así como también de sus rojos y amarillos. Las melaninas son polímeros heterogéneos compuestos de metabolitos de tirosina (Hudon, 1994). Los pigmentos carotenoides, obtenidos por los animales de sus dietas, confieren la mayoría de los brillantes colores rojo, amarillo y naranja, muy apreciados no sólo en acuicultura, sino también en la industria avícola (Toyomizu *et al*., 2001)

B.- CAROTENOIDES

Los carotenoides constituyen los pigmentos naturales más comunes, responsables no sólo de los variados matices encontrados en la naturaleza, sino también de variadas funciones que serán revisadas. Más de 650 diferentes carotenoides naturales son conocidos (Pfander, 1987). Estos pigmentos se encuentran en bacterias, hongos, plantas y animales. Sólo las plantas, bacterias, hongos y algas son capaces de sintetizar carotenoides; los animales no cuentan con capacidad de sintetizarlos y deben obtenerlos de la dieta (Schiedt, 1998). En el reino animal, después de las melaninas, los carotenoides son los pigmentos más ampliamente distribuidos. Los invertebrados y la mayoría de los vertebrados muestran gran diversidad y habilidad para modificar la estructura de los carotenoides en la dieta; sin embargo, en mamíferos la ocurrencia y distribución de carotenoides es muy limitada (Schiedt, 1998).

B.1.- ESTRUCTURA QUÍMICA DE LOS CAROTENOIDES

Los carotenoides son en su mayoría compuestos tetraterpenoides formados por ocho unidades isoprenoides, siendo el esqueleto de la molécula un largo sistema central de enlaces dobles alternados (Britton, 1995). Los hidrocarburos correspondientes a la fórmula empírica $C_{40}H_{56}$ son conocidos como carotenos y los derivados conteniendo una o más funciones oxigenadas (aldehido, ácido

caboxílico, epoxi, hidroxi, ceto, metoxi) se conocen como xantofilas (Schmidt *et al*., 1994). Los carotenoides pueden también ser acíclicos o poseer un anillo de seis miembros (ocasionalmente de cinco miembros), a uno o ambos extremos del esqueleto molecular (Schmidt *et al*., 1994). Pueden también estar presentes en forma libre, así como en forma de ésteres, glicósidos, sulfatos y caroteno-proteínas (Matsuno y Hirao, 1989).

B.2.- PROPIEDADES FÍSICAS Y QUÍMICAS DE LOS CAROTENOIDES

Las propiedades físicas y químicas de los carotenoides son consecuencia de su estructura química. El sistema de dobles enlaces conjugados, su más notable característica, es el cromóforo absorbente de luz que confiere a estos pigmentos su atractivo color. La mayoría de los carotenoides absorbe luz en el rango de 400-500 nm, exhibiendo su máximo a tres longitudes de onda que resultan en un espectro de tres bandas. A mayor número de dobles enlaces conjugados, mayor el valor de λ máxima. Sin embargo, la ciclación de la molécula resulta en cambio hipsocrómico (desplazamiento del λ máxima hacia una más baja longitud de onda), efecto hipocrómico (descenso en absorbancia) y pérdida de estructura fina (espectro con picos menos definidos). El licopeno, un carotenoide acíclico insaturado, es rojo y presenta λ máxima a 444, 470 y 502 nm; el β-caroteno bicíclico, si bien contiene el mismo número de dobles enlaces conjugados como el licopeno, es amarillo-naranja y tiene un λ máxima a 450 y 477 nm y una mera inflexión a 425 nm. El γ-caroteno monocíclico es rojo anaranjado y exhibe un λ máxima y espectro intermedio entre los del licopeno y β-caroteno. Otros carotenoides, en lugar de exhibir un espectro de tres picos, presentan un espectro que consiste en una banda única, ancha y simétrica. Este es el caso de los ceto-carotenoides, tales como la astaxantina, cantaxantina y equinona. Tanto, la longitud de onda de máxima absorción (λ_{max}) y la forma del espectro (estructura espectral fina) son características del cromóforo de la molécula que proveen valiosa información para identificar carotenoides (Britton, 1995)

Aparte de sus propiedades de absorción de luz, la cadena polieno también confiere a los carotenoides su distintiva reactividad química, de ser muy susceptible a la oxidación e isomerización. Un gran número de isómeros geométricos pueden existir; sin embargo, los carotenoides son naturalmente de la forma trans (E), si bien la presencia de isómeros cis (Z), usualmente en pequeñas cantidades, deberá ser siempre considerada (Schmidt *et al*., 1994). La inestabilidad de los carotenoides respecto a la oxidación, es una característica importante de la molécula en relación a la química de los radicales libres (Britton, 1995)

En lo que respecta a su solubilidad, los carotenoides son como otros compuestos isopropenoides superiores, siendo los carotenos típicos hidrocarburos no polares y las xantofilas más polares, pero insolubles en agua (Schmidt *et al*., 1994). Su comportamiento lipofílico hace de ellos proclives a acumularse en compartimientos lipofílicos como las membranas o lipoproteínas. La tendencia lipofílica de estos compuestos influencia también en su absorción, transporte y excreción en el organismo (Stahl *et al.,* 1993). Sólo combinados con proteínas (caroteno-proteínas), los carotenoides son solubles en una fase acuosa. La presencia de caroteno-proteínas se observa en crustáceos tales como langosta, cangrejo y langostino (Shahidi *et al*., 1998).

Es de gran importancia entender las propiedades físicas y químicas de los carotenoides, tales como su tamaño, forma, polaridad, siendo estas determinantes para su habilidad de encajar correctamente en su medioambiente molecular y permitirles su óptimo funcionamiento (Britton, 1995)

B.3.- BIOSÍNTESIS DE CAROTENOIDES

La biosínteis de carotenoides se produce a través de la ruta de los isoprenoides o terpenoides. El primer paso que compromete la biosíntesis de carotenoides es un acoplamiento cabeza con cabeza de dos moléculas del C_{20} geranil-geranil difosfato (GGPP) que da lugar al incoloro fitoeno. En los

siguientes pasos, la introducción de dobles enlaces crea las propiedades absorbentes de luz, que determinan el color de los carotenoides. Finalmente, ocurren diversas variaciones estructurales, que según Britton, (1998) resultan en los cientos de carotenoides.

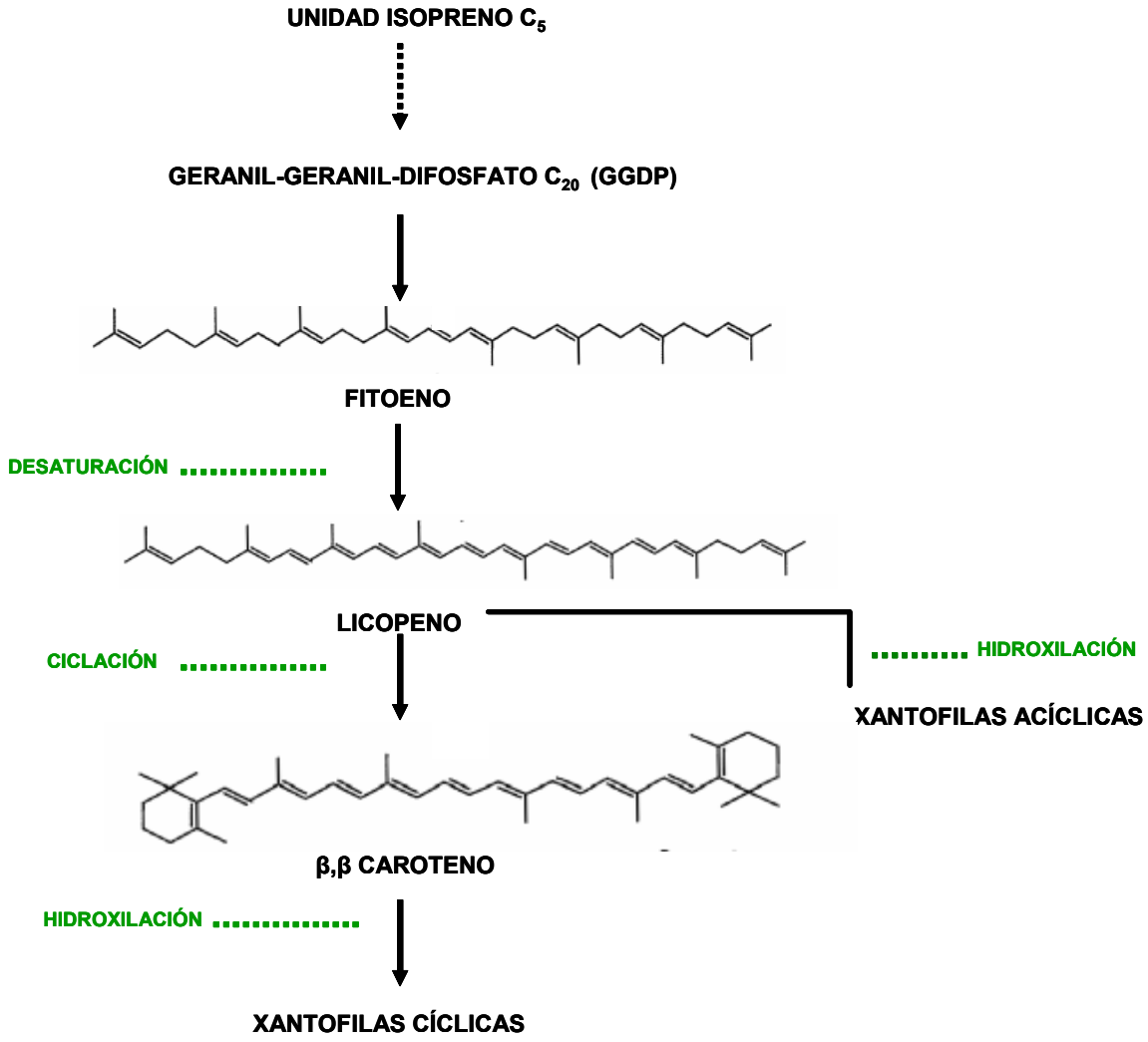


Figura 2. Ruta resumida de biosíntesis de carotenoides

B.4.- FUNCIONES DE LOS CAROTENOIDES

Las funciones de los carotenoides pueden ser divididas en dos grupos: (1) fenológicas y (2) fisiológicas (Latscha, 1991). En la naturaleza, el rol más perceptible de los carotenoides es el de conferir la diversidad de colores observados en plantas, frutos, flores y en el reino animal. El color no es simplemente un efecto cosmético, ya que influencia fuertemente en las señales de comunicación para atraer, alertar y como camuflaje. En la reproducción, los carotenoides son frecuentemente usados por los animales para producir señales sexuales asociadas con la elección de la pareja (Kodric-Brown, 1985, 1987; Hill, 1991; Badyaev y Hill, 2000). En plantas superiores, los carotenoides no sólo confieren coloración, sino también características de sabor y fragancia (tabaco, flores y frutas), a través de metabolitos de carotenoides (Enzell, 1985).

Las funciones fisiológicas de los carotenoides en plantas se relacionan con su rol en el proceso fotosintético. En la foto-protección, los carotenoides disipan la energía lumínica usada en la fotosíntesis e inhiben la formación de especies de oxígeno reactivo (ROS), siendo ambos mecanismos de defensa contra la excesiva energía solar. Además los carotenoides participan en la colección de luz y la transferencia de energía a la clorofila, para la función de fotosíntesis (Deming-Adams *et al*., 1996).

En animales, una de las más importantes funciones de los carotenoides es su rol como precursores de vitamina A (Krinsky *et al*., 1993). Este campo ha sido extensamente estudiado debido a la importancia fisiológica de la vitamina A en el crecimiento, la visión y la reproducción. No todos los carotenoides son precursores de vitamina A; sólo 60 han sido mencionados como precursores de retinoides (Pfander, 1987). Los más importantes precursores son: α-caroteno, γ-caroteno, β-criptoxantina, equinona, β-apo-12'-carotenal y por supuesto β-caroteno. Desde el punto de vista estructural, los precursores de retinoides deberán tener por lo menos un anillo β no sustituído Sin embargo, se ha encontrado que ciertas xantofilas, tales como cantaxantina, astaxantina,

zeaxantina, luteína y tunaxantina, son también precursores de retinoides en peces y ratas (Matsuno, 1991).

Los carotenoides también participan en los mecanismos de protección contra la oxidación, atrapando dos de las moléculas ROS (Sies, 1986; Halliwell, 1996): ($^1O_2$) un estado excitado de una forma parcialmente reducida de oxígeno, inestable y altamente reactiva, y radicales peroxil, generados en el proceso de oxidación de lípidos (Stahl y Sie, 2003). Los carotenoides reaccionan más eficientemente con radicales peroxil (Stahl y Sie, 2003). En general, los carotenoides son considerados antioxidantes, previniendo enfermedades causadas por estrés oxidativo (Chew y Park, 2004). También debe indicarse que la luz conduce a la formación de ROS; carotenoides (con absorción máxima cerca de 450 nm) filtran la luz azul, constituyendo una barrera defensiva contra el daño foto-oxidativo de los tejidos (Stahl y Sie, 2003).

Los carotenoides también participan en la mejora del crecimiento (Inborr y Lignell, 1997), en la función inmune y resistencia a enfermedades en animales superiores y el hombre (Bendich y Shapiro, 1986; Chew, 1992; Jyounchi *et al*., 1993; Chew y Park, 2004), en la función reproductiva, además de participar en señales sexuales, se ha observado la mejora del desempeño reproductivo de vacunos alimentados con dietas suplementadas con β-caroteno (Lotthammer, 1978).

C.- CAROTENOIDES EN ACUICULTURA

En la acuicultura los carotenoides han sido incluidos en dietas de crustáceos, salmónidos y otros peces, principalmente como pigmentantes, para conferir la coloración deseada a estos organismos cultivados. El consumidor subconscientemente relaciona el color del producto con valor nutritivo, salud, frescura y sabor; por lo tanto constituye un decisivo criterio de calidad que debe ser mantenido y optimizado. Los carotenoides no sólo contribuyen a mejorar la

calidad por mejora del color, sino que también contribuyen a posicionar en las mentes de los consumidores una mejor imagen de los productos acuícolas, dado el hecho que en años recientes se ha generado creciente información del efecto positivo de los carotenoides para la salud humana. Aparte de sus propiedades que benefician la calidad, los carotenoides parecen también mejorar ciertos parámetros productivos en las especies cultivadas. Sin embargo, mucho de la investigación se ha enfocado sólo en su rol como pigmentantes.

En los crustáceos decápodos tales como la langosta, camarón, langostino y cangrejo, los principales carotenoides encontrados son los cetocarotenoides, siendo la astaxantina la más abundante (Schiedt, 1998). La formación de los complejos caroteno-proteína en crustáceos conduce a coloraciones marrón, púrpura, verde o azul del exoesqueleto del animal vivo. Una vez que los carotenoides se exponen a temperaturas superiores a 60º C, se producen reacciones de desnaturalización irreversibles que liberan astaxantina, dando como resultado el característico color rojo-anaranjado de la astaxantina que exhiben los crustáceos (Shahidi *et al*., 1998) Las especies del género *Penaeus,* los crustáceos más explotados en la acuicultura, requieren de la inclusión en su dieta de una fuente de carotenoides para alcanzar la deseable coloración corporal. Varios estudios llevados a cabo con *Penaeus monodon* y *Penaeus japonicus* han demostrado que estas dos especies pueden alcanzar la apropiada coloración suplementando la dieta con una fuente de astaxantina o con fuentes de β-caroteno, cantaxantina o zeaxantina (Tanaka *et al*., 1976; Yamada *et al*., 1990; Chien y Jeng, 1992; Okada *et al*., 1994; Boonyaratpalin *et al*., 2001). Ya que los crustáceos tienen la habilidad metabólica de introducir modificaciones estructurales a los carotenoides dietarios, en particular la introducción de grupos hidroxi en los carbonos C (3) y C (3') y grupos ceto en C (4) y C (4'), ellos pueden convertir β-caroteno, cantaxantina o zeaxantina en astaxantina (Schiedt, 1998), como se aprecia en la Figura 3. Los carotenoides dietarios convertidos en astaxantina son depositados en el organismo del camarón en forma libre, en asociación con

proteínas (caroteno-proteínas) y en formas esterificadas, que son predominantemente mono-ésteres y di-ésteres (Foss *et al*., 1987 y Yamada *et al*., 1990). Si bien la mayoría de trabajos de investigación a la fecha se han enfocado en el efecto de la deficiencia de carotenoides en la coloración de especies de Penaeus, existe creciente evidencia que sugiere que los carotenoides influenciarían también en el crecimiento (Dall, 1995; Petit *et al*., 1997), la supervivencia (Liñán-Cabello *et al*., 2003; Supamattaya *et al*., 2005), la reproducción (Liñán-Cabello *et al*., 2003) la actividad antioxidante (Meyers y Latcha, 1997) y el estrés (Chien *et al*., 1999; Chien *et al*., 2003; Supamattaya *et al*., 2005).

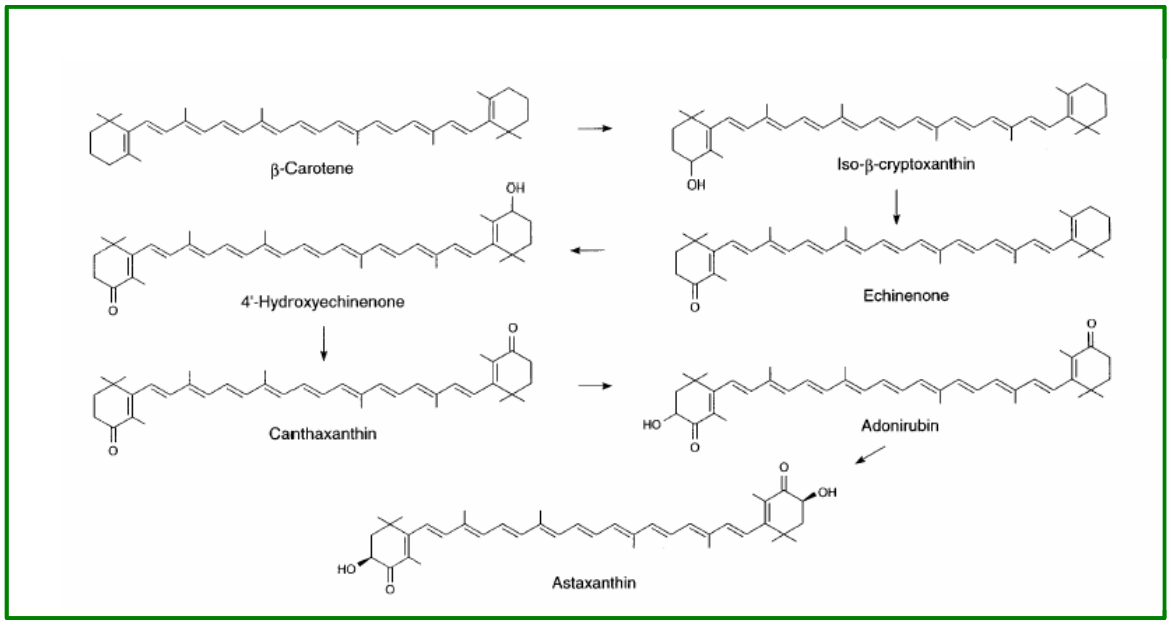


Figura 3. Formación de astaxantina a través de β-caroteno en el langostino *Penaeus japonicus* y en la langosta *Panulirus japonicus* (Buchecker, 1982)

En lo que respecta a equinodermos, siendo los erizos de mar las principales especies cultivadas. Los carotenos dietarios juegan un importante rol por ser los compuestos que dan la coloración característica a las gónadas. El principal carotenoide encontrado en el tejido gonadal es la equinona, que

comprende hasta el 83 % del total de carotenoides (Plank *et al*., 2002), la que se sintetiza a partir de β-caroteno (Shina *et al*., 1978; Goodwin, 1984; Matsuno *et al*., 2001). Los carotenoides empleados en la alimentación de los erizos de mar son mayormente naturales, ya que formas sintéticas de β-caroteno o astaxantina no mejoran el color gonadal (Goebel y Parker, 1998; Havardsson y Imsland, 1999). Con dietas en base a algas o la adición de β-caroteno obtenido del alga *Dunaliella salina*, secada por atomización, se ha logrado incrementar la intensidad de color gonadal (McBride *et al*., 1997; 1999; Robinson *et al*., 2002; Pearce *et al*., 2004). Además de mejorar la pigmentación, los carotenoides dietarios tienen un rol biológico en los erizos, participando en la reproducción, el desarrollo inicial y en la defensa biológica (Tsushima *et al*., 1997; Kawakami *et al*., 1998; Shpigel *et al*., 2004)

En los moluscos, es muy limitada la información disponible y la mayoría de los estudios con carotenoides han incidido en temas como su aislamiento e identificación (Shahidi *et al*., 1998). En un estudio conducido por Vershinin, (1996) con ocho especies de moluscos marinos y de agua dulce, sólo xantofilas trans fueron halladas en órganos no reproductivos de todas las especies, mientras que los carotenos estuvieron restringidos al hepato-páncreas. En *Octopus vulgaris*, un muy apreciado candidato a cultivo, tres estereoisómeros de astaxantina fueron identificados como los principales carotenoides (Moaka *et al*., 1989); sin embargo, no se dispone de información del posible rol de la astaxantina en estas especies. La más probable función de los carotenoides en moluscos podría ser la estabilización de la fluidez de la membrana celular (Vershinin, 1996)

D.- CAROTENOIDES EN PECES

Los carotenoides más difundidos en los peces son la luteína, zeaxantina, astaxantina y la tunaxantina (Matsuno y Tsushima 2001), mostradas en la Figura 4, siendo la tunaxantina el carotenoide predominante en peces marinos (Shahidi, 1998). Ya que los peces no son capaces de sintetizar carotenoides,

tienen que obtenerse de la dieta; en el caso de peces cultivados, las dietas se suplementan con carotenoides naturales o sintéticos.

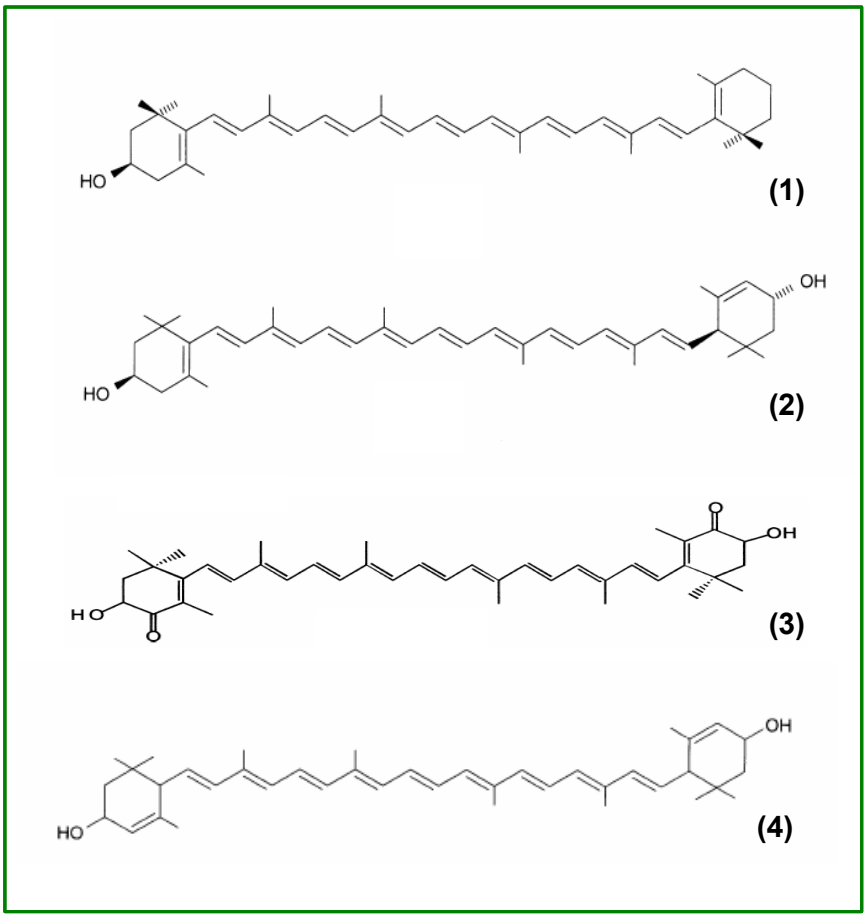


Figura 4. Estructura química de los principales carotenoides en peces luteina (1), zeaxantina (2), astaxantina (3) y tunaxantina (4).

D.1.- FUENTES DE CAROTENOIDES

Entre el grupo de carotenoides sintéticos, la astaxantina es el principal pigmento usado en acuicultura a nivel mundial (Higuera-Ciapara *et al*., 2006), siendo la cantaxantina (Figura 5) la fuente predominante en salmónidos

(Torrissen, 1986; Choubert *et al*., 1994; Metusalach *et al*., 1996). Sin embargo, ya que la astaxantina es el carotenoide presente naturalmente en el músculo de los salmónidos, la astaxantina sintética es el pigmento preferido ya que produce el verdadero color observado en estas especies. En cuanto a la cantaxantina, esta le confiere al filete una coloración más amarillo-anaranjada (Johnson, 1992). La astaxantina producida industrialmente presenta una molécula idéntica a aquella presente en organismos vivos, siendo una mezcla de los isómeros (3S, 3S), (3R, 3S) y (3R, 3R) en proporciones 1:2:1, respectivamente (Figura 6)

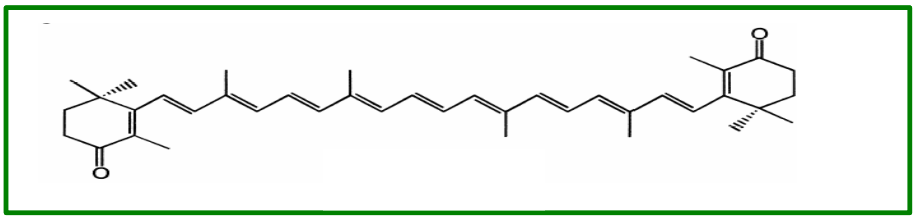


Figura 5. Estructura química de la cantaxantina.

El alto costo de los carotenoides sintéticos y la creciente demanda por una pigmentación natural, ha estimulado el uso de fuentes naturales de carotenoides con potencial de industrialización. Sin embargo, estos productos participan a la fecha de sólo una pequeña fracción del mercado, debido a su limitada producción (McCoy, 1999). Es este el caso de algunos microorganismos tales como microalgas, bacterias y levaduras que se ha informado sintetizan astaxantina (Johnson y Schroeder, 1995; Amstrong, 1997) y otros carotenoides de interés. La microalga de agua dulce *Hematococcus pluvialis* tiene la habilidad de acumular grandes cantidades de astaxantina, a nivel de 1.5 a 5.0 % W/W en base seca (Johnson y Schroeder, 1995; Krishna y Mohanty, 1998). El *Hematococcus* contiene principalmente mono-ésteres de astaxantina ligados a ácidos grasos 16:0, 18:0, 18:2 y 18:1 (Restrøm *et al*.,

1981), teniendo los no esterificados, los mono y di-ésteres una configuración pura (3S, 3´S) (Grung *et al*., 1992). Sin embargo, esta alga exhibe ciertas características desfavorables para su cultivo, tales como tasa lenta de crecimiento y ciclo de vida complejo, en comparación con otras micro algas exitosamente cultivadas a escala comercial, como la *Dunaliella spp*. y la *Spirulina spp*. (Cifuentes *et al*., 2003). La *Chlorella vulgaris* también ha dado resultados positivos en pigmentación de peces (Gouveia *et al*., 1996; 2002), mientras que la micro alga *Chlorococcum sp* parece ser una fuente promisoria de astaxantina, cantaxantina y adonixantina (Higuera-Ciapara *et al*., 2006). Es preciso señalar que además de conferir coloración apropiada, las micro algas tendrían un efecto positivo en el crecimiento, como fue mostrado en un estudio con larvas de *Penaeus monodon* (Darachai *et al*., 1999)

En el campo de las levaduras, *Phaffia rhodozyma* es probablemente la más importante, ya que contiene astaxantina como su principal carotenoide (Andrewes y Star, 1976), constituyendo aproximadamente 83 a 87 % del total de pigmentos (Shahidi *et al*., 1998). El isómero óptico (3R, 3´R) de astaxantina predomina en las levaduras rojas, opuesta a la configuración normal (3S, 3´S) presente en otras fuentes (Andrewes y Star, 1976). Los isómeros ópticos tienen el mismo grado de utilización y su configuración óptica es mantenida después de su deposición en el músculo de la trucha arco iris (Foss *et al*., 1984). Johnson *et al*., (1980) han informado que *Phaffia rhodozyma* también es una buena fuente de proteínas y lípidos. La inclusión de esta fuente de carotenoides, aparte de su efecto positivo en la pigmentación de peces, mejora la función hepática y el potencial defensivo contra el estrés oxidativo (Nakano *et al*., 1995; 1999)

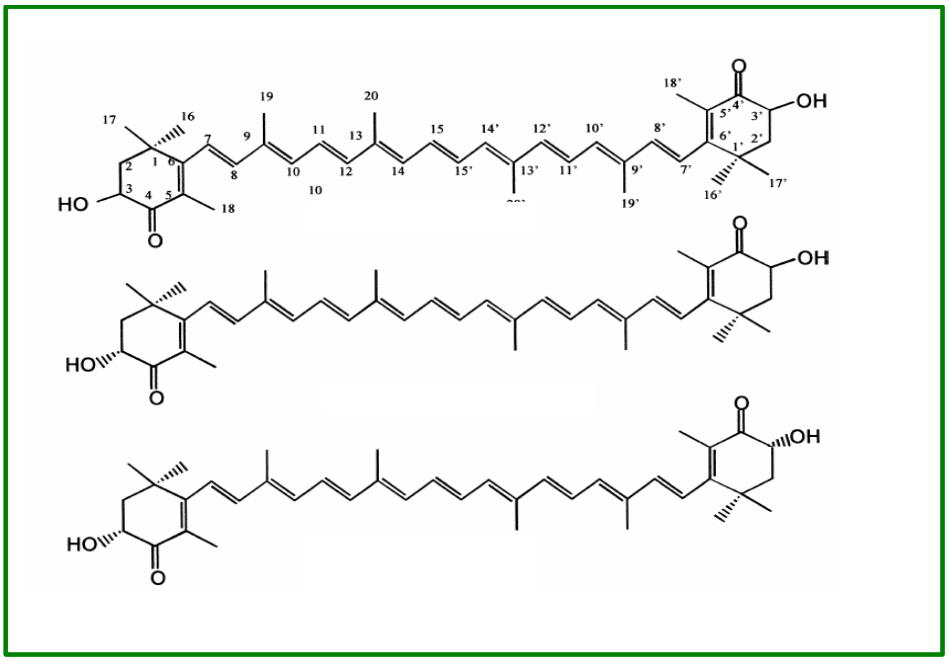


Figura 6. Isómeros configuracionales de la astaxantina (3S, 3´S) astaxantina; (3R, 3´S) astaxantina; (3R, 3´R) astaxantina.

Los desechos del procesamiento de crustáceos (camarón, krill, cangrejo y langostino) tienen también potencial como fuentes de carotenoides. En vista del hecho que cerca de 70 % del peso bruto de la captura constituyen desechos de procesamiento (Wilkie, 1972; Simpson y Haard, 1985), la necesidad de reducir los problemas medioambientales causados por el gran volumen de estos desechos (Torrissen y Naevdal, 1984; Shahidi y Synowiecki, 1991; Sahidi, 1995) y su contenido de carotenoides, hacen de los desechos de crustáceos un material atractivo para su industrialización, siendo la astaxantina el carotenoide predominante (Shahidi *et al*., 1994; Higuera-Ciapara *et al.,* 2006). Por lo general, este material está compuesto de sales minerales (15-35%), proteínas (25-50%), quitina (25-35%), de acuerdo con Lee y Peniston, (1982). Los subproductos de crustáceos han sido utilizados en la coloración de tegumentos y músculo de especies de importancia económica, con buenos resultados (Satio y Regier, 1971; Spinelli *et al*., 1974; Torrisen *et al*., 1982;

Meyers, 1977; Coral, 1997). Desventajas de esta fuente de carotenoides son la variabilidad en la concentración de pigmento y los elevados contenidos de ceniza y quitina, que reduce significativamente su digestibilidad por los peces y limita grandemente el nivel de inclusión en la formulación de dietas.

Las plantas también muestran potencial como fuentes de carotenoides. Ensayos con pimentón rojo han dado buenos resultados, si bien se observó una más baja eficacia en comparación con astaxantina disponible comercialmente (Carter *et al.,* 1994; Yanar *et al*., 1997); además, los pigmentos de la óleo resina de páprika confieren una coloración menos deseable a la trucha arco iris, en comparación con la cantaxantina (Katar *et al*., 1999). En un estudio llevado a cabo con *Sparus aurata*, la dorada, alimentada con una dieta conteniendo harina de gluten (rica en zeaxantina) la coloración de la frente y el opérculo alcanzaron el amarillo característico de sus contrapartes silvestres (Robaina *et al*., 1997). Es de interés continuar investigando otras probables fuentes vegetales de carotenoides para peces. El calendula (marigold), rico en luteína, puede ser una alternativa interesante, dado su eficaz uso en la avicultura para la coloración de la yema del huevo y de la piel.

D.2.- ABSORCIÓN Y TRANSPORTE DE CAROTENOIDES

Los carotenoides son compuestos hidrofóbicos que no se solubilizan fácilmente en el medio acuoso imperante en el tracto gastrointestinal de los peces, por lo que los procesos de digestión, absorción y transporte están asociados con los lípidos (Castenmiller y West, 1998)

La absorción intestinal de los carotenoides envuelve varios pasos, incluyendo el desarreglo de la matriz del alimento, formación de emulsiones de lípidos y solubilización dentro de micelas de sales biliares, antes de su transporte al entericito donde tiene lugar la absorción (Furr *et al*., 1997; Tyssandier *et al*., 2001). En salmónidos, se absorbe aproximadamente 35 % de la astaxantina en la dieta (Torrissen *et al*., 1986; Storebakken *et al*., 1992;

Struksnaes *et al*., 2005) principalmente a lo largo del intestino proximal (Torrissen *et al*., 1986; Al-Khalifa y Simpson, 1998; (Torrissen *et al*., 1989; Hardi *et al*., 1990; White *et al*., 2002), proceso que toma aproximadamente 18 a 30 horas (March *et al*., 1990; Choubert *et al*., 1994b). En comparación con otros nutrientes de peces, la absorción de carotenoides se considera lenta; además, muchos autores sugieren que la absorción intestinal de las micelas es un proceso de difusión pasiva (Choubert *et al*., 1994b; Parker, 1996; Castenmiller y West, 1998; Van den Berg, 1999). Los carotenoides son absorbidos sin previa conversión metabólica, excepto los ésteres de xantofilas que se hidrolizan antes de la absorción, ya que no se observan ésteres en plasma o músculo blanco de salmónidos (Schiedt, 1998; White et *al*., 2003b). Los ésteres de astaxantina encontrados en la piel de salmónidos son el resultado de la re-esterificación de carotenoides libres con ácidos grasos endógenos (Foss *et al*., 1987) Los estudios de absorción con especies de salmónidos se han efectuado mediante el monitoreo de los niveles sanguíneos de carotenoides (Choubert *et al*., 1994b; Kiessling *et al*., 1995; Gobantes *et al*., 1997; White *et al*., 2002, 2003b). Estos niveles son afectados por el metabolismo y los procesos de absorción y excreción (Castenmiller y West, 1998). Por lo tanto, este acercamiento es meramente informativo y no constituye una cuantificación de la absorción de carotenoides por el intestino (White *et al*., 2003a)

Los salmónidos absorben preferentemente los carotenoides más polares, particularmente astaxantina, más que cantaxantina, zeaxantina o los carotenos (Foss *et al*., 1984; Guillou *et al*., 1992; Schiedt *et al*., 1985). Las formas esterificadas y no esterificadas de carotenoides parecen también influenciar en la absorción. Muchos estudios han conducido a resultados contradictorios, con ciertos autores reclamando que las formas libres son mejor absorbidas que las formas éster (Schiedt *et al*., 1985; Foss *et al*., 1987; Storebakken *et al*., 1987; Choubert y Heinrich, 1993), mientras otros informan que ambas formas son igualmente absorbidas (Barbosa *et al*., 1999; Bowen *et al*., 2002). La dorada japonesa parece absorber más eficientemente el

dipalmitato de astaxantina sintético que la astaxantina no esterificada, si se toma en cuenta resultados de pigmentación de piel obtenidos (Ito *et al*., 1986). Es interesante mencionar que los ésteres de astaxantina predominan en las dietas de los especimenes silvestres de salmónidos y dorada japonesa, ya que estas especies se alimentan mayormente de crustáceos, por lo tanto deben ser capaces de utilizar eficientemente las formas esterificadas.

En lo que respecta a transporte, debido a la naturaleza hidrofóbica de los carotenoides, éstos no pueden circular en forma libre en el plasma, ligándose con lipoproteínas plasmáticas (Aas *et al*., 1999). En peces, los carotenoides son transportados mayormente con lipoproteínas de alta densidad (HDL), de acuerdo con Nakamura *et al*. (1985) y en limitada medida (5 a 7%) con lipoproteínas de baja densidad (LDL), según Ando *et al*., (1985). En trucha arco iris y otras especies del género *Oncorhynchus*, se ha observado presencia de astaxantina y cantaxantina en fracciones lipoproteicas séricas (Choubert *et al*., 1992,1994ª; Choubert y Heinrich, 1993). En hembras maduras se ha encontrado cantidades significativas de carotenoides ligados a vitelogenina (VtG), una proteína sérica específica de la hembra (Ando y Hatano, 1988). Durante la maduración sexual del salmón *Oncorhynchus keta*, HDL y VtG han sido asociados con el transporte de carotenoides durante su re-distribución del músculo a los tegumentos y del músculo a los ovarios, respectivamente (Ando *et al*., 1988). La albúmina, una proteína soluble, abundante en el cuerpo de los vertebrados y que constituye la principal proteína de transporte para ácidos grasos y otros compuestos hidrofóbicos (Sheridan, 1988; Peters, 1996), se ha sugerido como importante en el transporte de carotenoides en el salmón del Atlántico (Aas *et al*., 1999)

D.3.- METABOLISMO Y DEPOSICIÓN DE CAROTENOIDES

En peces, las rutas reductora y oxidativa juegan importantes roles en el metabolismo de carotenoides (Schiedt, 1998). Se ha sugerido que el metabolismo de carotenoides tiene lugar en los tejidos donde sus metabolitos

son encontrados (Storebakken y No, 1992), es decir, en hígado (Hardy *et al*., 1990; Metusalach et al., 1996) o en intestino (Aas *et al*., 1999). En salmónidos, aproximadamente 50 % de la astaxantina presente en la dieta absorbida puede ser metabolizada (Torrissen *et al*., 1989; Storebakken y No, 1992; Struksnaes *et al*., 2005). Los primeros trabajos en el área de metabolismo (Tanaka, 1978) establecieron dos clasificaciones para peces, basados en su capacidad metabólica. La primera clase comprendía a las especies incapaces de oxidar el anillo ionona, por lo tanto debían ingerir los derivados oxidados específicos. La segunda clase comprendía a peces con capaces de oxidar el anillo ionona en las posiciones 4 y 4´, siendo un ejemplo de éstos el pez dorado *Carassius auratus* y la carpa roja, *Cyprinus Carpio*, ambos capaces de convertir zeaxantina y luteína en astaxantina (Matsuno and Tsushima, 2001)

Los salmónidos son capaces de reducir, mas no oxidar los carotenoides obtenidos de la dieta. Estas reacciones metabólicas reductoras involucran el retiro paso a paso del grupo ceto en las posiciones 4 y 4´ del anillo ionona (Matsuno and Tsushima, 2001). La piel de este grupo de peces presenta predominantemente ésteres de astaxantina, cuando se suministra astaxantina ya sea libre o esterificada (Katsuyama *et al*., 1987; Bjerkeng *et al*., 2000). En un estudio llevado a cabo con *Salvelinus alpinus*, se encontró, aparte de mono y di ésters de astaxantina, pequeñas cantidades de astaxantina no esterificada y de xantofilas amarillas (iodaxantina, tunaxantina, luteína y zeaxantina), todos metabolitos esperados de astaxantina (Bjerkeng *et al*., 2000). Cuando cantaxantina fue incluida en dietas de salmónidos, prevaleció el β-caroteno, seguido de echinenona y finalmente cantaxantina. Se informó también de la presencia de isocriptoxantina en la piel de esta misma especie (Bjerkeng *et al*., 1990; Metusalach *et al*., 1995). Todos estos carotenoides mencionados son metabolitos de cantaxantina, siendo β-caroteno el producto final.

La seriola una especie cultivada en Japón y que se caracteriza por mostrar bandas amarillo brillante cerca de la línea lateral, tendría también la capacidad de reducir los carotenoides ingeridos (Fujita *et al*., 1983; Miki *et al*.,

1985). El color amarillo observado en los tegumentos está formado predominantemente por tunaxantina (Hirao, 1967), si bien la astaxantina es el carotenoide prevaleciente en su dieta natural. Estas observaciones sugieren que la astaxantina debe ser convertida por reducción en tunaxantina, retirándose el grupo ceto de las posiciones 4 y 4´ del anillo ionona y la conversión del anillo ß en anillo ε, como se aprecia en la Figura 7 (Miki *et al*., 1985). Cuando ejemplares adultos de seriola fueron alimentados con una dieta conteniendo luteína, también se observó un incremento en la tunaxantina de la piel (Schiedt, 1998). Como consecuencia, tanto la astaxantina como luteína podrían ser precursores de tunaxantina, siguiendo la ruta mostrada en la Figura 6, siendo 3´epiluteína el carotenoide común en ambas rutas. En el black bass, la tunaxantina es también el carotenoide predominante de la piel, no encontrándose astaxantina a pesar que su dieta está basada en crustáceos ricos en este carotenoide (Yamashita y Matsuno, 1992). *Caranx delicatissimus*, presenta más de 90 % de tunaxantina, luteína y zeaxantina en sus tegumentos y cuando esta especie es alimentada con *Spirulina maxima* (rica en zeaxantina) el contenido de estos carotenoides se incrementa (Shahidi *et al*., 1998). Tanto en striped jack como en el black bass, se ha sugerido una ruta metabólica de astaxantina o zeaxantina a tunaxantina.

Con relación a los peces de piel roja-rosada, como la dorada japonesa, los ésteres de astaxantina son los carotenoides predominantemente depositados en la piel y en menor medida la tunaxantina (Tanaka *et al.,* 1976; Allahpichay *et al.,* 1984; Nakazoe *et al.,* 1984). La inclusión en la dieta de astaxantina natural o sintética devolvió el característico y muy apreciado color de la piel, perdido al ser cultivado. La alimentación de la dorada japonesa con dietas suplementadas con β-caroteno y cantaxantina mostró un descenso en el nivel de carotenoides de la piel; sin embargo cuando se suministró zeaxantina o luteína, se observó un cierto incremento, si bien no comparable a los niveles alcanzados cuando se alimentó con una fuente esterificada de astaxantina (Nakazoe *et al.,* 1984). La dorada japonesa es capaz de reducir, pero no de oxidar los carotenoides dietarios. El incremento observado cuando ambas

zeaxantina y luteína fueron suplementadas en la dieta podría deberse a una deposición de estos carotenoides en la piel o tal vez debido a un proceso metabólico de reducción tanto de luteína como zeaxantina a tunaxantina; transformación que también ha sido sugerida en la seriola (Allahpichay *et al.,* 1984; Miki *et al.,* 1985)

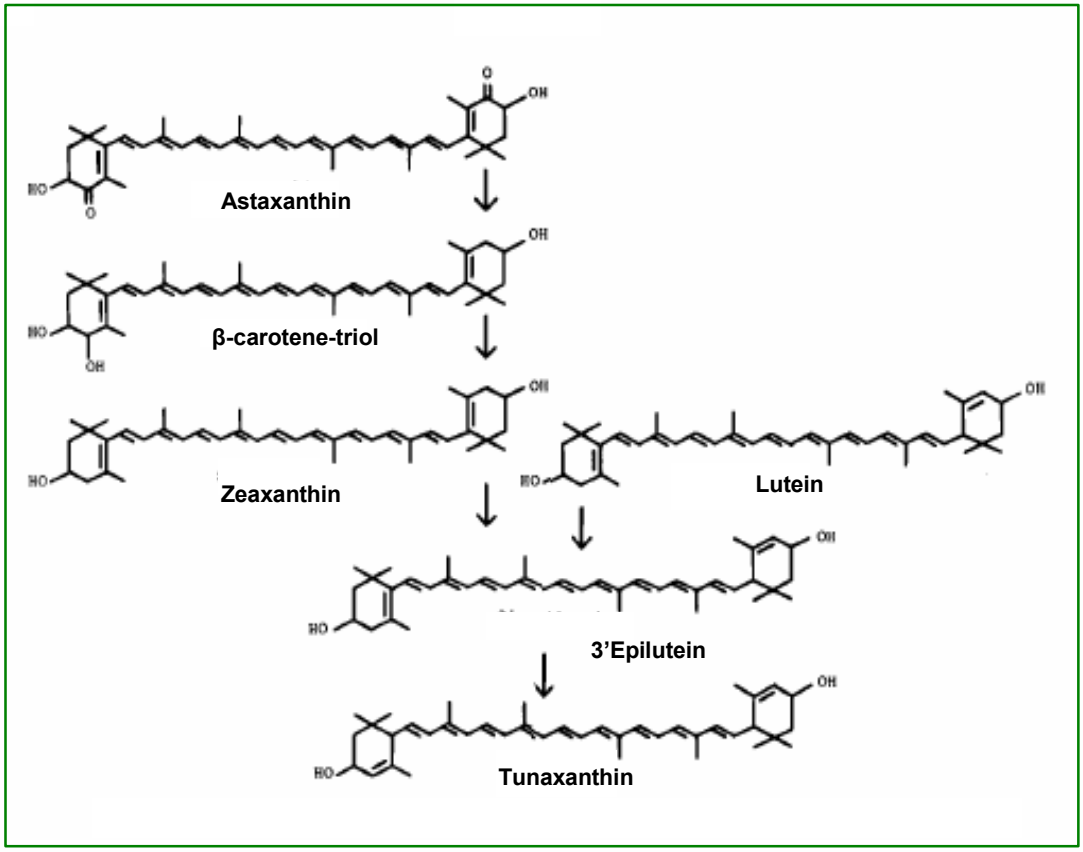


Figura 7. Posible ruta metabólica de astaxantina y luteína a tunaxantina en seriola (Miki *et al*., 1985).

D.4.- FUNCIONES

En peces, las funciones que desempeñan los carotenoides son similares a las previamente discutidas para otras especies animales: ser precursores de vitamina A (Schiedt *et al*., 1985; Guillou et al., 1989; Christiansen *et al*., 1994; white et al., 2003a); influenciar la función reproductiva (Craik, 1985; Christiansen y Torrissen, 1996; Verakunpiriya *et al*., 1997; Chou y Chien, 2001; Vasallo-Agius *et al*., 2001a, b); acción antioxidante (Bjerkeng y Johnsen, 1995; Shimidzu *et al.,* 1996; Nakano *et al*., 1999; Bell *et al*., 2000); mejora del sistema inmune (Nakano et al., 1995; Amar et al., 2003) y en la estructura del hígado (Segner *et al*., 1989; Page *et al*., 2005) Si bien se ha expresado que las funciones atribuidas a los carotenoides en peces son todavía especulativas (Choubert *et al*., 2005), otros investigadores consideran a estos compuestos como micronutrientes que los peces no son capaces de sintetizar y por lo tanto deben incluirse en la dieta (Baker, 2001)

Existe controversia en relación al rol de los carotenoides en el crecimiento de peces. Varios estudios han reportado una influencia positiva, mientras que otros no encontraron efecto. En larvas y juveniles de salmón del Atlántico, la inclusión de astaxantina y cantaxantina sintéticas no sólo mejoró el crecimiento sino también la supervivencia (Torrissen, 1984; Christiansen *et al*., 1994, 1995; Christiansen y Torrissen, 1996). En la carpa, la inclusión de β-caroteno en la dieta también mejoró la supervivencia y el crecimiento (Goswami, 1993), mientras que en la tilapia roja (*Oreochromis niloticus*), la inclusión de harina de cabezas de langostino en la dieta dio lugar a efecto positivo en el crecimiento. Cuando se usan fuentes naturales de carotenoides se debe tener en consideración la presencia de otros componentes que pueden estimular el crecimiento. Resultados obtenidos con larvas y juveniles de salmón del Atlántico, así como informes de trabajos con langostinos y erizos de mar (Petit *et al*., 1997; Myuki *et al*., 1997) sugieren que los carotenoides pueden tener un efecto positivo en el crecimiento, principalmente durante las fases iniciales de desarrollo. No se han encontrado efectos claros de concentraciones ni de la duración de su inclusión.

E.- CAROTENOIDES EN BOCINEGRO

El bocinegro es una especie de la familia *Sparidae*. Se distribuye geográficamente desde las Islas Británicas hasta Senegal, en el Atlántico este; desde Carolina del Norte a Argentina en el Atlántico oeste, así como también en los mares Mediterráneo y Adriático (Kentouri *et al*., 1995; Pajuelo y Lorenzo, 1996; Mihelakakis *et al.,* 2001). Los Juveniles se encuentran principalmente en profundidades de 20 m, con fondos arenosos, donde las presas de pequeño tamaño son abundantes; los individuos adultos prefieren aguas más profundas, cerca de los 250 m de profundidad, donde tienen acceso a la crustáceos más grandes, moluscos y peces pequeños (Labropoulou *et al*., 1999). Esta especie es altamente apreciada en la pesquería comercial, por su apariencia y calidad de carne, así como por la preocupación que causa el declive en sus capturas (Vaughan y Prager, 2002). Este hecho, conjuntamente con los exitosos estudios sobre el desarrollo del bocinegro bajo condiciones de cultivo (Kentouri *et al*., 1994, 1995; Hernández-Cruz *et al*., 1999), así como su crecimiento y supervivencia comparable con la dorada (*Sparus aurata*) y lubina (*Dicentrarchus labrax*) (Divanach *et al*., 1993), hacen de esta especie un candidato potencial para la industria acuícola en la región Mediterránea. Sin embargo, existen todavía ciertas limitaciones para el ingreso de esta especie en la acuicultura, como la pérdida de la coloración rojo-rosada de su piel, así como el oscurecimiento de la misma (Kentouri *et al*., 1995). Tegumentos gris oscuros se observan en el bocinegro de cultivo; esta característica del pez bajo cultivo influye dramáticamente en la aceptabilidad y valor de mercado de esta especie.

E.1.- COLORACIÓN DE LA PIEL DEL BOCINEGRO

El color de la piel de los peces es el resultado de la presencia de diferentes tipos de chromatóforos, células con pigmentos subdivididas en por lo menos seis diferentes tipos, dependiendo del pigmento presente (Fujii, 2000), siendo éstos: melanóforos (negro o marrón), eritróforos (rojo), xantóforos (amarillo), cianóforos (azul), leucóforos (blanquecinos) e iridoforos

(iridiscentes). Varias combinaciones de estos cromatóforos, en variadas proporciones, determinan la coloración del tegumento del pez (Fujii, 2000). En un estudio con bocinegro alimentado con una fuente de carotenoides, se observó principalmente melanóforos y xantóforos/eritróforos en la piel; por el contrario, cuando se suministró una dieta carente en carotenoides, se encontró principalmente melanóforos y muy pocos xantóforos/eritróforos, así como ausencia de coloración rojo-rosa en la piel (Chatzifotis *et al*., 2005). De igual forma en la dorada japonesa, una especie cercana al bocinegro, se asume que los eritróforos son las células pigmentantes predominantes en la piel, ya que el principal pigmento extraído de piel de esta especie fue di-éster de astaxantina (Tsukuda *et al*., 1966; Lin *et al*., 1997). Por lo tanto, la inclusión de carotenoides en dietas de bocinegros bajo condiciones de cultivo es un factor fundamental para recobrar la característica coloración del tegumento de esta especie (Foto 1).

Resultados prometedores se han logrado con el uso de astaxantina esterificada y no esterificada (Chebbaki, 2001; Cejas et al., 2003; Tejera et al., 2003,2005; Chatzifotis *et al*., 2005; Kalinowski *et al*., 2005). Sin embargo, se requiere información sobre las dosis apropiadas de astaxantina, sobre el momento oportuno de su inclusión, así como sobre los posibles carotenoides presentes en la piel de estas especies. Aparte de la astaxantina, otros carotenoides dietarios tales como β-caroteno y licopeno han sido empleados, sin conseguir la coloración de piel deseada (Chatzifotis *et al*., 2005). Se ha informado que la fase de desarrollo del pez (Foss *et al*., 1984; Katsuyama *et al*., 1987; Bjerkeng *et al*., 1992); los componentes dietarios, lipídicos y ácidos grasos (Torrissen *et al*., 1990; Bjerkeng *et al*., 1997; Bell *et al*. 1998; Nickell y Bromage, 1998a; Bjerkeng *et al*., 1999), la genética y tasa de recambio metabólico (March *et al*., Hudon *et al*., 1994; Bjerkeng *et al*., 1997) influencian la digestión, absorción, transporte y deposición de carotenoides; por lo que ellos deberán tomarse en consideración al estudiar el metabolismo de estos compuestos.

Foto 1. Bocinegros silvestres capturados en las Costas de la Isla de Fuerteventura.

Además de la respuesta a componentes dietarios, los peces pueden también cambiar de coloración como reacción a condiciones medio ambientales, estado fisiológico y estímulo estresante (Szisch *et al*., 2002). Estos factores pueden generar dos procesos básicos: cambio fisiológico de coloración, cambios rápidos y/o cambios morfológicos de color. El primero es la agregación o dispersión de gránulos pigmentados, principalmente melanosomas, y es controlado por el sistema nervioso simpático y/o el sistema endocrino. Los cambios morfológicos se relacionan con el tipo, número o densidad de los melanóforos y/o la cantidad neta de melanina (Bagnara *et al*., 1973; Fujii, 1993; Sugimoto, 1993, 2002; Szisch *et al*., 2002). Un prolongado cambio de color fisiológico puede conducir a un cambio morfolófico. Condiciones de cultivo tales como densidad, antecedentes del tanque, espectro

de luz, intensidad de luz y estresantes relacionados a la acuicultura, pueden también influenciar la coloración de piel del bocinegro. La mayoría de las condiciones de cultivo parecen causar cambios fisiológicos de color, si bien al persistir condiciones inapropiadas en el tiempo, cambios morfológicos pueden ocurrir, dando lugar a oscurecimiento de la piel del Bocinegro debido a que síntesis de melanina tiene lugar.

2.- OBJECTIVOS

El objetivo general de la presente tesis es de mejorar la coloración de la piel del bocinegro de cultivo, y alcanzar una coloración similar al de su contraparte en hábitat natural, considerando la importancia que tienen las características de color de la piel en el valor de mercado de esta especie. Para alcanzar esta premisa el presente trabajo de investigación se centró en los siguientes objetivos:

1.- Determinar el efecto de la inclusión en la dieta de diferentes fuentes de carotenoides sobre la coloración de la piel del bocinegro.

Estudios fueron realizados para evaluar la sensibilidad de la coloración de la piel del bocinegro de cultivo, a la inclusión de harina de carcasa de langostino, (como fuente de astaxantina esterificada), canthaxantina sintética y astaxantina sintética (Capítulos 4 y 6). Asimismo, se evaluó el efecto de diferentes concentraciones, de las fuentes de carotenoides mencionadas, sobre la coloración de la piel del bocinegro mediante colorimetría, empleando las ecuaciones de la Comisión Internacional de Estándares de Luminosidad (CIE).

2.- Determinar el efecto de la inclusión de carotenoides en la dieta sobre el desempeño productivo del bocinegro cultivado.

Parámetros de crecimiento y eficiencia alimenticia fueron registrados para bocinegros alimentados con dietas suplementadas con canthaxantina sintética, harina de carcasa de langostino, y astaxantina sintética (Capítulos 4, 5 y 6).

3.- Determinar el período de suplementación óptimo con diferentes fuentes de carotenoides para alcanzar una coloración de la piel del bocinegro de cultivo similar al de la especie en su hábitat natural.

Bocinegros de cultivo fueron alimentados con dietas suplementadas con harina de carcasa de langostino o astaxantina sintética por diferentes períodos de tiempo, antes de la cosecha, y la coloración de piel alcanzada fue comparada a la de las especies silvestres (Capítulos 5 y 6).

4.- Evaluar el efecto de la alimentación con carotenoides sobre la concentración de carotenoides en la piel de bocinegro.

Extraer y cuantificar los carotenoides totales de la piel de bocinegros de cultivo, alimentados con diferentes fuentes de carotenoides por diferentes períodos de tiempo (Capítulos 5 y 6). Establecer la posible relación entre los parámetros de coloración y la concentración de carotenoides en piel.

5.- Separar, cuantificar, e identificar tentativamente los carotenoides en la piel de bocinegros cultivados y silvestres.

Una serie de métodos de cromatografía de capa fina y cromatografía líquida de alta presión fueron evaluados para separar, cuantificar e identificar tentativamente los carotenoides presentes en la piel de bocinegros cultivados y silvestres (Capítulos 6 y 7).

MATERIAL Y MÉTODOS:

En el presente trabajo se llevaron acabo cuatro experimentos 1, 2, 3, 4 descritos en los capítulos 4, 5, 6 y 7 respectivamente, de la presente tesis. A continuación se describirá el material y métodos empleados en estas cuatro experiencias.

A.- ANIMALES Y CONDICIONES EXPERIMENTALES

Los bocinegros de cultivo utilizados en los experimentos fueron obtenidos a través de puestas naturales en la planta experimental de cultivos marinos del Instituto Canario de Ciencias Marinas (ICCM), Gran Canaria, España. Al inicio de todo experimento, los peces fueron preseleccionados en función al peso medio deseado, y siendo repartidos en sus respectivos tanques experimentales, de manera que no hubiese diferencias significativas entre las biomasas de los mismos al inicio de cada experiencia. En la Tabla 1 se muestran las condiciones de cultivo en las que se llevó acabo cada experimento. Los tipos de tanques utilizados se muestran en las Fotos 1 y 2. En todos los experimentos se trabajó con circuito abierto y agua de mar natural.

Tabla 1. Condiciones experimentales empleadas en los experimentos 1, 2 y 3.

CONDICIONES EXPERIMENTALES	**EXP. 1**	**EXP. 2**	**EXP. 3**
Volumen tanques (l)	100	500	500
Flujo de agua (l/min)	3	6	6
Material de tanques	Fibra de vidrio		
Color y forma de los tanques	Gris-circular		
Temperatura (º C)	17 – 19		
Oxígeno (mg/l)	8-10		
Foto-período	12 horas luz : 12 horas oscuridad		

Foto 1. Tanques experimentales de 100 l utilizados en la experiencia 1.

Foto 2. Tanques experimentales de 500 l utilizados en las experiencias 2 y 3.

Además de bocinegros de cultivo, ejemplares del medio natural capturados en aguas del Archipiélago Canario, concretamente en Fuerteventura fueron empleados en los experimentos 2, 3 y 4. Distribuidos en 4 tanques circulares de 1 m^3, grises, y de fibra de vidrio (Foto 3). Al igual que los peces de cultivo estos se mantuvieron en la planta experimental de cultivos marinos del ICCM, a circuito abierto, y las condiciones de foto-periodo, temperatura y oxígeno del agua fueron las mismas que los peces de cultivo.

Foto 3. Tanques experimentales de 1000 l utilizados para los peces del medio natural capturados en la isla de Fuerteventura.

B.- DIETAS EXPERIMENTALES Y ALIMENTACIÓN

B.1 FORMULACIÓN

Las dietas suministradas en las experiencias 1, 2 y 3 fueron formuladas tal como se muestran en las tablas 2, 3 y 4 respectivamente, previo análisis de los insumos a ser utilizados. La harina y aceite de pescado se obtuvieron de

Proaqua, España, la harina de carcasa de langostino de Sopropeche, Francia y los carotenoides sintéticos de DSM, Madrid, España. Todas las dietas experimentales fueron formuladas para tener un mismo nivel de proteínas y lípidos. En cuanto a las vitaminas y minerales estos también fueron los mismos en todas las dietas utilizadas (Tabla 2).

Tabla 2. Premix de vitaminas y minerales utilizados en todas las dietas experimentales.

VITAMINAS (mg/kg of diet)		**MINERALES (mg/g of diet)**	
Acetato de Retinol	25	$(H_2PO_4)Ca$	1.605
Menadiona	20	$CaCO_3$	4.0
Colecalciferol	5	$FeSO_4.7H_2O$	1.5
Cianocobalamina	0.5	$MgSO_4.7H_2O$	1.605
Biotina	1	K_2HPO_4	2.8
Acido fólico	10	$Na_2PO_4H_2O$	1
Piridoxina	40	$Al(SO_4)_3.6H_2O$	0.02
Riboflavina	50	$ZnSO_4.7H_2O$	0.24
Tiamina	40	$CuSO_4.5H_2O$	0.12
Cloruro de colina	90	KI	0.02
Pantotenato de cálcico	117	$CoSO_4.7H_2O$	8
Ácido nicotínico	200	$MnSO_4H_2O$	8

B.2 PROCESAMIENTO

En cuanto a su elaboración, fue llevada acabo en la planta experimental de cultivos marinos del ICCM haciendo uso de una mezcladora y una peletizadora (CPM Mod. 8.3, California, USA) a través de la matriz de granulado correspondiente.

B.3 ALIMENTACIÓN

En el experimento primero la alimentación fue llevada acabo a saciedad aparente tres veces al día, seis veces por semana. En los experimentos 2 y 3 la alimentación se realizó también a saciedad aparente, dos veces al día, seis veces por semana. Antes de iniciarse todas las experiencias los bocinegros de cultivo recibían una dieta sin carotenoides (Proaqua, España). Respecto a los bocinegros capturados del medio natural, su alimentación fue a base de comida natural (mejillones), y su alimentación a saciedad aparente dos veces al día durante una semana que fue el tiempo requerido para su aclimatación.

Tabla 3. Ingredientes y composición proximal de las dietas utilizadas en el experimento 1.

Ingredientes	**Control**	**CTX40**	**CTX100**	**SM20**	**SM40**
Harina de pescado	70.9	70.9	70.9	66.3	61.7
Harina de carcasa de langostino [a]				8.0	16.0
Almidón gelatinizado[b]	18.3	18.2	18.2	14.6	11.0
Premix vitaminas	2.0	2.0	2.0	2.0	2.0
Premix minerales	2.0	2.0	2.0	2.0	2.0
Carboximetil celulosa[c]	0.5	0.5	0.5	0.5	0.5
Aceite pescado	6.4	6.4	6.4	6.6	6.8
Cantaxantina [d]		0.04	0.10		
Composición (% peso seco)					
Proteína	51.6	50.9	52.9	52.8	53.2
Lípidos	13.3	15.2	13.9	14.1	14.3
Ceniza	10.8	9.2	10.4	12.4	13.7
Humedad	11.0	9.7	11.8	12.3	12.0
Astaxantina (mg/kg)	9.3			21.1	38.0
Cantaxantina (mg/kg)		38.3	100.6		

[a] Sopropeche shrimp shell meal, Francia

[b] Merigel 100 Amylum Group

[c] Carboxymethil cellulose Sigma

[d] CAROPHYLL red 10%, DSM.

Tabla 4. Ingredientes y composición proximal de las dietas utilizadas en el experimento 2.

Ingredientes	**Control**	**SM**
Harina de pescado	75.3	65.6
Harina de carcasa de langostino [a]		16.0
Aceite de pescado	7.8	8.0
Almidón gelatinizado [b]	12.4	5.9
Premix vitaminas	2.0	2.0
Premix minerals	2.0	2.0
Carboximetil celulosa[c]	0.5	0,5
Composición (% peso seco)		
Proteina	51.6	53.2
Lípidos	13.3	14.3
Ceniza	10.8	13.7
Humedad	11.0	12.0
Astaxantina (mg/kg)		21.2

[a] Sopropeche shrimp shell meal

[b] Merigel 100 Amylum Group

[c] Carboxymethil cellulose Sigma

Tabla 5. Ingredientes y composición proximal de las dietas utilizadas en el experimento 3.

Ingredientes	**Control**	**ASTX100**
Harina de pescado	68.8	68.8
Almidón gelatinizado [a]	18.0	18.0
Premix de vitaminas	2.0	2.0
Premix de minerals	2.0	2.0
Carboximetil celulosa [b]	0.5	0.5
Aceite de pescado	8.7	8.7
Astaxantina		0.125
Composición (% peso seco)		
Proteínas	51.6	52.4
Lípidos	13.3	14.9
Ceniza	10.8	9.8
Humedad	11.0	10.1
Astaxantina (mg/kg)	9.33	92.60

[a] Merigel 100 Amylum Group

[b] Carboxymethil cellulose Sigma

[C] CAROPHYLL Pink (8% astaxanthin) DSM

C.- MUESTREOS

Previo a todos los muestreos, los peces permanecieron en ayuno 24 horas, y antes tomar datos requeridos por cada experimento, los animales fueron anestesiados con 2-phenoxy etanol. El color de la piel fue medido haciendo uso de un colorímetro portátil (Minolta Chroma Meter CR-200, Minolta Co. Ltd., Osaka, Japón). Antes de realizar la lectura, se calibró con una placa estándar (Minolta Co. Ltd., Osaka, Japan). Las lecturas se tomaron en la parte lateral izquierda como se muestra en la Foto 4. La determinación del color se realizó a partir de la lectura de tres parámetros L*, a* y b* CIE (1976). L* es la luminosidad del color que varía desde 0 a 100 para negro y blanco respectivamente. a* fluctúa entre el rojo y el verde para valores positivos y negativos respectivamente. b* fluctúa entre el amarillo y el azul para valores positivos y negativos respectivamente. A partir de los valores de a* y b* se calculó el color y el croma utilizando las siguientes fórmulas:

$$Color_{ab}=\arctan(b^*/a^*)$$ (Hunt, 1977)

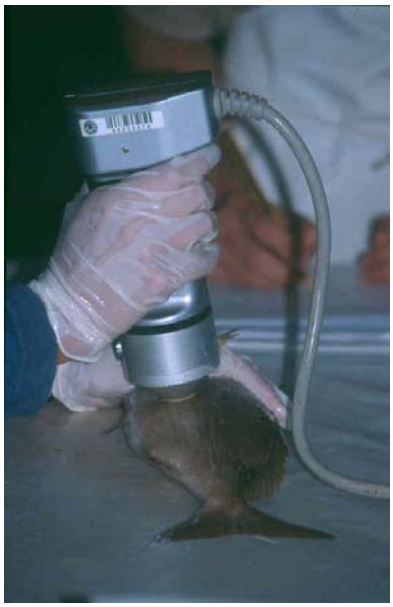
$$Croma_{ab}= (a^{*2}+ b^{*2})^{1/2}$$ (Hunt, 1977)

El color viene a ser una medida angular donde 0º es rojo; 90º amarillo, 180º verde y 270º azul, y el croma o intensidad del color.

C.1.- EXPERIMENTO 1

El primer experimento, cuyo tiempo de duración fue de 105 días, se llevaron acabo tres muestreos, al inicio, al día 75 y al final de la experiencia, tomándose sólo datos de peso y coloración de la piel. Para el color, tres zonas del lado lateral izquierdo fueron muestreadas siendo estas las siguientes: anterior lateral (zona I), anterior dorsal (zona II) y aleta caudal (zona III).

Foto 4. Mediciones de color de la piel del bocinegro con el colorímetro.



C.2.- EXPERIMENTO 2

En el segundo experimento, con un tiempo de duración de 180 días, se llevaron acabo cuatro muestreos, al inicio, al día 60, 120 y al final de la experiencia. Los datos de peso y coloración de la piel se tomaron en todos los puntos de muestreo, en cuanto al color, en esta experiencia sólo se muestreo en la zona anterior lateral (zona I). Para muestras de pez entero se tomaron 6 peces por tratamiento al final del experimento. Para análisis de carotenoides totales en piel se muestrearon 9 peces por tratamiento en la zona izquierda anterior lateral.

C.3.- EXPERIMENTO 3

En el tercer experimento con un tiempo de duración de 90 días se llevaron acabo tres muestreos, al inicio, al día 30 y al final de la experiencia. Se tomaron datos de peso y coloración de la piel en todos los puntos de muestreo, en cuanto al color en esta experiencia al igual que en la segunda, sólo se

muestreo en la zona anterior lateral (zona I). Para análisis de carotenoides totales en piel se muestrearon 9 peces por tratamiento en la zona izquierda anterior lateral.

C.4.- PECES DEL MEDIO NATURAL

Los peces del medio natural fueron muestreados sin ser anestesiados previamente. Para la obtención de datos de color todos los peces fueron muestreados en la parte izquierda en la zona lateral anterior. En lo que concierne a análisis de carotenoides, 10 peces fueron sacrificados, obteniéndose muestras de piel de la misma zona donde se obtuvieron los datos de color.

D.- PARÁMETROS BIOLÓGICOS E INDICES DE UTILIZACIÓN DEL ALIMENTO

Las siguientes fórmulas fueron utilizadas para estudiar el efecto de las dietas sobre los parámetros de crecimiento y rendimiento de las dietas de bocinegro. El peso se presenta en términos absolutos o bien como porcentaje del peso inicial (crecimiento relativo). El crecimiento también se puede expresar como la tasa de crecimiento específica (SGR) que ofrece información sobre el crecimiento diario del pez.

- Crecimiento relativo = $\frac{\text{Peso medio final} - \text{Peso medio inicial}}{\text{Peso medio inicial}} * 100$

- SGR = $\frac{\text{Ln Peso medio final} - \text{Ln Peso medio inicial}}{\text{Número de días}} * 100$

En lo concerniente al rendimiento de las dietas, el índice de conversión (IC) se define como la relación entre el peso del alimento ingerido y el incremento del peso resultante.

- IC = Alimento ingerido (g)
  Incremento de peso (g)

E.- MÉTODOS ANALÍTICOS DE COMPOSICIÓN

Las dietas, piel y peces enteros de los cuales se llevó acabo un análisis bioquímico, fueron guardados a -80º C, en atmósfera de nitrógeno hasta su análisis. En el presente apartado se describen los métodos de análisis utilizados con las diferentes muestras. Todos los análisis se realizaron por triplicado con excepción de los carotenoides totales tanto en piel como en piensos, estos se ejecutaron por duplicado (Barua *et al*., 1993)

Previo al análisis bioquímico, las muestras fueron homogeneizadas, en el caso de las dietas con un mortero de porcelana. Los peces enteros se liofilizaron (CD8, Heto Lab., Dinamarca) durante 5 o 6 días dependiendo del tamaño del pez y posteriormente se homogeneizaron con un molinillo eléctrico. En cuanto a la piel, utilizando unas tijeras afiladas, se procedió a cortar en tiras lo más finas posibles.

E.1.- HUMEDAD

La humedad de las muestras se determinó por desecación en estufa a 105º C hasta peso constante (AOAC, 1995).

E.2.- CENIZA

El contenido de ceniza fue determinado por incineración de la muestra en un horno de mufla a una temperatura de 450º C hasta peso constantes (AOAC, 1995).

E.3.- PROTEÍNAS

Las proteínas se calcularon a partir de la composición en nitrógeno total de las muestras determinada mediante la técnica de Kjeldhal (AOAC, 1995). El método consiste en la digestión de las muestras con ácido sulfúrico concentrado a 420º C en presencia de un catalizador de cobre, seguido de una destilación.

E.4.- LÍPIDOS

El método utilizado para la extracción de lípidos totales es el descrito por Folch *et al.,* (1957), haciendo uso de una mezcla de cloroformo-metanol (2:1 v/v) conteniendo 0.01% de BHT. Una vez evaporado el solvente con una corriente de nitrógeno, los lípidos fueron pesados. Los lípidos fueron almacenados en atmósfera de nitrógeno y disueltos en cloroformo para evitar su oxidación, y luego poder analizar los ácidos grasos.

E.5.- ÁCIDOS GRASOS

Para la determinación de los ácidos grasos, los lípidos totales se transesterificaron con ácido sulfúrico al 1% en metanol según metodología de Christie, (1982). Los FAMES se diluyeron en hexano y su separación, identificación y cuantificación se realizó mediante cromatografía de gases bajo las condiciones descritas por Izquierdo *et al*., (1990) en la Tabla 5.

E.6.- CAROTENOIDES

Para la extracción de carotenoides se llevaron acabo dos metodologías una para la extracción de carotenoides de las dietas experimentales (Barua *et al.,* 1993) y otra para la extracción de carotenoides de la piel (Schiedt *et al*., 1995). En cuanto a la cuantificación de la concentración de carotenoides es espectro-foto-métrica.

Tabla 5. Condiciones operativas para la determinación de ácidos grasos

Aparato	Shimadzu GC-14-A (Shimadzu instrument division, Kyoto, Japón.
Integrador	Shimadzu C-R5A
Columna	Capilar de sílice fundida, 30*0.32 mm D.I (Supelco, Inc., Bellefonte, EE.UU)
Gas portador	Helio
Presión de los gases	He=1, H_2 =0.5, N_2= 0.5 y aire 0.5 kg/cm^2
Detector	FID A 250 º C
Temperatura en inyector	250º C
Horno	Temperatura inicial 180º C durante 10 minutos, tasa de incremento de temperatura 2.5º C por minuto, temperatura final 215º C durante 12 minutos.

Para las dietas se realizó primero una extracción con etilo acetato: metanol (1:1), luego sólo con etilo acetato y finalmente con hexano. Todos los sobrenadantes conteniendo los carotenoides extraídos se evaporaron a sequedad en atmósfera de nitrógeno. El extracto de carotenoides fue resuspendido en un volumen de hexano cuya absorbancia este comprendida entre 0.2 y 0.8. Antes de llevar acabo la extracción carotenoides presentes en las dietas experimentales se llevo una digestión enzimática en los piensos con

carotenoides sintéticos como son: las dietas de CTX40 y CTX100 del primer experimento y la dieta de ASTX100 del tercer experimento. Esto es debido a que los carotenoides sintéticos vienen en una matriz de almidón para una mejor utilización de los mismos. La digestión se lleva acabo adicionando 10 mg de Maxatase (International Biosynthetics, Rijswijk, Netherlands) a la dieta y luego se añade 10 ml de agua. Toda la mezcla se agita y se deja incubando en un baño de agua a 50º C por 30 minutos (Weber, 1988).

Para la piel, se realizaron extracciones sucesivas con acetona hasta no observar color en el sobrenadante. Los sobrenadantes se evaporaron, bajo atmósfera de nitrógeno, hasta obtener aproximadamente 5 ml. Para retirar el agua de los sobrenadantes una cantidad similar de los mismos se adiciona de hexano, seguido por 2 ml de agua. La mezcla se agita con cuidado, hasta obtener 2 fases. Se retira la fase acuosa (inferior) y se le adiciona hexano para extraer los carotenoides restantes, esta operación se repite hasta que las extracciones con hexano no presentan color. La fase de hexano (superior) la cual contiene los carotenoides, se lava de 2 a 3 veces con agua para quitar los posibles restos de acetona. Finalmente, el hexano se evapora a sequedad bajo atmósfera de nitrógeno y el extracto de carotenoides se resuspende en un volumen de hexano cuya absorbancia este comprendida entre 0.2 y 0.8.

Una vez extraídos los carotenoides de un determinado peso de muestra, se procede a la cuantificación, previa medición de la absorbancia se filtran los carotenoides haciendo uso de pipetas Pasteur rellenas de algodón. A continuación se obtiene el espectro de absorción y el valor de la extinción E, a la longitud máxima de absorción (λ_{max}). En las dietas con harina de carcasa de langostino y en la dieta con astaxantina sintética del experimento 3 el λ_{max} fue a 470 nm. Para las dietas con cantaxantina del primer experimento el λ_{max} fue de 466 nm. El valor de extinción utilizado para la astaxantina y la cantaxantina fue de 2100 y 2200 respectivamente (Britton, 1995).

La concentración de carotenoides se calculó a través de la siguiente fórmula:

$$\mu g/g = 10000*V*A / W* E_{1\%,1cm}$$

Donde: V es el volumen total del extracto de carotenoides, W es el peso de la muestra, A es la absorbancia de la muestra y $E_{1\%,\ 1cm}$ es la absorbancia específica de una solución al 1% medida en una celda de 1 cm.

E.6.1- SAPONIFICACIÓN DE CAROTENOIDES

Para la eliminación de los ácidos grasos unidos a ciertos carotenoides se llevó acabo la saponificación de la muestra según la metodología descrita por Schmidt *et al.,* 1994 con algunas modificaciones. Una solución nueva de NaOH en agua (43% w/w) fue adicionada al extracto de carotenoides, provenientes de la piel de bocinegros, disueltos en etanol. Si los carotenoides no se disuelven del toso unas gotas dietileter son adicionadas. Aproximadamente, 0.5 ml de NaOH se adicionan cada 5 ml de solución de carotenoides, para dar una concentración final de NaOH de 7-8%. La solución se guarda de un día para otro a temperatura ambiente, bajo atmósfera de nitrógeno y el tubo es envuelto en papel aluminio para proteger los carotenoides de la luz. Al día siguiente, se procede a la recuperación de los carotenoides saponificados. Para estos el mismo volumen de dietileter es adicionado y la solución es agitada. Se forman dos fases y la superior conteniendo los carotenoides saponificados es retirada y se lava con agua. Finalmente, el solvente se evapora a sequedad bajo atmósfera de nitrógeno y el extracto se resuspende en la fase móvil a ser utilizada posteriormente y filtrado a través de un filtro de teflón (PTFE) de 0.45 µm.

E.6.2- SEPARACIÓN DE CAROTENOIDES POR CROMATOGRAFÍA CAPA FINA (TLC)

Una de las metodologías utilizadas en la separación y cuantificación de carotenoides fue la TLC. Utilizando placas de vidrio de 20x20 cm revestidas de silicagel (G60 Merck glass plates). Como líquido de desarrollo o fases móviles se utilizaron las siguientes mezclas: Hexano/acetona (4:1) y hexano/dietil eter (20:1). La cámara de desarrollo debe estar saturada, para lo cual se cubre la parte interior de la misma con papel de filtro y se deja aproximadamente 30 minutos con la fase móvil correspondiente. Como se debe evitar la luz cuando se trabaja con carotenoides la cámara de desarrollo debe estar también cubierta por la parte exterior con papel aluminio. Para la aplicación de la muestra en las placas se utilizaron capilares de vidrio, aplicando una línea delgada conteniendo una cantidad conocida de muestra, aproximadamente 3 μg. Una vez aplicada la muestra, la placa se introduce en la cámara de desarrollo y cuando el solvente ha alcanzado 1 cm por debajo del final de la placa, se retira de la cámara y se marca con un lápiz la posición del la fase móvil y los carotenoides separados. Una vez que el solvente de la placa se evapore, los valores de Rf son tomados. El Rf viene a ser la relación entre la distancia recorrida por cada carotenoide separado y la distancia recorrida por la fase móvil.

Terminado el desarrollo cromatográfico en TLC, se raspa de la placa de forma individual, cada uno de los componentes se diluyen con acetona hasta no observar color en la silica. El volumen total de acetona es filtrado haciendo uso de pipetas Pasteur rellenas de algodón, se evapora a sequedad bajo atmósfera de nitrógeno y luego el extracto de carotenoides es evaporado a sequedad bajo atmósfera de nitrógeno. El extracto de carotenoides se resuspende en un volumen de hexano cuya absorbancia este comprendida entre 0.2 y 0.8. De esta manera la absorbancia es medida y las cantidades relativas (%) de cada carotenoide separado son estimadas.

E.6.3- SEPARACIÓN DE CAROTENOIDES POR CROMATOGRAFÍA LÍQUIDA DE ALTA RESOLUCIÓN (HPLC)

Los carotenoides en la piel también fueron separados e identificados mediante HPLC de fase reversa (Thermo liquid chromatograph) equipado con cuatro bombas y un detector "photo diode array" (PDA). Las xantofilas rojas presentes en la piel de bocinegros fueron separadas según el método de Yuan and Chen, (1998). Con una columna C_{18} (5µm; 150 x 4.6 mm, Thermo) y a una temperatura ambiente, con un sistema de fases móviles que consistían en lo siguiente: **A:** diclorometano, **B:** metanol, **C:** acetonitrilo and **D:** agua. Del minuto 0 al 8, isocrático, A: B: C: D = 5: 85: 5.5: 4.5. Del minuto 8 al 14, gradiente, A: B: C: D = 5: 85: 5.5: 4.5 a A: B: C: D = 22: 28: 45: 4.5. Del minuto14 al 50, isocrático A: B: C: D = 22: 28: 45: 4.5. Todos los solventes utilizados fueron de calidad HPLC (Sigma Chemical Co). El flujo fue de 1 ml/ min y el espectro de absorción se midió entre 250 y 700 nm. Los picos se detectaron a una longitud de onda de 480 nm para facilitar la detección de ketocarotenoides.

Los carotenoides amarillos de la piel de bocinegro fueron separados, previa saponificación, utilizando la misma columna descrita para las xantofilas rojas. La fase móvil consistió de lo siguiente: diclorometano: metanol: acetonitrilo: agua (5: 70: 20.5: 4.5) durante 15 minutos con un flujo de 1 ml/ min. El espectro de absorción se midió entre 250 y 700 nm. Los picos se detectaron a una longitud de onda de 440 nm para facilitar la detección de la tunaxantina.

E.6.4- IDENTIFICACIÓN DE CAROTENOIDES

Para la identificación de los posibles carotenoides presentes en la piel del bocinegro se utilizó principalmente las características espectrales que muestran en la zona visible como en la zona ultravioleta (UV/Vis) y se compararon con aquellos reportados por otros autores, además se uso co-

cromatografía con estándares puros, aunque para nuestro caso los estándares comerciales son escasos. Para los carotenoides que presentan un espectro conformado por tres bandas es posible usar el parámetro (%III/II). En este convenio se expresa se expresa la altura de la banda de absorción a la longitud de onda mayor (III) como porcentaje de la altura intermedia (II) que normalmente es el máximo de absorción. Este ratio es útil para comparar el espectro entre carotenoides (Britton, 1995).

F.- ANALISIS ESTADISTICOS

Los resultados obtenidos se han expresado como media ± desviación estándar de la media. Los datos procedentes de diferentes tratamientos de un mismo experimento se compararon estadísticamente utilizando el análisis de varianza (ANOVA). Detectadas las diferencias significativas a través del ANOVA, las diferencias entre medias fueron comparadas mediante el test de Duncan, como criterio general se tomó un 5% de nivel de significación.

Cuando las varianzas eran heterogéneas y/o los datos no presentaban una distribución normal se intentaba hacerlas homocedásticas transformando las variables en sus logaritmos o bien con la función arco seno. Si la heterogeneidad persistía en los datos, se empleaba el test no paramétrico de Kruskal-Wallis.

CONCLUSIONES

Efecto de la inclusión en la dieta de diferentes fuentes de carotenoides sobre la coloración de la piel del bocinegro

1. La adición de hasta 100 mg de cantaxantina sintética por kilogramo de alimento no mejoró la coloración de la piel del bocinegro de cultivo.

2. La harina de carcasa de langostino representó una buena fuente de astaxantina esterificada al remplazar el 16% de harina de pescado, confiriendo al bocinegro de cultivo una coloración similar al de los peces silvestres.

3. La astaxantina sintética en niveles de 100 mg/kg de alimento, mejoró la coloración de la piel del bocinegro de cultivo.

4. Los parámetros de color de la piel fueron mejorados con la inclusión de una fuente de astaxantina en la dieta del bocinegro de cultivo. Sin embargo, la inclusión de una fuente de astaxantina en la dieta afectó negativamente la luminosidad de la piel.

5. La zona anterior lateral de la piel del bocinegro demostró ser el mejor indicador de una adecuada coloración y por lo tanto se propone como área de medición de color en estudios futuros.

Efecto de diferentes fuentes de carotenoides sobre el desempeño productivo del bocinegro de cultivo

6. La suplementación de dietas de bocinegro con cantaxantina sintética y astaxantina sintética, en niveles de hasta 100 mg/kg de alimento, por un período de 100 días no tuvo efecto sobre el crecimiento o eficiencia alimenticia de esta especie.

7. La suplementación con harina de carcasa de langostino mejoró significativamente los parámetros productivos, cuando fue suplementada en niveles de 16%, en reemplazo de harina de pescado por un período mayor a 120 días.

Efecto del tiempo de suplementación, con diferentes fuentes de carotenoides, sobre la coloración de la piel del bocinegro de cultivo

8. Es necesario un periodo de suplementación de 120 a 180 días antes de la cosecha, con harina de carcasa de langostino, en reemplazo del 16% de harina de pescado, para que el bocinegro de cultivo adquiera una coloración de la piel similar a la de los peces silvestres.

9. Un período de suplementación de 30 días antes de la cosecha, es necesario, para que el bocinegro adquiera una coloración de piel similar al silvestre, cuando una fuente de astaxantina sintética es utilizada en un nivel de 100mg/kg alimento.

Efecto de la alimentación con carotenoides sobre la concentración de carotenoides en la piel de bocinegro

10. La concentración de carotenoides en la piel del bocinegro se incrementa con un mayor periodo de suplementación con harina de carcasa de langostino o astaxantina sintética.

11. Una correlación positiva, de tendencia logarítmica, se encontró entre los parámetros de coloración de la piel, a*, b*, y croma y la concentración de carotenoides en la piel.

12. Una correlación negativa, de tendencia logarítmica, se observó entre los parámetros de coloración de la piel, luminosidad y color, y la concentración de carotenoides en la piel.

Separación, cuantificación, e identificación tentativa de los carotenoides en la piel de bocinegros cultivados y silvestres

13. La coloración de la piel del bocinegro es determinada por la concentración relativa de carotenoides rojo y amarillo. Una sobre suplementación de astaxantina en la dieta del bocinegro de cultivo, le confiere una coloración de piel más rojiza de lo deseado, al ser comparada con los peces silvestres.

14. Los principales carotenoides presentes en la piel de bocinegros de cultivo, alimentados con una dieta suplementada con astaxantina, y en especimenes silvestres, fueron esteres de astaxantina y esteres de tunaxantina.

15. Para mantener la coloración rojiza, característica de la piel del bocinegro, la astaxantina debe ser un constituyente primordial de las dietas, en vista de la inhabilidad de esta especie en oxidar carotenoides. Además, al presentar el bocinegro la habilidad de reducir la astaxantina dietaria a tunaxantina, probablemente por la ruta metabólica del β-catoteno triol, otros precursores de tunaxantina, en lugar de astaxantina, pueden ser evaluados en estudios futuros.