SKIN COLOUR CHANGES IN ORNAMENTAL KOI (*Cyprinus carpio*) FED DIFFERENT DIETARY CAROTENOID SOURCES

TAN PHAIK SHIANG

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by

TAN PHAIK SHIANG

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PERUBAHAN WARNA KULIT PADA IKAN KOI HIASAN (*Cyprinus carpio*) YANG DIBERI MAKAN DENGAN PELBAGAI SUMBER DIET BERKAROTENOID

ABSTRAK

Harga ikan koi meningkat sejajar dengan keamatan warna kulitnya. Warna and pigmentasi ikan disebab oleh penyerapan dan penempatan karotenoid. Ikan tidak berupaya untuk sintesis karotenoid sendiri, oleh itu, karotenoid mesti dimasukkan ke dalam diet mereka.

Tiga eksperimen telah dijalankan untuk menyiasat perubahan warna kulit ikan koi hiasan (Kohaku) dengan memberi diet yang diperkaya dengan karotenoid daripada alga (*Haematococcus pluvialis* dan *Spirulina*) dan kelapa sawit (dalam bentuk serbuk dan minyak). Diet yang ditambah dengan karotenoid sintetik (astaxanthin dan kantaxanthin) dan satu diet kawalan yang tidak ditambah sebarang karotenoid digunakan untuk perbandingan.

Dalam eksperimen pertama, ikan koi (12.89 $\pm$ 0.02g) diberi diet yang ditambah dengan sumber karotenoid sebanyak 100ppm. Eksperimen ini telah dijalankan dengan sistem air yang statik dalam jangkamasa 10 minggu. Ikan koi yang diberi diet karotenoid tambahan menunjukkan pewarnaan badan yang lebih daripada ikan diet kawalan yang tiada penambahan karotenoid. Parameter tumbesaran ikan koi dipengaruhi oleh sumber karotenoid. Ikan koi yang diberi diet yang ditambah dengan spirulina atau Carosol masing-masing, mendapat parameter tumbesaran yang lebih tinggi daripada ikan koi yang diberi diet yang lain. Terdapat perbezaan yang signifikan ($P< 0.05$) dalam jumlah karotenoid dan jenis karotenoid yang terdapat pada kulit, otot dan hati ikan koi.

Jumlah karotenoid yang terdapat dalam kulit ikan yang diberi diet yang ditambah dengan spirulina atau Carophyll Pink menunjukkan nilai tinggi yang signifikan, 12.08mg kg^{-1} dan 11.76mg kg^{-1}, masing-masing. Pemerhatian morfologi kromatofor dalam sisik ikan koi yang diberi diet berlainan dijalankan dengan mengguna mikroskop cahaya berkuasa tinggi.

Dalam eksperimen kedua, ikan koi (254.89 $\pm$ 16.82g) diberi diet yang ditambah dengan sumber karotenoid pada 250ppm atau 500ppm masing-masing. Eksperimen ini telah dijalankan dalam sistem air statik untuk jangkamasa 8 minggu. Parameter tumbesaran ikan koi tidak dipengaruhi oleh penambahan karotenoid ke dalam diet mereka. Ikan koi yang diberi diet karotenoid tambahan menunjukkan pewarnaan badan yang lebih baik daripada ikan koi yang diberi diet kawalan. Parameter warna seperti L*, c* dan h* diukur dengan menggunakan Minolta Colour Reader (CR-100) pada tiga bahagian yang terdapat warna seperti putih, jingga dan merah. Berdasarkan skala warna CIELCH (CIE, 1976), L* menunjukkan kecahayaan, C* menunjukkan Kroma dan h* menunjukkan sudut warna. Ikan koi yang diberi diet yang ditambah dengan 500ppm Carophyll Pink atau spirulina telah menambahkan kemerahan. Sebahagian ikan koi yang diberi diet karotenoid tambahan 500ppm, bahagian kepala menjadi kekuningan dan ini menujukkan bahawa karotenoid telah melebihi batasan. Terdapat perbezaan signifikan ($P < 0.05$) dalam jumlah karotenoid dan jenis karotenoid individu yang disimpan di dalam gonad dan serum darah. Nilai jumlah karotenoid yang terdapat dalam serum darah dan gonad ikan koi yang diberi diet 250ppm spirulina tambahan merupakan nilai tertinggi.

Dalam eksperimen ketiga, ikan koi (49.36 $\pm$ 0.32g) diberi diet yang ditambah dengan sumber karotenoid sebanyak 250ppm. Eksperimen ini telah dijalankan dalam system air yang bergerak untuk jangkamasa selama 12 minggu. Parameter tumbesaran ikan koi tidak dipengaruhi oleh penambahan karotenoid pada diet mereka. Tiada perbezaan signifikan ($P > 0.05$) pada jumlah karotenoid dalam kulit semua ikan koi. Bagaimanapun, terdapat perbezaan yang signifikan pada jenis karotenoid individu untuk ikan koi yang diberi diet berlainan. Kandungan astaxantin dalam ikan koi diberi diet yang ditambah dengan 250ppm Carophyll Pink adalah lebih tinggi daripada diet yang ditambah dengan 250ppm Carophyll Red atau Caromin sepanjang tempoh pemberian makanan. Parameter warna (L*, a*, b*, c* and h*) diukur pada sebekah kiri bahagian dorsal. L* adalah kecahayaan, a* menunjukkan merah-hijau chromaticity dan b* kuning-biru chromatociti (CIELAB, 1976). Terdapat perbezaan yang signifikan ($P<0.05$) bagi nilai L* dan b* dalam kulit ikan koi. Secara kesimpluan, diet yang ditambah dengan sebarang karotenoid boleh mempengaruhi atau meninggikan parameter tumbesaran dan pewarnaan dalam ikan koi. Pigmentasi ikan koi bergantung pada sumber karotenoid yang sesuai (sintetik atau alga) dengan kepekatan yang sesuai (100-250 ppm) dan jangkamasa pemeliharaan yang sesuai (8-12 minggu) adalah faktor yang penting untuk pewarnaan dan parameter tumbesaran ikan koi yang lebih baik.

SKIN COLOUR CHANGES IN ORNAMENTAL KOI *(Cyprinus carpio)* FED DIFFERENT DIETARY CAROTENOID SOURCES

ABSTRACT

Koi value increases with intensity of skin colour. Fish coloration and pigmentation is due to absorption and deposition of carotenoids. Fishes are unable to synthesize their own carotenoids and therefore these must be included in their diet.

Three trials were undertaken to investigate skin colour changes in ornamental koi (Kohaku) by feeding a dietary carotenoid supplement of algal (*Haematococcus pluvialis* or Spirulina) or palm carotenoid (powder and oil form). Diets containing synthethic carotenoids (Astaxanthin or Canthaxanthin) and a control diet with no carotenoids added were used for comparison.

In the first experiment, koi (12.89 $\pm$ 0.02g) were fed diets supplemented with carotenoid sources at a level of 100ppm. The experiment was conducted in a static water system for a period of 10 weeks. Koi fed with diets supplemented with carotenoids exhibited better body coloration than those fed the control diet without added carotenoids. Growth performance of koi were affected by the dietary carotenoids source. Koi fed diets supplemented with spirulina or Carosol respectively, showed higher growth than koi fed other diets. There were differences ($P< 0.05$) in skin total carotenoid and individual carotenoid types in the skin, muscle and liver of koi. The total carotenoids on the skin of koi fed with Spirulina or Carophyll Pink supplemented diets showed significantly higher value, 12.08mg kg^{-1} and 11.76mg kg^{-1}, respectively. Visual

observations of the morphology of chromatophores on the scale of koi fed with different diet were done under high power light microscope.

In the second experiment, koi (254.89 ± 16.82g) were fed diets fortified with carotenoids at a level of 250ppm or 500ppm, respectively. The experiment was conducted in static water system for a period of 8 weeks. Growth performances of koi were not affected by the addition of carotenoids to their diet. Koi fed added dietary carotenoids exhibited better body coloration than koi fed the control diet with no added carotenoids. Colour parameter such as L*, c* and h* were measured using a Minolta Colour Reader (CR-100) at three different colour regions (white, orange and red) on the koi body. Based on the CIELCH colour scale (CIE, 1976), L* indicates Lightness, C* indicates chroma and h* indicates the hue angle. Koi fed diets supplemented with 500ppm Carophyll Pink or Spirulina increased their redness. Some of the head region of koi fed carotenoids supplemented at 500ppm turned yellowish and this indicated an overdose of dietary carotenoids. There were significant differences ($P < 0.05$) in total carotenoid and individual carotenoid types deposited in koi gonad and blood serum. The total carotenoids concentrations in blood serum and gonad of the koi fed 250ppm Spirulina supplemented diet were the highest.

In the third experiment, koi (49.36 ± 0.32g) were fed diets fortified with carotenoid sources at a level of 250ppm in a water flow through system for a period of 12 weeks. Growth performances of the koi were not affected by the addition of carotenoids to their diet. There were no significant differences ($P > 0.05$) in total carotenoid on the skin of all the koi. However, there were

significant differences (P< 0.05) in individual carotenoid types of koi fed different diets. The astaxanthin content of koi fed diets supplemented with 250ppm Carophyll Pink was significantly higher than diet supplemented with 250ppm Carophyll Red or Caromin throughout the feeding trial. Colour parameter (L*, a*, b*, c* and h*) were measured at the left dorsal regions. L* indicated Lightness, a* indicates red-green chromaticity and b* indicates yellow-blue chromaticity (CIELAB, 1976). There were significant differences (P<0.05) for L* and b* of koi skin. In conclusion, certain dietary carotenoids may affect or increase the growth performances and colouration in koi. Koi pigmentation depends on proper carotenoid source (synthetic or algal) with suitable carotenoid levels (100-250ppm) and appropriate feeding period (8-12 weeks) which are the crucial factors for better colouration and growth performances of koi.

CHAPTER 1 INTRODUCTION

The culture and export of ornamental fishes in Malaysia has expanded rapidly in recent years. The requirement of feed has increased following the high production of ornamental fishes. Nutrition is the most important factor in keeping ornamental fishes healthy. In order to develop efficient and economical feed formulas for aquaculture, basic information of nutrient requirement and chemical composition of feed ingredients in relation to their acceptability and the ability of fish to digest and utilise nutrients from various sources is required. Thus, it is necessary to conduct research and development work to develop effective diets which are of lower cost for the industry.

Differences in species, varieties, colours and patterns of ornamental fishes play an important role in attracting home owners to keep these fishes in their house. Ornamental fishes also derive the name as “life jewels” because of its ornamental values. That means it must be beautiful and decorative as it helps to beautify our living environment. In other words, coloration of ornamental fish plays the most important role.

Coloration and hence, pigmentation plays an important role in both ornamental and food fishes such as goldfish, koi, salmon, trout, sea bream and prawns. Normally, the “quality” of the fish is based on the pigmentation. The colour of fish might also bring some other meanings for certain people. For example, red-coloured fishes such as koi and goldfish will be more attractive

and higher priced as the Chinese, Taiwanese and Japanese consider them more auspicious.

Ornamental carp or koi is now available in many countries spanning Europe, USA and the Far East. Availability of koi is maintained by local producers in these countries, but trade of high quality koi plays a major role in meeting a growing world-wide demand. European countries import large quantities of koi, mainly from Israel, at an annual return of about US$ 10 million. However, top-quality koi are imported mainly from Japan. Their price may reach several thousand US dollar for each fish. Colours and patterns are the major factors in making a high quality koi. Based on growing demand, it seems that global koi production can still increase in both quantity and quality.

Koi is an economically important species of ornamental fish cultured in Malaysia. A major challenge towards meeting the growing demand for koi is to understand the genetic control of colour traits, but so far very few studies have been carried out. Other ways to enhance the coloration of koi are hormone stimulation, natural environment stimulation, feeding dietary pigment enriched feed and injection of dyes. However, the most economical and practical way is through feeding of pigment enriched feeds. Fishes are given special diets containing additional pigments to trigger and enhance coloration of fishes and this has lead to enhanced quality of the coloured ornamental koi.

The cost of each pigment and carotenoid source is different. Carophyll Pink ™ is the most effective pigment for colour enhancement tested so far. Due

to this reason, Carophyll Pink ™ is also the most expensive among the various commercially available pigment sources. Therefore, a lot of researches have been carried out in food fish to replace Carophyll Pink with other cheaper carotenoid sources but very few studies have been conducted in ornamental fish such as koi. Even though small amounts of carotenoids are added in commercial feeds, it is very expensive and sometimes can amount to 15% or more of ornamental fish feed cost.

The palm-based carotenoid source is cheaper and is currently only used in food applications. To date, no research has been done using the palm-based carotenoid in fish diets for pigmentation purposes and this is the first study. This study had been carried out to compare the efficacy of carotenoids extracted from palm oil with that of established carotenoid sources currently used in aquafeeds.

The present study has been designed to determine a proper dose-response relationship regarding the pigmentation of koi. The aim was to benefit feed formulators and the production of ornamental koi by improving koi colour quality. Therefore, the objective for this study was to determine the effects of different levels and various sources of carotenoid on the colour enhancement, pigmentation efficiency and growth rate of Japanese koi (*Cyprinus carpio L.*).

CHAPTER 2 LITERATURE REVIEW

2.1 Carotenoids

According to the Britannica Encyclopaedia, carotenoids refer to any group of non-nitrogenous and bio-chromes yellow, orange or red pigments that are almost universally distributed in living organisms. Carotenoids can be synthesized by bacteria, fungi, lower algae and green plants. They are most conspicuous in the petals, pollen and fruit (e.g., carrots, tomatoes, sweet potatoes and citrus fruits) of the flowering plants. Carotenoids can also be seen in the autumn foliage of deciduous shrubs and trees. Carotenoids in the leaves of green plants are serving as accessory pigments in photosynthesis. They trap the solar energy and pass it to the primary photosynthetic pigment, chlorophyll. Carotenoids play a major role in the biological colouration of animals.

Carotenoids are an important group of natural lipid soluble pigments that are found in all families of the plant and animal kingdoms. There are about 600 different natural carotenoids that have been identified (Lorenz 1998a; Latscha, 1991). Carotenoids derive their names from the fact that they constitute the major pigment in the carrot according to a trivial name system and an IUPAC system (Bauernfeind & Klaui, 1981; Simpson, 1982). Carotenoids represent the most widespread and structurally diverse pigmenting agents. Carotenoids and their derivatives are important in animals as the basis of the visual pigments responsible for light detection and colour discrimination (Britton, 1983). Carotenoids are responsible for many of the brilliant yellow to red colours in

plants and animals, as well as the variety of bluish, greenish, purplish, brownish and blackish colours seen in many fish and crustaceans (Latscha, 1990).

Carotenoids are structurally related to retinol and β-carotene, the main sources of vitamin A for animals (Latscha, 1991). β-carotene can be converted to two identical molecules of retinol and vitamin A (Boileau *et al.*, 1999). The beautiful yellow, orange and red colours found in the skin of ornamental fish are the result of a group of carotenoid pigments, although colourless pteridines may also be present (Chan *et al.*, 1990). Fish, in the same way as other animals, must ingest carotenoids as precursors for vitamin A, as well as depositing pigments. Aquatic animals are unable to perform a *de novo* synthesis of carotenoids in their body and therefore they must acquire these pigments from their diet (Bagnara & Hadley, 1973; Simpson, 1982; Lorenz, 1998b; Latscha, 1991). In nature, fish gain their colour through their natural diets such as algae because only plants and protists are able to synthesize carotenoids.

Bicyclic carotenoids like β–carotene or xanthophylls are ingested from plants and may be converted to astaxanthin, the most commonly occurring carotenoids in aquatic animals (Simpson *et al.*, 1981; Goodwin, 1986; Torrissen, 1989; Latscha, 1990). Based on this, carotenoid synthesised from plants and algae can be added into diets for colour enhancement. Fish differ greatly in their ability to ingest and transform various carotenoids. The specificity of the carotenoid pattern of fish and the selectivity of carotenoids uptake has been studied and it is known that they concentrate carotenoids in their skin, ovary, liver, muscle, and other tissues (Bagnara & Hadley, 1973).

Dietary carotenoids are widely used to provide pigmentation of fish in cases where the fish can no longer obtain it from natural feeds. Although the usage of carotenoids in improving pigmentation is commonly adopted, the proper type of carotenoids, the correct amounts and the feeding period are still a research issue for different species of fishes in their various growing stage. In intensive modern aquaculture systems, dietary carotenoids must be included as part of the diets formulation, especially in diets for ornamental fishes. Katayama *et al.* (1973) proposed that aquatic animals can be divided into three classes based on their biosynthetic capabilities:

Group I. Red carp type: Animals that can convert lutein, zeaxanthin or intermediates to astaxanthin, but β-carotene is not the major precursor of astaxanthin. They can store astaxanthin in the diet directly to their body. Goldfish, red carp and fancy red carp belongs to this group.

Group II. Prawn type: Animals that can convert β-carotene and zeaxanthin to astaxanthin. Generally crustaceans belong to this group.

Group III. Sea bream type: Animals that cannot convert β-carotene, lutein or zeaxanthin to astaxanthin but can transfer pigments from diet to their body tissue pigment, as free form or esterified. Sea bream and red sea bream are the examples of this group.

Simpson (1982) reported that a number of recent studies on pigmented carp have shown the conversion to astaxanthin of β-carotene, of lutein, of zeaxanthin and of isocrytoptoxanthin, echinenone, and canthaxanthin (Figure 2.1). Astaxanthin is mainly responsible for the bright reddish colour of ornamental fishes. Figure 2.1 shows the carotenoid conversions that have been postulated for red carp. Koi is listed under this group.

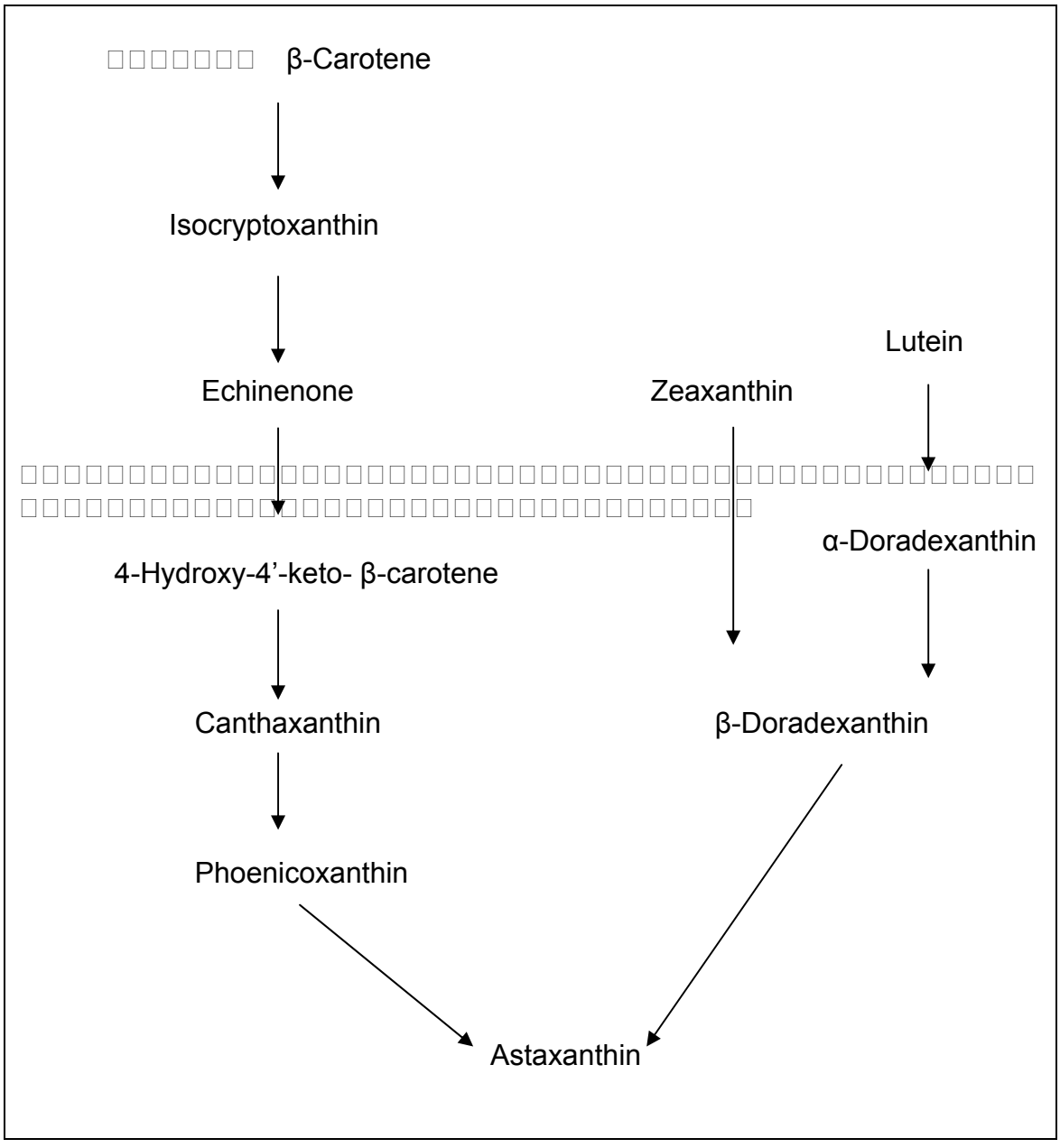


Figure 2.1 Postulated pathways of carotenoid conversions for red carp type fish modified from Simpson, 1982.

2.1.1 Structure of carotenoids

According to Bagnara and Hadley (1973), the general group of carotenoid pigments are divided into two main categories, the carotenes (hydrocarbon class) and the xanthophylls (alcoholic, oxygenated class). The carotenes have a chain of carbon atoms united by alternate single and double bonds with an ionone ring at each end of the chain (Figure 2.2). The alternation of single and double bonds is responsible for the colour of carotenoids. The carotenes contain only carbon and hydrogen atom and the addition of oxygen will form hydroxyl groups and gives xanthophylls.

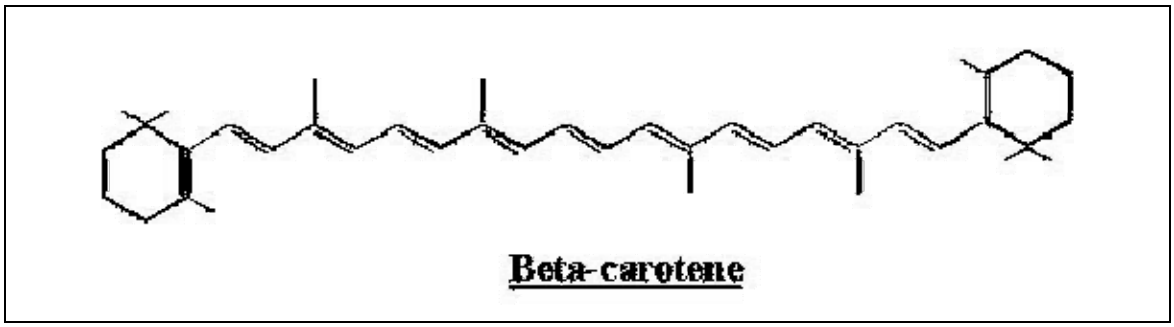


Figure 2.2 β- carotene

Torrissen (1986, 1988 & 1989) found that astaxanthin and canthaxanthin are two major carotenoids found in the flesh of salmonid: Atlantic salmon (*Salmo salar*) and rainbow trout (*Salmo gairdneri*). These two carotenoids are supplemented in feeds to achieve the desired coloration of the flesh to meet consumer preferences and are the major commercially available carotenoid source for aqua feeds formulation.

Astaxanthin

Astaxanthin, like other carotenoids, has been shown to have biological and nutritional functions in fish (Torrissen, 1984; Torrissen *et al*. 1989b; Torrissen & Christiansen, 1995a). Astaxanthin is the carotenoid responsible for the pink-red pigmentation of wild fishes and shrimps. Astaxanthin is the main carotenoid pigment of red and pink coloured aquatic animals (Simpson e*t al*. 1981; Torrissen, 1986). According to the technical report of Aquasearch (2000), astaxanthin has chemical features that result in the existence of several forms of astaxanthin that can be found in nature, such as stereoisomers, geometric isomers and free or esterified forms (Figure 2.3).

Stereoisomers - Astaxanthin has two chiral or asymmetric centres. These are the carbons numbered 3 and 3' on the two rings in the structure. Chemists identify chiral centres as being either R or S. R and S are from the Latin words *rectus* and *sinister*, meaning right and left. The two chiral centres in astaxanthin, carbons 3 and 3' can each exist either in the R or the S form. Thus, there are a total of three stereoisomers: 3S, 3'S; 3R, 3'S or 3R, 3'R.

Geometric isomers - Carbon-carbon double bonds can have the atoms attached to them arranged in different ways. If the two groups are attached on the opposite side of the double bond, they are termed *E*. If the two groups are attached on the same side of the double bond, they are termed *Z*. *E* and *Z* are

from the German words *entgegen* and *zusammen*, meaning together and opposed. Older texts may refer to Z as "*cis*" and E as "*trans*".

Free or esterified – Astaxanthin has two hydroxyl (OH) groups, one on each terminal ring. These can be "free" (unreacted) hydroxyls, or can react with an acid to form an ester. If one hydroxyl reacts with a fatty acid, the result is termed as a mono-ester. If both hydroxyl groups are reacted with fatty acids, the result is termed as a *di*-ester. Adding of a fatty acid to form an ester makes the esterified end of the molecule more hydrophobic. The hydrophobicity (difficulty in dissolving in water) of *di*-esters are higher than mono-ester followed by the "free" form.

Difference between synthetic astaxanthin and natural astaxanthin

Synthetic astaxanthin is produced as the free (unesterified) xanthophylls and as a 1:2:1 mixture of the three stereoisomers. Astaxanthin from natural sources tends to occur predominantly as either the 3S, 3'S; or 3R, 3'R (*all – E*) isomers, while the 3R, 3'S (*meso*) isomer is the most abundant in synthetic astaxanthin.

Astaxanthin is deposited in different forms, level and tissues by different fish species. Free astaxanthin is deposited mainly in the flesh, blood serum and other internal organs, while esterified astaxanthin predominates in the skin, teguments and eggs of salmon (Torrisen e*t al*., 1989b).

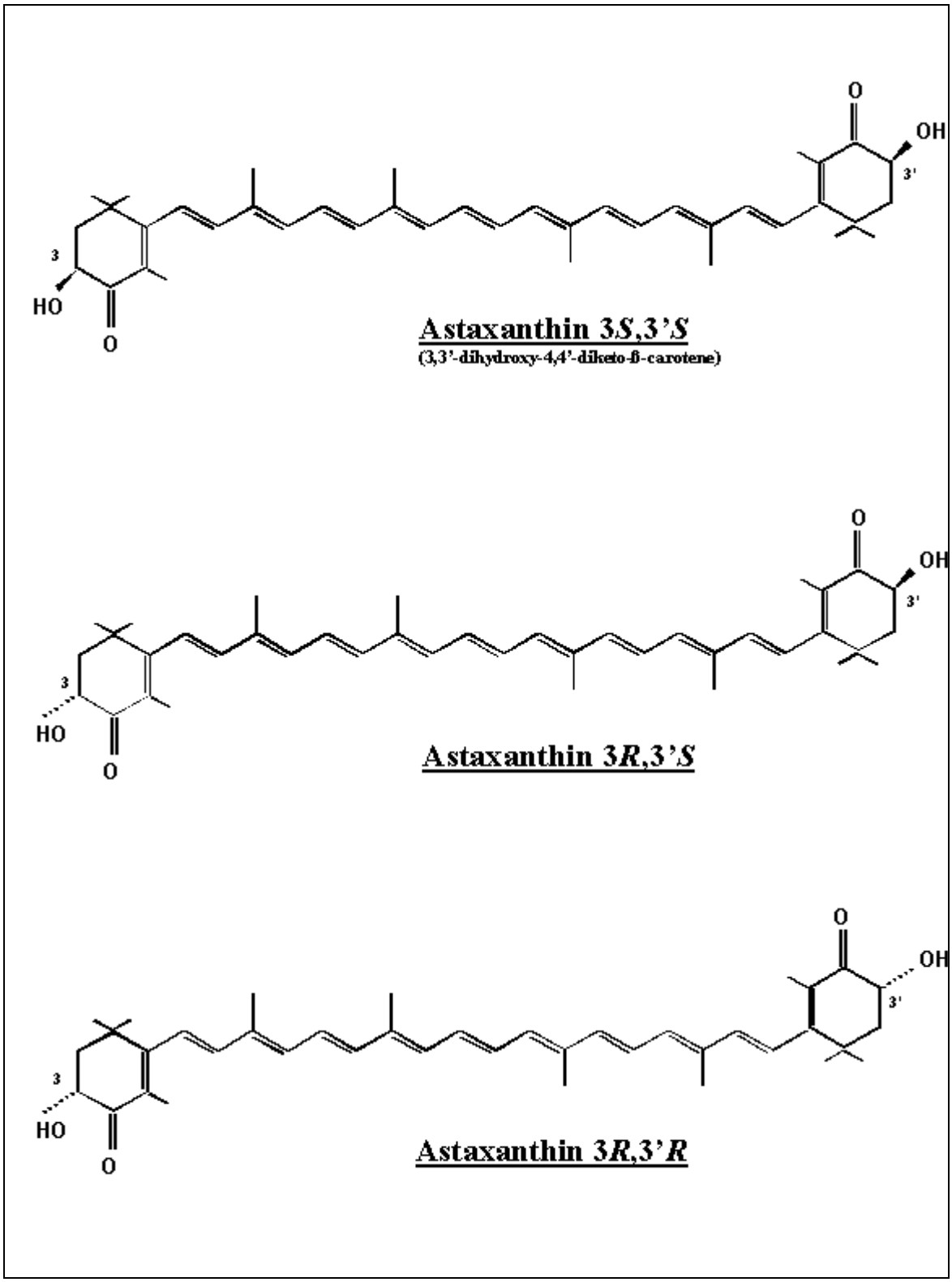
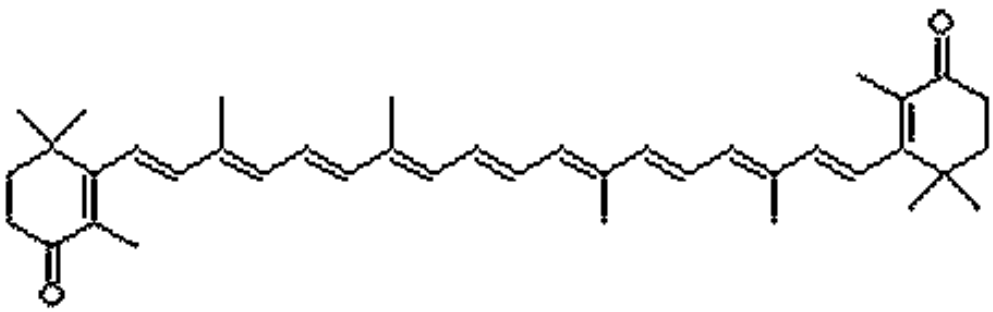


Figure 2.3 Three stereoisomers of astaxanthin.

Canthaxanthin

Canthaxanthin is a naturally occurring carotenoid found in nature in tissues of various bird species, fungal species *Cantharellus cinnabarinus* (chanterelle), insects, fish, crustacean, fungi, algae and many other organisms. Canthaxanthin is widely applied as a feed additive delivering red pigmentation (Figure 2.4). Canthaxanthin is used in poultry pigmentation to impart a red colour to egg yolks and to broiler skin. Used in conjunction with yellow pigments, canthaxanthin increases yolk colour intensity to meet market demands for golden-orange yolks (Roche). In the pigmentation of salmonid fishes, canthaxanthin is supplied in the feed in order to impart a desirable colouration to the flesh. Addition of this pigment to grow-out salmonid feeds ensures that flesh products attain the colour according to consumer expectations (Storebakken e*t al*. 1987). As well as having vitamin A activity, canthaxanthin is a well known free-radical scavenger.



Canthaxanthin

Figure 2.4 Canthaxanthin

2.1.2 Carotenoid Sources

Hoffman La Roche (Basel, Switzerland) started commercial production of synthetic canthaxanthin under the trade name “Carophyll Red” in 1964 for colouring food and feeds. The other manufacturer, BASF group, also produced synthetic canthaxanthin under the trade name Lucanthin® Red. Beside this synthetic canthaxanthin, free astaxanthin also been synthesized by Hoffman La Roche under the trade name “Carophyll Pink”. Over 6000kg of carotenoid pigments were used in salmonid culture at a cost over $1000 U.S. per kg for synthesized product (Torrissen *et al*. 1989b).

Some of the commercial aquafeed producer is currently adding Carophyll Pink into their dry-pelleted diets (Torrissen *et al*. 1989b). A group of scientific researchers did several studies on Atlantic salmon and rainbow trout using Carophyll Pink and Carophyll Red to improve flesh pigmentation (Choubert & Storbakken, 1989; Bjerkeng & Berge, 2000; Baker *et al*., 2002). Based on the results of studies on salmonids, astaxanthin seems to be absorbed and deposited better than canthaxanthin. (Torrissen, 1986; Storbakken *et al.*, 1986; Storbakken *et al.*, 1987; Foss *et al.*, 1987b). Hatlen (1995) also tested the pigmentation on Arctic charr using Carophyll Pink in different concentrations.

Harpaz *et al.* (1998) compared the effect of three different carotenoid sources (dried algal cells *Dunaliella salina*, Carophyll Pink and alfalfa meal) on

growth and pigmentation of crayfish and found that crayfish fed feeds fortified with carotenoids exhibited better coloration. Growth and survival of the crayfish were not affected by carotenoids.

According to the NatuRose Technical Bulletin published by Cyanotech Corporation, NatuRose™ is a natural source of astaxanthin derived from a unique strain of the microalga, *Haematococcus pluvialis* grown under controlled conditions on the island of Hawaii. The largest percentage of carotenoid fraction of NatuRose consists of astaxanthin, with about 15% of the remaining fraction consisting of canthaxanthin, lutein and beta-carotene (Lorenz, 2000). This is the algae first studied by the scientist Girod Chantrans two hundred years ago and has recently received much attention due to its capability to synthesize and accumulates large amounts of astaxanthin during and at the end of the growth phase.

NatuRose™ is used as a pigmentation enhancer and as a nutrition source for a variety of freshwater and marine species and poultry animals (Lorenz, 2000). The astaxanthin ester composition of *Haematococcus* algae meal is similar to that of crustaceans (Lambertsen and Braekken, 1971, Maoka *et al.*, 1985, Foss *et al.*, 1987a). The market price of alga products of astaxanthin is about US$300 per kg and β-carotene is about US$600 per kg (Borowitzka, 1994).

Turujman *et al.* (1997) indicated that farmed salmon should be fed a diet containing natural astaxanthin to achieve the same astaxanthin profile as their

wild counterparts. This study showed that farmed salmon could be easily distinguished from the wild salmon. The farmed salmon are fed synthetic astaxanthin, which contains primarily the 3R-3'S isomer and unable to convert it to the more common and natural 3S-3'S form. This study also concluded that the 3S-3'S is the main form found in wild Pacific and Atlantic salmon species and this is the same form found in *H. pluvialis*.

Scientific studies of *Haematococcus* microalgae by research institutions and international feed companies have concluded that this microalgae is a safe and effective natural alternative for pigmenting aquatic animals (Lorenz, 2000). Miki (1991) found that the more stable esterified form of astaxanthin is believed to be an adaptive feature to be able to store astaxanthin in tissues without excessive oxidation. Esterified astaxanthin is the main form found in *H. pluvialis*. Naturose has been successfully used as a source of pigments in aquaculture in such species as shrimp, rainbow trout, coho and Atlantic salmon.

Scientific research done by Torrissen (1989) compared two different pigment sources (Carophyll Pink and *Haematococcus* astaxanthin) also indicated that the algal astaxanthin can be used as an alternative pigment source for Atlantic salmon (Lorenz, 2000). Choubert & Heinrich, (1993) used *H. pluvialis* in comparison with synthetic astaxanthin and canthaxanthin to test pigmentation on rainbow trout, *Oncorhynchus mykiss*. Their results indicated that physical colour measurements in fish fed with algal incorporation diets showed that increased pigmentation of the trout muscle caused an increase in Chroma (C*) and a reduction in hue (h*) and lightness (L*). Recent studies on

Haematococcus indicated that properly disrupted cells have similar bioavailability as commercially formulated astaxanthin (Barbosa *et al.*, 1999).

NatuRose has produced demonstrable results in the pigmentation of koi and many varieties of ornamental fish (Lorenz, 2000). Koi breeders carried out fish trials to determine the ability of the koi to assimilate xanthophylls from *Haematococcus* algae and they found that koi fed with astaxanthin from *Haematococcus* algal meal had a dark red coloration, whereas the control group that had no astaxanthin had a pale orange skin coloration (Lorenz, 2000). Based on the unpublished data, a group of fish breeder from Aquarium Center, Inc. of Randallstown Maryland, Reef Propagations Inc. and fish farms in Hawaii have added or mixed NatuRose into the ornamental fish feeding diets. A significant improvement in colour and pigmentation could be seen in freshwater and marine ornamental fish, such as tetras, cichlids, gouramis, goldfish, koi, danios, swordtails, Rosy Bards, rainbow fish, discus and clown anemone fish (Lorenz, 2000).

Spirulina is a multicellular, filamentous blue-green algae belonging to the phylum Cyanophyceae (Torrissen *et al*. 1989b). Spirulina can be considered as the most concentrated natural sources of nutrition known for both terrestrial and aquatic animals. Wagener and Rebello (1987) stated that spirulina which contain valuable ingredients of protein required in animal feed is an excellent substitute for animal protein in human nutrition. Animal feed grade spirulina powder is used as a feed ingredient for pigmentation of prawns, marine fish and ornamental fish. Spirulina have relatively expensive market prize at around $17

to $25 per kg depends on the quality (Henson, 1990). The commercially produced microalgae, *Spirulina platensis* or *S. maxima* are a remarkably rich source of amino acids, carotenoids and vitamin (Henrikson, 1989 cited in Henson, 1990).

Henson (1990) stated that Japanese fish farmers are discovering the potential benefits of using spirulina algae as a feed supplement. They discovered five key benefits of using spirulina, such as better growth rates, increased survival rates, improvement in quality and enhanced coloration of the cultured fish while reduced medication requirements and waste in the effluent (wastewater treatment). They found that spirulina increases feed palatability while providing essential nutrients.

Producers of fish fry starter feeds in Japan are including spirulina in their premium feeds. Results show that the palatability and flavour of spirulina trigger the feeding response of the fish fingerlings therefore allowing it to get the nutrition it needs to survive. Taiyo Fisheries has conducted a sixty days feeding trial with yellowtail fingerlings with results showing both an increase in growth and decrease in mortality rates with a 37% increase in harvested tons (Kato, 1988). Fish fed with spirulina have improved flavour (Hirano, 1985; Suyama, 1984), flesh texture (Henson, 1990), firm flesh and bright skin colours (Mori, 1987). Japanese growers of ornamental koi carp, mackerel, yellowtail and sea bream observed colour improvement with spirulina supplemented diets (Matsuno, 1979). Fish with firm texture and sharp colours can command a higher selling price.

Spirulina reduces toxicity of medications and may itself have anti-viral properties (Henson, 1990). Many medications can cause kidney damage resulting in reduced vitality or even death. Spirulina supplemented diets reduce pollution of the environment by increasing feed utilization due to uneaten and undigested feed. Moreover, spirulina also help to reduce wastewater pollutants, and finally eliminating costly treatment systems. Overall, spirulina will increase the effectiveness of existing systems.

Boonyaratpalin and Unprasert (1989) indicated that spirulina influence the pigmentation of red tilapia. Likewise, spirulina added in the diet of ornamental fish such as goldfish and fancy red carp enhanced pigmentation (Miki *et al.*, 1986; Borowitzka, 1994), whereas Choubert (1979) did not observe pigmentation in rainbow trout fed diets containing spirulina. Gouveia *et al.* (2003) compared various microalgal biomass carotenoid sources (*Chlorella vulgaris*, *H. pluvialis* and *Spirulina maxima*) with synthetic astaxanthin (Carophyll Pink) and the found that microalgal biomass, especially *C. vulgaris*, may contribute to enhanced skin colour of koi and goldfish.

Carosol^{TM} 3% is in a spray dried water dispersible form of natural mixed carotenes which has been extracted and purified from fruits of the oil palm tree. Carosol^{TM} 3% contains natural alpha-carotene, beta-carotene, gamma-carotene, lycopene and other carotenoids. It is a unique mixture in natural proportions and commonly found in fruits and vegetables. Concentrated Caromin^{TM} is an opaque reddish vegetable oil suspension of natural occurring mixture of carotenoids, extracted and concentrated from fruits of the oil palm

tree. Caromin™ has been used to formulate colour emulsions by food colorant manufacturers worldwide. Besides being a natural food colorant, Caromin™ has vitamin A activity and confer protection against free radical damages to the human cells. There is a lack of scientific studies on these two carotenoid sources in aquatic animals. As far as we know, these two carotenoid sources have not been evaluated for use as a pigmentation agent in fish feeds.

2.1.3 Function of carotenoids

2.1.3.1 Biological Functions

Czeczuga (1979) reported that fishes with higher level of carotenoids were more resistant to bacterial and fungal diseases than fishes with low carotenoid levels. Torrissen (1984) showed that carotenoids supplied in the diet increased the growth rate in Atlantic salmon. This strongly indicated that carotenoids have a biological function. Segner *et al.* (1989) found an improved liver histology and performance in fish fed high astaxanthin levels in the diet. Other carotenoids functions include (Torrissen 1989):

1) Fertilization hormone
2) Source of pigments for chromatophores
3) Function in respiration
4) Protection from light
5) Resistance to elevated temperature and ammonia
6) Provitamin A

Other than pigmentation, studies indicate that carotenoids also have a biological function involved in growth and reproduction. Several researchers have reported positive effects on growth for different fishes fed diets supplemented with carotenoids, especially astaxanthin (Torrisen, 1984 and 1986; Christiansen *et al.*, 1994). The mobilization of carotenoids from the flesh to the skin and ovaries during maturation also shows that carotenoids may have a function in reproduction. Torrissen and Torrissen (1985) detected a decrease in the carotenoids content of the flesh of Atlantic salmon about six months prior to spawning and proved the mobilization of carotenoids from the flesh to the skin and ovaries during maturation. Torrissen (1984, 1989b) showed the presence of astaxanthin and canthaxanthin in the plasma of feeding rainbow trout, indicating that serum is the transport medium.

Astaxanthin has also been shown to increase egg survival and percentage of fertilized eggs, to protect eggs against extreme conditions (Craik, 1985) and to stimulate growth (Torrissen, 1984). Craik (1985) assumed that the colour of eggs provides an indication of the quality of eggs. It has been suggested that carotenoids may have a respiratory function because the eggs of species that undergo development in poorly oxygenated water have more pigment than those which develop in well-oxygenated water and large eggs tend to have higher carotenoid concentration than small eggs. It has been proposed that astaxanthin has a positive effect on reproduction. Carotenoid content has also been linked to the ability of the egg to tolerate harsh environment conditions.

A number of studies published by Christiansen in Norway has examined the effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (Christiansen & Torrissen, 1997), growth and survival of Atlantic salmon juveniles (Christiansen & Torrissen, 1996), and first-feeding fry (Christiansen *et al.*, 1995a), antioxidant status and immunity in Atlantic salmon (Christiansen *et al.*, 1995b) and effects of astaxanthin and vitamin A on growth and survival during the first feeding of Atlantic salmon (Christiansen *et al.*, 1994). A recent ground-breaking study (Christiansen & Torrissen, 1995b; Christiansen *et al.*, 1996) demonstrated that Atlantic salmon fry have a definitive growth and survival requirement for astaxanthin in their diet. Fish fed diets with astaxanthin below 5.3ppm were found to have marginal growth; those fed levels above 5.3ppm had significantly higher lipid levels accompanied by lower moisture levels. When fry were fed astaxanthin concentrations below 1ppm, survival rates plummeted. More than 50% of the fry fed diets with less than 1.0ppm astaxanthin died during the experimental period and survival of those groups receiving higher concentrations had survival rates greater than 90%. Thus, Atlantic salmon have the distinction as being the first salmonid species for which astaxanthin has been shown to be an essential vitamin, with absolute minimum levels being about 5.1ppm. Higher astaxanthin levels of 13.7ppm in the feed continued to improve the fish lipid levels. Their results also strongly suggested a provitamin A function for astaxanthin over the same fry-feeding period (Christiansen *et al.*, 1994). Furthermore the results indicated astaxanthin as a fertilization hormone and photo protective element (Christiansen & Torrissen, 1997). Astaxanthin also has function in respiration and stress protection from elevated temperatures or ammonia (Christiansen *et al.*, 1995b).

2.1.3.2 Pigmentation Functions

Studies have shown that there are many sources of dietary carotenoids that can be used for the coloration of cultured fish. Carrot and hibiscus (Shahreza, 1994), astaxanthin and canthaxanthin (Torrissen, 1986; Ito *et al.*, 1986; Storbakken *et al.*, 1987; Choubert & Storbakken, 1989; Choubert & Heinrich, 1993; Lim, 1999; Barbosa *et al.*, 1999; Baker *et al.*, 2002) and microalgae (Harpaz *et al.*, 1998; Law, 2000, Gouveia *et al.*, 2003) can be used in fish diets to enhance the colours of fishes.

Synthetic astaxanthin has long been used in aquaculture to enhance the flesh colour of cultured rainbow trout and salmon (Torrissen, 1984, 1986 and 1989; Choubert & Storbakken, 1989; Choubert & Heinrich, 1993; Barbosa *et al.*, 1999; Baker *et al.*, 2002). Torrissen (1989) had shown evidence pointing to the vital role of carotenoids in the physiology and health of plants and animals. This study observed higher growth rates in Atlantic salmon. Astaxanthin has also been incorporated into feeds for ornamental fishes.

Ito *et al.* (1986) tested the effects of feeding red sea bream (*Pagrus major*) different astaxanthin sources. In the group fed free astaxanthin, the carotenoid content of the skin improved for 1 month, but reached a saturation point and did not increase further. In the group fed astaxanthin ester, the carotenoids in the skin was significantly higher after the first and second sampling periods. After two months, the group fed astaxanthin esters had 1.7-fold higher astaxanthin content in the skin than the group fed free astaxanthin

(13.23 mg kg^{-1} compared to 7.94 mg kg^{-1}, respectively). Thus, dietary astaxanthin esters are more efficiently utilized than free astaxanthin for deposition and coloration of skin of red sea bream.

The findings of Smith *et al.* (1992) on coho salmon suggested that feeding low astaxanthin concentrations throughout the grow-out period from fry to market size resulted in the most efficient use of pigment and produced uniform pigmentation. In addition, an increased dietary dose has been shown to have no significant effect on the variation in pigmentation in Atlantic salmon (Torrissen *et al.*, 1995), while an increase in the duration of feeding carotenoids has been reported to reduce inter-fish variation in pigmentation in Atlantic salmon (Torrissen *et al.*, 1995), rainbow trout and Chinook salmon (March and MacMillan, 1996).

Storebakken *et al.* (1987) also showed similar results in the study on pigmentation of Atlantic salmon. The retention coefficients decreased as the pigment dose in the diet increase and this phenomenon showed that there is an optimal level of dietary carotenoids which maximised deposit accumulation of carotenoid in the fish. The excess carotenoid given would be a waste and it will increase the cost of production.

Storebakken *et al.* (1987) also reported that astaxanthin tends to be better deposited than canthaxanthin in Atlantic salmon. Recently, published data showed that canthaxanthin can be equally as effective as astaxanthin, if not better, in pigmenting Atlantic salmon (Buttle *et al.*, 2001). Given the

relatively lower market price for canthaxanthin, this has attracted enormous interest. On the other hand, canthaxanthin has been perceived as being less “natural” than astaxanthin, primarily because astaxanthin is by far the most abundant carotenoid in the flesh of wild Atlantic salmon (Shahidi *et al.*, 1998). Stability of canthaxanthin through secondary processing (freezing/smoking) has also been of concern (Sheehen *et al.*, 1998).

Torrissen (1989) assumed that pink to red pigmentation in flesh could be achieved by pigmentation of astaxanthin and canthaxanthin. The study of Choubert and Storebakken (1989) on dose response to astaxanthin and canthaxanthin pigmentation of rainbow trout fed various dietary carotenoids showed that astaxanthin is more efficiently utilized and the flesh pigmentation increased by increasing the dietary carotenoids concentration in different ratio of different pigment.

Due to lack of scientific studies, most of the information on ornamental fish, especially koi, is unpublished data, university project reports and testimonies from hobbyists, aquaculturists and fish farmers.

Shahreza (1994) added hibiscus flower and carrot into goldfish practical diet to test the skin colour enhancement and the result indicated that carotenoids accumulated in the body of goldfish especially in the head region. The study on the effect of Carophyll Pink on the skin coloration of Angelfish (*Pterophyllum scalare*), Japanese carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) also showed the same result (Lim, 1999). Paripatananont