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# ABSORPTION AND UTILISATION OF NATURAL AND SYNTHETIC ASTAXANTHIN FORMS IN SALMONID NUTRITION

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# ABSORPTION AND UTILISATION OF NATURAL AND SYNTHETIC ASTAXANTHIN FORMS IN SALMONID NUTRITION

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by

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A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

## DOCTOR OF PHILOSOPHY

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# ABSTRACT

**Daniel Allan White "The absorption and utilisation of natural and synthetic astaxanthin forms in salmonid nutrition."**

Consumer preference for commercially reared fish products that resemble their wild counterparts has resulted in the supplementation of pigments called carotenoids into aquafeeds to promote a pink-red colour in the flesh of salmonid fish. To date synthetic forms of these pigments have been commonly utilised to achieve this desired colouration, with the carotenoid astaxanthin being the regular choice for the feed manufacturer. However, increase in consumer demand for farmed fish products reared on natural feed additives has evoked an interest in natural sources of astaxanthin that could be successfully used to pigment salmonid fish efficiently.

In the current study, the microalga *Haematococcus pluvialis* has been assessed as a potential feed supplement to pigment the flesh of rainbow trout (*Oncorhynchus mykiss*). More specifically, those natural characteristics that may well limit the absorption and utilisation of astaxanthin from this source have been assessed individually and discussed from a physiological standpoint. The cell wall of *Haematococcus pluvialis* when cracked efficiently presents no limitation to the absorption and utilisation of astaxanthin from this source. Indeed, the cell wall remnants help to prevent oxidation of astaxanthin in the feed compared to cell wall free extracts of carotenoid from the same source. However, esterified astaxanthin (which this algae predominantly contains) is not absorbed as efficiently as unesterified synthetic astaxanthin. Furthermore, the extent of esterification is negatively related to the absorption of astaxanthin. Regional variation in ester hydrolysis along the gastrointestinal tract combined with gut transit time of the ingested feed may explain these limitations. However, despite limitations in absorption, the muscle deposition of astaxanthin supplied as esters does not significantly differ from the unesterified form. The optical purity of astaxanthin esters from this source does not prejudice the final deposition of astaxanthin in fish tissues.

An *in vitro* model has been developed to assess the absorption of astaxanthin at the intestinal level in salmonid fish in order to define absorption characteristics of carotenoids under different abiotic and biotic conditions. The absorption of astaxanthin seems to occur in a linear passive manner into the intestinal tissue. Although size of the fish does not affect the absorption of astaxanthin, temperature does have a significant effect. Although there were no significant differences in absorption between Atlantic salmon (*Salmo salar*) and rainbow trout, absorption tended to be greater in the latter species and merits further study.

# LIST OF CONTENTS

CHAPTER 3.0

THE SPOROPOLLENIN CELL WALL OF *HAEMATOCOCCUS PLUVIALIS*: EFFECTS ON ABSORPTION AND DEPOSITION OF ASTAXANTHIN IN RAINBOW TROUT

## CHAPTER 6.0

## EVALUATION OF AN *IN VITRO* PERFUSION SYSTEM TO ASSESS THE UPTAKE OF ASTAXANTHIN IN ISOLATED INTESTINE FROM RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM)

## CHAPTER 7.0

## USE OF EVERTED GUT-SACS TO COMPARE THE ABSORPTION OF ASTAXANTHIN IN SALMONID FISH

# LIST OF FIGURES

## CHAPTER 1.0

## CHAPTER 2.O

## CHAPTER 3.0

## CHAPTER 4.0

## CHAPTER 5.0

## CHAPTER 7.0

# LIST OF TABLES

## CHAPTER 6.0

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# AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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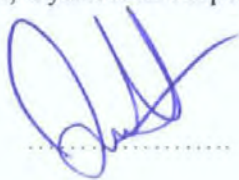
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# CHAPTER 1.0

## GENERAL INTRODUCTION

### 1.1 Salmonid aquaculture and nutritional aspects of pigmentation

As harvest limitations in global fisheries have become increasingly apparent aquaculture has developed into a rapidly expanding global industry with increasing importance. Meyers (1994) reviewed the status of the global aquaculture industry and its significance. Among the carnivorous finfish species cultured, fifty percent are salmonids with a ratio of 1:1 salmon and trout. In the period of 1986-1989, increases of 100-180% occurred in the production of farmed Atlantic salmon (*Salmo salar*) and Pacific salmon (*Oncorhynchus* spp.), with concurrent increases of around 20-70% for other farmed finfish and crustaceans. World production of all farmed salmon (in 1999) was estimated to be over 750,000 metric tonnes per annum, with figures expected to approach 1 million metric tonnes by the year 2001 and 1.3 million tonnes by the year 2005 (Lorenz & Cysewski 2000). The most prevalent growth has occurred within the Norwegian Atlantic salmon industry where from 1979 to the early 1990's production expanded from approximately 4,000 to over 150,000 metric tonnes. Bjerkeng (1997) noted that this production of Atlantic salmon had increased to 220,000 metric tonnes. Chile, the other major producer of farmed salmonids, increased production of farmed salmon and sea trout from 200,700 metric tonnes in 1998 to 222,900 metric tonnes in 1999, an 11% increase (Egan 2000). The United Kingdom and Canada U.K., also recognised as significant contributors to world wide salmonid culture, increased production from 115,000 metric tonnes in 1998 to 119,000 metric tonnes in 1999, a 3% increase (Egan 2000).

1

This growth in the aquaculture industry has been facilitated in part by the parallel developments that have occurred within the salmonid feed industry, since successful fish culture is dependent on sound nutrition and feeding regimes (Cho 1990). In this respect, there has been considerable progress and advances in the last 30 years concerning the nutritional requirements of fish, particularly salmon and trout. Fish generally have a high requirement for protein that provides the key essential amino acids needed for growth and tissue repletion. Additionally, most carnivorous fish including salmonids have the ability to spare the use of protein for energy purposes by preferentially metabolising lipids and to a limited extent carbohydrate in the feed (GrisdaleHelland & Helland 1997; Sveier *et al.* 1999). Lipids also supply the essential fatty acids such as the *n-3* and *n-6* series that are important for health and efficient growth performance at all stages of development. Modern aquafeeds for salmon and trout are formulated to be nutrient dense with respect to protein and energy. Therefore, it is standard practise to produce diets containing high oil inclusion (>30%) and based on premium quality fishmeal concentrates and selected ingredients such as soybean meal and corn glutein. These macro ingredient components are complemented with vitamin and mineral supplements to provide a completely balanced feed that satisfies the nutritional requirements of the species in question.

It is essential that farmed fish resemble their wild counterparts in terms of size; shape; taste and texture. Furthermore, the final colour of fish flesh is an important consideration (Simpson & Kamata 1978; Goodwin 1986). The consumer prefers red-coloured products of salmonid fishes (Ostrander *et al.* 1976; Hatano *et al.* 1987; Rounds *et al.* 1992; Sigurgisladottir *et al.* 1994; Skonberg *et al.* 1998). Further to this preference, redness adds to enjoyment of eating salmonids (Sylvia *et al.* 1995) and may be an indicator of quality (Sylvia *et al.* 1996). Indeed, aquaculture practices that produce high-value salmonid species employ feeding strategies that result in the development of a pink to red colour in

the flesh of fish. This has been achieved in practise by the incorporation of pigments known as carotenoids, in commercial fish feeds at typical concentrations ranging from 40 to 80 mg kg$^{-1}$ (Nickell & Bromage 1998a). Although these carotenoids are naturally widespread in nature (primarily in plants), salmonids, amongst other animals, cannot synthesise these molecules *de novo*, hence the reason for their dietary inclusion (Hata & Hata 1973; Simpson & Kamata 1978; Ando *et al.* 1986b; Ando *et al.* 1992; Guillou *et al.* 1992a; Storebakken & No 1992; Blanc & Choubert 1993; Choubert & Storebakken 1996).

In nature, wild salmonids are pigmented through ingestion of crustaceans which contain the carotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'dione) (Schiedt *et al.* 1986; Skrede & Storebakken 1986), yet synthetically manufactured astaxanthin and the carotenoid canthaxanthin (β,β-carotene-4,4'-dione) are commonly supplemented into salmonid feed to promote a desired flesh colouration (Schiedt *et al.* 1986; Bjerkeng 1992; Bjerkeng *et al.* 1992; Bell *et al.* 1998; Akhtar *et al.* 1999).

## 1.2    Chemistry of carotenoids

Carotenoids are pigments synthesised by photosynthetic microorganisms and plants, but not by animals (Furr & Clark 1997). However, animals can modify the structure of ingested carotenoids through various metabolic routes. These carotenoids represent over 600 compounds in nature and all possess similar chemical features; an polyisoprenoid structure; a long conjugated series of double bonds within the centre of the molecule (usually 9-13 double bonds) and near symmetry around the central double bond (Britton 1995; see Figure 1.1).

(a)

(b)

(c)

(d)

(e)
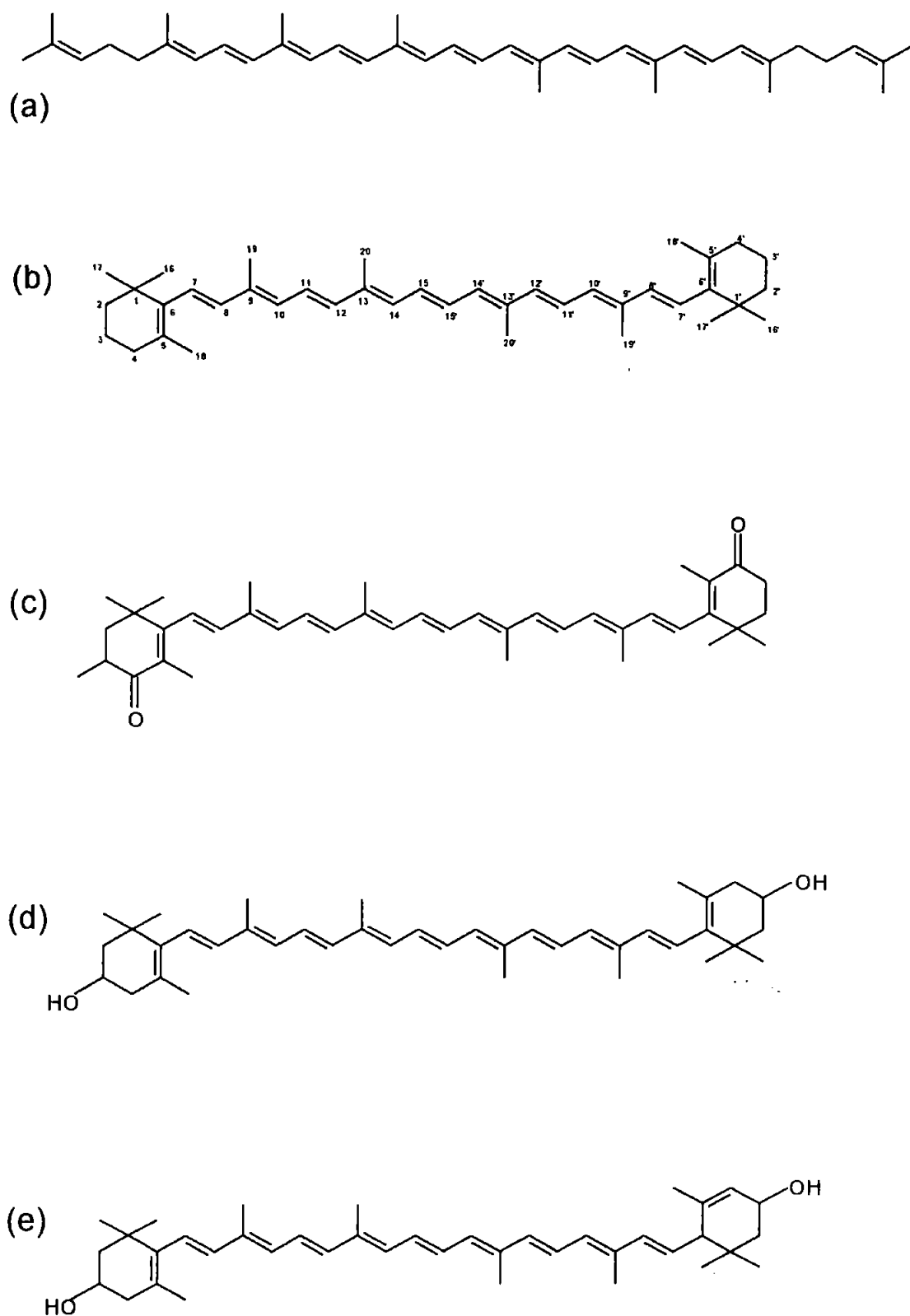
Figure 1.1    Structural formula of carotenoids other than astaxanthin mentioned in this

study (a) lycopene (b) β-carotene (c) canthaxanthin (d) zeaxanthin (e) lutein. The

numbering of carbon atoms used in nomlenculture of carotenoids is given for β-carotene.

The system of alternating single and double bonds that form the central structure of the carotenoid molecule is known as the chromophore. It is the length and structure of the chromophore which gives the carotenoids as a group their distinctive molecular shape, chemical reactivity, and light (ultra violet and/or visible) absorbing properties (Van Breeman 1996), which leads to their colourful appearance (usually yellow to red in colour). Carotenoids contain 3-11 or more conjugated double bonds and may be present in numerous *cis* (*Z*) isomeric or *trans* (*E*) geometric forms (Bjerkeng *et al.* 1997; see Figure 1.2). The all-*E*-isomer is considered to be the predominant native form where *Z* isomers are generally artefacts. Furthermore, carotenoids may be present in various optical configurations i.e. they exist as various enantiomers. Astaxanthin has two chiral centres giving rise to three optical isomers; an enantiomeric pair and a meso form; (3*S*,3'*S*); (3*R*,3'*R*) and (3*R*, 3'*S*) (Bjerkeng, 1992; see Figure 1.3). Carotenoids that contain one or more oxygen functions e.g. astaxanthin and canthaxanthin, belong to a group collectively known as the xanthophylls (Ruban *et al.* 1993).

## 1.3 Carotenoid Function

It is believed that carotenoids first emerged in primitive archaebacteria as lipid based compounds that reinforced cell membranes (Vershinin 1999). However, apart from the desired colouration that carotenoids impart in salmonid flesh, the functional purpose of carotenoids in fish species generally remains to be explained fully. It is well documented that carotenoids act as precursors for vitamin A in fish (Schiedt *et al.* 1985; Al-Khalifa & Simpson 1988; Christiansen *et al.* 1994; Torrissen & Christiansen 1995; Christiansen & Torrissen 1996). However, reviewed evidence suggests that these pigments could have a positive effect in relation to fecundity, fertility and larval development.
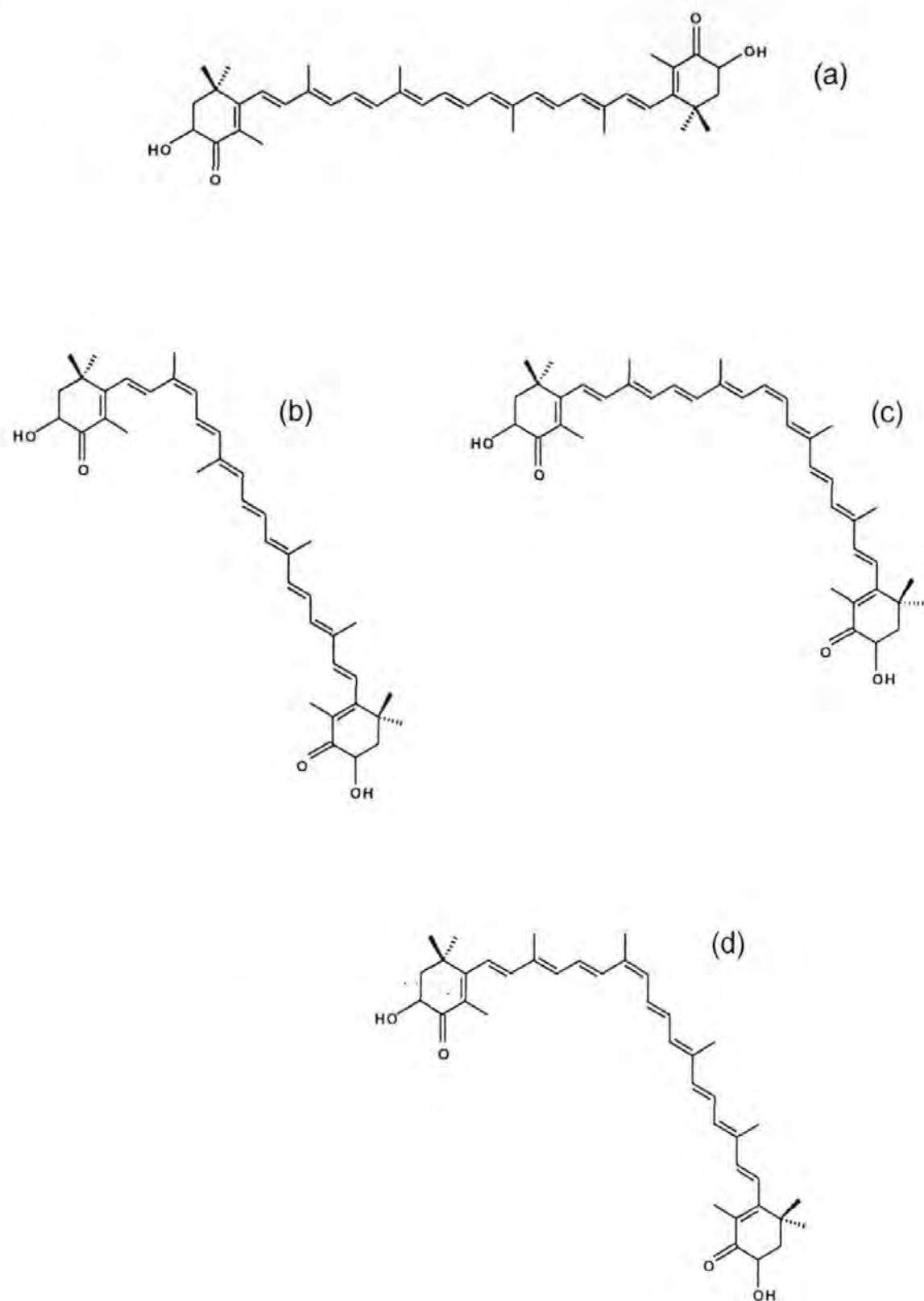
Figure 1.2    Geometric isomers of astaxanthin: (a) All-*E*-astaxanthin  (b) 9*Z*-astaxanthin
(c) 15*Z*-astaxanthin (d) 13*Z*-astaxanthin.

**1 (3S,3'S)**



**2 (3R,3'S)**



**3 (3R,3'R)**
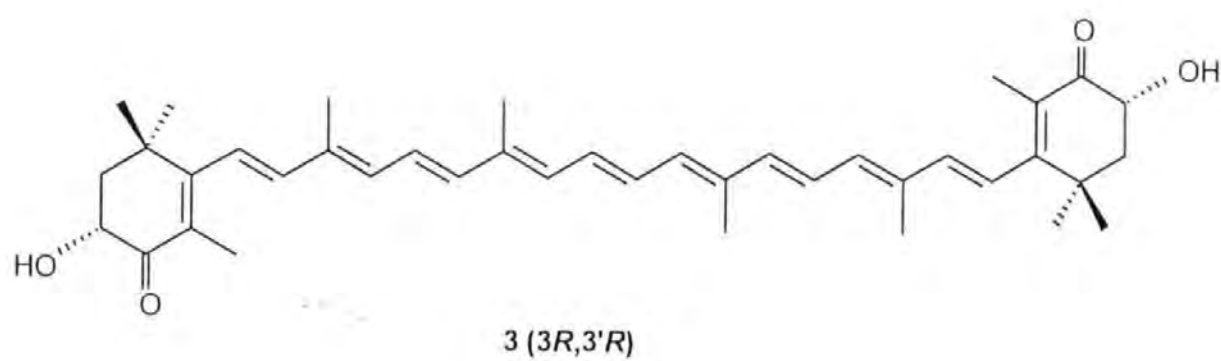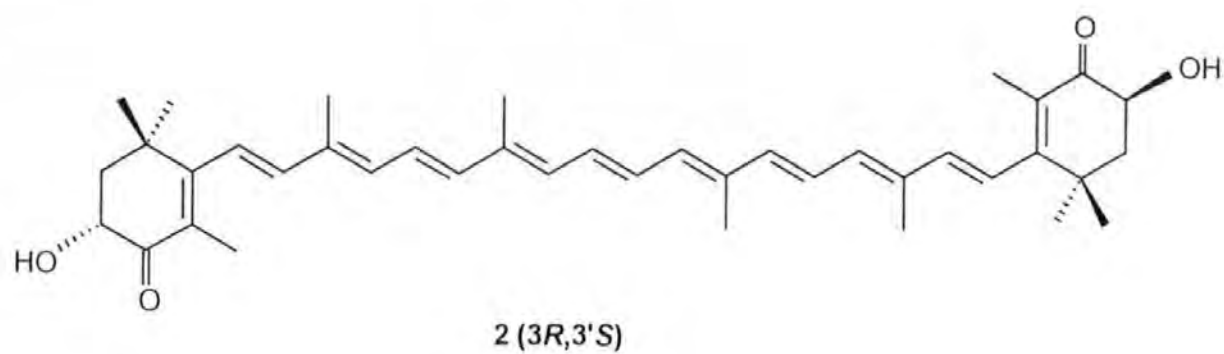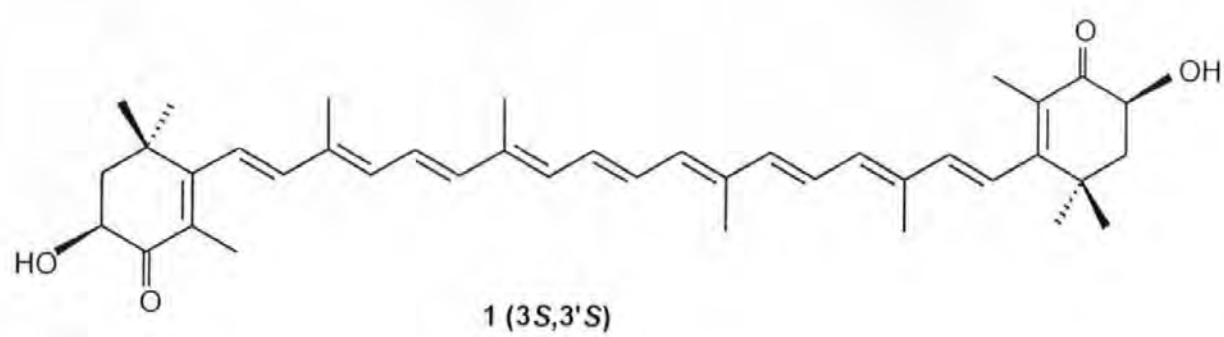
Figure 1.3    Structures of astaxanthin optical isomers (1) all-*E*-(3*S*,3'*S*)- (2) all-*E*-(3*R*,3'*S*; meso)- (3) all-*E*-(3*R*,3'*R*).

Positive correlations have been noted between carotenoid supplementation in feeds and growth rate of fish during the start-feeding period in Atlantic salmon (Christiansen *et al.* 1994; Torrissen 1984). Dietary astaxanthin has been shown to improve the condition of broodstock and enhance total egg production in spawning striped jack, *Pseudocaranx dentex* (Vassalo-Agius *et al.* 2001). Indirect evidence has demonstrated that at time of sexual maturation, deposited pigment in salmonid flesh is mobilised and transferred to the skin in males and the eggs in females, leading to the hypothesis that they play an important role during reproduction (Torrissen *et al.* 1989). In addition, dietary supplementation with astaxanthin has been shown to improve liver ultrastructure in some fish species (Segner *et al.* 1989). This positive effect may be associated with a carotenoid's singlet-oxygen quenching ability inhibiting free radical formation (Tacon 1981). Several studies have shown the potential of carotenoids to prevent free radical production and lipid peroxide production (Miki 1991; Nakano *et al.* 1995; Kobayashi & Sakamoto 1999; Nakano *et al.* 1999). Indeed, astaxanthin has been shown to be an excellent antioxidant that perfectly quenches excited states as well as ground state radicals (Beutner *et al.* 2001).

However, in Atlantic salmon, pigmentation of eggs had no effect of on subsequent fertilisation rates and survival from the eyed stage to hatching (Christiansen & Torrissen 1997) or during the embryonic stage (Torrissen 1984). Choubert *et al.* (1998) found that feeding diets supplemented with canthaxanthin and astaxanthin to rainbow trout had no effect on frequency of maturing females; timing of ovulation; relative fecundity; egg weight; fertilisation rate and fingerling growth. This led the authors to suggest that any positive effect via dietary supplementation with carotenoids would only be noted under "abnormally poor" culturing conditions.

## 1.4    Sources of carotenoid

Dietary sources of synthetic astaxanthin and canthaxanthin have been used extensively for the pigmentation of salmonid fish species. These sources are incorporated into beadlets containing a complex mixture of gelatin, carbohydrates starch and antioxidants. Although canthaxanthin was commercially available some years prior to astaxanthin, the latter carotenoid has become the pigment of choice in most salmonid production facilities in Europe due to the more natural colour it imparts once deposited in salmonid flesh. The most economically important commercial source of synthetic astaxanthin (CAROPHYLL®️ Pink, Hoffmann-La Roche, Basel, Switzerland; Bjerkeng et al. 1997) consists of a racemic mixture (1:2:1) of the (3R,3'R), (3R,3'S; meso) and (3S,3'S) isomers, respectively. In recent years both the consumer and fish farmer have become increasingly wary over the introduction of chemically synthesised additives into the food chain. This has encouraged a general trend for using natural sources of feed nutrients (Johnson & An 1991). Table 1.1 provides a summary of a number of feed trial type studies that have assessed the absorption and utilisation of carotenoids by salmonids from an array of different carotenoid sources.

### 1.4.1    Crustaceans and by-products

Crustaceans and crustacean waste produce contain relatively high concentrations of these pigments and have warranted considerable interest as an alternative natural source of carotenoids for salmonid feeds (Saito & Regier 1971; Spinelli et al. 1974; Kuo et al. 1976; Sivtseva & Dubrovin 1982; Choubert & Luquet 1983; Meyers & Thibodeaux 1983; Tidemann et al. 1983; Arai et al. 1987; Mandeville et al. 1991; Virtue et al. 1995).

Table 1.1    Comparative absorption and deposition of carotenoid from different sources in different salmonid species

| Reference | Pigment source | Salmonid species | Trial Length (days) | Dietary carotenoid concentration (mg kg⁻¹)† | Dietary Lipid (%) | Apparent Digestibility (%) | Plasma/serum carotenoid concentration (µg ml⁻¹) | Flesh carotenoid concentration (mg kg⁻¹)‡ | Flesh retentio (%) |
|---|---|---|---|---|---|---|---|---|---|
| | **SYNTHETIC** | | | | | | | | |
| Storebakken & Choubert (1991) | Synthetic astaxanthin | Rainbow trout | 56 | 25-50 | 17 | - | - | 2 | 11 |
| | Synthetic canthaxanthin | Rainbow trout | 56 | 25-50 | 17 | - | - | 2 | 7 |
| Choubert & Storebakken (1996) | Synthetic astaxanthin | Rainbow trout | 15 | 12.5-200 | 17 | 61-79 | - | - | - |
| | Synthetic canthaxanthin | Rainbow trout | 15 | 12.5-200 | 17 | 54-69 | - | - | - |
| March & MacMillan (1996) | Synthetic astaxanthin | Rainbow trout | 189 | 40-100 | - | - | 6-9 | 4 | - |
| | Synthetic astaxanthin | Chinook salmon | 175 | 40-100 | - | - | 3-6 | 4 | - |
| | Synthetic astaxanthin | Atlantic salmon | 280 | 40-100 | - | - | 3-4 | 4 | - |
| | **ALGAE** | | | | | | | | |
| Sommer et al. (1991) | Haematococcus pluvialis, whole | Rainbow trout | 100 | 40 | 10 | - | - | 1 | - |
| | Haematococcus pluvialis, broken | Rainbow trout | 100 | 40 | 10 | - | - | 2 | - |
| | Synthetic astaxanthin | Rainbow trout | 100 | 40 | 10 | - | - | 4 | - |
| Sommer et al. (1992) | Haematococcus pluvialis, broken | Rainbow trout | 100 | 20-80 | 18 | - | - | 1-3 | - |
| | Synthetic astaxanthin | Rainbow trout | 100 | 80 | 18 | - | - | 5 | - |
| Choubert & Heinrich (1993) | Haematococcus pluvialis | Rainbow trout | 28 | 100 | 7 | - | - | 6 | 1.5 |
| | Synthetic astaxanthin | Rainbow trout | 28 | 100 | 7 | - | - | 12 | 4.1 |
| | Astaxanthin: canthaxanthin (1: 1) | Rainbow trout | 28 | 100 | 7 | - | - | 13 | 3.1 |
| Gouveia et al. (1998) | Chlorella vulgaris | Rainbow trout | 42 | 64 | 15-20 | 71-83 | - | (15-21) | 4-5 |
| | Canthaxanthin: astaxanthin (5:3) | Rainbow trout | 42 | 64 | 15-20 | 79-87 | - | (14-21) | 3-5 |
| | Synthetic astaxanthin | Rainbow trout | 42 | 24 | 15-20 | 63-75 | - | (8-12 ) | 5-8 |

Table 1.1 (continued)

| Reference | Pigment source | Salmonid species | Trial Length (days) | Dietary carotenoid concentration (mg kg$^{-1}$)$^\dagger$ | Dietary Lipid (%) | Apparent Digestibility (%) | Plasma/serum carotenoid Concentration (µg ml$^{-1}$) | Flesh carotenoid concentration (mg kg$^{-1}$)$^\ddagger$ | Flesh retentio (%) |
|---|---|---|---|---|---|---|---|---|---|
| Barbosa et al. (1999) | *Haematococcus pluvialis* | Rainbow trout | 5 | 100 | 9-24 | - | 7-9 | - | - |
| | Synthetic astaxanthin | Rainbow trout | 5 | 100 | 9-24 | - | 5-8 | - | - |
| | **CRUSTACEAN** | | | | | | | | |
| Peterson et al. (1966) | Crayfish extract | Brook trout | 40 | 53 | - | - | - | 1 | - |
| | Raw crayfish | Brook trout | 25 | 53 | - | - | - | 1 (inc. skin, fins and flesh) | - |
| | Paprika extract | Brook trout | 35 | 257 | - | - | - | 3.22 (inc. skin, fins and flesh) | - |
| Spinelli & Mahnken (1978) | *Pleuroncodes planipes* (oil extract) | Coho salmon | 120 | 30-90 | - | - | - | - | 3-5 |
| Torrissen et al. (1981) | Shrimp waste (*Pandalus borealis*) | Rainbow trout | 87 | 5 | 29-30 | 45-71 | | 1-2 | - |
| Meyers & Thibodeaux (1983) | Crawfish meal | Rainbow trout | 90 | 20% inc. | - | - | - | 4 | - |
| | Crawfish meal extract in oil | Rainbow trout | 90 | 15% inc. (0.6mg Ax g$^{-1}$ oil) | - | - | - | 5 | - |
| Choubert & Luquet (1983) | Shrimp meal | Rainbow trout | - | 10-12 | 9-17 | - | - | 1 | - |
| Arai et al. (1987) | *Euphausia superba*- Oil extract | Coho salmon | 28 | 70 | 13-15 | - | - | 2 | - |
| Mori et al. (1989) | Krill oil | Coho salmon | 42 | 8-16 | 14-15 | - | 1.6-3.0 | 2 | - |
| | Synthetic astaxanthin | Coho salmon | 42 | 8-16 | 14-15 | - | 0.7-2.2 | 2 | - |

Table 1.1 (continued)

| Reference | Pigment source | Salmonid species | Trial Length (days) | Dietary carotenoid concentration (mg kg$^{-1}$)[†] | Dietary Lipid (%) | Apparent Digestibility (%) | Plasma/serum carotenoid concentration (μg ml$^{-1}$) | Flesh carotenoid concentration (mg kg$^{-1}$)[‡] | Flesh retention (%) |
|---|---|---|---|---|---|---|---|---|---|
| Coral *et al.* (1998) | *Pleuroncodes planipes* (oil extract) | Rainbow trout | 42 | 75 | 10 | - | - | 1-4 | - |
| | **YEAST** | | | | | | | | |
| Johnson *et al.* (1980) | *Phaffia rhodozyma*, intact | Rainbow trout | 87 | 55 | - | - | - | 2 | - |
| | *Phaffia rhodozyma*, broken | Rainbow trout | 48 | 55 | - | - | - | 11 | - |
| | *Phaffia rhodozyma*, partially digested | Rainbow trout | 42 | 55 | - | - | - | 3 | - |
| | *Phaffia rhodozyma*, fully digested | Rainbow trout | 42 | 55 | - | - | - | 12 | - |
| Gentles & Haard (1991) | *Phaffia rhodozyma* (mechanically milled; enzyme treated; spray-dried or extracted) | Rainbow trout | 56 | 112 | 15 | - | - | (14-28) | - |
| Choubert *et al.* (1995) | *Phaffia rhodozyma* (extracted) | Rainbow trout | 28 | 50-100 | 12-13 | >70 | - | 1-4 | 3-4 |
| Coral *et al.* (1998) | *Phaffia rhodozyma* | Rainbow trout | 42 | 75 | 11 | - | - | 1-4 | - |
| Whyte & Sherry (2001) | *Phaffia rhodozyma*, untreated | Atlantic salmon | 150 | 64 | 30 | - | - | 3-4 | - |
| | *Phaffia rhodozyma*, heat treated | Atlantic salmon | 150 | 64 | 30 | - | - | 3-4 | - |
| | *Phaffia rhodozyma*, heat and chemically treated | Atlantic salmon | 150 | 66 | 30 | - | - | 3-4 | - |
| | Synthetic astaxanthin | Atlantic salmon | 150 | 69 | 30 | - | - | 3-4 | - |

[†] Values based on total carotenoid content according to wet weight.  % inclusion levels have been used where concentration values are not available.  [‡] Values in parentheses represent mg kg$^{-1}$ dry muscle.

12

However, further processing of shrimp waste is required to extend this product's short shelf life and make it economically viable (Torrissen *et al.* 1981). Furthermore, crustacean meals are low in protein and high in minerals such as chitin and calcium carbonate, which restrict their use in feed formulations (Meyers & Rutledge 1971). Nonetheless, comparative studies of crustacean by-products and synthetic astaxanthin have demonstrated that this natural source can produce acceptable pigmentation levels in salmonids (Mori *et al.* 1989).


1.4.2   Yeast


Natural microbial sources have been identified as potential pigment sources for salmonids. For example, the yeast *Phaffia rhodozyma* has a high nutritional value (>20% protein and lipid) and contains the carotenoid astaxanthin (Johnson *et al.* 1977; Sanderson & Jolly 1994; Calo *et al.* 1995). Choubert *et al.* (1995) compared carotenoid retention in the flesh of rainbow trout fed diets supplemented with synthetic astaxanthin, *Phaffia rhodozyma* and canthaxanthin. Flesh retention, when expressed as a percentage of carotenoid intake, was higher for the astaxanthin group compared with the groups fed the yeast preparation. However, no differences were observed when retention was expressed according to the Apparent Digestibility Coefficients (ADC) of the astaxanthin sources, demonstrating that poor carotenoid retention was a result of low digestibility. This poorer digestibility may have been due to the naturally occurring cell wall that *Phaffia rhodozyma* possesses. This can impede pigment uptake (will reduce carotenoid digestibility) unless sufficient rupturing of the cell (enzymatic treatment) is undertaken prior to dietary supplementation (Johnson *et al.* 1980). However, Atlantic salmon have been shown to deposit astaxanthin from *Phaffia rhodozyma* regardless of whether cells had been treated (heat and chemical

treatment) in comparison to the synthetic source of astaxanthin (Whyte & Sherry 2001).


### 1.4.3 Algae


Algae are a large and diverse group of organisms that synthesise carotenoids. A number of studies regarding the use of algal supplements as carotenoid sources have been published (Ben-Amotz *et al.* 1989; Stahl *et al.* 1993 Gouveia *et al.* 1998).


The microalga *Haematococcus pluvialis* contains large amounts of astaxanthin (1.5-3.0% dry weight) although manipulation and production of mutant strains can result in early-enhanced (2.2-3.2 fold) astaxanthin accumulation (Tripathi *et al.* 2001). When strains of *Haematococcus pluvialis* are exposed to growth-limiting conditions (nitrogen and phosphate limitation; addition of NaCl and high temperature or light intensity) vegetative cells begin producing astaxanthin and concurrently undergo changes in cell morphology resulting in the formation of large red aplanospores (Boussiba & Vonshak 1991; Harker *et al.* 1996a) and the development of a thick sporopollenin cell wall that is resistant to oxidative degradation (Burczyk 1987). This encysted cell wall may impede absorption of pigment when used as a carotenoid source in animal diets (Johnson & An 1991). Additional carotenoids produced under these conditions include canthaxanthin, echionene, adonirubin and β-carotene, although their contribution usually represents a small percentage of the total carotenoid (Grung *et al.* 1992).


Additionally, 95% of the accumulated astaxanthin in this alga source is in an esterified form primarily consisting of monoesters, as opposed to diesters (Renstrøm & Liaaen-Jensen 1981). These esters consist of 16:0, 18:1 and 18:2 fatty acids esterified onto the 3'

hydroxyl group of astaxanthin (Lorenz & Cysewski 2000). Furthermore, astaxanthin synthesised by *Haematococcus pluvialis* is optically pure, existing as the 3*S*,3'*S* isomer (Renstrøm *et al.* 1981) compared to the commonly used synthetic source which is a racemic mixture of isomers.

Gastrointestinal ester hydrolysis is thought to be a pre-requisite to absorption of esterified carotenoids and vitamins into the systemic blood circulation (Mahadevan *et al.* 1963; Muller *et al.* 1976; Tyczkowski & Hamilton 1986a; Storebakken *et al.* 1987; Wingerath *et al.* 1995; Schweigert 1998). Subsequently, differences in digestibility may exist for astaxanthin esters compared to the free form since hydrolysis may be a limiting step. Moreover, reduced digestibility of carotenoid esters may result in poor flesh retention values for carotenoids in salmonids (Schiedt *et al.* 1986; Foss *et al.* 1987; Storebakken *et al.* 1987).

Several studies have shown comparatively poorer efficiency in pigmentation of salmonids when using *Haematococcus pluvialis* as a source of astaxanthin in comparison to the synthetic free form of astaxanthin (Sommer *et al.* 1991; Sommer *et al.* 1992; Choubert & Heinrich 1993). In all cases it was suggested that poorer pigmentation was a result of reduced bioavailability of astaxanthin from *Haematococcus pluvialis* due to encysted cell wall encapsulation.

However, contradictory evidence was presented by Barbosa *et al.* (1999) who compared the serum astaxanthin concentrations in trout fed diets supplemented with two different sources of astaxanthin (*Haematococcus pluvialis* and synthetic astaxanthin) at two different dietary lipid levels (9 and 24%). For the higher energy diet serum astaxanthin

15

concentrations were not significantly different between fish fed the algal and synthetic sources of astaxanthin. However, when dietary lipid level was low serum astaxanthin concentrations were significantly higher in fish fed diets supplemented with the algal source compared to the synthetic form. This suggests that differences noted in flesh retention between these two dietary sources as discussed previously were not a direct result of reduced bioavailability due to cell wall encapsulation or necessary carotenoid ester cleavage prior to blood absorption.

Clearly, confounding evidence has been presented concerning the use of *Haematococcus pluvialis* as a pigment source for salmonids. If indeed use of this dietary astaxanthin source results in unfavourable flesh pigmentation compared to the synthetic free form, then the evidence may suggest that limitations in digestibility and absorption are the cause of these noted discrepancies.

## 1.5    Flesh deposition of carotenoids

Salmonids, unlike other fish species, deposit ingested astaxanthin (40-80%) unchanged in their flesh (Foss *et al.* 1984; Schiedt *et al.* 1985; Storebakken *et al.* 1985; Torrissen *et al.* 1989; Bjerkeng 1992). Additionally, astaxanthin is deposited in its free form in salmonid flesh regardless of source (Choubert & Luquet 1983; Henmi *et al.* 1987). Henmi *et al.* (1989) demonstrated that in salmon muscle the carotenoids astaxanthin and or canthaxanthin binds with one β-ionine ring to a hydrophobic binding site which exists on the surface of the actomyosin protein within the myofibrils. Astaxanthin has one hydroxyl group and one carbonyl group on each of the β-ionine rings at opposite ends of its structure, where canthaxanthin has one carbonyl group on each of it β-ionine rings. These

hydroxyl and carbonyl groups form hydrogen bonds with the myofibril proteins. Subsequently, the strength of the carotenoid-protein association is dependent on the number of hydrogen bonds. Since astaxanthin can form two hydrogen bonds for each of it β-ionine rings, it combines more strongly to actomyosin than other carotenoids.

Reported values for astaxanthin in the flesh of farmed salmon are between 4-10mg kg$^{-1}$ (Torrissen *et al.* 1989). However, a number of abiotic and biotic factors are thought to influence the deposition of carotenoids in salmonids (Nickell & Bromage 1998a) including size or age (Spinelli & Mahnken 1978; Torrissen et al. 1989; March *et al.* 1990; Bjerkeng *et al.* 1992; Hatlen *et al.* 1995a); temperature (Storebakken *et al.* 1986; March *et al.* 1990) and sexual maturation (Crozier 1970; Sivtseva & Dubrovin 1982; Torrissen & Torrissen 1985; Ando *et al.* 1992).

Several authors have demonstrated a dose response relationship between dietary astaxanthin and pigmentation in salmonids, whereby a plateau in pigmentation capacity is reached at a feed astaxanthin concentration of ca. 60mg kg$^{-1}$ (Choubert & Storebakken 1989; Torrissen *et al.* 1989; Storebakken & No 1992; Olsen & Mortensen, 1997). Consequently, increasing dietary astaxanthin concentration further would be uneconomic. It has been suggested that variations in the number and size distribution of muscle fibres may influence astaxanthin deposition (variability in availability of binding sites) thus limiting flesh retention and causing the plateau effect (Nickell & Bromage 1998b). However, Johnston *et al.* (2000) found no significant correlation between muscle fibre density and flesh astaxanthin concentration in Atlantic salmon. Other authors have explained this dose response effect in part by a limitation in absorption efficiency or digestibility of the carotenoid (Choubert & Storebakken 1989; Torrissen *et al.* 1990;

Hatlen *et al.* 1995b).

Added to an apparent dose response effect, deposition efficiency between carotenoids used for salmonid pigmentation (canthaxanthin and astaxanthin) also differs. It has been repeatedly shown that rainbow trout deposit astaxanthin more efficiently in their flesh than canthaxanthin (Foss *et al.* 1984; Torrissen 1986, 1989b; Bjerkeng *et al.* 1990; Choubert & Storebakken 1989; No & Storebakken 1992). However, it has been shown more recently that Atlantic salmon absorb and/or deposit canthaxanthin more favourably in their flesh compared to astaxanthin (Buttle *et al.* 2001). It is plausible that such differences result from a greater affinity for one carotenoid to bind to the flesh of salmonids in comparison to the other. However, Henmi *et al.* (1989, 1991) demonstrated that astaxanthin and canthaxanthin bound non-specifically to actomyosin in salmon muscle. This suggests that discrepancies between these carotenoids in terms of deposition do not result from limitations at the muscle binding sites. Perhaps these differences may be explained in part by more efficient absorption of astaxanthin compared to canthaxanthin (or *visa versa*) as has been suggested for rainbow trout (Torrissen 1986; Guillou *et al.* 1992a; Choubert *et al.* 1994a).

Despite evidence to support the notion that absorption of pigments from consumed feed into the systemic circulation may well be limiting, contradictory findings have been reported that suggest metabolism of pigments as opposed to absorption is responsible for poor flesh pigmentation (March *et al.* 1990). However, March & MacMillan (1996) presented data (for Atlantic salmon) that again suggested the absorptive capacity of the intestine limited the amount of astaxanthin that can be advantageously added to salmonid diets. Nonetheless, interest based on increasing digestibility and/or bioavailability of these

carotenoids is at the forefront of current scientific research investigations.

## 1.6    Carotenoid metabolism

March *et al.* (1990) and March & MacMillan (1996) have indicated that rapid metabolism rather than failing absorption is responsible for poor pigmentation in salmonids, although no concise quantitative estimates are available on the proportion of absorbed astaxanthin that is metabolised.

Salmon and rainbow trout differ from goldfish since they cannot oxidise 3,3'-dihydroxy carotenoids (e.g. zeaxanthin) to astaxanthin (Hata & Hata 1973). Astaxanthin and canthaxanthin are reductively metabolised in salmonids (Schiedt *et al.* 1988ab, 1989; Bjerkeng *et al.* 1990, 1992; see Figure 1.4). · In Arctic charr (*Salvelinus alpinus*) the recorded major reductive metabolite of astaxanthin was idoxanthin, comprising 47-75% of total flesh fillet carotenoids (Aas *et al.* 1997; Hatlen *et al.* 1995b). Bjerkeng *et al.* (1999a) inferred that this reduction of astaxanthin to idoxanthin predominantly takes place in the liver of the salmonid fish. This is in agreement with the findings of Hardy *et al.* (1990) who administered radiolabelled canthaxanthin orally to rainbow trout and noted that the liver was the major organ that contained reductive metabolites. However, the possibility exists that concentration of metabolites formed in other tissues and organs may take place in the liver, as suggested by Metusalach *et al.* (1996).
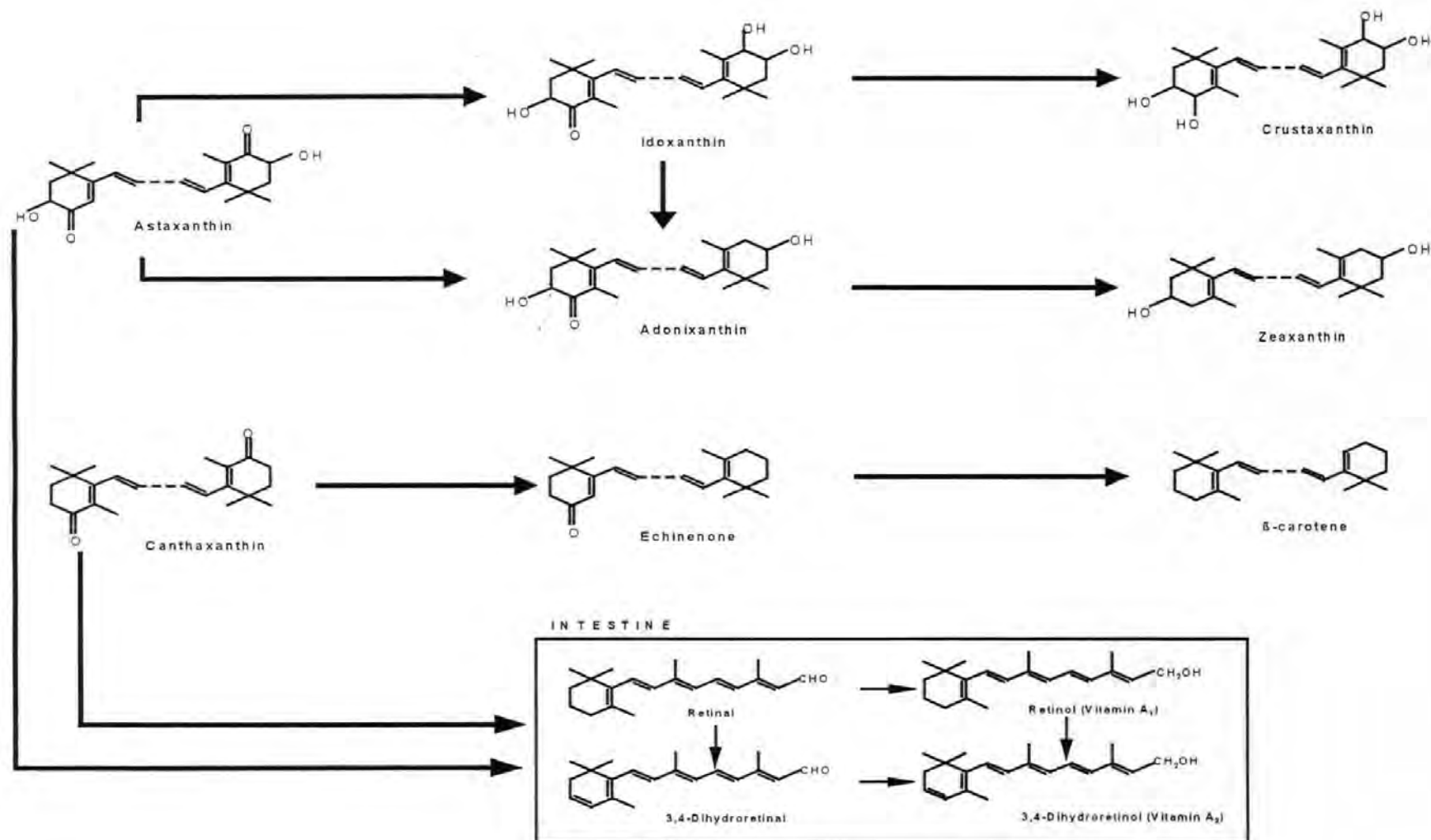
Figure 1.4    Reductive metabolism of astaxanthin and canthaxanthin in salmonid fish.  Taken and adapted from Torrissen *et al*. (1989).

In rainbow trout astaxanthin and canthaxanthin are reductively metabolised into yellow xanthophylls and carotenes. Astaxanthin is reduced to zeaxanthin ($\beta,\beta$-carotene-3,3'-diol) via $\beta$-adonixanthin (3,3-dihydroxy-$\beta,\beta$-carotene-4-one) and canthaxanthin is reduced to $\beta$-carotene ($\beta,\beta$-carotene) via echionene (Schiedt *et al.* 1985). Furthermore, rainbow trout can also convert ketocarotenoids into vitamin A in the intestinal wall, although *in vivo* this usually only occurs when the diet is not sufficiently supplemented with vitamin A (Lambertesen & Braekkan 1969; Schiedt *et al.* 1985; Al-Khalifa & Simpson 1988). Aas *et al.* (1999) reported the rapid conversion of astaxanthin into idoxanthin in Atlantic salmon fed a single dose of radiolabelled astaxanthin. The rapid appearance of idoxanthin in the blood suggested that the intestine could be an important site for astaxanthin metabolism. Reductive metabolites of astaxanthin and canthaxanthin are also found in the skin of rainbow trout (Schiedt *et al.* 1985; Bjerkeng *et al.* 1990) although it is not clear whether reductive metabolism occurs in the skin itself.

The cost of synthesised carotenoids and their necessary dietary implementation is high. Approximately 10-15% of the price of salmon feeds is due to the substantial investment required for dietary carotenoid inclusion (Torrissen *et al.* 1989; Hardy *et al.* 1990). In Norway, supplementation of synthetic astaxanthin in salmon diets accounts for 15-20% of the total feed costs (Torrissen & Ingebrigtsen 1992). Furthermore, only up to 15% of astaxanthin consumed is actually retrieved from fish muscle tissue (Torrissen *et al.* 1989; Storebakken & No 1992; Bjerkeng *et al.* 1999a). Consequently, understanding the mechanism of carotenoid absorption and the factors that may have an influence on the process are fundamental for improving flesh pigmentation in salmonids (Nickell & Bromage 1998a).

## 1.7 Carotenoid digestibility and absorption

Of great consideration when formulating and producing fish feeds are the quality of raw materials and the digestibility of the feed ingredients (Spyradakis *et al.* 1989; Cho 1990). Indeed, knowledge of a supplemented carotenoid's digestibility is critical for evaluation of its pigmentation efficacy (Choubert *et al.* 1995). The digestibility of a compound after ingestion is a good primary indicator of absorption efficiency as it indicates the quantity of compound that has been absorbed from the feed matrix. The current literature shows large inconsistencies between digestibility and flesh retention values in salmonids which suggests considerably higher levels of these pigments are absorbed by salmon than are retained in the flesh. However, carotenoid digestibility values may have been over estimated due to the degradation of carotenoid in faecal samples (Meyers 1994).

The bioavailability of carotenoids can be expressed as the proportion of carotenoid ingested that can be absorbed and is available for use and/or storage (Schweigert 1998). Bioavailability of carotenoids is influenced by a variety of factors. These include species of carotenoid; molecular linkage; amount of carotenoids consumed; matrix in which the carotenoid is incorporated; effectors of absorption and bioconversion; nutrient status of the host; genetic factors; host-related factors and mathematical interactions (Castenmiller & West 1998). Furthermore, the efficiency of digestion and gut transit time are likely to influence the proportion of any micro-nutrient that is absorbed from feed (Jackson 1997).

Bioavailability is considered a primary parameter when assessing the pigmentation efficiency of a specified carotenoid. Most scientific feed trials employ an initial assessment of carotenoid absorption by taking digestibility measurements, and/or measurements of blood concentration in the fishes systemic circulation. Indeed, high

22

correlation has been recorded between dietary carotenoid concentration and carotenoid levels in the blood (Choubert *et al.* 1994a; Kiessling *et al.* 1995 Storebakken & Goswami 1996). This evaluation can be conducted after continuous feeding to ascertain steady state concentrations of carotenoid in the blood. Alternatively, a post-prandial blood carotenoid profile can be formed after a single ingested dose of one or more carotenoids (Choubert *et al.* 1987; Gobantes *et al.* 1997). However, caution should be displayed when using blood concentrations as indicative of bioavailability since they are affected by absorption of carotenoid; removal of carotenoid from serum for storage/deposition; bioconversion, or excretion (Castenmiller & West 1998; Van het Hof *et al.* 2000). Additionally, inherent heterogeneity of subjects/animals results in large inter-individual variability in bioavailability assessment (Jackson 1997).

## 1.8    Mechanism of carotenoid uptake by the intestine

Digestibility values are influenced by the quantity of carotenoid that is absorbed within the gastrointestinal tract of the animal once ingestion has taken place. According to Torrissen (1986) and Al-Khalifa & Simpson (1988) the absorption of carotenoids occurs mainly along the proximal and mid-intestine of salmonids, although other authors have found little difference in carotenoid absorption along the entire length of the intestine (Guillou *et al.* 1992a). In humans, carotenoids appear to be absorbed by duodenal mucosal cells by a mechanism involving passive diffusion (Parker 1996). The pyloric section of the intestine in salmonids represents the human duodenal region

The mechanism of carotenoid absorption in humans at the gastrointestinal level has been extensively reviewed (Erdman *et al.* 1993; Parker 1996; Furr & Clark 1997; Van den Berg 1999). Figure 1.5 and the following summary is based on these publications.

INGESTED
ASTAXANTHIN

U.W.L

← BILE

MICELLES

Chylomicron formation

Lymph

Astaxanthin

Reductive metabolism

Vitamin A1 & A2
Other polar metabolites

Portal blood?

Sloughed cells

% Unabsorbed?

Specific cell wall
and/or intracellular
transport?
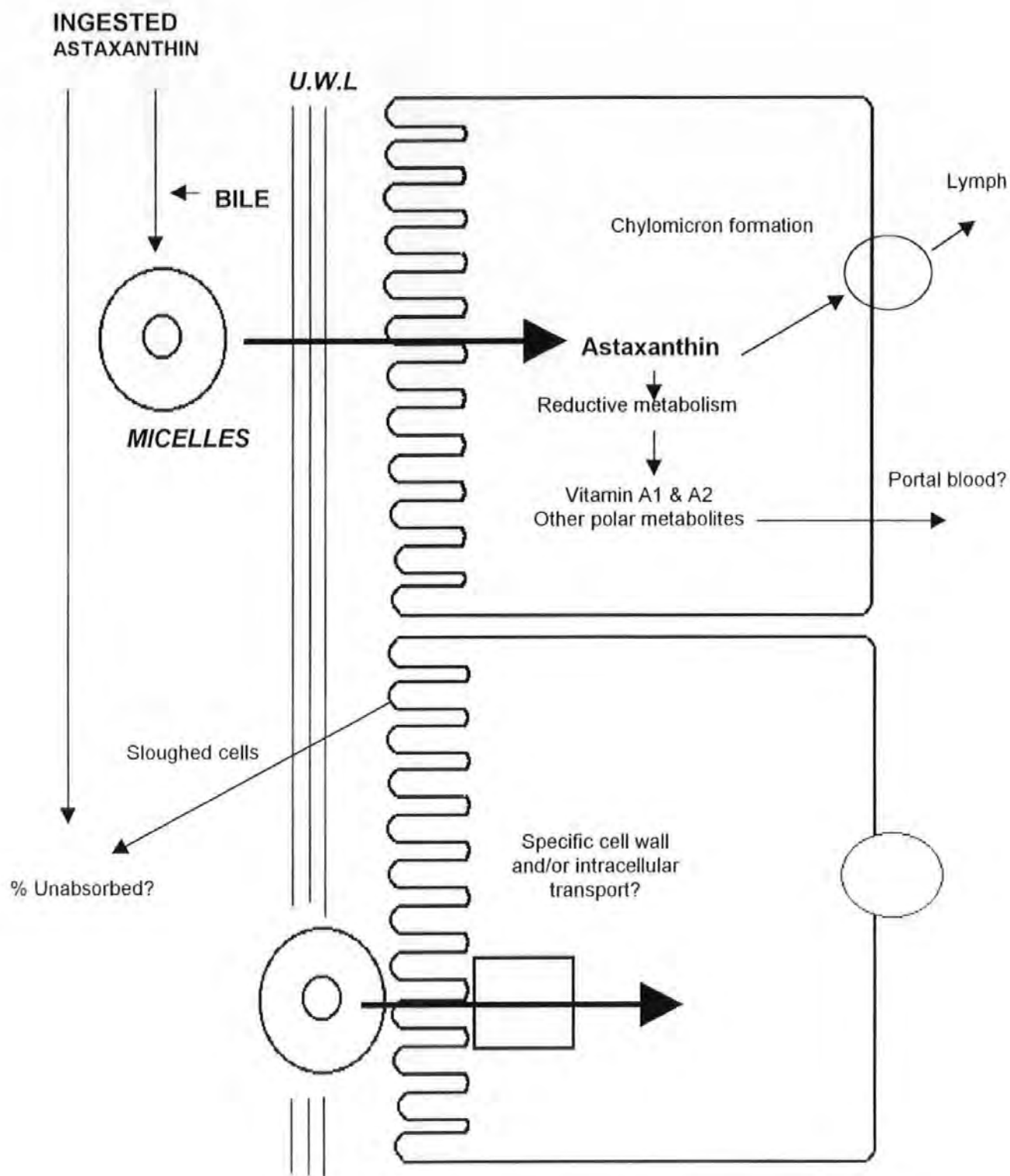
Figure 1.5    Proposed uptake of astaxanthin from lipid micelles across the unstirred water layer (U.W.L) into intestinal mucosal cells; possible metabolic events and movement of astaxanthin into lymph and portal blood.

Prior to absorption, carotenoids have to be released from the food matrix in which they are ingested. Initially this is achieved by the action of gastric hydrolysis of dietary proteins and lipids. Once released, lipophilic carotenoids will naturally dissolve in the oily phase of lipid droplets produced by gastric hydrolysis. Natural gastric action then renders the lipid droplets into a fine emulsion that passes into the duodenum region of the gastrointestinal tract. Solubility of a carotenoid and distribution within the emulsion is determined by polarity of the pigment, where more polar xanthophylls are located at the surface and the least polar carotenoids incorporated into the triacylglycerol core. Carotenoid distribution will affect the transfer of carotenoids into mixed bile salt micelles. This is an essential step since carotenoid absorption does not take place without micelle formation. Lipid micelles, whose formation is dependant on bile flow from the gall bladder (stimulated by dietary fat), are thought to act as a vehicle for carotenoid transfer across the unstirred water layer (system of water lamellae along the microvillus surface of the intestine). Once this layer is traversed, carotenoids are absorbed unchanged in a passive manner by mucosal cells.

It is likely that this passive diffusion process is dependent on the carotenoid concentration gradient between the micelle and the plasma membrane of the enterocyte. As carotenoids are hydrophobic in nature, direct contact between the micelle and the cell membrane is probably required.

If carotenoids are not metabolised in the enterocyte (usually applies to provitamin A carotenoids) they are packaged into chylomicron like structures and secreted into lymph and subsequently enter the systemic blood circulation. However, the intracellular mechanisms controlling these processes are not well understood.

Tyczkowski & Hamilton (1986b) demonstrated differential absorption for different carotenoids (zeacarotene and lutein) along the intestinal tract of chickens, suggesting

regulatory mechanisms for carotenoid absorption. However, the absorption of carotenoids in chickens is thought to be independent of lipid absorption since cases of lipid malabsorption and hypocarotenoidemia are not correlated (Osborne *et al.* 1982). Non-specific esterases have been found along the intestinal brush border membrane and within the cytoplasm of enterocytes in Nile tilapia, *Oreochromis niloticus* (Tengjaroenkul *et al.* 2000). If these findings apply to salmonids, this may suggest carotenoid-ester metabolism may take place intra- and extracellularly.

## 1.9    Digestibility and micelle formation

Formation of bile salt micelles is thought to be essential for the uptake of fatty acids, or indeed carotenoids, since they acts as a "shuttle" between the intestinal contents and the aqueous-microvillus interface of the intestine (Westergaard & Dietschy 1976). Micelles are thought to penetrate the unstirred water layer, a series of water lamellae adjacent to the microvillus surface which form a natural barrier to carotenoid uptake (Furr & Clark 1997). Furthermore, it has been recorded that micelle formation is more important for the absorption of insoluble non-polar lipids, such as $\alpha$-tocopherol, compared to polar lipids such as oleic acid (MacMahon & Thompson 1969; MacMahon & Thompson 1970). In addition, the presence of polar lipid in micelles enhances the solubility of non-polar lipids (El-Gorab & Underwood 1973). These findings imply that the formation of lipid micelles formed following intraluminal triglyceride lypolysis of the feed matrix is a restricting factor in the absorption of carotenoids (Borel *et al.* 1996).

The degree to which a specific carotenoid is incorporated into micelles may influence its relative digestibility. Choubert *et al.* (1994a) demonstrated that the clearance rate and potential absorption mechanism (passive absorption) of canthaxanthin and astaxanthin in

the blood of immature trout was similar, yet circulating blood levels of astaxanthin were 2.3 times greater than that of canthaxanthin. Discrepancies in uptake may have been due to greater solubilisation of astaxanthin compared to canthaxanthin in micelles.

However, it has been demonstrated *in vitro* that once critical micelle concentration of the bile salt(s) has been achieved (usually a low value) carotenoid absorption is sufficiently facilitated (Hollander & Ruble 1978). Moreover, when detergent concentrations are higher than this critical value, the uptake of β-carotene has been shown to decline sharply (El-Gorab *et al.* 1975).

## 1.10    Dietary lipids and carotenoid absorption

Dietary lipid stimulates the production of bile which facilitates intraluminal solubilisation of carotenoids (micelle formation) prior to absorption, without which apparent carotenoid absorption is reduced (Erdman *et al.* 1993; Williams *et al.* 1998). In fact a number of studies have found a positive relationship between lipid inclusion and apparent digestibility of carotenoids (Torrissen *et al.* 1990; Choubert *et al.* 1991; Gouveia *et al.* 1998) and pigmentation of salmonids (Abdul-Malak *et al.* 1975). Moreover, it has been shown that the absorption and deposition of astaxanthin is influenced by the source and composition of the oil in relation to fatty acid content (Christiansen *et al.* 1991). This may infer that differences between absorption of different fatty acid classes exist, a feature that has been recorded in turbot, *Scophthalmus maximus* (Koven *et al.* 1994; Koven *et al.* 1997).

Bjerkeng *et al.* (1999b, 2000) demonstrated that flesh retention of astaxanthin was significantly higher in salmon fed capelin and Peruvian high PUFA oil compared to herring and sandeel oil. Salmon fed diets containing Peruvian high PUFA oil also had

27

significantly higher fillet carotenoid content than fish fed the other diets. However, plasma astaxanthin concentrations were positively related to monounsaturated fatty acids content and negatively to the content of $n$-3 fatty acids. There is evidence from analytical studies that suggests that PUFA's are more effectively absorbed than saturated fatty acids during lipid digestion in fish (Lie *et al.* 1987; Koven *et al.* 1997). In contrast, it has been recorded that rats absorb astaxanthin to a greater extent from olive oil emulsions compared to corn oil emulsions, suggesting the presence of polyunsaturated fatty acids (PUFA) actually reduce uptake in mammals (Clark *et al.* 2000). Furthermore, contradictory evidence was presented by Torrissen (1985) who found no significant differences in pigmentation between fish fed diets supplemented with capelin oil "high" in free fatty acids and those fed an oil lower in fatty acids. Nonetheless, differences between the two oil sources may have not been great enough to promote a noticeable affect.

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## 1.11 Blood transport of carotenoids

Measurements of blood carotenoid concentration following carotenoid ingestion indicate the amount of carotenoid that has been absorbed into the systemic circulation and is potentially available for utilisation. In humans, carotenoids are transported in plasma exclusively by lipoproteins with the distribution among lipoprotein classes determined largely by the physical properties of the carotenoid (Erdman *et al.* 1993; Parker 1996). The available literature supports the contention that carotenoids must be bound to blood protein before transport in the blood of salmonids presumably to solubilise the hydrophobic pigment. For example, in Chum salmon (*Oncorhynchus keta*) astaxanthin is mainly bound to high-density lipoprotein (Ando *et al.* 1985; Nakamura *et al.* 1985) and very high-density lipoprotein fractions (Ando *et al.* 1986b). Canthaxanthin has been found in all lipoprotein fractions in immature rainbow trout (Choubert *et al.* 1992; Chavez *et al.*

1998). In female chum salmon during spawning, appreciable amounts of astaxanthin are found bound to vitellogenin, a likely carrier of carotenoid from the flesh to the ovaries (Ando *et al.* 1986c). In Atlantic salmon, astaxanthin in blood plasma is mainly associated with a protein other than lipoproteins, presumably albumin (Aas *et al.* 1999). These findings could suggest that binding of astaxanthin to blood proteins may limit internal distribution of astaxanthin and deposition in salmonid flesh. However, evidence has been presented for rainbow trout indicating that the saturation of lipoprotein fractions is not a limiting factor for transport of carotenoid (Chavez *et al.* 1998). Similar findings have been found in humans for the carotenoid β-carotene (Mathews-Roth & Gulbrandsen 1974).

## 1.12 Differences between carotenoids and their bioavailability

A number of different carotenoids may be present in fish feeds due to feed supplementation with different sources of carotenoid and the origin of other dietary ingredients. It has been demonstrated that different carotenoids may have different relative bioavailability (Schiedt *et al.* 1985; Guillou *et al.* 1992ab; Clark *et al.* 2000). In rainbow trout astaxanthin is more efficiently absorbed into the blood in comparison to canthaxanthin (Torrissen 1989a; Choubert *et al.* 1994a), and zeaxanthin (Guillou *et al.* 1992a).

Some authors have suggested that carotenoid polarity will influence the absorption of these pigments. For example, astaxanthin (two hydroxyl and ketone groups), is absorbed (blood plasma values) in rainbow trout to a greater extent than other carotenoids (canthaxanthin, zeaxanthin, β-cryptoxanthin, β-carotene and lycopene) which are more hydrophobic (Tyssandier *et al.* 1998). The authors suggested that less hydrophobic carotenoids are absorbed more efficiently, or, are cleared from the plasma at a slower rate. Gobantes *et al.*

29

(1997) recorded post-prandial serum concentrations of astaxanthin and canthaxanthin in immature rainbow trout force fed individual doses of one of these carotenoids. The maximum level of canthaxanthin in serum was 1.6 times lower than that of astaxanthin. It was suggested that this finding was due to canthaxanthin being less polar than astaxanthin and that this might effect an animal's ability to form intestinal micellar solutions with one carotenoid compared to another. Evidence from *in vitro* studies supports the theory that carotenoid polarity affects the solubility of these pigments in aqueous solutions of mixed micelles (El-Gorab & Underwood 1973). Additionally, variation in carotenoid polarity leads to differences in regional distribution of these molecules within the lipid micelle, where polar and non-polar carotenoids are associated with the surface and the core of the micelle, respectively (Borel *et al.* 1996). Distribution of carotenoids within the lipid micelle and the effect that this would have on the absorption characteristics of a carotenoid in fish are not clear.

## 1.13 Carotenoid isomers and bioavailability

Optical isomers of dietary astaxanthin are utilised equally by salmonids, and no epimerization occurs once deposited in the flesh (Foss *et al.* 1984; Storebakken *et al.* 1985; Arai *et al.* 1987). However, there is considerable interest in the bioavailability of different geometric isomers of carotenoids in various animal models, and the subsequent differences between them (Stahl *et al.* 1993; Hébuterne *et al.* 1995; Stahl *et al.* 1995; Østerlie *et al.* 2000). Bjerkeng *et al.* (1997) noted that flesh carotenoid concentration in rainbow trout tended to be higher in those fish fed diets supplemented with all-*E*-astaxanthin compared to those fish fed a diet supplemented with a mixture of isomers. Furthermore, apparent digestibility coefficients (ADC) of total astaxanthin were higher for trout fed the all-*E*-astaxanthin diet compared to trout fed a diet supplemented with the stereoisomer mixture.

It was suggested that this indicated a competitive mechanism of uptake for the different stereoisomers. These findings were supported in an extension study where higher total carotenoid concentrations were found in the blood of trout fed diets supplemented with all-*E*-astaxanthin compared to those trout fed diets supplemented with the stereoisomer mixture (Østerlie *et al.* 1999). This suggested more efficient intestinal absorption of the all-*E*-isomers compared to the *Z*-isomers. Similarly, preferential accumulation of all-*trans* β-carotene compared to 9-*cis*-β-carotene in human plasma has been demonstrated (Gaziano *et al.* 1995). Furthermore, *cis-trans* isomerisation has been demonstrated during the absorption of β-carotene, resulting in the near absence of post-prandial plasma 9-cis-β-carotene after its oral administration in humans (You *et al.* 1996).

Selective accumulation of all-*E*-astaxanthin has been recorded in the muscle of salmon, and in blood samples of both salmon and halibut (Bjerkeng & Berge 2000). These differences may be due to variation in intestinal micellar loading between isomers. Indeed Bjerkeng & Berge (2000) recorded higher Apparent Digestibility Coefficients for all-*E*-astaxanthin compared to the 9*Z* and 13*Z* isomer for both salmon and halibut.

However, contradictory evidence has been presented in humans where it has been shown that lycopene is isomerized from the *trans* to *cis* form pre-absorption in the gastric lumen (Re *et al.* 2001). It was suggested that this might be significant as *cis* lycopene has greater bioavailability than the *trans* form due to increased solubility in micelles. Indeed, *cis*-isomers of carotenoids are less likely to crystallise/form aggregates than the extended trans configuration (Britton 1995; Castenmiller & West 1998) which would lead to increased incorporation into a bile-acid micelle.

## 1.14 Synergistic/antagonistic effects between carotenoids on bioavailability

It is not uncommon practise for feed companies to supplement fish feeds with both astaxanthin and canthaxanthin. Indeed it has been documented that combined consumption of astaxanthin and canthaxanthin seems to have a synergistic effect on flesh pigmentation (Foss *et al* 1987; Torrissen 1989b). However, interaction at the intestinal level (competition during micellar incorporation, intestinal absorption, lymphatic transport) may reduce absorption of carotenoids (Van het Hof 2000). Indeed, numerous authors have suggested combined consumption of one or more carotenoids may inhibit the uptake/bioavailability of one or both pigments (Gärtner *et al.* 1996; Hageman *et al.* 1999). For example, White *et al.* (1994) investigated the pharmacokinetics of β-carotene and canthaxanthin after individual and combined doses in the serum of human subjects. These authors noted that ingestion of a concurrent β-carotene dose reduced the peak serum canthaxanthin concentration (ca 39%) and the area under the serum canthaxanthin concentration time curve at twenty four (ca. 38%) and seventy two (ca. 34%) hours. However, this antagonism was not reciprocal, as canthaxanthin did not inhibit the appearance of β-carotene. Similarly, combined doses of β-carotene and lutein results in reduced serum area under the curve values for lutein (54-61% of controls) in human subjects (Kostic *et al.* 1995). However, lutein reduced the area under the curve value for β-carotene in some subjects but enhanced it in others, suggesting individual responses can differ markedly. Results from these studies could suggest that; there are specific mechanisms for the intestinal carotenoid absorption; there is preferential absorption of specific carotenoid groups and/or there is competition between carotenoids for mixed micelles incorporation. Clearly, carotenoids do interact with each other during intestinal absorption, metabolism and serum clearance. Alternatively, it has been shown in rats that the absorption of pro-vitamin A carotenoids e.g. β-carotene may be increased due to other

32

carotenoids (canthaxanthin and zeaxanthin) inhibiting the action of $\beta$-carotene dioxygenase (Grolier *et al.* 1997).

Pozo *et al.* (1988) recorded increased deposition of canthaxanthin in the flesh of rainbow trout when diets were supplemented with both canthaxanthin and vitamin E suggesting that this was a result of vitamin E reducing oxidative degradation of canthaxanthin in the trout gut. Similar findings have been shown for astaxanthin and vitamin E in Atlantic salmon (Christiansen *et al.* 1991; Bjerkeng, *et al.* 1999). Bjerkeng *et al.* (1999a) suggested that vitamin E inhibits the catabolism of astaxanthin, hence sparing the compound for deposition.

## 1.15 Evidence from *in vitro* studies

*In vitro* applications designed for the study of chemical and solute uptake across animal intestine have been established for many years (Fisher & Parsons 1949; Darlington & Quastel 1953; Wilson & Wiseman 1954) and are favoured by some in comparison to *in vivo* protocols of absorption assessment on both ethical and technical grounds. The only work that has been published regarding *in vitro* salmonid intestinal models and carotenoid absorption was a brief experiment carried out by Al-Khalifa & Simpson (1988). The authors observed the tissue (pyloric caeca and ileal intestine) conversion of astaxanthin dispersed in aqueous solution (Tween 20) into vitamin A. Although this study indicated the usefulness of an *in vitro* approach, no further studies have investigated the potential to develop similar models for salmonids to assess factors that influence absorption and subsequently digestibility of astaxanthin. Other *in vitro* studies that have been published on carotenoids are focused on the uptake of $\beta$-carotene in mammalian models (Olson 1964; El-Gorab *et al.* 1975; Garrett *et al.* 1999ab).

33

## 1.16 Aims and Objectives

The following thesis/project is based on a series of studies and experiments carried out in order to meet three general objectives:

1. Develop methodology to assess carotenoid absorption in salmonids in order to evaluate the efficacy of commercially important sources of carotenoid for salmonid pigmentation.

2. To determine what natural characteristics of the green microalga *Haematococcus pluvialis* (Flowtow) limit its potential as a dietary source of natural astaxanthin in salmonid feeds in comparison to other established synthetic forms of this carotenoid.

3. To develop a viable *in vitro* technique to assess the absorption of carotenoids at the gastrointestinal level which can then be used to evaluate the influence of abiotic and biotic factors which could potentially influence the absorption of carotenoids.

The first of these objectives was met in a series of *in vivo* investigations where measurements of digestibility (Apparent Digestibility Coefficients) and serum (blood) absorption of carotenoid were taken and determined for different carotenoid sources. Furthermore, novel kinetic strategies were developed for analysis of post-prandial serum astaxanthin concentrations taken from groups of voluntarily feeding fish. The potential of this method for a rapid assessment of carotenoid bioavailability is discussed (see General Discussion).

The second of the main objectives was again evaluated in a series of *in vivo* feed trial investigations using the rainbow trout as a salmonid model and designed to answer the following questions:

(a)     To what extent does the sporopollenin cell wall of *Haematococcus pluvialis* limit the digestibility and absorption of astaxanthin in rainbow trout? Is rupturing of the cell wall sufficient to render pigment available or is complete extraction of the carotenoid necessary prior to feed supplementation?

(b)     Is the gastrointestinal tract of rainbow trout effective in the hydrolysis of astaxanthin esters and subsequent absorption of astaxanthin? Is there regional variation along the intestine in relation to hydrolytic capacity that may present a physiological limitation to the use of an esterified source of astaxanthin for the pigmentation of salmonids?

(c)     Does the extent of esterification limit the absorption of astaxanthin?

The final objective was met by evaluating two potential *in vitro* techniques designed to either examine solute uptake and translocation across the intestinal barrier (everted gut perfusions) or solute uptake by intestinal tissue (everted gut sacs).

# CHAPTER 2.0

## GENERAL MATERIALS AND METHODS

### 2.1 Experimental Diets-composition and analysis

The diets used in the nutrition trials were produced by EWOS Technology Centre

(Livingston, Scotland, U.K.) or Trouw Aquaculture Ltd. (Wincham, U.K.). These basal

diets were formulated to meet the nutritional requirements of rainbow trout. A typical feed

pellet size of 2-5mm was employed depending on the starting weight of fish for successive

experiments. Specific details concerning dietary composition and carotenoid content can

be found in the Materials and Methods sections of respective studies.

#### 2.1.1 Preparation of *Haematococcus pluvialis* feed supplements

Extraction and isolation of carotenoid fractions i.e. astaxanthin monoesters, diesters or total

carotenoid extracts from *Haematococcus pluvialis* spores was performed by the Carotenoid

Research Group, Liverpool John Moores University, Liverpool.

A volume (ca. 5ml) of *Haematococcus pluvialis* culture was centrifuged (MSE Mistral

1000, Sanyo Gallenkamp PLC., Loughborough, Leicestershire, U.K.) at 1200 x *g* for 5min.

The supernatant was discarded and the cells re-suspended in distilled water. The cells

were then centrifuged (1200 x *g*) again for a further 5min. The supernatant was then

discarded and the cells were re-suspended in re-distilled acetone. Cells were then carefully

transferred to a bijou bottle containing 0.5cm diameter glass beads (²/₃ full). To ensure

complete recovery of all cells the centrifuge tube was rinsed with re-distilled acetone into

the bijou bottle. The cells were then homogenised for 5min using a tissue homogeniser (Status X620, Phillip Harris Ltd., Leicestershire, U.K.). Following homogenisation, the sample was filtered through absorbent cotton fibres to remove cell debris. To ensure all material was removed, the bijou bottle and the lid were rinsed with re-distilled diethyl ether, which was then filtered and added to the sample. The filtrate was dried and stored at $-20°C$ until separation of astaxanthin forms by TLC (see below) and spectrophotometric quantification of carotenoid content (see below).

### 2.1.2   Addition of carotenoid to diets (top coating)

Some of the experimental diets utilised in the current studies were supplied as basal feeds without carotenoid supplementation so that various carotenoid sources could be added in controlled amounts to meet target levels. In this procedure experimental feeds were placed in 3kg batches into a preheated oven (50°C) for 30min. The required amount of CAROPHYLL® Pink (F. Hoffmann-La Roche, Basel, Switzerland) was added to 25ml of distilled water and warmed to 40°C in a sonicating water bath until the gelatine matrix had dissolved and the pigment had dispersed homogeneously (ca. 10-15min). The dispersed pigment was then poured into a glass beaker containing the desired quantity of oil (calculated according to 26 % feed inclusion), plus 90ppm of the antioxidant ethoxyquin. The pigment, oil and water were mixed thoroughly using a magnetic stirrer bar for 10min to form an emulsion. *Haematococcus pluvialis* products were dispersed directly into the oil without the addition of water owing to the absence of a gelatine matrix. The resulting emulsion was heated on a magnetic stirrer-hot plate to 40°C and then poured slowly and continuously onto the warmed experimental feeds whilst being mixed at a steady speed (Hobart Feed Mixer, Hobart Ltd., Southgate, U.K.). After all oil had been delivered to the feed, the diets were left to mix for a further 20min to ensure complete absorption of

37

residual oil. Diets were then left to air dry for 12 hours in the dark before being analysed for carotenoid concentration (see below) and bagged for storage at –20°C.

### 2.1.3 Protein determination

The protein content of fish feeds was determined by the Kjeldahl method. Typically 50-100mg of ground feed was weighed into borosilicate digestion tube containing 1 Kjeldahl catalyst tablet (3g $K_2SO_4$; 105mg $CuSO_4.5H_2O$ and 105mg $TiO_2$; Thompson & Capper Ltd., Runcorn, Cheshire, U.K.). Concentrated $H_2SO_4$ (10ml) was then added to each borosilicate tube prior to the digestion process. Digestion was performed on Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) for 30min at 220°C and a further 60min at 380°C. Samples were then removed from the heating block and allowed to cool to room temperature (ca. 20°C).

Samples were then diluted with distilled water and neutralised with 40% NaOH (Gerhardt Vapodest Distillation Unit). Following neutralisation, samples were steam distilled and the ammonia produced was collected in a 250ml conical flask containing 50ml of saturated orthoboric acid ($H_3BO_3$) plus BDH 4.5 indicator. The resulting distillate was then titrated with 0.1M HCL and the percentage protein in the dry sample calculated by using the following equation:

$$\text{Crude protein (\%)} = \frac{100 \times (S^t - B^t \times 0.10 \times 14 \times 6.25)}{W} \qquad \text{Equation (2.1)}$$

Where;

$S^t$ = sample titre (ml)

$B^t$ = blank titre (ml)

W = sample weight (mg)

0.10 = Molarity of HCL

14 = relative atomic mass of nitrogen

6.25 = factor describing nitrogen to protein conversion


### 2.1.4 Lipid determination


Feed lipid content was determined gravimetrically (following pre-treatment of the feed with acid hydrolysis) essentially according to Bligh & Dyer (1959). Ground feed (ca 2g) was placed in a polypropylene tube (50ml) and 10ml of HCL (6M) and 10ml of methanol were added. The tubes were then sealed and placed in the oven for 30min at 70°C. After the samples had cooled to room temperature 18ml of dichloromethane (DCM) was added before being shaken vigorously and left to stand for a further 60min. The tubes were shaken again and then centrifuged (10min at 2010 × $g$). An aliquot of the resultant hypophase (1ml) was removed with a glass syringe and placed into a pre-weighed vial. The solvent was removed by evaporation under a gentle stream of nitrogen followed by heating at 105°C for 1 hour. After the samples had cooled to room temperature in a dessicator, the vials were re-weighed and the lipid content of the sample determined from the following equation:


$$\text{Lipid (\%)} = 100 \times 18 \times \frac{wv^f - wv^i}{W} \qquad \text{Equation (2.2)}$$

Where:

$wv^f$ = weight of vial final (g)

$wv^i$ = weight of vial initial (g)

W = weight of sample (g)

39

### 2.1.5 Moisture determination

Determination of moisture in the feed was carried out according to methodology previously described (A.O.A.C. 1990). Ground feed samples (ca.5-10g) were fully dried at 105°C to a final constant weight in a fan assisted-exhaust oven (Pickstone E 70F; RE Pickstone Ltd., Thetford, Norfolk, U.K.). The percentage moisture content was calculated using the following equation:

$$\text{Moisture (\%)} = 100 \times \frac{W^f}{W^i} \qquad \text{Equation (2.3)}$$

Where:

$W^f$ = dried weight of sample (g)

$W^i$ = initial weight of sample (g)

### 2.1.6 Ash determination

Ash content of feed was determined in accordance with A.O.A.C. (1990). Ground feed (0.45-0.5g) was accurately weighed into a pre-weighed ceramic crucible and ashed at 550°C for 12 hours in a muffle furnace (Carbolite GLM 11/7, Carbolite Furnaces Ltd., Bamford, Sheffield, U.K.). The crucible and contents were then re-weighed and the percentage ash content of the sample calculated from the following equation:

$$\text{Ash (\%)} = 100 \times \frac{C^f - C^i}{W} \qquad \text{Equation (2.4)}$$

Where:

$C^f$ = final weight of crucible and contents (g)

$C^i$ = initial weight of crucible (g)

W = sample weight (g)

### 2.1.7   Energy determination

The energy values of the experimental feeds were determined by bomb calorimetry according to A.O.A.C. (1990).

Ground diet (ca. 0.5g) was pressed into a cylindrical pellet attached to a 6cm length of gun cotton using a vice and stainless steel pellet press. The pellet was then suspended on a nickel wire (attached to anode and cathode to complete the firing circuit) in an adiabatic bomb (Gallenkamp and Co. Ltd., Loughborough, U.K.). Distilled water (1ml) was added to the bomb to ensure absorption of combustion gases and the bomb was then assembled and pressurised to 30 bar with 100% oxygen. The bomb was then immersed into a calorimeter water jacket that inclusive of water weighed 3kg. The initial water temperature was then measured twice to ensure that there was no ambient temperature variation, before firing the bomb to ignite the combustion gases. The temperature rise of the water jacket was accurately recorded every two minutes until a maximum temperature was reached (most samples had a typical run time of 8mins) and the temperature rise was calculated. The same procedure was repeated for 0.5g of benzoic acid and 0.5g of a benzoic acid standard. All analyses were carried out in triplicate. The energy content of the sample was then calculated according to the following equation:

Energy content (kJ g$^{-1}$) = $\underline{\Delta^t S / (\Delta^t B \times E^B)}$        Equation (2.5)

$$W$$

Where:

$\Delta^t S$ = temperature change induced by combustion of sample

$\Delta^t B$ = temperature change induced by combustion of 0.5g of benzoic acid

$E^B$ = energy value of 0.5g of benzoic acid standard

W = weight of the sample


### 2.1.8 Inert marker analysis


Yttrium oxide was included in the fish feed as an inert dietary marker (0.1%) and was analysed in both feed and faecal samples (following acid digestion) in order to determine Apparent Digestibility Coefficients.


Approximately 100mg of finely ground (pestle and mortar) feed or faecal material were weighed into a closed vessel 120ml teflon RPFA bomb (CEM Instruments, N.C., U.S.A.), and 10ml of concentrated nitric acid (69%) was added. The teflon bomb was then sealed and placed in a digestion microwave (700W MDS-2000, CEM instruments) for 5min at 10% power; 1min at 20% power and 10min at 50% power, followed by a 5min cooling period. The samples were then removed from the digestion microwave and allowed to cool to room temperature (ca. 20°C). The acid digests were then decanted into a 25ml volumetric flask and made to volume with distilled water. Samples were stored in 50ml polypropylene tubes until Induction Coupled Plasma (ICP) analysis for yttrium content. All digestions were carried out in triplicate.

Analysis of yttrium was performed on an ICP emission spectrometer (Varian Liberty 200, Australia) and calculated on integrated analytical software (ICP AES) by linear correlation with external standards of known concentration (10, 20, 40 ppm). The content of yttrium oxide in both the diets and associated faeces was then calculated from the molecular weight of yttrium oxide and known weight of initial sample. All analyses were carried out in triplicate.

## 2.2     Nutrition Trials

### 2.2.1   Experimental fish

All female, pigment naive rainbow trout (fed non-pigmented diets) were used in the experimental trials. These were hand-graded and obtained from Hatchland's Trout Farm (Devon, U.K.). Fish were randomly assigned into groups of 30-40 fish per tank at an initial mean weight of 85-150g (depending on trial and conditions).

Atlantic salmon utilised for *in vitro* everted gut sac experiments (ca. 400-500g) were obtained from Lovatt Fisheries (Inverness, Scotland, U.K.). Fish were maintained in the saltwater system described below.

### 2.2.2   Experimental System

All feed trials were carried out within the aquarium facilities at the University of Plymouth (Plymouth, Devon, U.K.). The experimental trout systems consisted of six square 400l fibre-glass tanks supplied with re-circulated fresh water at a flow rate of ca. 10L min$^{-1}$ through an external spray bar (maintained unidirectional flow). Outflow and water level

were controlled with an internal standpipe. The system was maintained at $15 \pm 1°C$ with a chiller and temperature controller (R & R Electronics, Cornwall, U.K.) and was kept under a 12 hours light/dark photo period regime using artificial light from fluorescent tubes. Removable pre-filter media (meshed sponge) was used to trap faecal and other particulate matter from outflow water and was washed once per week throughout experimental trial periods to prevent excess nitrogen levels in the ambient water. Following this initial filtration, water passed through an internal high surface area biofilter consisting of a trickle plate and plastic bio-spheres which contained denitrifying bacteria to convert toxic ammonia into nitrites and nitrates. In addition, water circulated through a filtration vessel containing charcoal filter media in order to maintain optimal water chemistry.

Atlantic salmon were maintained in re-circulating saltwater systems in tanks with 800L capacity and temperature of $12 \pm 1°C$. Water flow, filtration and biofilter systems were similar to those described above. Photoperiod was also controlled under artificial light with a 16-hour light: 8-hour dark regime.

### 2.2.3 Feeding

Prior to the feeding trial fish were usually acclimated for a minimum of two weeks in which time they were sparingly fed rations of commercial unpigmented feed (Trouw Aquaculture, Wincham, U.K.).

Typically, each experimental diets was hand fed to triplicate groups of fish one or two times daily at a feeding ration of 1-2% BW $day^{-1}$ for an 8-12 week period. Alterations to feed quantity were made daily assuming a feed conversion ratio (FCR) of 1.0 with appropriate adjustments made for mortalities. This feeding ration may have been

reassessed for biomass after four weeks (fish were re-weighed) depending on the experiment in question. Over feeding was minimised by careful observation of fish feeding behaviour. Specific details concerning feeding strategies can be found in the Material and Methods sections of respective studies.

## 2.2.4 Faecal Stripping

In order to determine apparent digestibility coefficients (ADC) for astaxanthin, faecal material was required from fish. Fish were removed from their respective tanks and anaesthetised in a 200mg ml$^{-1}$ solution of phenoxy-2-ethanol (Sigma, Poole, Dorset, U.K.) in domestic mains water. Once sufficiently anaesthetised, faecal samples were stripped from the fish by applying gentle pressure along the ventral abdominal region according to Austreng (1978) and collected in metallic trays. Faecal samples for whole groups/tanks of fish were pooled to provide a sufficient quantity of dried material for carotenoid analysis. The pooled samples were immediately frozen (-20°C) for 1h before being freeze-dried for a minimum of 24 hours to remove moisture. Samples in the freeze dryer were shielded from ambient light by using a black plastic covering. Once dry, the samples immediately underwent carotenoid extraction (see below).

## 2.2.5 Blood Sampling

Serum carotenoid concentrations were routinely used as an indicator of carotenoid absorption in the nutrition feed trials. Carotenoid concentration was measured in serum in preference to plasma due to a 10% increased carotenoid recovery (Nierenberg 1984; Stacwicz-Sapuntzakis *et al*. 1987). Fish were first anaesthetised in a 200mg ml$^{-1}$solution of phenoxy-2-ethanol (Sigma, Poole, Dorset, U.K.) in tap water. Blood was then taken

from the caudal vein of the fish using a 2ml non-heparanised disposable syringe equipped with 23.5 gauge needles (Becton Dickinson, Dublin, Ireland) and placed in 2ml eppendorf tubes. Blood samples were permitted to clot for 24 hours at +4°C before being centrifuged at 13000 × $g$ (Micro-Centaur MSB010.CX2.5, Sanyo Gallenkamp PLC, Uxbridge, U.K.) for 15min to separate out blood serum.

## 2.3 Assessment of growth and feed utilisation

A number of parameters were used to define the growth performance and utilisation of feed by fish at the end of the nutrition trials. These were as follows:

### 2.3.1 Specific Growth Rate (SGR)

Specific Growth Rate (SGR) describes the daily growth of fish and is defined as the percentage increase in live weight over a certain period of time:

$$SGR\ (\%\ d^{-1}) = 100 \times \frac{\ln w^f - \ln w^i}{T} \qquad \text{Equation (2.6)}$$

Where:

$w^f$ = final weight (g)

$w^i$ = initial weight (g)

$T$ = trial duration (days)

46

### 2.3.2  Feed Conversion Ratio

The feed conversion ratio (FCR) describes the amount of feed consumed that results in live weight gain. Alternatively, it indicates the extent to which feed is utilised for growth:

$$FCR = \frac{\Sigma f}{\Sigma w} \qquad \qquad \text{Equation (2.7)}$$

Where:

$\Sigma f$ = total amount of feed fed

$\Sigma w$ = total biomass gain

## 2.4  Carotenoid analyses

### 2.4.1  Conventions adopted

Due to the reactive nature of carotenoids a number of precautions as described by Schiedt & Liaaen-Jensen (1995) were taken to avoid the breakdown of the compounds during extraction and analysis:

- All procedures were carried out under red/subdued ambient light. Reactions were carried out in darkness and equipment such as freeze-dryers were covered in a black lining.

- Since high temperatures promote carotenoid isomerisation, high temperatures and extreme temperature fluctuation were avoided by carrying out reactions at room temperature or below. Solvents with low boiling points were used so that

concentration of samples in solvent could be performed in water baths with temperature not exceeding 40°C. Smaller sample volumes were evaporated with a gentle stream of nitrogen. All dried samples were stored under a blanket of nitrogen at –20°C.

- Contact of samples with acids and alkalis was avoided by preventing the use of strongly acidic reagents and alkalis in the laboratory during routine carotenoid extraction and analyses.

- All solvents used for the quantification and analysis of carotenoids in samples were of HPLC grade. Other materials used throughout the studies were at least of ANALAR grade if not higher depending on availability and supply. The addition of antioxidants i.e. butylated hydroxytoluene (BHT, 2,6-di-t-butyl-p-cresol) to solutions (namely methanol) during extraction was routine. Dichloromethane was used regularly in place of chloroform due to the high solubility of carotenoids in this solvent.

- Pipettes and other instrumentation were regularly calibrated before use.

- Carotenoid standards were prepared on a frequent basis to avoid artefact formation resulting from oxidation or isomerisation.

## 2.4.2    Extraction from Feed and Faeces

The feed and faeces extraction technique incorporated procedures from the methods of Weber (1988) and Bligh & Dyer (1959). Approximately 4g (± 0.2g) of finely ground (Braun blender, Braun Consumer Service, Isleworth, Middlesex, U.K.) feed was placed in a 50ml polypropylene conical tube containing 100mg of Maxatase (P-3000 encapsulated, International Biosynthetics, Rijswik, Netherlands). Distilled water (15ml) was added to the sample, which was then vortexed for 30 seconds. The tube and contents were placed in a preheated (50°C) sonicating water bath for 35min, ensuring that the sample was vortexed

48

vigorously every 5min. The sample was then removed from the heated bath, placed in the dark, and allowed to cool to ambient temperature (ca. 20°C). Methanol (15ml+500ppm BHT) and dichloromethane (12ml) were added to the sample which was again vortexed. The sample was then centrifuged (2010 × $g$) for 15min at 5°C. An aliquot (1ml) of the resultant hypophase was pipetted into an amber vial and blown down under a gentle stream of nitrogen. Feed samples were re-suspended in hexane (4ml), carefully decanted into sealable eppendorf tubes, and centrifuged (Micro-Centaur MSB010.CX2.5, Sanyo Gallenkamp PLC, Uxbridge, U.K.) at 13 000 × $g$ for 5min to precipitate insoluble lipid prior to carotenoid quantification. Faecal samples were subjected to the following semi-purification process due to the presence of large amounts of insoluble matter.

A preparative chromatography column was assembled by filling a disposable pasteur pipette (15cm) with a slurry of n-hexane and silica gel (Keiselgel 60 GF$_{254}$) supported on a glass wool wad. The pipette was then attached to a glass quick-fit water vacuum assembly, and filled with n-hexane to further moisten the silica packing. Carotenoid extracts from faeces were re-suspended in n-hexane (2ml) and transferred to the top of the column with a pasteur pipette. This procedure was repeated several times to ensure all carotenoid had been transferred to the column. Lipids and beta-carotene were eluted from the extracts by running 20ml of 6% ether in hexane through the column under vacuum. Insoluble material present in the faeces precipitated at the top of the column packing. Carotenoids were then eluted with 10ml of 100% ether and collected in a 20ml glass tube. Purified extracts were blown down under a gentle stream of nitrogen and stored at –20°C in amber vials prior to analysis.

### 2.4.3 Extraction from serum

500µl of serum was pipetted into a 3ml glass Bijou bottle containing 1ml of ethanol (precipitates protein). After mixing, 1ml of diethyl ether and 1ml of n-hexane were added to the sample, which was then mixed again. Samples were sealed and allowed to stand in the dark for 10min to ensure efficient carotenoid extraction. The resultant hyperphase was pipetted (500µl) into an amber vial and then evaporated under a gentle stream of nitrogen. Samples were stored at –20°C prior to analysis by High Performance Liquid Chromatography (HPLC).

### 2.4.4 Extraction from fish muscle

The flesh extraction method utilised was a modified version of the procedure described by Bjerkeng *et al.* (1997). Rainbow trout fillets were de-skinned and pooled into groups (5 fillets per group). The pools of flesh were then diced and placed in a blender (Waring blender, Phillip Harris Ltd., Leicestershire, U.K.) and homogenised into a fine paste consistency. Approximately 5g of this homogenate was placed in a 50ml polypropylene tube to which 5ml of distilled water and 5ml of methanol (containing 500ppm BHT) was added. The contents of the tube were then homogenised (Status X620, Phillip Harris Ltd., Leicestershire, U.K.) before the addition of 15ml of dichloromethane. The tube and contents were then mixed vigorously for 30 seconds and placed in the dark for 10min. The tube and contents were then shaken vigorously for a further 30 seconds and then centrifuged (2010 x *g*) at 5°C for 15min. An aliquot of the resultant hypophase (3ml) was withdrawn with a glass syringe and dispensed into a 4ml amber vial before being evaporated under a gentle stream of nitrogen. Samples were then stored at –20°C prior to HPLC analysis. All analyses were carried out in duplicate.

50

### 2.4.5 Extraction from liver and gastrointestinal tract

Fish livers were excised from the fish and homogenised whole (Ultra-turrax T8, IKA Labortechnik, Staufen, Germany) into a slurry from which 2g was used for carotenoid extraction. Samples of gastrointestinal tract (0.5-1.5g) were taken according to the gastrointestinal region represented. All samples were placed in 20ml sealable glass vials and homogenised (Status X620, Phillip Harris Ltd., Leicestershire, U.K.) with 2ml of distilled water and 2ml of methanol (+500ppm BHT) for 30 seconds. Dichloromethane (6ml) was then added to the vial contents, which were then mixed vigorously for a further 30 seconds. Vials were placed in the dark for 10min to ensure complete extraction of carotenoid. Samples were removed from the dark, vortexed for a further 30 seconds and then centrifuged at 2010 $\times$ $g$ for 15min at 5°C. An aliquot of the resultant hypophase (3ml) was withdrawn with a glass syringe and dispensed into a 4ml amber vial before being blown down under a gentle stream of nitrogen. Samples were stored at –20°C until HPLC analysis.

### 2.4.6 Extraction from skin

Extraction of carotenoids from skin was as described by Schiedt *et al.* (1995). Frozen skin samples (100-200g) were cut with scissors and dehydrated with twice the quantity of anhydrous $MgSO_4$ for 30min. The sample was then covered in acetone and homogenised (Waring blender, Phillip Harris Ltd., Leicestershire, U.K.) before filtration through a glass filter (porosity 2). The skin samples were re-extracted with two more volumes of acetone to ensure complete carotenoid recovery. The acetone was then evaporated from the sample using a rotary evaporator to leave a dry lipid-carotenoid residue.

The free polar carotenoids were then separated from less polar material (incl. carotenoid esters and fat) by partition between dimethyl sulphoxide (DMSO) and hexane (1:1v/v). The lipid extract of the skin was dissolved in an appropriate volume of hexane to give a 5% solution (w/v) to which an equal volume of DMSO was added. Free astaxanthin (100%) partitioned into the DMSO phase and 90-95% of the lipid remained in the hexane phase that also contained xanthophyll esters. The two phases were then separated and the hexane phase was washed with water whilst adding small amounts of ethanol to prevent emulsions forming, before being evaporated. Carotenoids were re-extracted from the DMSO phase by first adding distilled water, saturated NaCl solution and ethanol to give a mixture of DMSO: water: NaCl solution: ethanol of 1:0.55: 0.25: 1. Carotenoids were then extracted from the mixture with 2 to 3 volumes of ether/hexane (1:1) until colourless. The ether/hexane extract was then washed with water before being evaporated to dryness.

### 2.4.7    Separation of pigments -Thin-Layer Chromatography (TLC)

TLC was carried out to determine the percentage contribution of diesters and monoesters of astaxanthin as well as free astaxanthin in the *Haematococcus pluvialis* products, diets and faeces. TLC on the product itself as well as some diets was performed at Liverpool John Moores University. TLC identification of carotenoid species in faecal material was carried out at the University of Plymouth.

For semi-preparative work 0.5mm thick self-prepared TLC plates were used. Commercial aluminium backed plates (Merck, Silica gel 60 GF$_{254}$) were used for analytical studies to ensure high resolution and accurate determination of Rf values for the constituent carotenoids. Normal-phase silica TLC was used for all analytical and preparative work

Semi-preparative plates were prepared by adding 60g of Kieselgel 60 GF$_{254}$ to 120ml of distilled water. The mixture was then shaken vigorously to form a slurry (quantity sufficient for the preparation of five, 20x20cm silica plates). Using a plate spreader the silica slurry was applied to acetone-washed glass plates at a thickness of 0.5mm. The plates were then dried at 120°C for at least three hours prior to use. To prevent tailing of carotenoids plates were acidified with solution of 2.5% (w/v) citric acid in methanol. Once coated the plates were allowed to dry fully before any sample was applied.

To separate pigments and astaxanthin forms an origin line was drawn approximately 1.5cm above the bottom edge of the plate. The sample was then re-suspended in a small volume of re-distilled diethyl ether and then applied to the plate along the origin. This was achieved by using a capillary tube or a drawn-out Pasteur pipette. The TLC plate was then placed in a developing tank containing acetone in n-hexane (30/70 v/v) and the tank covered in a dark cloth. Once developed, the plate was removed from the tank and the solvent front marked. The colour and Rf value of each pigment band was recorded. The Rf value of each band was calculated using the following equation:

$$Rf = x / y \qquad \text{Equation (2.8)}$$

Where:

x = distance of band from origin

y = distance of solvent front from origin.

Where available, appropriate carotenoid standards (kindly donated by Hoffmann La Roche, Basel, Switzerland) were co-chromatographed with the sample. Rf values were lutein

(0.34); free astaxanthin (0.37); astacene (0.43); astaxanthin monoesters (0.47); canthaxanthin (0.48); astaxanthin diesters (0.79); β-carotene (0.98).

Starting with the lowest Rf value, each band was rapidly scraped off of the plate and placed in a small glass sinter. Carotenoid was removed from the silica under vacuum by the addition of a small volume of di-ethyl ether and collected in a 20ml glass tube. The sample was then decanted into a 4ml amber vial ensuring the glass tube was washed with small volumes of ether. Samples were then blown down under a gentle stream of nitrogen and stored at −20°C. Samples were analysed for astaxanthin content spectrophotometrically (see below).

### 2.4.8    Preparation of carotenoid standards

To prepare an astaxanthin standard approximately 3mg of crystalline astaxanthin (Sigma, Poole, Dorset, U.K.) was carefully weighed into a 100ml amber volumetric flask. Choloroform (10ml) was then added to the flask and the solution briefly sonicated. The solution was then made to volume with n-hexane and the solution gently everted several times to ensure homogeneity. An aliquot of this solution (5ml) was then pipetted into a second 100ml amber volumetric flask containing 4ml of chloroform and was made to volume with n-hexane (n-hexane: chloroform; 95.5:4.5). This solution was used as the external standard. The standard was stored at 5°C prior to concentration determination when it was brought to room temperature and again sonicated.

To prepare a canthaxanthin standard approximately 3mg of crystalline canthaxanthin (Sigma, Poole, Dorset, U.K.) was carefully weighed into a 100ml amber volumetric flask. 10ml of chloroform was then added to the flask and the solution briefly sonicated. The

solution was then made to volume with n-hexane and the solution gently everted several times to ensure homogeneity. An aliquot of this solution (5ml) was then pipetted into a second amber 100ml volumetric flask containing 4ml of acetone and was made to volume with n-hexane (n-hexane: chloroform: acetone; 95.5:0.5:4.0). This solution was used as the external standard. The standard was stored at 5°C prior to concentration determination when it was brought to room temperature and again sonicated

### 2.4.9 Spectrophotometric carotenoid quantification

Some samples (namely feed and faecal samples) were assessed for carotenoid concentration using a UV-Vis spectrophotometer (Helios, UNICAM Ltd., Cambridge, U.K.). Samples were assessed for astaxanthin (unesterified, monoester or diester) at $\lambda 470$nm in n-hexane using an $E_{1\% \, 1cm}$ of 2100 (Britton 1995). Corrections were made for absorption contributed by carotenoids other than astaxanthin (determined following separation of carotenoid extracts by TLC). Furthermore, background absorption contributed by other dietary ingredients was corrected for by carrying out extractions and quantification on basal feed (non-carotenoid supplemented). HPLC confirmed that basal feeds did not contain significant quantities of carotenoids (<1ppm).

Astaxanthin and canthaxanthin standards were measured at 470nm ($E_{1\% \, 1cm}$ = 2100) and 466nm ($E_{1\% \, 1cm}$ = 2260), respectively against suitable blanks. Determination of carotenoid concentration was carried out using the following formula:

$$\text{Carotenoid concentration } (\mu g \, ml^{-1}) = \frac{\text{Absorbence at } \lambda max \times 10000}{E_{1\% \, 1cm}} \qquad \text{Equation (2.9)}$$

Where:

λmax = wavelength corresponding to maximum absorption for a carotenoid in a particular solvent

$E_{1\% 1cm}$ = Extinction coefficient for a specific carotenoid in a particular solvent.

### 2.4.10 High Performance Liquid Chromatography (HPLC) Analysis

Two different HPLC stationary phases were employed for carotenoid analysis throughout the experimental trials. Blood (serum) and flesh samples were analysed using nitrile and silica stationary phases, respectively. Figure 2.1 illustrates typical chromatograms produced using both stationary phases.

The isocratic HPLC system comprised a Spherisorb S5-CN nitrile column (Phenomenex, Cheshire, U.K.; length 250mm; internal diameter 4.6mm; particle size 5μm) with 20% acetone in hexane as mobile phase (1.5ml min⁻¹ flow rate). Alternatively, a Lichrosorb 5-SIL 60A column (Phenomenex, Cheshire, U.K.; length 125mm; internal diameter 4mm; particle size 5μm) with 14% acetone in hexane as mobile phase (1.2ml min⁻¹ flow rate) was used. These were coupled with a P332 single channel detector and 325 pump (Kontron Instruments, Watford, Hertfordshire, U.K.). The detector wavelength was maintained at 470nm for a 12min run time throughout analysis of all samples, where all-$E$ astaxanthin exhibited a retention time of ca. 7 or 11min on the nitrile and silica columns, respectively. Kontron Kroma 2000 software was utilised to integrate samples against external carotenoid standards.

(a)



(b)
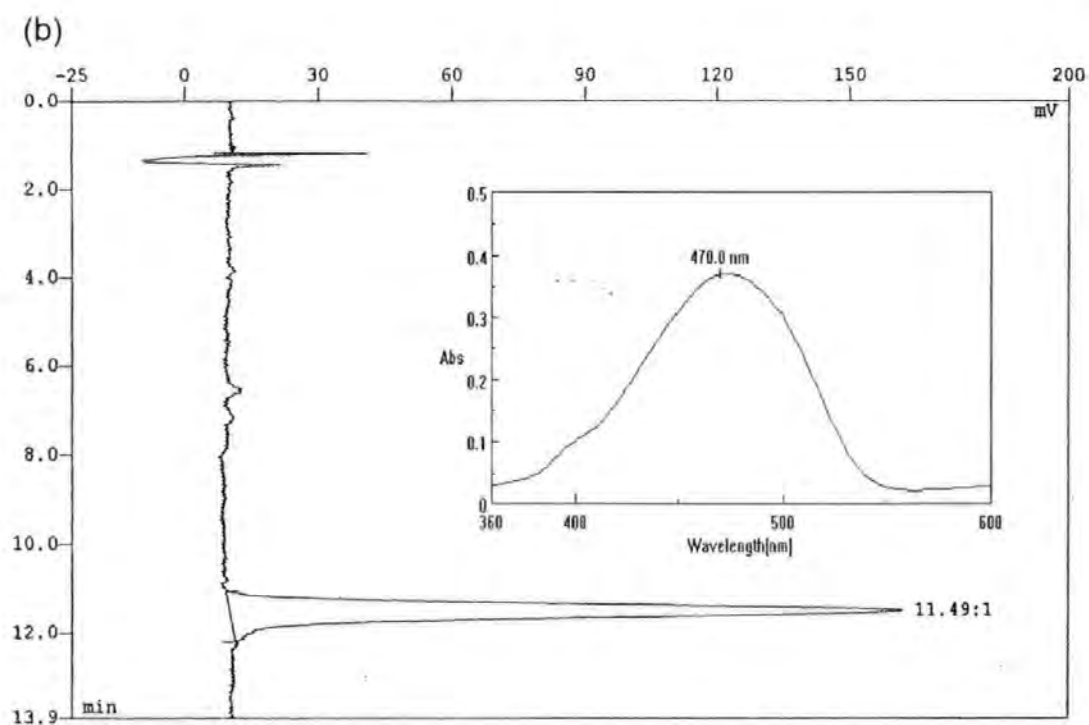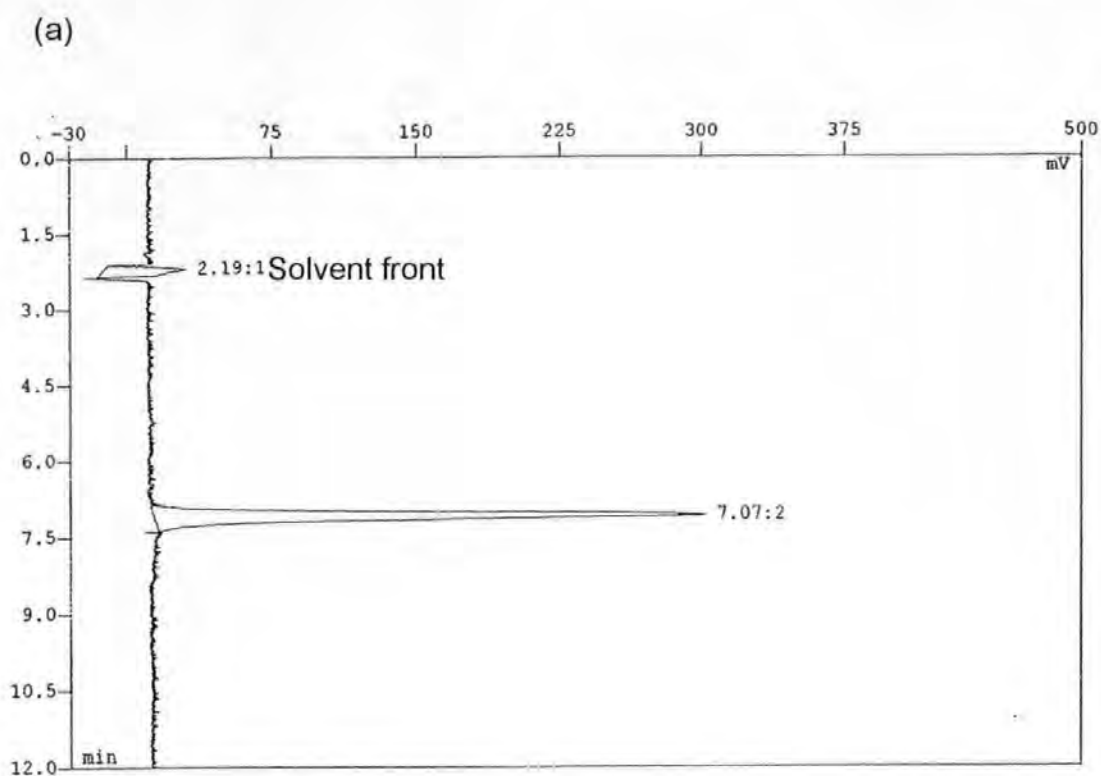


Figure 2.1    Typical HPLC chromatograms of all-*trans*-astaxanthin (standard) on a (a)

nitrile (retention time ca. 7min) and (b) silica column (retention time ca. 11min). A typical

spectral scan of an astaxanthin standard (λmax 470nm) has been included in chromatogram

(b).

## 2.5    Assessment of carotenoid absorption and utilisation

### 2.5.1    Apparent Digestibility Coefficient (ADC)

The ADC value can be calculated to indicate the percentage of a particular ingredient or additive that has been absorbed by an animal from ingested feed. The ADC for astaxanthin was calculated using the following equation by utilising yttrium as a non-absorbable marker in the feed:

$$ADC\ (\%) = 100 - 100 \times \left[ \frac{d^{yt}}{f^{yt}} \right] \times \left[ \frac{f^{ax}}{d^{ax}} \right] \qquad \text{Equation (2.10)}$$

Where:

$d^{yt}$ = yttrium concentration in the diet (mg kg$^{-1}$)

$f^{yt}$ = yttrium concentration in the faeces (mg kg$^{-1}$)

$f^{ax}$ = astaxanthin concentration in the faeces (mg kg$^{-1}$)

$d^{ax}$ = astaxanthin concentration in the diet (mg kg$^{-1}$)

### 2.5.2    Flesh Retention

Calculation of astaxanthin retention is an indicator of the percentage of astaxanthin fed that is actually retained in the fish muscle. The relative net apparent retention (NAR) of astaxanthin can be determined from initial and final flesh astaxanthin concentrations, the accurate determination of feed intake and biomass gain. The ratio of 0.61 is applied to the biomass to compensate for the muscle to live weight ratio:

58

$$NAR\ (\%) = \frac{(0.61 \times W^f \times F^{ax}) - (0.61 \times W^i \times I^{ax})}{\Sigma f \times d^{ax}} \qquad \text{Equation (2.11)}$$

Where:

$W^f$ = final biomass

$W^i$ = initial biomass

$I^{ax}$ = mean initial flesh astaxanthin concentration (mg kg$^{-1}$)

$F^{ax}$ = mean final flesh astaxanthin concentration (mg kg$^{-1}$)

$\Sigma f$ = total feed fed

$d^{ax}$ = dietary astaxanthin concentration (mg kg$^{-1}$)

## 2.6    Statistics

Statistical evaluation of data was performed using the software package StatGraphics Plus 4.0 (Manugistics Inc., Rockville, Maryland, U.S.A.). Regression analysis accompanied by graphical representation was generally carried out using SigmaPlot 4.0 (SPSS Inc., Chicago, Illinois, U.S.A.). Specific details concerning statistical analyses of different data sets are presented within individual studies.

59

# CHAPTER 3.0

# THE SPOROPOLLENIN CELL WALL OF *HAEMATOCOCCUS PLUVIALIS*: EFFECTS ON ABSORPTION AND DEPOSITION OF ASTAXANTHIN IN RAINBOW TROUT.

## 3.1 Introduction

Distinctive colouration of farmed salmonid flesh owing to the dietary supplementation of carotenoids in salmonid feeds is critical for consumer acceptance (Torrissen *et al.* 1990). Subsequently, salmon farmers strive to achieve the market demand for a fish muscle astaxanthin concentration of between 6-7 mg kg$^{-1}$ (Torrissen & Christiansen 1995).

Carotenoid-containing products such as krill (Mori *et al.* 1989; Torrissen *et al.* 1989); shrimp wastes and oil extracts (Saito & Regier 1971; Torrissen *et al.* 1989); crayfish oil extracts (Peterson *et al.* 1966); red crab wastes and oil extracts (Spinelli *et al.* 1974; Spinelli & Mahnken 1978) as well as the yeast *Phaffia rhodozyma* (Johnson *et al.* 1977) have been used successfully as feed supplements to pigment salmonids.

The microalga *Haematococcus pluvialis* (Flowtow) is also a natural source of astaxanthin. This algae possesses the ability to accumulate large amounts of carotenoid (Choubert & Heinrich 1993) typically associated with the formation of aplanospores or cysts when the algae is subjected to growth limiting conditions e.g. depletion of nitrogen in the growth medium and/or exposure to high irradiances (Boussiba & Vonshak 1991; Harker *et al.* 1996a). These cysts are similar to those found in many microalgae and in pollen from higher plants (VanWinkle Swift & Rickoll 1997). Furthermore, these cysts are surrounded

60

by a tough sporopollenin cell wall that renders them resistant to chemical attack (Good &

Chapman 1979; Burczyk 1987) to KOH and acetolysis, but they can be attacked by

chromic acid. Indeed, resistance to acetolysis is used in part to characterise such cell walls.

Subsequently, these cell walls may impede absorption of carotenoid once ingested by an

animal (Johnson & An 1991). Indeed, several studies have demonstrated comparatively

poorer pigmentation efficiency when using *Haematococcus pluvialis* as a source of

astaxanthin with reference to the commercial synthetic source, CAROPHYLL® Pink

(Sommer *et al.* 1991; Sommer *et al.* 1992; Choubert & Heinrich 1993). In all cases poorer

pigmentation was in part explained by reduced bioavailability of carotenoid as a direct

result of the encysted cell wall.

Several methods have been proposed to disrupt algal cells (Farrow and Tabenkin 1966;

Ruane 1977; Nonomura 1987) although most methods are not very efficient for disrupting

the sporopollenin wall of *Haematococcus* cysts. Methods that have been evaluated include

cryogenic grinding (Burbrick 1991) autoclaving and homogenisation (Mendes-Pinto *et al.*

2001). It is important to consider the efficiency of such processing techniques with regards

to homogeneous treatment of the sample and subsequently the bioavailability of

astaxanthin for salmonid pigmentation.

The following study is a description of two experimental feed trials designed to assess the

degree to which cell wall encapsulation affects the digestibility, absorption and final flesh

deposition of astaxanthin in rainbow trout. The first of these was designed to assess

whether removal of the cell wall is necessary prior to feed supplementation in order to

achieve satisfactory absorption and deposition of astaxanthin compared to the synthetic

source. The second experiment investigated the potential of a confidential (patent

pending) cell wall cracking procedure at two different levels on the relative bioavailability of pigment again compared to the synthetic form of astaxanthin.

## 3.2     Materials and Methods

### 3.2.1   Experimental design

In the following experiments each dietary treatment was assigned to triplicate tanks containing groups of fish.   Subsequently, each tank is considered as a replicate ($n=3$). Following sampling and analysis of individual fish tissues, statistical comparison of results between treatments was based on mean values from each tank ($n=3$) to avoid pseudo-replication and false interpretation of results (Morris 1999).

### 3.2.2   Basal Diets

For both experiments, the basal feeds containing no added carotenoid and formulated to meet the nutritional requirements of rainbow trout were supplied by Trouw Aquaculture Ltd. (Wincham, UK). Table 3.1 outlines the dietary ingredients and proximate composition of the diets.

### 3.2.3   Experimental diets (Experiment 1)

Three experimental diets were utilised with an astaxanthin concentration target of 60ppm. Diet A contained the synthetic source of astaxanthin, CAROPHYLL® Pink (F. Hoffmann La Roche, Basel, Switzerland).   Diet B was supplemented with a commercially available source of *Haematococcus pluvialis* (NatuRose, Cyanotech Corporation, Kailua-Kona,

Hawaii, U.S.A.) and Diet C contained a total carotenoid extract that had been extracted from *Haematococcus pluvialis* cells. Mechanical extraction of algal pigments prior to dietary supplementation was performed as described in Chapter 2 (section 2.1.1). Pigment sources were added to the pre-pelleted feed with additional oil (cod liver oil; Seven Seas Ltd., Hull, U.K.) in a top coating procedure (section 2.1.2) to bring the diets to a commercial level of 26% oil. Carotenoid extraction and analysis of feeds was carried out as described previously (section 2.4.2). Thin layer chromatography was performed on feed extracted pigments to determine relative percentage contributions of astaxanthin forms (monoester, diester, unesterified) according to Kobayashi *et al.* (1991; see section 2.4.7). Table 3.2 outlines the astaxanthin composition of each diet.

### 3.2.4   Fish, feeding and sampling (Experiment 1)

Rainbow trout (98.1 ± 0.4g) were obtained from Hatchlands Trout Fisheries (Devon, UK) and randomly assigned into individual tanks (40 fish per tank), maintained at 15°C under a 12h light/dark photoperiod. Triplicate groups/tanks of fish were fed either experimental diets in a single morning ration (1.2-1.5 % BW day$^{-1}$) for 10 weeks days with alterations made daily assuming a feed conversion ratio of 1.0. Flesh fillets were taken from trout at week 4 (5 fish per tank) and at the end of the trial (10 fish per tank) for carotenoid analyses (see section 2.4.4). The perceived colour of these fillets was also determined using the colour graded *Salmo*Fan™ (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Three individuals made separate determination for each fillet under artificial ambient light. Fish were also anaesthetised and stripped of faecal material (Austreng 1978) at week 8 of the trial. Faeces were pooled per replicate, freeze-dried and analysed for carotenoid and inert marker concentrations (see sections 2.1.8, 2.4.2 and 2.4.9).

At week 10, feeding of the experimental diets ceased but fish were fed for a further 4 days

on a non-pigmented diet (same basal formulation as in Table 3.1) to permit blood clearance

of astaxanthin (March *et al.* 1990). Fish were then fed a single ration (1.3% BW day$^{-1}$) of

the respective pigmented diet and blood samples were taken (see sections 2.2.5) from fish

(5 fish per tank) at five time intervals up to 24h post feeding for carotenoid analyses

(section 2.4.10).


### 3.2.5   Experimental diets (Experiment 2)


Encysted-astaxanthin rich cells of *Haematococcus pluvialis* were broken using pressure

treatment (details confidential-patent pending; A J Young pers. Comm). A wet slurry of

freshly harvested cells were exposed to two different pressures (5000 and 20000psi). At

the lower pressure (5000psi) the algal cell wall was ruptured (cell remained largely intact)

and at the higher pressure (20000psi) the cell was fully disrupted with no cell structure

remaining (determined histologically; see below). Ethoxyquin (100ppm) was carefully

mixed with the cell slurry following cracking and prior to spray drying (Lab Plant SD-05

laboratory spray dryer). Analysis of carotenoid content and composition of the cracked

products (before and after drying) revealed ca. 100% recovery of astaxanthin, with no

detectable formation of astacene (an indicator of oxidised astaxanthin).


Four experimental diets were utilised with an astaxanthin concentration target of 60ppm.

Diet CP contained the synthetic source of astaxanthin, CAROPHYLL® Pink (F. Hoffmann

La Roche, Basel, Switzerland). Diet 5000 was supplemented with *Haematococcus*

*pluvialis* cracked under low pressure. Diet 20000 contained *Haematococcus pluvialis* cells

cracked under high pressure and Diet NAT contained a commercially available source of

*Haematococcus pluvialis* (NatuRose, Cyanotech Corporation, Kailua-Kona, Hawaii,

U.S.A.). Addition of pigment sources, carotenoid extraction, TLC and analysis was carried out as described previously. Table 3.3 outlines the astaxanthin composition of each diet.

### 3.2.6 Fish, feeding and sampling (Experiment 2)

Rainbow trout ($106.2 \pm 0.1g$) were randomly assigned into individual tanks (30 fish per tank). Triplicate groups of fish were fed experimental diets in a single morning ration (1.2-1.5 % BW $day^{-1}$) for 8 weeks with alterations made daily assuming a feed conversion ratio of 1.0. On the final day of the trial fish were stripped of faecal material (Austreng 1978) which was freeze dried and then analysed for carotenoid and inert marker concentrations (as described previously). Flesh fillets were taken from all fish (30 fish per tank) at the end of the trial for carotenoid analysis.

### 3.2.7 Electron Microscopy

Aplanospores of *H. pluvialis* (produced by a combination of nitrogen-deprivation and exposure to high irradiances) cracked under different pressures and cells of *H. pluvialis* from the NatuRose™ product were examined by scanning electron microscopy using a JEOL JSM-840 microscope (Welwyn Garden City, U.K.) at an accelerating voltage of1-15KV. The samples were lyophilised (Chris Alpha 1-4, B-Braun Biotech Intl., Epsom, U.K.) at -52°C, and coated with a thin gold-palladium film (10-15nm) using a Polaron E5000 sputter unit (Thermo VG Scientific, East Grinstead, U.K.). Figure 3.1 shows magnified pictures of the various cracked cells taken using electron microscopy.

### 3.2.8 Statistical analysis

Data were subjected to tests for normal distribution (standardised skewness and kurtosis) and variance checks (Cochrans and Barlett's test) before comparison. Net apparent retention (NAR) of astaxanthin was calculated according to Torrissen (1995) incorporating a flesh to body weight ratio of 0.61 (Wathne *et al.* 1998). Statistical analysis and regression/curve fitting of data was carried on StatGraphics Plus 4.0 and SigmaPlot 4.0, respectively. Flesh astaxanthin concentrations, NAR and apparent digestibility coefficients (ADC) were compared across treatments using the analysis of variance at the 95% confidence limit.

### 3.2.9 Kinetic modelling

After ingestion of a single meal the time course of serum astaxanthin concentration shows two phases: a lag phase followed by the uptake phases. The following simple model was used to describe these kinetics.
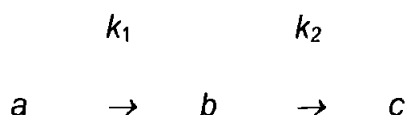
$$a \xrightarrow{k_1} b \xrightarrow{k_2} c$$

In this model $c$ represents the serum astaxanthin concentration, and the first order rate constants, $k_1$ and $k_2$ apply to the lag and uptake phases, respectively. The following set of ordinary differential equations (3.1, 3.2 and 3.3) are derived from the model:

$$\frac{da}{dt} = -k_1 \cdot a \qquad \text{Equation (3.1)}$$

66

$$\frac{db}{dt} = k_1 \cdot a - k_2 \cdot b \qquad \text{Equation (3.2)}$$

$$\frac{dc}{dt} = k_2 \cdot c \qquad \text{Equation (3.3)}$$

These were solved using Maple 6 (Waterloo Maple Inc., U.S.A.), using the limit values: $a = a_0$ at $t = 0$; $b = 0$ at $t = 0$; and $c = c_0$ at $t = 0$. Equation (3.4) shows the relationship between $c$ and time, $t$.

$$c = a + c - \frac{k_2\, k_1\, a\, \left( e(-t\, k_1)/k_1 - e(-k_2\, t)/k_2 \right)}{-k_1 + k_2} \qquad \text{Equation (3.4)}$$

Equation (3.4) was fitted to the time courses of serum astaxanthin concentration using the simplex method (Ebert $et\ al.$ 1989). The maximum uptake rate of astaxanthin in each case was calculated using the best-fit values for $k_2$, $a_0$, and $c_0$ by multiplying $k_2$ by $(a_0 + c_0)$.

## 3.3    Results

Within an experiment, no significant differences ($P > 0.05$) were recorded for mean feed conversion ratios (FCR) or specific growth rates (SGR) for rainbow trout between dietary treatments (Table 3.4). Although the initial starting weights of trout were similar in both experiments (ca. 100g) fish fed diets in Experiment 2 displayed lower FCR (ca. 0.8) and higher SGR (ca. 1.5) values than those fish fed in Experiment 1 (ca 0.1 and 1.2,

respectively) suggesting more efficient growth. This is supported by the increase in live weight of fish over time for fish in Experiment 1 (mean final weight 286.7 ± 2.3g; ca. 29g week$^{-1}$) compared to fish in Experiment 2 (mean final weight 265.5 ± 1.8g; ca. 33g week$^{-1}$). Furthermore, fish in Experiment 1 grew at a slower rate between weeks 0-4 (17.3 ± 0.2g week$^{-1}$) compared to weeks 4-10 (20.0 ± 0.3g week$^{-1}$; $n$=3, ±SEM) with an average wet weight of 167.2 ± 0.6g for fish at week 4.

In Experiment 1, no significant differences (P>0.05) were noted between mean apparent digestibility coefficients (ADC) for astaxanthin across dietary treatments (Table 3.5) with all mean values between 73-75% ($n$=3). Similarly, no significant differences were recorded between mean ADC values for astaxanthin across dietary treatments in Experiment 2 with mean ADC values between 59-70% ($n$=3). Mean ADC values for astaxanthin in Experiment 1 were notably higher than those in Experiment 2 for similar dietary treatments e.g. Diet A (73.6 ± 0.7) and Diet CP (69.1 ± 4.6) although this difference was not significant (P>0.05; $n$=3, ±SEM).

In Experiment 1, no significant differences (P>0.05) were recorded between final muscle astaxanthin concentrations in rainbow trout fed the diet supplemented with unesterified astaxanthin, Diet A (6.2 ± 0.6µg g$^{-1}$), *Haematococcus pluvialis*, Diet B (6.0 ± 0.5µg g$^{-1}$) or cell free carotenoid extract, Diet C (5.2 ± 0.1µg g$^{-1}$; $n$=3, ± SEM) (Figure 3.2). Similarly, no significant differences (P>0.05) were found between final NAR values for fish fed Diet A (11.3 ± 1.1%), Diet B (10.3 ± 1.3%) or Diet C (9.4 ± 0.6%). However, in Experiment 2 the final mean muscle astaxanthin concentrations in rainbow trout were significantly higher (P<0.05) in fish fed Diet CP (2.8 ± 0.2µg g$^{-1}$) compared with those fed Diet 5000 (2.3 ± 0.1µg g$^{-1}$) and Diet 20000 (2.3 ± 0.1µg g$^{-1}$), but not significantly higher than those

fish fed Diet NAT ($2.5 \pm 0.2\mu g \ g^{-1}$; $n=3$, $\pm$SEM). In confirmation of this, the mean NAR values for Diet CP ($7.2 \pm 0.4\%$) were also significantly higher than those noted in fish fed Diet 5000 ($4.2 \pm 0.1\%$) and Diet 20000 ($3.9 \pm 0.1\%$), although were not significantly higher than the mean retention values recorded in fish fed Diet NAT ($5.0 \pm 0.4\%$).

Examination of flesh astaxanthin concentrations after 4 and 10 weeks of feeding in Experiment 1 show a positive increase in deposition over time (Figure 3.3). Furthermore, the rate of astaxanthin deposition was notably greater in the second half of the trial (weeks 4-10) compared to weeks 0-4 where fish fed Diet A, Diet B, and Diet C only deposited 1.3 $\pm 0.2\mu g \ g^{-1}$; $1.3 \pm 0.1\mu g \ g^{-1}$ and $1.1 \pm 0.04\mu g \ g^{-1}$ of astaxanthin, respectively ($n=3$, $\pm$SEM). No significant differences ($P>0.05$) were perceived in coloration between the fillets of fish fed the different dietary treatments (assessed using the *Salmo*Fan™) with a range of 25-31. Evaluation of the relationship between *Salmo*Fan™ score and flesh concentrations of astaxanthin for fish taken at weeks 4 and 10 in Experiment 1 (Figure 3.4) demonstrated a significant relationship ($P<0.001$) between the two measurements. However, the correlation coefficient between the two parameters (0.80) only indicated a moderately strong association.

Examination of the serum astaxanthin concentrations in fish fed a single meal of either of the experimental diets (Figure 3.5) suggested significant quantities of astaxanthin were still present in the serum of fish at time 0 (ca. $0.8-1\mu g \ ml^{-1}$) despite feeding fish non-pigmented feed for four days. Nevertheless, kinetic assessment of maximum serum astaxanthin absorption rates showed that the mean absorption rate in fish fed Diet A ($0.44 \pm 0.07\mu g \ ml^{-1}h^{-1}$) was numerically but not significantly higher ($P>0.05$) compared to those in fish fed Diet B ($0.28 \pm 0.11\mu g \ ml^{-1}h^{-1}$) and Diet C ($0.26 \pm 0.02\mu g \ ml^{-1}h^{-1}$). Furthermore, at time

24h, mean serum astaxanthin concentrations (minus values at time 0) were not significantly different (P>0.05) in fish fed Diet A ($1.07 \pm 0.13 \mu g \ ml^{-1}$), Diet B ($1.27 \pm 0.11 \mu g \ ml^{-1}$) or Diet C ($1.10 \pm 0.25 \mu g \ ml^{-1}$).

Table 3.1     Ingredients and formulation of the experimental diets for both experiments

| Ingredients: | Percentage inclusion |
|---|---|
| [a]LT Fishmeal | 61.3 |
| Wheat gluten | 5.6 |
| Wheat | 21.6 |
| Fish oil | 10 |
| [b]Vitamin premix | 0.8 |
| [b]Mineral premix | 0.7 |
| Yttrium oxide | 0.01 |

**Proximate composition:**

| | |
|---|---|
| Protein | $46.0 \pm 0.3$ |
| Fat | $27.3 \pm 0.1$ |
| Ash | $10.4 \pm 0.1$ |
| Moisture | $6.1 \pm 0.04$ |
| Energy | $21.7 \pm 0.1$ MJ kg$^{-1}$ |

[a]Norse LT 94, Egersund, Norway. [b]Vitamins/mineral premixes as formulated by Trouw Aquaculture Ltd. (Wincham, U.K.). Supplementary oil (Cod Liver Oil, Seven seas Ltd., Hull, U.K.) was added to basal formulation, providing a carrier for the astaxanthin sources and to increase final oil level to a commercial level of 26% (w/w). Analyses of diet composition ($n$=3, $\pm$ SEM).

Table 3.2    Astaxanthin forms and inclusion concentration in diets from Experiment 1.

| Dietary treatment | Astaxanthin inclusion rate $(mg\ kg^{-1})$ (mean ± SEM, $n$ =3) |
|---|---|
| **Diet A** – Unesterified astaxanthin | 61.7 ± 2.5 |
| **Diet B** – Processed cells of *H. pluvialis* | |
| Astaxanthin monoesters | 44.2 |
| Astaxanthin diesters | 15.5 |
| Unesterified astaxanthin | 3.1 |
| Total astaxanthin | 62.8 ± 0.5 |
| **Diet C** – Cell-free total carotenoid extract from *H. Pluvialis* | |
| Astaxanthin monoesters | 42.2 |
| Astaxanthin diesters | 15.4 |
| Unesterified astaxanthin | 2.9 |
| Total astaxanthin | 60.5 ± 2.0 |

Relative concentrations of astaxanthin forms determined from percentage contribution as evaluated by TLC (see Materials and Methods).

Table 3.3        Astaxanthin forms and inclusion concentration in diets from Experiment 2

| Dietary treatment | Astaxanthin inclusion rate $(\text{mg kg}^{-1})$ (mean $\pm$ SEM, $n$ =3) |
|---|---|
| **Diet CP** – Unesterified astaxanthin | 58.5 $\pm$ 0.4 |
| **Diet 5000** – Processed cells of *H. pluvialis* | |
| Astaxanthin monoesters | 42.6 |
| Astaxanthin diesters | 19.0 |
| Unesterified astaxanthin | 4.7 |
| Total astaxanthin | 66.3 $\pm$ 0.3 |
| **Diet 20000** – Processed cells of *H. pluvialis* | |
| Astaxanthin monoesters | 51.9 |
| Astaxanthin diesters | 17.7 |
| Unesterified astaxanthin | 4.5 |
| Total astaxanthin | 74.2 $\pm$ 0.4 |
| **Diet NAT** – Processed cells of *H. pluvialis* | |
| Astaxanthin monoesters | 44.4 |
| Astaxanthin diesters | 15.1 |
| Unesterified astaxanthin | 3.3 |
| Total astaxanthin | 62.8 $\pm$ 1.0 |

Relative concentrations of astaxanthin forms determined from percentage contribution as evaluated by TLC (see Materials and Methods).

Table 3.4     Growth Parameters for rainbow trout fed diets in both experiments.

| | Experiment 1 | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|
| | Diet A | Diet B | Diet C | Diet CP | Diet 5000 | Diet 20000 | Diet NAT |
| Specific Growth Rate (SGR). | 1.22 ± 0.02 | 1.27 ± 0.04 | 1.23 ± 0.04 | 1.54 ± 0.01 | 1.54 ± 0.01 | 1.57 ± 0.01 | 1.58 ± 0.02 |
| Feed Conversion Ratio (FCR). | 1.09 ± 0.02 | 1.04 ± 0.06 | 1.10 ± 0.06 | 0.81 ± 0.01 | 0.81 ± 0.01 | 0.79 ± 0.01 | 0.78 ±0.02 |

Values represent means ($n$=3, ±SEM). No significant differences were recorded between dietary treatments within an experiment for either SGR or FCR.

Table 3.5    Apparent Digestibility Coefficients for Astaxanthin in Rainbow Trout Fed the Dietary Treatments for Both Experiments.

| Dietary Treatment | ADC |
| --- | --- |
| **Experiment 1** | |
| Diet A | $73.6 \pm 0.7$ |
| Diet B | $74.5 \pm 1.5$ |
| Diet C | $74.5 \pm 1.3$ |
| | |
| **Experiment 2** | |
| Diet CP | $69.1 \pm 4.6$ |
| Diet 5000 | $61.1 \pm 5.1$ |
| Diet 20000 | $59.8 \pm 4.5$ |
| Diet NAT | $59.5 \pm 6.4$ |

Values represent means ($n$=3, ±SEM). No significant differences were recorded between treatments within an experiment for ADC.
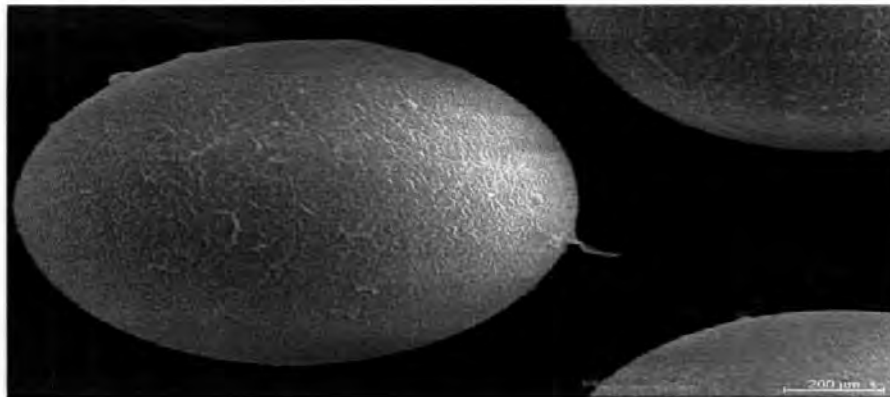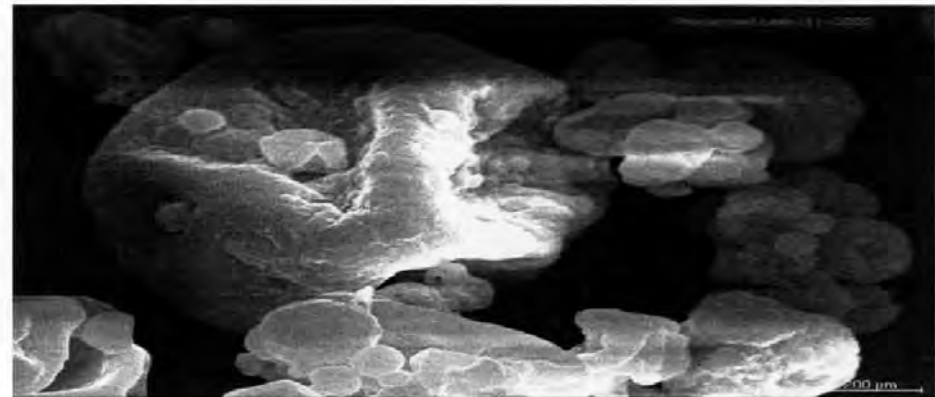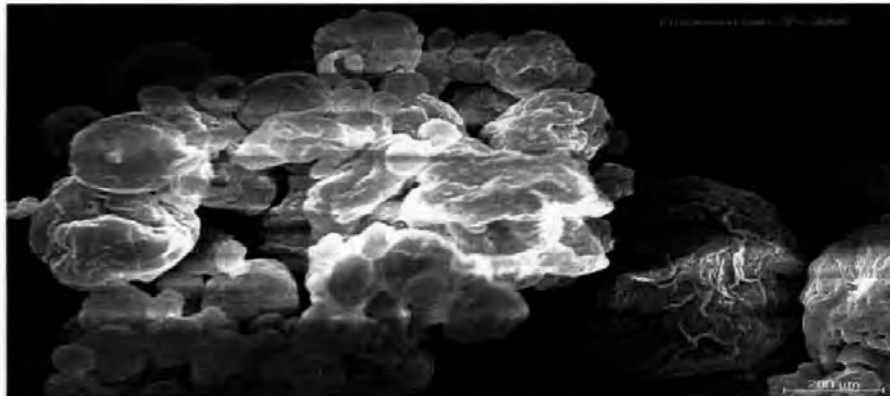
(a)  (b)  (c)  (d)
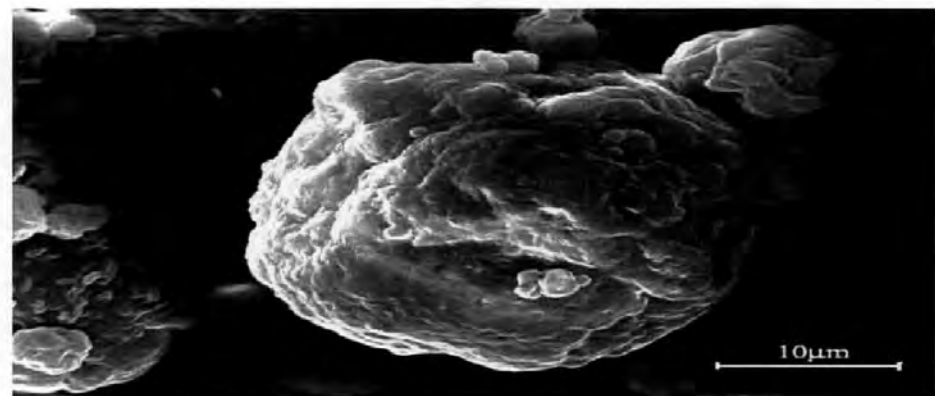
Figure 3.1  Scanning electron micrographs (× 2000) of *Haematococcus pluvialis* cells (a) intact cell; (b) cracked under 5000psi; (c) cracked under 2000psi and (d) NatuRose™ cell cracked using a proprietary process.
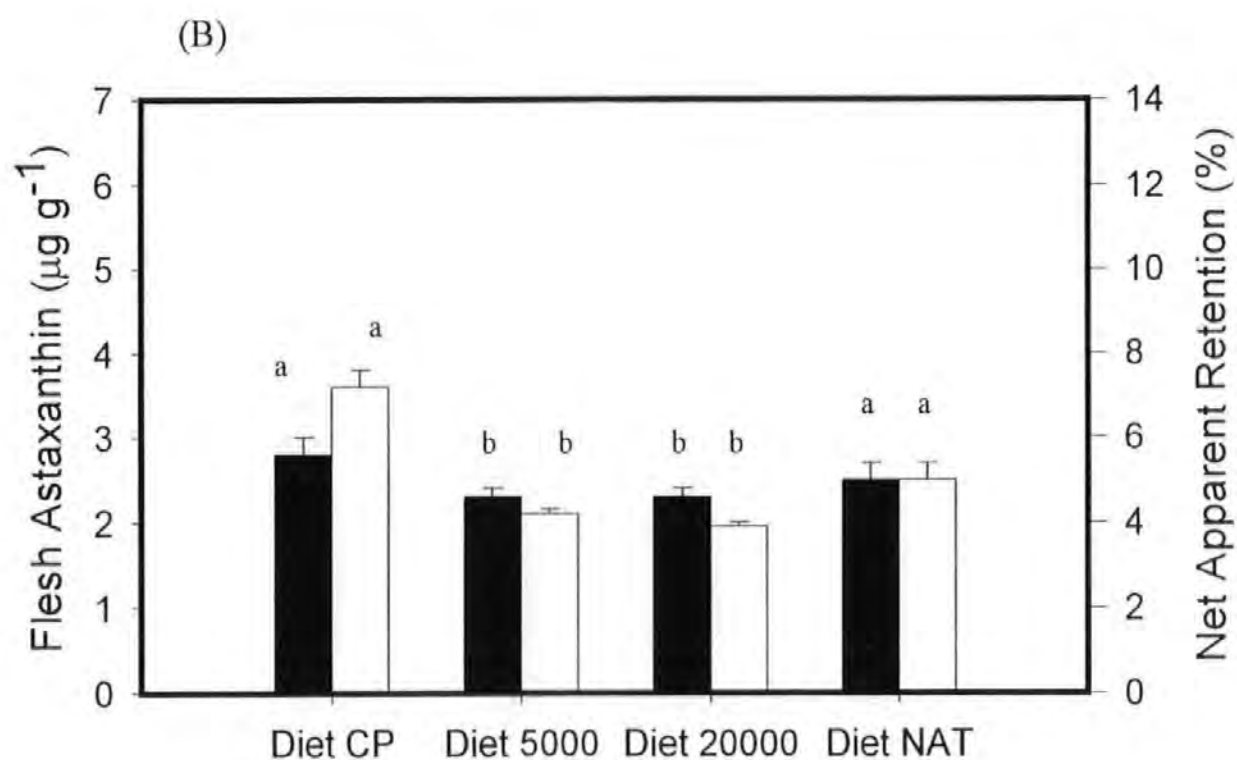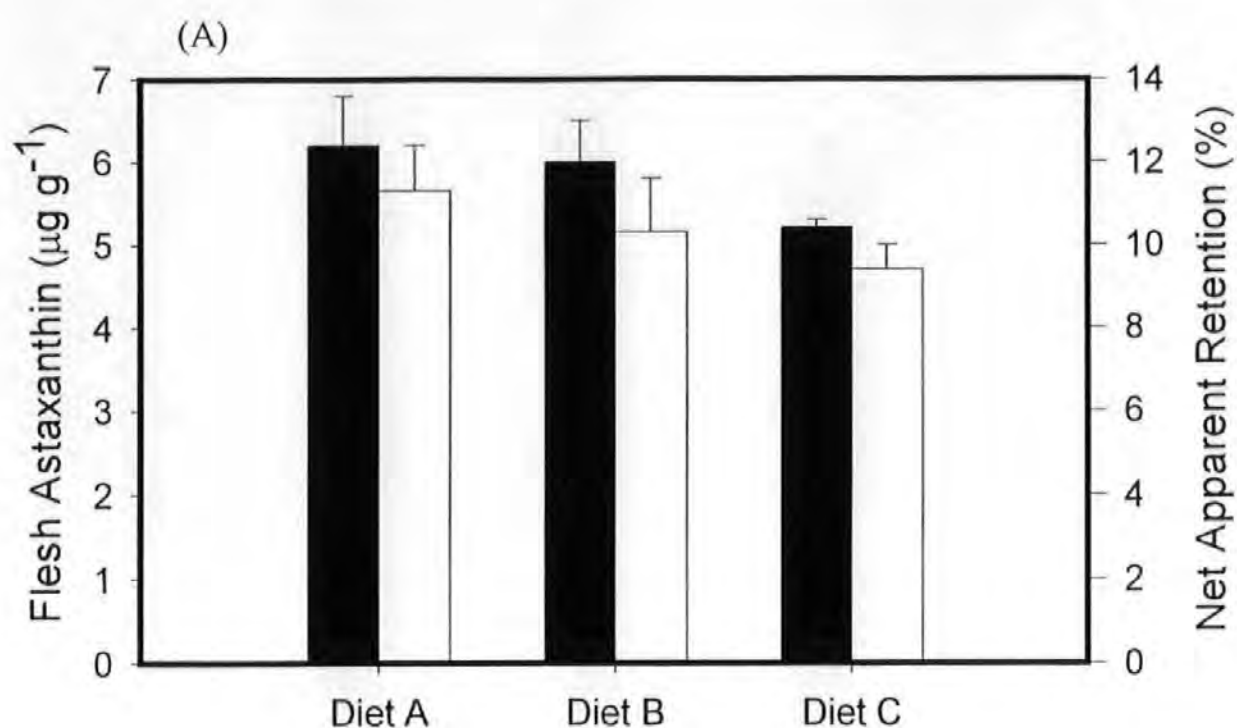
Figure 3.2    Flesh astaxanthin concentrations (solid bars) and NAR values (open bars) for fish fed diets in Experiment 1 (A) and Experiment 2 (B). Columns represent means ±SEM (*n*=3). [ab]Columns bearing different superscripts are significantly different (P<0.05)
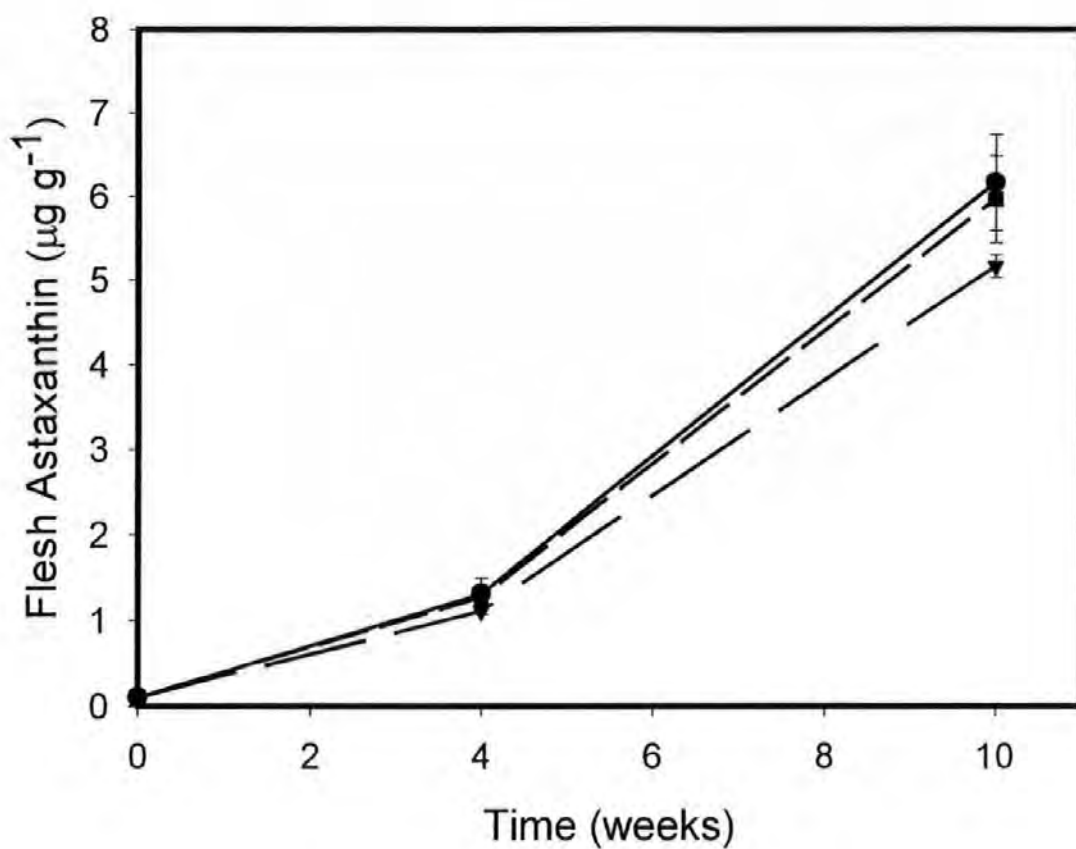
Figure 3.3    Astaxanthin deposition in the flesh of trout fed diets in Experiment 1 after 4 and 10 weeks of feeding.  Values represent means ($n=3$, ± SEM) for Diet A (●; solid line), Diet B (■; short dash) and Diet C (▼; long dash).  No significant differences were recorded between treatments.
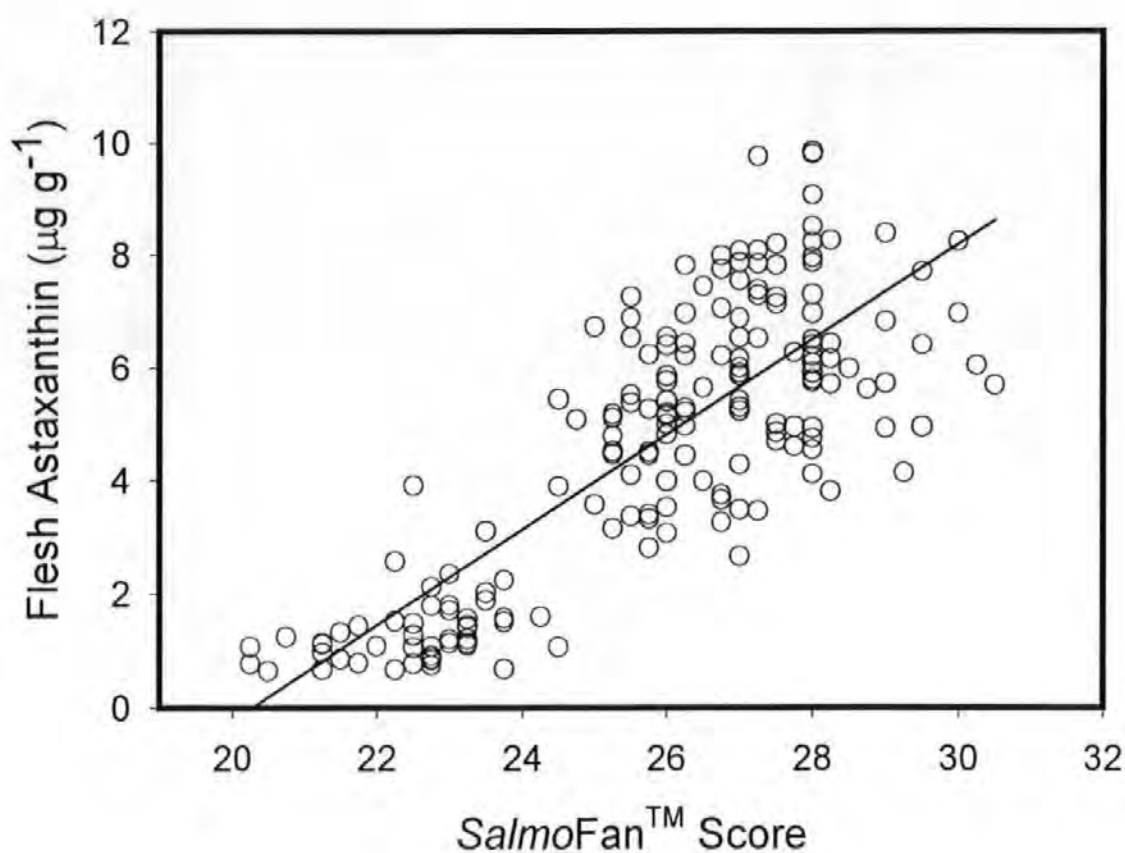
Figure 3.4    Relationship between flesh astaxanthin concentrations and *Salmo*Fan™ Score for fish fed diets in Experiment 1. Equation for the regression analysis was *Salmo*Fan™ score= 22.2366 + 0.767496×flesh astaxanthin (correlation coefficient=0.80; $R^2$=64.63%). Values represent individual fish sampled at weeks 4 and 10.
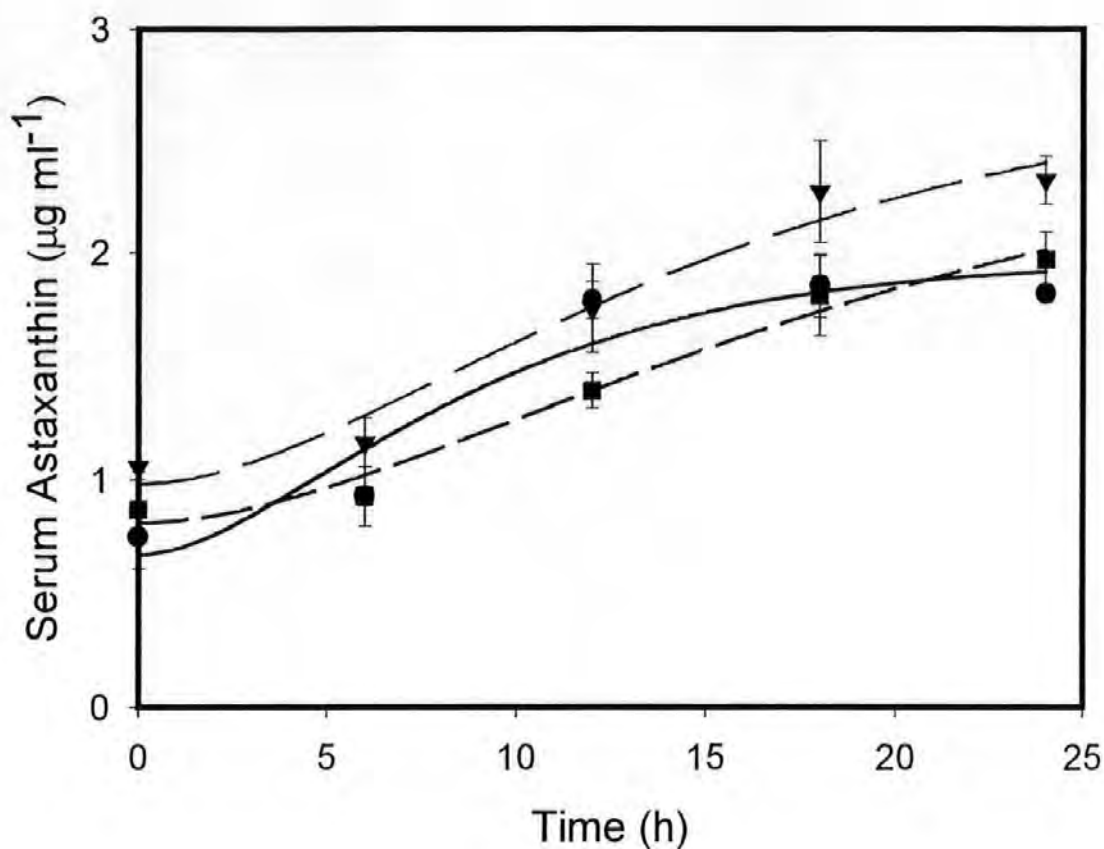
Figure 3.5    Post-prandial serum profiles for trout fed a single meal supplemented with either synthetic unesterified astaxanthin, Diet A (●; solid line); *Haematococcus pluvialis*, Diet B (■; short dash) or cell free carotenoid extract, Diet C (▼; long dash). Values represent the means of replicates ($n=3$, ± SEM). Maximum absorption rates determined by multiplying the respective apparent uptake rate constant ($k_2$) by ($a_0 + c_0$). Time 0 represents approximately 1h after fish were fed.

## 3.4    Discussion

No apparent differences were recorded for growth of rainbow trout across treatments within experiments, indicating that the use of different astaxanthin supplements from *Haematococcus pluvialis* presents no deleterious effects on feed utilisation and growth performance. However, the differences in feed utilisation and growth rate of fish between the two experiments was unexpected since fish were essentially fed diets of identical proximate composition and nutritional value and were maintained under the same ambient conditions during the experimental trials. Such variation can only be explained by differences, possibly genetic, in the groups of fish brought into the experimental facilities for each experiment.

Astaxanthin concentrations in diets varied throughout both experiments. In experiment 1, Diet B (encysted *Haematococcus pluvialis*) had a stable astaxanthin profile yet both Diet A (synthetic/unesterified astaxanthin) and Diet C (cell free carotenoid extract) decreased in astaxanthin concentration by almost 20% over the ten-week trial period. Similarly, in experiment 2, Diet CP decreased in astaxanthin concentration by almost 30%. However, the other diets supplemented with *Haematococcus pluvialis* products did not decrease significantly in astaxanthin content throughout the experiment. This suggests that the natural cell wall of *Haematococcus pluvialis* serves to prevent oxidation of dietary astaxanthin when added to aquafeeds, irrespective of whether the sporopollenin cell wall has been cracked. However, this variation in feed carotenoid levels hinders the interpretation of astaxanthin retention values in the flesh, especially those in fish fed unesterified astaxanthin that may be underestimated as a consequence. It is questionable whether the ability to produce feeds with stable carotenoid supplements is an advantage since the turnover of feeds in today's intensive aquaculture facilities is rapid.

81

The Apparent Digestibility Coefficients (ADC) for astaxanthin in both experiments were in keeping with previously reported values of between 60-80% (Choubert & Storebakken 1996; Bjerkeng *et al.* 1997). A lack of significant difference in ADC values between dietary treatments used in experiment 1 and experiment 2 suggests that if rupturing of the cell wall of *Haematococcus pluvialis* cells is adequate then the intestinal absorption of astaxanthin is not limited. However, although there were no significant differences in ADC values in experiment 2, there was a trend for fish fed diets supplemented with unesterified astaxanthin to have higher ADC values. Similarly, Storebakken *et al.* (1987) reported average apparent digestibility values for unesterified astaxanthin in Atlantic salmon (64%) that were higher than for astaxanthin dipalmitate (47%) although this difference was not significant. Furthermore, Foss *et al.* (1987) reported higher digestibility values for unesterified astaxanthin fed to rainbow trout (91-97%) compared to astaxanthin dipalmitate (42-67%). Variation in results and lack of significant differences may have been due to factors such as oxidation of pigment in faecal samples, incomplete extraction of carotenoid and destruction of carotenoids in the intestinal tract.

The significant quantities of astaxanthin remaining in the serum of trout fed on a non-pigmented diet for four days suggests that longer periods of feeding are required to ensure minimal astaxanthin concentrations. It is not clear whether residual serum astaxanthin concentrations would have had an effect on uptake rate of astaxanthin into serum. However, if the concentration gradient between the feed and the serum were an important factor influencing uptake by passive diffusion (Kiessling *et al.* 1995), then astaxanthin in the serum would act to reduce this gradient subsequently reducing the uptake rate. Nevertheless, observation of the post-prandial astaxanthin serum profiles for the different trout groups demonstrated that all sources of astaxanthin were absorbed into the blood. In

addition, those fish fed synthetic astaxanthin had higher mean serum astaxanthin absorption rates compared to those fish fed *Haematococcus pluvialis* and a cell free extract from the same alga, although this difference was not significant. This suggests that the uptake of astaxanthin derived from *Haematococcus pluvialis* may be rate limited. However, since the uptake of astaxanthin from the cell free extract was almost identical to that from the cracked cells, the necessary hydrolysis of astaxanthin esters contained within this alga may well be the rate-limiting step (Torrissen *et al*. 1989). However, at 24 hours after ingestion there was no significant difference in the mean serum astaxanthin concentrations between fish fed the different astaxanthin sources.

Muscle astaxanthin concentrations at the end of the trial in experiment 2 are in keeping with values previously reported in other feeding trials of similar duration and feed carotenoid inclusion level (Sommer *et al*. 1991; Sommer *et al*. 1992) although those in experiment 1 (5-6µg g$^{-1}$) were higher. In contrast, Choubert & Heinrich (1993) achieved levels of flesh pigmentation of 6.2mg carotenoid kg$^{-1}$ in rainbow trout fed diets supplemented with *Haematococcus pluvialis*, although feed inclusion levels used were higher (100mg kg$^{-1}$) than in the current study. The moderate pigmentation levels in experiment 2 compared to experiment 1 may be explained in part by the lower ADC values and the shorter trial duration, although higher values were expected. In contrast to previous studies (Sommer *et al*. 1991; Sommer *et al*. 1992; Choubert & Heinrich 1993), no significant difference in flesh pigmentation were recorded between fish fed the commercial synthetic (CAROPHLL® Pink) and commercial natural (*Haematococcus pl*.; NatuRose) sources of astaxanthin. Flesh retention values for astaxanthin showed similar results. However, pigmentation efficiency for algal cells supplemented into Diet 5000 and Diet 20000 (cracked in a confidential procedure) was significantly lower than the synthetic source. There were no differences between the contribution of monoesters, diesters and

unesterified astaxanthin in the various sources of algal cells. This may suggest inefficiency in the cracking procedure used on the *Haematococcus pluvialis* culture supplemented into these two diets, compared to the procedure used to crack the commercial source of algal cells. However, this does demonstrate the necessity to efficiently crack the sporopollenin cell wall of *Haematococcus pluvialis* prior to dietary supplementation in order to achieve desired levels of flesh pigmentation.

The rate of astaxanthin deposition in the muscle of those fish fed experimental diets in Experiment 1 was clearly greater in the second period of the trial (wk 4-10) compared to the first period (wk 0-4). Similarly, the rate of growth for fish was greater in the second period. Other authors have suggested that salmonids under 150g pigment poorly which is supported by the current findings (Abdul-Malak *et al.* 1975; Torrissen 1985; Arai *et al.* 1987). This suggests that there may be a relationship between the growth or age of fish and the ability to deposit pigment, maybe due to a physiological constraint in the number of binding sites within the flesh (Nickell & Bromage 1998b). However, other authors who have examined the relationship between muscle fibre density and pigment deposition in Atlantic salmon have found no relationship (Johnston *et al.* 2000). Moreover, perhaps larger fish are able to absorb more pigment from the diet. This potential relationship between fish size and carotenoid absorption merits further study.

Measurements of carotenoid concentration in the flesh should not be used as criterion of perceived colour (Little *et al.* 1979). Comparative measurements of flesh carotenoid deposition in the whole muscle using a colour fan have been used previously (Little *et al.* 1979; Skrede *et al.* 1990; Johnston *et al.* 2000; Buttle *et al.* 2001). Results in the current experiment suggest a linear relationship between flesh pigment concentration and visual colour according to the *Salmo*Fan™. The correlation between perceived flesh colour and

flesh carotenoid concentration in the current study was similar to that found previously for rainbow trout (r=0.80) and sea trout (r=0.77; Foss *et al.* 1987). Similarly, other authors have found a good correlation between the astaxanthin concentration in salmon flesh and the Roche Colour Card for Salmonids (Skrede *et al.* 1990; Christiansen *et al.* 1995b). However, Smith *et al.* (1992) did not observe a linear relationship between scores taken from the Roche Colour Card for Salmonids and the flesh astaxanthin concentration in Coho salmon, but the astaxanthin concentrations in their study tended to be higher than reported here. Differences between findings from these studies may be due to species differences and problematic differentiation of colour differences at higher concentrations. Torrissen *et al.* (1989) stated that the relationship between visual score and carotenoid level is linear only at low levels (up to 3 to 4 mg kg$^{-1}$) in farmed fish. Fish farmers and producers of salmonids commonly use such visual assessments as opposed to chemical analyses for practical purposes. However, presentation of the sample, ambient illumination; variability between individuals in their subjective assessment of differences in colour and the masking effect of inter-muscular fat can bias visual perception. Subsequently, instrumental measurements of colour such as assessment of the CIELCH colour space are more accurate in evaluating pigmentation (Skrede & Storebakken 1986; Choubert *et al.* 1997; Olsen & Mortensen 1997; Hatlen *et al.* 1995b).

There is an apparent lack of literature concerning different techniques to disrupt encysted cells of *Haematococcus pluvialis* and their efficacy, probably owing to commercial and proprietary constraints. Indeed those methods that have been proposed to disrupt algal cells are under patent and are not very efficient for disrupting the sporopollenin cell wall of *Haematococcus* cysts (Farrow & Tabenkin 1966; Ruane 1977; Nonomura 1987). However, Mendes-Pinto *et al.* (2001) attempted to assess a range of physical and chemical processes on the recovery of astaxanthin from these algal cells. These authors examined

the effects of autoclaving; acid and alkali treatment; enzymatic treatment; spray drying and mechanical disruption with a cell homogeniser. Pigment availability from processed cells was assessed by extraction of astaxanthin into acetone. It was demonstrated that both mechanical disruption and autoclaving of the astaxanthin-rich algal biomass were effective treatments resulting in a high yield of astaxanthin. Such an *in vitro* assessment of availability requires further confirmation of carotenoid availability *in vivo* before real conclusions can be made on the efficacy of a cell disruption process.

Results from this study suggest *Haematococcus pluvialis* can be efficiently utilised as a source of pigment for rainbow trout providing the cell wall is sufficiently ruptured prior to feed supplementation in order to aid carotenoid bioavailability. Furthermore, extraction of the pigment from the cell wall does not result in improved levels of pigmentation, is impractical on a large scale (Mendes-Pinto *et al.* 2001) and would only add to production costs of the product. Indeed, the cell wall, despite being cracked, seems to prevent oxidation of the product when added to the feed, an additional advantage compared to both the carotenoid extract and the synthetic source. Notable differences in rates of serum astaxanthin absorption from the synthetic, unesterified source compared to that of carotenoid extract from *Haematococcus pluvialis*, warrants examination of the role of the salmonid gastrointestinal tract in context of carotenoid ester hydrolysis, carotenoid absorption and subsequent assimilation. In addition, the utilisation of individual acyl esters and the differences between them requires further study.

# CHAPTER 4.0

# PHYSIOLOGY OF THE INTESTINE MAY LIMIT THE ABSORPTION OF ASTAXANTHIN FROM ESTERIFIED SOURCES IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM).

## 4.1    Introduction

In the wild, salmonids achieve a natural flesh pigmentation through the ingestion of crustaceans that contain astaxanthin (3,3'-dihydroxy-$\beta$,$\beta$-carotene-4,4'dione; Schiedt *et al.* 1986; Skrede & Storebakken 1986; Scalia *et al.* 1989).  In intensive salmonid rearing, feeds are commonly supplemented with synthetic astaxanthin and the carotenoid canthaxanthin ($\beta$, $\beta$-carotene-4, 4'-dione; Bjerkeng 1992; Bjerkeng *et al.* 1992; Bell *et al.* 1998; Akhtar *et al.* 1999).  Nonetheless, consumer demand for strictly natural food additives has led to growth in the use of natural sources of feed ingredients (Johnson & An 1991).

The microalga *Haematococcus pluvialis* (Flowtow) contains a high quantity of astaxanthin (between 1.5-3.0% dry weight).  However, up to 95% of astaxanthin from this source is esterified (ca. 70% monoesters, ca. 25% diesters; Lorenz & Cysewski 2000).  Following ingestion of astaxanthin esters, intestinal hydrolysis is required before absorption of astaxanthin can occur into the systemic circulation of the fish (Schiedt *et al.* 1986; Storebakken *et al.* 1987).  Synthetic astaxanthin dipalmitate is poorly utilised in comparison to free astaxanthin (Foss *et al.* 1987; Storebakken *et al.* 1987) and some authors have reported poorer pigmentation when using feeds supplemented with *Haematococcus pluvialis* compared to those supplemented with free astaxanthin (Sommer *et al.* 1991;

87

Sommer *et al.* 1992).  The rate of hydrolysis of astaxanthin esters to free astaxanthin appears to be the limiting factor, and this may explain observed differences in deposition (Torrissen *et al.* 1989).

Several factors may affect the rate of astaxanthin ester hydrolysis.  These factors are influenced by, or are dependent on the physiological characteristics of the gastrointestinal tract.  Firstly, the action of digestive enzymes on nutrients and contact time at absorptive sites in the intestine are affected by transit rate of the feed bolus (Choubert & Storebakken 1996).  It has been stated that digestion efficiency and gut transit time are likely to influence the proportion of any feed supplemented micro-nutrient that is absorbed (Jackson 1997).

Secondly, there may be differences in esterase activity and subsequently hydrolysis along the length of the salmonid intestine.  Indeed, the greater part of enzymatic hydrolysis, nutrient release and uptake in fish is reported to take place generally in the lumen of the anterior intestine (Fänge & Grove 1979; Buddington & Doroshov 1986; Lie *et al.* 1987; Vernier 1990).  Regional variation in intestinal hydrolysis has been recorded for other forms of nutrients such as L-ascorbyl-2-phosphate in rainbow trout (Miyasaki *et al.* 1993) and lipids in turbot, *Scophthalmus maximus* (Koven *et al.* 1994, 1997).

Finally, there may be differences in carotenoid absorption along the length of the salmonid intestine.  Some authors have suggested that the anterior intestine region is primarily responsible for carotenoid absorption (Torrissen 1986; Al-Khalifa & Simpson 1988) while other authors have reported no variation (Guillou *et al.* 1992a).

The aims of this study were four fold. First, to compare the "steady state" serum astaxanthin concentrations in rainbow trout, *Oncorhynchus mykiss* (Walbaum) following daily feeding with esterified or free astaxanthin to indicate the efficacy of absorption under standard feeding conditions. Secondly, to quantitatively compare the absorption rates of astaxanthin in the serum of fish fed a single meal containing each astaxanthin source. It was envisaged that this would indicate a limitation in absorption as a result of the requirement for ester hydrolysis. Thirdly, to assess whether there are any differences along the trout intestine with respect to the hydrolysis of esterified astaxanthin. Finally, to compare the deposition of astaxanthin from these two sources in the flesh as this is ultimately the determinant of a product's pigmentation efficacy.

## 4.2    Materials and Methods

### 4.2.1    Experimental design

In the following experiment each dietary treatments was assigned to triplicate tanks containing groups of fish. Subsequently, each tank is considered as a replicate ($n=3$). Following sampling and analysis of individual fish tissues, statistical comparison of results between treatments was based on mean values from each tank ($n=3$) to avoid pseudo-replication and false interpretation of results (Morris 1999).

### 4.2.2    Experimental feeds

Experimental diets, prepared at EWOS Technology Centre (Livingston, Scotland, U.K.), contained either free astaxanthin (Diet FR) added to dry ingredients as water dispersible beadlets (8% w/v astaxanthin content; CAROPHYLL® Pink, F Hoffmann-La Roche,

Basel, Switzerland), or esterified astaxanthin (Diet EST) added to the feed mixture as a dry powder form of *Haematococcus pluvialis* (NatuRose, Cyanotech Corporation, Kailua-Kona, Hawaii, U.S.A.). Basal dietary ingredients included fishmeal (59%); soya product (10.5%); wheat (13.5%) and fish oil (17%). Origin of ingredients is proprietary information. Feeds were analysed in duplicate (at EWOS) for protein by the Kjeldahl method (EC directive 93/28/EEC), for fat by extraction with petroleum spirit after acid hydrolysis (EC directive 84/4/EEC) and moisture by oven drying at 103°C (EC directive 71/393/EEC). Proximate composition of diets was protein (45%); lipid (25%) and dry matter (93%). Target dietary total carotenoid concentration was 50.6mg $kg^{-1}$ and 50.0mg $kg^{-1}$ for Diet FR and Diet EST, respectively. As a percentage of total astaxanthin in Diet EST, monoesters (ca. 50%) were predominant compared to diesters (ca. 40%) and free/unesterified astaxanthin (ca. 10%). This was confirmed by thin layer chromatography (see section 2.4.7) according to Kobayashi *et al.* (1991).

### 4.2.3   Fish and feeding trial

Female rainbow trout (Hatchlands Trout Farm, Devon, U.K.) were held in a re-circulation system (six 400L glass fibre tanks) maintained at 15°C and exposed to a 12h light/dark photoperiod (see section 2.2.2). Fish (85 ± 9g) were randomly assigned into groups of 30 per tank.

For determination of the steady state astaxanthin concentrations in the serum of the fish, test diets were fed in a single morning meal (1.5% BW $day^{-1}$) for 56 days, with rations being adjusted assuming a feed conversion ratio (FCR) of 1.0. Fish in three of the tanks were fed Diet FR while fish in the other three tanks were fed Diet EST. On the final day and within 1 hour of fish being fed, samples of blood (see below); flesh; liver and

90

gastrointestinal tract regions with digesta (Figure 4.1) were taken for astaxanthin analysis (5 fish per replicate tank).

To determine the time course of serum astaxanthin absorption after a single meal, the remaining fish were then fed a carotenoid-free diet with the same basal formulation as the experimental diets at 1.5% BW day$^{-1}$ for 14 days in attempt to clear astaxanthin from the serum. They were then fed a single meal of the respective astaxanthin-supplemented diets to appetite. Blood samples were taken (3 fish per replicate tank) at eight times up to and including 72h after the meal (Figure 4.4). The fish were not fed further meals following this single carotenoid-supplemented meal.

### 4.2.4   Analysis of feed, digesta, gastrointestinal tract and blood

Carotenoids were extracted from diets (at EWOS Technology Centre, Livingston, Scotland) essentially according to Schierle & Härdi (1994). In brief, following enzymatic digestion of the feed (Maxatase P-3000 encapsulated, International Biosynthetics, Rijswik, Netherlands), carotenoids were extracted with ethanol/dichloromethane (1:1 v:v) and purified on a 4cm glass open-top silica (Silica gel 60) column. Extracts from diets were analysed spectrophotometrically for total carotenoid content in heptane (Diet FR) or dimethyl sulfoxide (Diet EST) at 470nm ($E_{1\%\ 1cm}$ =1910) or 492nm ($E_{1\%\ 1cm}$ =2220), respectively.

Freeze-dried digesta were pooled on a replicate basis ($n$=3) and analysed using methodology modified after Weber (1988) (see section 2.4.2). Forms of astaxanthin (monoesters, diesters, and free astaxanthin) were separated by TLC according to

Kobayashi *et al.* (1991; see section 2.4.7) and analysed spectrophotometrically in *n*-hexane at 470nm using an $E_{1\% \, 1cm}$ value of 2100 (Britton 1995; see section 2.4.9).

Carotenoids were extracted from fish serum; muscle; liver and cleaned gut samples according to extraction methodology previously described (see sections 2.4.3; 2.4.4 and 2.4.5). Samples were stored at -20°C until HPLC analysis (see section 2.4.10).

### 4.2.5 Statistical analysis

Data were subjected to tests for normal distribution (standardised skewness and kurtosis) and variance checks (Cochrans and Barlett's test). Statistical evaluation of data was carried out on StatGraphics Plus 4.0. Regression analysis and curve fitting was carried out on SigmaPlot 4.0. Astaxanthin uptake rates and astaxanthin concentrations in flesh; liver; gut tissue; digesta; serum and were compared across treatments using one-way analysis of variance test at the 95% confidence limit.

### 4.2.6 Kinetic modelling

After ingestion of a single meal the time course of serum astaxanthin concentration shows three phases: a lag phase followed by the uptake and elimination phases (Figure 4.2). The following simple model was used to describe these kinetics.
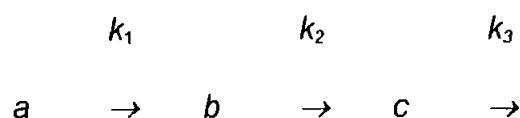
$$a \xrightarrow{k_1} b \xrightarrow{k_2} c \xrightarrow{k_3}$$

In this model $c$ represents the serum astaxanthin concentration, and the first order rate constants, $k_1$, $k_2$ and $k_3$, apply to the lag, uptake and elimination phases, respectively. The following set of ordinary differential equations (4.1-4.3) are derived from the model.

$$\frac{da}{dt} = -k_1 \cdot a \qquad\qquad \text{Equation (4.1)}$$

$$\frac{db}{dt} = k_1 \cdot a - k_2 \cdot b \qquad\qquad \text{Equation (4.2)}$$

$$\frac{dc}{dt} = k_2 \cdot b - k_3 \cdot c \qquad\qquad \text{Equation (4.3)}$$

These were solved using Maple 6 (Waterloo Maple Inc., U.S.A.), using the limit values: a $= a_0$ at $t = 0$; $b = 0$ at $t = 0$; and $c = c_0$ at $t = 0$. Equation (4.4) shows the relationship between $c$ and time, $t$.

$$c = \left[ \frac{a_0 \cdot k_1 \cdot k_2 \cdot \left( \frac{e^{(k_3-k_1)\cdot t}}{k_3-k_1} - \frac{e^{(k_3-k_2)\cdot t}}{k_3-k_2} \right)}{k_2-k_1} - \frac{a_0 \cdot k_2 \cdot k_1 - c_0 \cdot k_2 \cdot k_3 + c_0 \cdot k_1 \cdot k_2 + c_0 \cdot k_3^2 - c_0 \cdot k_1 \cdot k_3}{(k_3-k_1) \cdot (k_2-k_3)} \right] \cdot e^{-k_3 \cdot t}$$

Equation (4.4)

Equation (4.4) was fitted to the time courses of serum astaxanthin concentration using the simplex method (Ebert *et al.* 1989). The maximum uptake rate of astaxanthin in each case was calculated using the best-fit values for $k_2$, $a_0$, and $c_0$ by multiplying $k_2$ by $(a_0 + c_0)$. The latter represents the value that the serum astaxanthin concentration would reach at t = $\infty$ if there were no elimination of astaxanthin from the serum, i.e. if $k_3 = 0$ (see Figure 4.2).

## 4.3    Results

There were no significant differences (P>0.05) in growth after 56 days with final weights of 215 ± 3.2g and 219 ± 1.6g (± SEM, $n$=3) and feed conversion ratios (FCR) of 1.1 ± 0.08 and 1.0 ± 0.03 (± SEM, $n$=3) for fish fed diets supplemented with free (Diet FR) or esterified (Diet EST) astaxanthin, respectively.

In both fish fed Diet FR or Diet EST the ileal intestine had significantly higher (P<0.05) mean astaxanthin (unesterified form) concentrations compared to the stomach and posterior intestine (Table 4.1). No significant differences (P>0.05) were recorded between fish fed Diet FR or Diet EST for each of the gut sections. HPLC analysis could not confirm the presence of astaxanthin esters in the gut-tissue samples.

In the digesta of those fish fed Diet EST (Figure 4.3) astaxanthin was detected only in its free form in the ileal and posterior intestine (post pyloric region). Furthermore, in the ileal and posterior intestine the digesta contained a higher concentration of free astaxanthin (7.1 ± 1.9μg g$^{-1}$ and 5.2 ± 2.9μg g$^{-1}$, respectively) in comparison to the stomach (2.7 ± 0.6μg g$^{-1}$; ± SEM, $n$=3), though the total content of astaxanthin was lower. Astaxanthin forms in the stomach digesta were representative of the diet with slight increases in monoester (ca. 9%) and corresponding decreases in diester (ca. 7%). Astaxanthin concentrations in the digesta from the stomach, ileal and posterior intestine were 34.3mg kg$^{-1}$, 7.1mg kg$^{-1}$ and 5.2mg kg$^{-1}$, respectively, with the most notable reduction in astaxanthin concentration occurring between the stomach and the ileal intestine.

After 56 days of daily feeding the mean serum astaxanthin concentration of fish fed Diet FR (2.0 ± 0.3 μg ml$^{-1}$; ± SEM, $n$=3) was significantly higher to fish fed Diet EST (1.3 ±

0.1µg ml$^{-1}$; ± SEM, $n$=3) at the 90% confidence level (P=0.0582). However, following

ingestion of a single meal the maximum post-prandial serum astaxanthin absorption rates

of rainbow trout (Figure 4.4) were not significantly different (P>0.1) for fish fed Diet FR

(0.8 ± 0.24µg ml$^{-1}$h$^{-1}$) or Diet EST (1.02 ± 0.37µg ml$^{-1}$h$^{-1}$) (± SEM, $n$=3). Observation of

regression analysis for post-prandial data shows a peak serum astaxanthin time of ca.16h

for fish fed either dietary treatment. Comparison of mean serum astaxanthin values at 16h

for those fish fed a single meal of Diet FR (1.7 ± 0.3µg ml$^{-1}$) and Diet EST (1.0 ± 0.3µg

ml$^{-1}$) revealed no significant differences, P>0.1 ($n$=3, ± SEM), although mean values were

higher for fish fed Diet FR.


At the end of the trial no significant differences (P>0.05) were observed in the mean flesh

astaxanthin concentrations (Table 4.2) between trout fed Diet FR (2.1 ± 0.5µg g$^{-1}$) or Diet

EST (1.7 ± 0.4µg g$^{-1}$). Similarly, no significant differences were recorded between final

mean liver astaxanthin concentrations between trout fed Diet FR (0.5 ± 0.05µg g$^{-1}$) or Diet

EST (0.4 ± 0.02µg g$^{-1}$; $n$=3, ±SEM). Flesh concentrations of astaxanthin were notably

higher than astaxanthin concentrations recorded in the liver.

Table 4.1    Mean astaxanthin concentrations ($\mu$g g$^{-1}$ wet weight) in individual gastrointestinal regions of rainbow trout fed diets supplemented with free/unesterified (Diet FR) or esterified (Diet EST) astaxanthin.

| | Region 1 | Region 2 | Region 3 |
| | Stomach | Ileal intestine | Posterior intestine |
| --- | --- | --- | --- |
| Diet FR | $0.3 \pm 0.05^a$ | $0.8 \pm 0.14^b$ | $0.2 \pm 0.01^a$ |
| Diet EST | $0.2 \pm 0.04^a$ | $0.9 \pm 0.15^b$ | $0.3 \pm 0.14^a$ |

Values represent means ($n$=3, $\pm$ SEM). Values within a row bearing the same superscripts are not significantly different (P>0.05).

Table 4.2    Mean astaxanthin concentrations in serum ($\mu$g ml$^{-1}$), liver and fish muscle ($\mu$g g$^{-1}$ wet weight) from rainbow trout fed diets supplemented with free/unesterified (Diet FR) or esterified (Diet EST) astaxanthin for 56 days.

| | Serum | Liver | Fish Muscle |
| --- | --- | --- | --- |
| Diet FR | $2.0 \pm 0.3$ | $0.5 \pm 0.05$ | $2.1 \pm 0.5$ |
| Diet EST | $1.3 \pm 0.1$ | $0.4 \pm 0.02$ | $1.7 \pm 0.4$ |

Values represent means ($n$=3, $\pm$ SEM). Values within a column with different superscripts are significantly different (P<0.05).
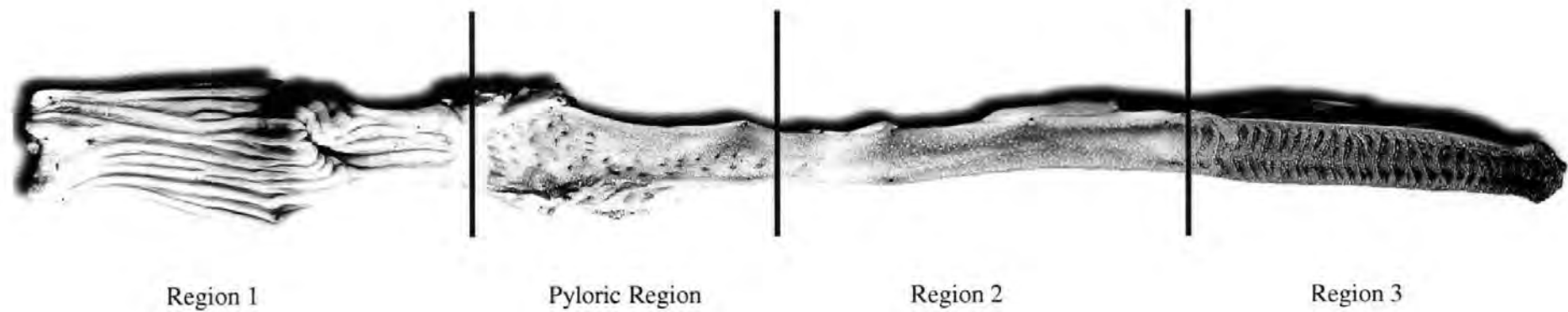
Figure 4.1    Representation of the various gastrointestinal regions sampled; region 1 (stomach), region 2 (ileal intestine), region 3 (posterior intestine).
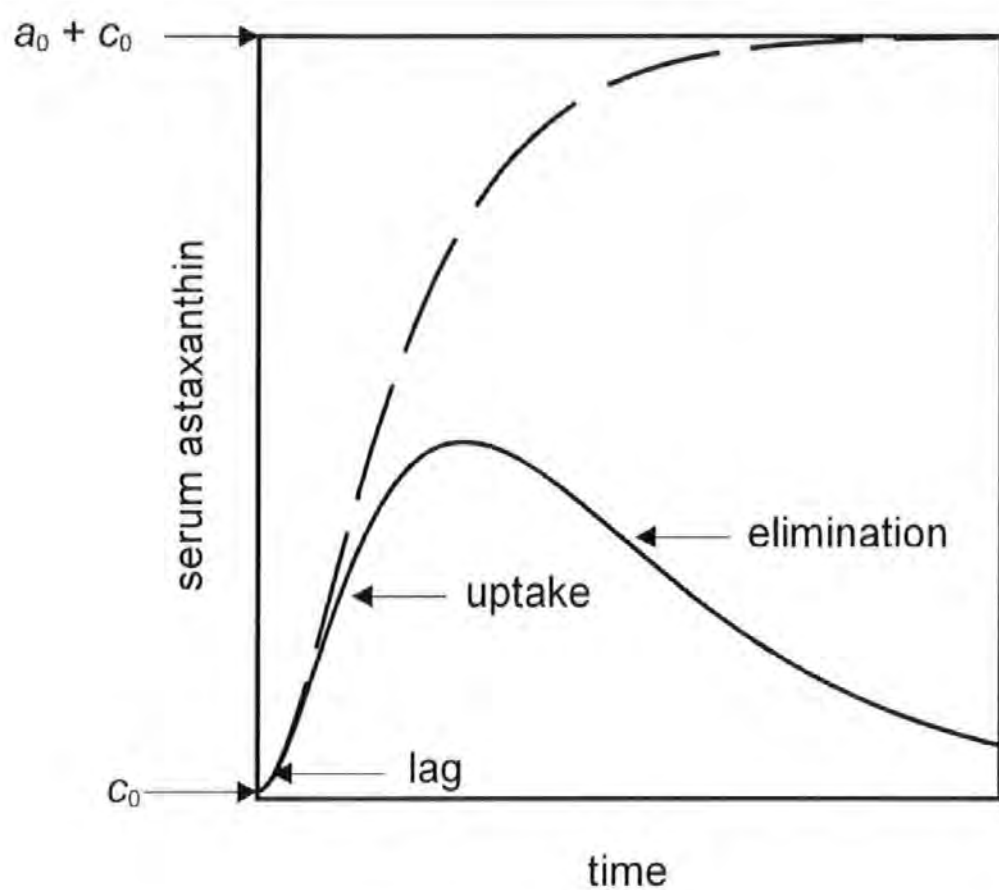
Figure 4.2    A simulated time course of serum astaxanthin after ingestion of a single meal. Details of the kinetic model used are described in the text. The dashed line represents a simulated time course using the same model and parameters except that $k_3 = 0$.
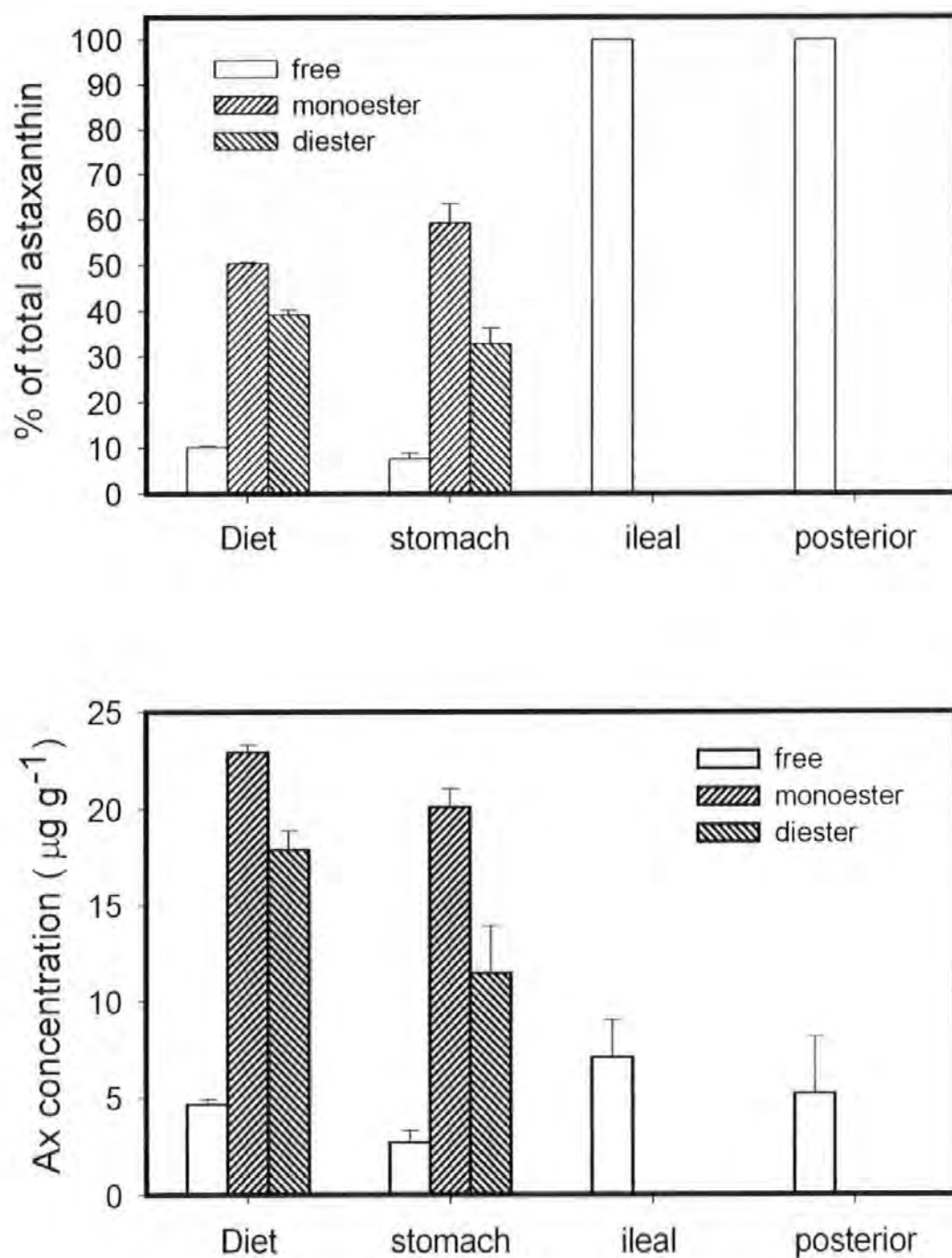
Figure 4.3    The relative percentage contributions and concentrations of astaxanthin (Ax) forms in the diet and digesta of fish fed esterified astaxanthin, Diet EST ($n$=3, ± SEM).
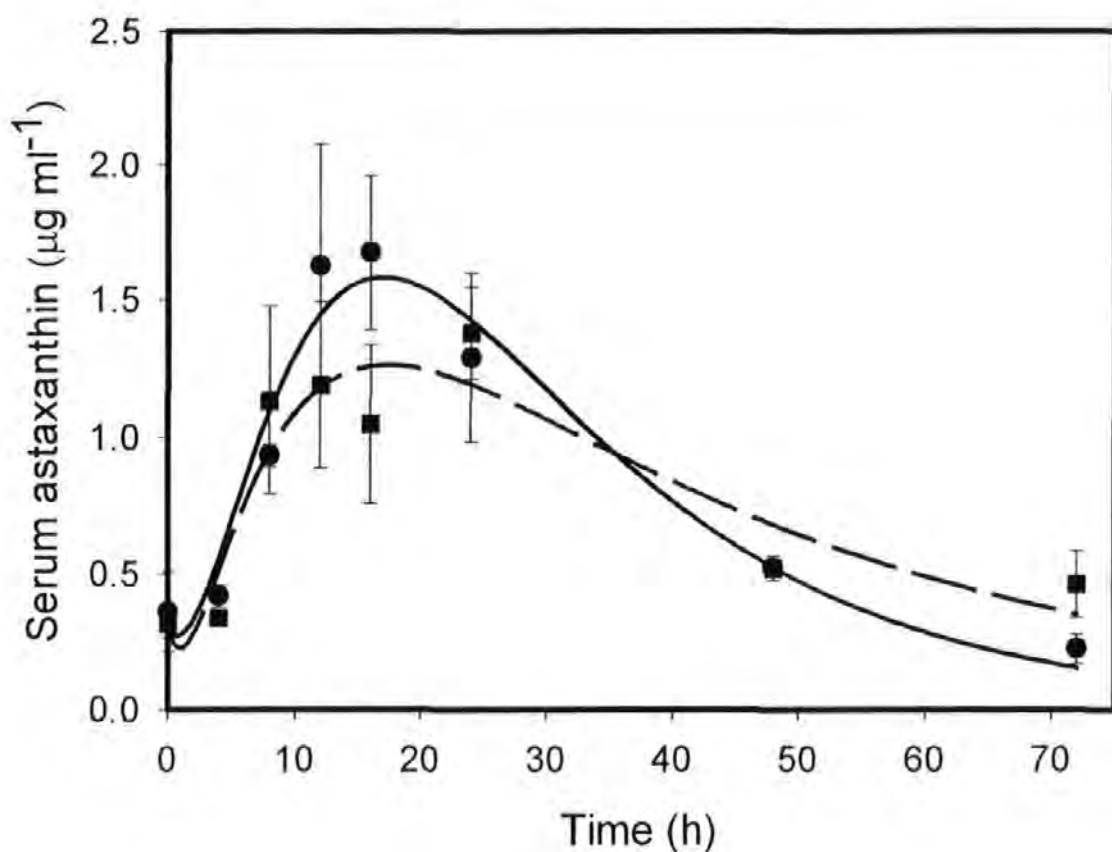
Figure 4.4    Post-prandial astaxanthin serum profiles for trout fed a single meal supplemented with either free astaxanthin, Diet FR (-●-) or esterified astaxanthin, Diet EST (--■--).    Values represent the means of replicates ($n=3$, ±SEM).    Maximum absorption rates of astaxanthin determined as described in Materials and Methods (kinetic modelling).  Time 0 represents approx. 1h after fish were fed.

## 4.4    Discussion

A number of natural sources of astaxanthin, including crustacean wastes and microalgae e.g. *Haematococcus pluvialis*, contain predominantly esterified astaxanthin. This is often a complex mixture of monoesters and diesters, with unesterified astaxanthin representing a small percentage of the total carotenoid. Astaxanthin is deposited in a free, unesterified form in the white muscle of salmonids, but re-esterification takes place (with fatty acids endogenous to the fish) on deposition in the skin (Schiedt *et al.* 1985). Consequently, hydrolysis of these esters within the salmonid gastrointestinal tract prior to absorption may be an important factor limiting uptake and subsequent deposition of natural astaxanthin esters (Choubert & Heinrich 1993).

Blood astaxanthin concentration in the current study displayed expected high inter-individual variation (Guillou *et al.* 1992a; Gobantes *et al.* 1997). Attempts to normalise serum astaxanthin concentrations (following ingestion of a single meal), according to the amount of astaxanthin fed to each respective tank, resulted in no significant reduction in standard errors of mean values. This may be due to insufficient data concerning individual food consumption. However, other authors have found weak correlation's with gut contents and blood astaxanthin concentrations (r=0.71) in Atlantic salmon (Kiessling *et al.* 1995). This may suggest that the amount of feed consumed is only partly responsible for the absorption of astaxanthin. Several approaches could be used and adapted to measure individual feed consumption by fish. Sequential feeding with different coloured foods has been used with relative success to define food intake in individual trout (Johnston *et al.* 1994). Other authors have used more refined means by including radioactive isotopes within salmonid feeds (Storebakken *et al.* 1981; Storebakken & Austreng 1988a,b)

The mean steady state serum astaxanthin concentrations recorded after 56 days of feeding fish in the current trial suggest the absorption of astaxanthin was not as efficient when fed as a mixture of esters compared to the free form at the 90% confidence level. This suggests that intestinal hydrolysis of astaxanthin esters probably was limiting under the regular feeding conditions used in this study. These findings are contrary to those of Barbosa *et al.* (1999) who found no significant difference in serum astaxanthin concentrations between rainbow trout fed for 5 days on diets supplemented with either *Haematococcus pluvialis* or synthetic astaxanthin. However, Barbosa *et al.* (1999) used higher feed carotenoid concentrations (100mg kg$^{-1}$) than those in the current study (50mg kg$^{-1}$) which may explain why blood astaxanthin concentrations recorded by those authors (ca. 5-9µg ml$^{-1}$) were higher than reported here. This, combined with the shorter feeding period used by these authors, may account for the discrepancy between these authors findings and our own. Findings in the current study are in keeping with those of other groups who have demonstrated that synthetic astaxanthin dipalmitate is poorly utilised in comparison to free, unesterified astaxanthin in salmonids (Foss *et al.* 1987; Storebakken *et al.* 1987). However, since these groups made no direct assessment of absorption of astaxanthin into serum, direct comparisons with the current study are difficult. Furthermore, the effective utilisation of dietary astaxanthin monoesters and diesters for the pigmentation of rainbow trout has been demonstrated (Bowen *et al.* 2001).

Kinetics of astaxanthin and canthaxanthin absorption in the serum of immature rainbow trout fed individual doses of carotenoid has been previously studied (Gobantes *et al.* 1997). However, no groups to date have studied the uptake kinetics of astaxanthin derived from *Haematococcus pluvialis*. It is important that such kinetics are defined for this source

since the rate of ester hydrolysis, and subsequently the rate at which astaxanthin is absorbed into the blood, is believed to be the limiting factor in the use of esterified sources of astaxanthin (Torrissen *et al.* 1989). The methodology used in this investigation to observe post-prandial blood astaxanthin concentrations is very similar to that previously described by Mori *et al.* (1989) who sub sampled fish at timed intervals from a group of Coho salmon (*Oncorhynchus kisutch*) fed a pigmented diet to satiation following a period of starvation. In the current study, analysis of post-prandial serum astaxanthin concentrations after ingestion of a single meal suggests that the absorption rates of astaxanthin are similar when supplied as free or esterified astaxanthin, in a single meal. Similar findings have been noted for Coho salmon (*Oncorhynchus kisutch*) fed free astaxanthin and an astaxanthin diester derived from krill (Mori *et al.* 1989). Results would suggest that the rate of absorption of astaxanthin into the blood was not limited by the requirement for hydrolysis when supplied as dietary esters in a single dose. However, serum peak astaxanthin concentrations (16h) were again higher in fish fed unesterified astaxanthin compared to those fish fed esterified astaxanthin. A lack of significant difference in uptake rates may have been due to variability in the data, caused by small sample sizes and satiation feeding. Certainly, the standard errors on values would support this. Further uptake rate assessments of this nature should subsequently address these factors.

Although the pyloric caeca were not examined for astaxanthin concentration because of difficulties associated with removing feed particulates (Brett & Higgs 1970) our results support the contention that the anterior/ileal intestine is largely responsible for carotenoid absorption (Choubert *et al.* 1987; Al-Khalifa & Simpson 1988; Torrissen *et al.* 1990). Indeed, the histological structure of the mid-intestine examined in the current investigation

is identical to that of the pyloric caeca but different to that of the posterior intestine (Bergot *et al.* 1975). This has lead to the reasoning that pyloric caeca are structures which increase the intestinal surface area for absorption (Buddington & Diamond 1987). Austreng (1978) demonstrated that a large proportion of protein and fat absorption took place in the anterior half of the small intestine, including the caeca. Since carotenoid absorption is thought to be intrinsically associated with fat absorption (Parker 1996; Furr & Clark 1997), and positive effects of dietary fat supplementation have been recorded on carotenoid digestibility (Torrissen *et al.* 1990; Choubert *et al.* 1991; Gouveia *et al.* 1998), it is not surprising that carotenoids are absorbed in the anterior intestine. However, *in vivo* studies that have examined the influence of pyloric caeca have found little relationship between the number and length of these structures and the digestibility of fat and protein (Ulla & Gjerdrem 1985) or the digestibility of canthaxanthin (Choubert *et al.* 1991).

Non detectable levels of astaxanthin monoesters and diesters in digesta taken from the ileal and posterior intestine suggest that the hydrolysis of esters prior to absorption also takes place in the pyloric region of the gastrointestinal tract. Other authors have investigated site dependency of esterase activity in animal and human gastrointestinal tract. For example, Augustijns *et al.* (1998) examined the distribution of esterase activity along the gastrointestinal tract of rat and pig by using homogenates of scraped intestinal mucosa from various parts of small intestine and colon using p-nitrophenyl acetate as a substrate. Not only did these authors find important interspecies differences with rat intestine possessing much higher activity than pig intestine, but also both species demonstrated a gradual decrease of esterase activity along the gut. Rigtrup & Ong (1992) demonstrated a retinyl ester hydrolase activity intrinsic to the brush-border membrane of rat small intestine as well as an esterase apparently originating from the pancreas. The esterase originating

from the pancreas preferentially hydrolysed short-chain retinyl esters and was stimulated by trihydroxy bile salts. These properties were similar to that of cholesterol ester hydrolase, known to bind to the brush border of the intestine. The second esterase, intrinsic to the brush border, preferentially hydrolysed long-chain retinyl esters and was stimulated by both trihydroxy and dihydroxy bile salts. Furthermore, Mathias *et al.* (1981) demonstrated that the majority of tocopheryl esters are hydrolysed in the intestinal lumen (pancreatic esterase) but some tocopheryl ester is hydrolysed within the enterocyte by a mucosal esterase associated with the endoplasmic reticulum. Non-specific esterases have been found along the intestinal brush border membrane and within the cytoplasm of enterocytes in Nile tilapia, *Oreochromis niloticus* (Tengjaroenkul *et al.* 2000). *In vitro* studies are required to determine if those esterases responsible for the hydrolysis of astaxanthin esters are specific and whether they originate from the pancreas of the fish or are intrinsic to intestinal cells.

Barrowman (1984), who reviewed the hydrolysis of fat-soluble vitamin esters, stated that there was no evidence for specific vitamin ester hydrolases in pancreatic juice. Rather that the activity against these esters resides in enzymes of broader specificity such as non-specific lipase and possibly pancreatic lipase. In agreement with this, Harrison (1993), based on evidence from reviewed literature, stated that there were no purified enzymes that have been demonstrated to be "specific" for long chain retinyl esters or even to hydrolyse these at a faster rate than other esters. Furthermore, the author went on to state that there are at least three enzymes that are potentially involved in the hydrolysis of dietary retinyl esters: pancreatic lipase, pancreatic carboxyl ester lipase and one or more retinyl ester hydrolases associated with the brush border membrane. Each may play a role according to their ability to interact with retinyl esters in different physio-chemical forms in which the

vitamin may exist in the intestine (emulsions, mixed micelles, liquid-crystalline vesicles). Since the absorption of fat-soluble vitamins and carotenoids is likely similar owing to their hydrophobic nature, it is reasonable to assume that carotenoid esters are hydrolysed in the same manner. Although, the current study suggests that the majority of carotenoid-ester hydrolysis takes place in the pyloric region of the trout intestine, the capacity of the hind intestine for esterase activity cannot be ruled out.

Regional differences in astaxanthin ester hydrolysis along the length of the trout intestine may result in an influence of gut transit rate on the absorption of astaxanthin from an esterified source. Frequent feeding may increase the gastrointestinal transit rate of the feed bolus limiting efficient digestion and absorption of nutrients, as has been suggested for dry matter, protein, lipid and energy (Staples & Nomura 1976; Pedersen 1987; Cho & Kaushik 1990). These findings may account for the disparity in the current study between steady state astaxanthin concentrations after regular feeding and rate of astaxanthin absorption after ingestion of a single meal, when supplied as esterified or unesterified astaxanthin. Furthermore, such effects may account in part for discrepancies between studies on the utilisation of esterified astaxanthin. For example, in those studies by Storebakken *et al.* (1987) and Foss *et al.* (1987) which recorded poor utilisation of astaxanthin diester, fish were fed every 20min for 18h and 24h (to excess) per day, respectively. In comparison, Bowen *et al.* (2001) fed a restricted ration (1.3-1.8% BW day[-1]) and found no differences in the utilisation of esterified forms of astaxanthin. Although Choubert & Storebakken (1996) found that feeding rate did not affect the digestibility of astaxanthin and canthaxanthin, further research is required on the effects of feeding rate on absorption of esterified carotenoids. Indeed, viable feeding strategies or feed ingredients that extend the

gastrointestinal residence time of feed may enhance the absorption of feed supplemented esterified astaxanthin.

The final flesh astaxanthin concentrations recorded in the current study were lower than expected considering the length of the feed trial and the feed astaxanthin concentrations. In another study, rainbow trout fed diets supplemented with *Haematococcus pluvialis* contained final muscle astaxanthin concentrations of 6.2mg kg$^{-1}$ after just four weeks of feeding (Choubert & Heinrich 1993). However, the carotenoid inclusion levels were higher in that study (100mg kg$^{-1}$) as was the initial starting weight of fish (140g). Starting weight of fish in the current investigation (ca 85g) may have resulted in initial poor deposition of pigment since other authors have stated that fish below 100-150g pigment poorly (Abdul-Malak *et al.* 1975; Torrissen 1985a). However, final flesh astaxanthin concentrations in the current study are similar to those reported by Sommer *et al.* (1992) following 50 days of feeding with a 60ppm diet (ca 1-2μg g$^{-1}$) and Storebakken & Choubert (1991) who fed rainbow trout diets supplemented with 50ppm astaxanthin for eight weeks.

After 56 days of feeding there was no significant difference in final muscle or liver astaxanthin concentrations between fish fed diets supplemented with free or esterified astaxanthin. However, there was a trend for the mean flesh and liver astaxanthin concentration of those fish fed Diet FR to be higher than those of fish fed Diet EST (similar to the "steady state" blood astaxanthin concentrations). These findings are similar to those of Bowen *et al.* (2001) who demonstrated that the isolated *Haematococcus* mono- and diesters of astaxanthin were as equally well utilised as the synthetic unesterified astaxanthin in terms of deposition into the white muscle in rainbow trout. Similarly a 'cell-

free' total carotenoid extract from *Haematococcus* was effective in pigmenting rainbow trout. However, these results contradict the findings of Schiedt & Leuenberger (1981) and Schiedt *et al.* (1985) who reported that synthetic astaxanthin dipalmitate was largely ineffective in pigmenting salmonids when compared to the unesterified form of the carotenoid. Furthermore, Choubert & Heinrich (1993) reported very low carotenoid retention rates (1.5%) for rainbow trout fed with *Haematococcus* and suggested that cleavage of the astaxanthin esters may be a limiting step for the deposition of astaxanthin. Sommer *et al.* (1991, 1992) also reported a lower efficiency for astaxanthin from cells of *Haematococcus* compared to synthetic astaxanthin. In the current study astaxanthin from both diets was equally utilised, suggesting that esterification *per se* is not a major factor in limiting the use of *Haematococcus pluvialis* as a pigment source. However, it is not clear whether the difference in mean flesh astaxanthin concentration between fish fed the two treatments in the current study would have been significant had the trial continued for longer. The poor deposition of astaxanthin in the liver compared to the flesh in fish fed the experimental diets suggests that the liver is not a major storage organ for astaxanthin, or, that the liver metabolises astaxanthin rapidly (Hardy *et al.* 1990). The presence of metabolites could not be determined in the current study.

Although the encysted wall of *Haematococcus pluvialis* used in this study was ruptured (95% of cells cracked in a proprietary milling process) it may have posed some further limitation to astaxanthin absorption. Future investigations should be conducted using diets supplemented with astaxanthin fractions isolated from the encysted algal cells. Furthermore, astaxanthin from the esterified source used in the current investigation (*Haematococcus pluvialis*) was a mixture of mono- and diesters (predominantly mono-esterified). This raises questions concerning the extent of esterification and its effects on

astaxanthin absorption and utilisation in salmonids. Separate assessment of monoester and diester fractions and the development of parallel *in vitro* digestion/hydrolysis studies will enhance understanding of carotenoid-ester hydrolysis mechanisms and may prove useful for product development and application.

# CHAPTER 5.0

# EXTENT OF ESTERIFICATION AND ITS INFLUENCE ON THE UTILISATION OF (3*S*,3'*S*)-ASTAXANTHIN IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM).

## 5.1 Introduction

Accumulation of astaxanthin in *Haematococcus pluvialis* is associated with the formation of aplanospores or cysts (induced by nitrogen limitation, high temperature or light intensity) where it develops a tough sporopollenin cell wall (Good & Chapman 1979; Burczyk 1987; Grung *et al.* 1992). Salmonids appear to lack the digestive enzymes necessary to break down the *Haematococcus* sporopollenin wall and intact astaxanthin-rich aplanospores of *Haematococcus* do not pigment the white muscle of salmonids (Sommer *et al.* 1991; Bowen *et al.* pers. comm.). However, previous work (see Chapter 4) has shown that efficient cracking of this cell wall renders pigment available for deposition in the flesh of rainbow trout. Furthermore, this deposition is comparable to that of the synthetic, unesterified source of astaxanthin (CAROPHYLL Pink; F. Hoffmann La Roche, Basel, Switzerland) which is commonly used within the aquafeed industry.

Another important factor that distinguishes algal astaxanthin from the synthetic form is the configuration of the carotenoid. In this algae, astaxanthin predominantly occurs (>99%) as the (3*S*,3'*S*) form (Renstrøm *et al.* 1981) whilst the synthetic, unesterified, product is a racemic mixture of the (3*R*, 3'*R*), (3*S*, 3'*S*) and the meso form (3*R*, 3'*S*) in the ratio 1:1:2 (Bernhard 1990). Foss *et al.* (1987) demonstrated that all three epimers of unesterified

110

astaxanthin are equally utilised by salmonids, suggesting that rainbow trout would not discriminate against the algal source of carotenoid according to its enantiomer composition. However, other feed trials with salmonids have shown discrimination against dipalmitate ester forms of $(3S,3'S)$-astaxanthin with the $(3R,3'R)$ form deposited in the flesh to a greater extent (Schiedt *et al.* 1985; Foss *et al.* 1987) suggesting that all optical ester forms of astaxanthin are not utilised equally.

A further characteristic of astaxanthin from *Haematococcus* is that a very high proportion (typically >95%) of the astaxanthin exists as a complex mixture of esters, primarily monoesters rather than diesters (Renstrøm & Liaaen-Jensen 1981; Grung *et al.* 1992; Harker *et al.* 1996b; Lorenz & Cysewski 2000). The carotenoid composition of the *Haematococcus* aplanospore is in part dependent upon the age of the culture so that the ratio of astaxanthin monoesters: diesters decreases with time (Harker *et al.* 1996b). The main fatty acid components of the astaxanthin esters from this alga are $C_{18:1}$ and $C_{20:1}$ (Renstrøm & Liaaen-Jensen 1981; Bowen & Young pers. comm.), which are major components of plant cell membranes.

Esterification of astaxanthin may be an important factor limiting flesh deposition (Choubert & Heinrich 1993). Indeed the rate of hydrolysis of astaxanthin esters to free astaxanthin appears to be limited which may explain observed differences in deposition (Torrissen *et al.* 1989). It has been shown that synthetic astaxanthin dipalmitate is poorly utilised in comparison to free astaxanthin in trout and sea trout, and in addition canthaxanthin in salmon (Foss *et al.* 1987; Storebakken *et al.* 1987). This is contrary to earlier observations where natural astaxanthin esters have been shown to be more effective in the pigmentation of rainbow trout compared to the free form of astaxanthin (Simpson &

Kamata 1979). Furthermore, a study by Mori *et al.* (1989) demonstrated that there was practically no difference between an astaxanthin diester from krill and synthesised free astaxanthin in their absorption and deposition by Coho salmon. Indeed, results from previous investigations (see Chapter 3 and 4) would agree with the latter examples since *Haematococcus pluvialis* products (which contain predominantly esterified astaxanthin) have been shown to be efficient sources of pigment compared to the synthetic/unesterified form. However, some digestibility and rate absorption assessments (into serum) for astaxanthin from the same studies have suggested that the absorption of esterified astaxanthin may well be limited, although not to a significant extent. Furthermore, it is difficult to interpret the significance of this limitation when astaxanthin esters are supplied in algal spores where the influence of the cracked cell wall cannot de differentiated from the limitation presented by the requirement for ester hydrolysis.

Interpretation of the absorption efficiency of astaxanthin esters is confounded when they are supplied within a complex mixture of carotenoids. *Haematococcus pluvialis* commonly contains other carotenoids such as lutein; echionene; canthaxanthin and β-carotene in addition to the various ester forms of astaxanthin (Grung *et al.* 1992). Bias in absorption due to interaction between carotenoids at the intestinal level cannot be ruled out (White *et al.* 1994; Gärtner *et al.* 1996; Kostic *et al.* 1995; Hageman *et al.* 1999). Furthermore, it is not clear whether the extent of esterification limits the availability and deposition of astaxanthin. It is plausible to assume that the hydrolysis of an astaxanthin monoester to its free form may well be faster relative to the hydrolysis of an astaxanthin diester. Such differences and their implications have not been assessed previously in *in vivo* situations.

The following investigation was designed to evaluate whether the extent of esterification limits the digestibility and absorption of astaxanthin. In addition, to determine whether (3$S$,3'$S$)-astaxanthin esters from *Haematococcus pluvialis* are utilised as efficiently as a racemic astaxanthin mixture. Finally to assess whether the extent of esterification influences utilisation in comparison to an unesterified synthetic form in pigmenting rainbow trout.

## 5.2    Materials and Methods

### 5.2.1    Experimental design

In the following experiment each dietary treatment was assigned to triplicate tanks containing groups of fish. Subsequently, each tank is considered as a replicate ($n$=3). Following sampling and analysis of individual fish tissues, statistical comparison of results between treatments was based on mean values from each tank ($n$=3) to avoid pseudo-replication and false interpretation of results (Morris 1999).

### 5.2.2    Experimental Diets

The basal feeds containing no added carotenoid and formulated to meet the nutritional requirements of rainbow trout and were supplied by Trouw Aquaculture Ltd. (Wincham, UK). Dietary treatments had identical proximate basal composition (Table 5.1) and target levels of 30ppm astaxanthin. Diet FR contained free astaxanthin (8% w/v dispersible beadlets; CAROPHYLL® Pink, F. Hoffman-La Roche); Diet MONO and Diet DI contained astaxanthin monoesters and astaxanthin diesters (isolated from *Haematococcus*

*pluvialis*), respectively. Diet HAEM was supplemented with a cell free carotenoid extract. Table 5.2 outlines the astaxanthin composition of the experimental diets. Carotenoid extraction from algal cells and astaxanthin ester isolation was performed as previously described (see sections 2.1.1 and 2.4.7). Astaxanthin fractions were added to the basal diets in a top coating procedure (see section 2.1.2) where the fat content of the diet was increased to a commercially acceptable level of 26%.

### 5.2.3 Fish feeding and sampling

Rainbow trout ($141 \pm 0.6$g) were randomly assigned into individual tanks (40 fish per tank) maintained at 15°C under a 12h light/dark photoperiod. Diets were fed in a single morning ration (1.3-1.8% BW day$^{-1}$) to triplicate groups/tanks of fish for 8 weeks, with alterations to feed quantity made daily assuming a feed conversion ratio (FCR) of 1.0. Faeces were then stripped from fish (see section 2.2.4) and analysed for astaxanthin (see sections 2.4.2 and 2.4.9) to determine apparent digestibility coefficients (ADC) as described previously (see sections 2.5.1). In addition, flesh and skin samples were taken from fish (5 fish per tank; 15 fish per treatment) and analysed for carotenoid by HPLC (see sections 2.4.4; 2.4.6 and 2.4.10); optical isomer content (see below) and evaluation of carotenoid retention (see section 2.5.2).

Following faecal stripping, fish were fed a carotenoid free diet (same basal formulation as experimental diets) for 14 days to reduce the level of carotenoid in the serum of fish to a minimum. Fish were then fed a single ration (1.3% BW) of the respective carotenoid supplemented diet. Blood samples were taken from fish (5 fish per tank; 15 fish per treatment) at six time intervals up to and including 72h for carotenoid analyses.

### 5.2.4 Analysis of astaxanthin chiral forms

Analysis of the various chiral forms of astaxanthin in feeds, fish muscle and skin samples was performed at Liverpool John Moores University. Analysis was achieved by derivatization of unesterified (see below) carotenoid to produce their dicamphanates. Astaxanthin was dissolved in dry pyridine (0.5ml) and reacted with 50mg of (-)-camphanoyl chloride (Sigma, Poole, UK) for 10min at room temperature (ca. 20°C). The resulting dicamphanates were extracted by partition into diethyl ether.

Analysis of dicamphanates was performed on normal-phase HPLC (Lichrosorb SI60; 250 × 4.6mm; particle size 5μm; detection wavelength 491nm). The solvent system used was n-hexane/ethyl acetate (75/25 v/v) at a flow rate of 0.5ml min$^{-1}$. Retention times for isomers were; (3R,3'R) 18.5min; (3R,3'S) "meso" 19.8min; (3S,3'S) 21.3min.

As astaxanthin is predominantly esterified in the skin of rainbow trout, anaerobic saponification was performed on these samples prior to optical isomer analysis. The method used was based on that originally developed by Schiedt et al. (1993). Typically 10-100μg of carotenoid esters was dissolved in dichloromethane (maximum 3ml) and mixed. Sodium methoxide (1ml; prepared by dissolving 1.5g of sodium in 100ml of methanol) per ml of astaxanthin solution was added to a side arm flask and the entire system flushed with nitrogen to remove all traces of oxygen. The sodium methoxide and astaxanthin solutions were frozen with liquid nitrogen and placed under vacuum. The solutions were then thawed at room temperature (ca. 20°C) in order to de-gas them. This freeze-thaw cycle was repeated at least three times. The sodium methoxide solution was

then mixed with the astaxanthin solution under high vacuum and constant stirring for 15min. The mixture was then acidified with 1ml of 1N $H_2SO_4$ $ml^{-1}$ of sodium methoxide, removed and diluted with ethanol and water. Astaxanthin was then extracted with hexane: diethyl ether (1:1 v/v), washed with water and dried under a gentle stream of nitrogen.

### 5.2.5 Statistical analysis

Data were subjected to tests for normal distribution (standardised skewness and kurtosis) and variance checks (Cochrans and Barlett's test) before comparison. Net apparent retention (NAR) of astaxanthin was calculated according to Torrissen (1995) incorporating a flesh to body weight ratio of 0.61 (Wathne *et al.* 1998). Statistical analysis and regression/curve fitting of data was carried on StatGraphics Plus 4.0 and SigmaPlot 4.0, respectively. Flesh astaxanthin concentrations, NAR and apparent digestibility coefficients (ADC) were compared across treatments using the analysis of variance at the 95% confidence limit.

### 5.2.6 Kinetic modelling

Kinetic determination of serum astaxanthin uptake rates for fish fed the various dietary treatments was carried out as previously described (see Chapter 4, section 4.2.5).

## 5.3 Results

No significant differences ($P>0.05$) were recorded between the mean initial and final weights of rainbow trout used in this study across dietary treatments (Table 5.3). Fish

weight increased by ca. 150g throughout the trial with final fish weights representative of typical pan-size fish (ca. 250g). Similarly, no significant differences were noted for the mean feed conversion ratios (FCR) across treatments.

The apparent digestibility coefficients (ADC) for the respective treatments (Figure 5.1) were Diet FR 65.73 ± 1.33%; Diet MONO 60.51 ± 2.80%; Diet DI 62.63 ± 0.74% and Diet HAEM 59.69 ± 2.22% ($n$=3, ±SEM). No significant differences (P>0.05) were recorded between the ADC values across treatments although there was a trend for higher ADC values in fish fed diet supplemented with free astaxanthin (Diet FR). However, there was no obvious difference in ADC values between the other treatments.

Following ingestion of a single ration (Figure 5.2) there were no significant differences (P>0.05) in the serum-astaxanthin absorption rates between fish fed Diet CP (0.35 ± 0.10µg ml$^{-1}$h$^{-1}$); Diet MONO (0.24 ± 0.09µg ml$^{-1}$h$^{-1}$); Diet DI (0.19 ± 0.02µg ml$^{-1}$h$^{-1}$) or Diet HAEM (0.23 ± 0.11µg ml$^{-1}$h$^{-1}$). However, the rate of absorption was clearly higher in fish fed free astaxanthin. Furthermore, there was a trend for fish fed monoesterified astaxanthin to have higher absorption rates than those fish fed diesterified astaxanthin.

The serum peak times for astaxanthin were similar for all treatment (24-32h). However, at 24h and 32h mean serum astaxanthin concentrations (minus values at time 0) were significantly higher ($P$=0.001 and $P$=0.018, respectively) in fish fed Diet FR (1.66 ± 0.05µg ml$^{-1}$ and 1.61 ± 0.06µg ml$^{-1}$, respectively) and Diet MONO (1.43 ± 0.20µg ml$^{-1}$ and 1.54 ± 0.10µg ml$^{-1}$, respectively) compared to fish fed Diet DI (0.92 ± 0.07µg ml$^{-1}$ and 0.99 ± 0.07µg ml$^{-1}$, respectively) and Diet HAEM (0.65 ± 0.09µg ml$^{-1}$ and 1.13 ± 0.20µg ml$^{-1}$, respectively)($n$=3, ±SEM).

117

The deposition of astaxanthin and total carotenoid in the flesh/muscle of rainbow trout (Figure 5.3) was not significantly different (P>0.05) for fish fed Diet CP (4.0 ± 0.4 and 4.3 ± 0.5μg g⁻¹, respectively); Diet MONO (4.3 ± 0.8 and 5.2 ± 1.0μg g⁻¹, respectively); Diet Di (3.5 ± 0.5 and 4.1 ± 0.7μg g⁻¹, respectively) or Diet HAEM (3.9 ± 0.3 and 5.1 ± 0.4μg g⁻¹, respectively). In addition to astaxanthin, trace amounts of lutein, zeaxanthin and idoxanthin (3,3',4'-trihydroxy-β,β-carotene-4one) were found in all fish examined, including those fed synthetic all-*trans* astaxanthin (Diet FR). No significant differences (P>0.05) were found between the different diets for the levels of these xanthophylls deposited in the white muscle of rainbow trout (data not shown). Similarly, net apparent retention values (Table 5.4) for both total carotenoid (ca. 12-14%) and astaxanthin (ca. 10-12%) were not significantly different (P>0.05) for fish fed the different dietary treatments.

The chirality of astaxanthin (Table 5.5) deposited in the muscle of fish fed all diets was representative of the chirality of astaxanthin in the diets. Subsequently, those fish fed diets supplemented with ester fractions from *Haematococcus pluvialis* (Diet MONO, Diet EST and Diet HAEM) deposited the (3*S*,3'*S*) astaxanthin form where fish fed the racemic mixture of astaxanthin (Diet CP) deposited the racemic mixture in the flesh. However, *in vivo* racemisation of astaxanthin was observed in the skin of fish fed all diets. For the diets supplemented with algal fractions an almost racemic ratio of 1:2:1 (3*S*,3'*S*; 3*R*,3'*S*; 3*R*,3'*R*) was recorded suggesting extensive epimerization of the (3*S*,3'*S*)-astaxanthin form.

Both 9-*cis* and 13-*cis* isomers of astaxanthin were detected in the white muscle of rainbow trout (data not shown). Whilst 9-*cis* astaxanthin was noted in fish fed all diets, the presence of 13-*cis* was restricted to those fish fed astaxanthin derived from algae (Diet

118

MONO, Diet DI and Diet HAEM). Levels of these isomers was small and only accounted for less than 4% of the total carotenoid recorded in the muscle of rainbow trout. Both of these isomers were found naturally in all diets following carotenoid extraction.

Table 5.1        Ingredients and proximate composition of the experimental diets

| Ingredients: | Percentage inclusion |
|---|---|
| [a]LT Fishmeal | 61.3 |
| Wheat gluten | 5.6 |
| Wheat | 21.6 |
| Fish oil | 10 |
| [b]Vitamin premix | 0.8 |
| [b]Mineral premix | 0.7 |
| Yttrium oxide | 0.01 |
| **Proximate composition:** | |
| Protein | $45.9 \pm 0.4$ |
| Fat | $27.3 \pm 0.1$ |
| Ash | $10.4 \pm 0.1$ |
| Moisture | $6.1 \pm 0.03$ |
| Energy | $21.4 \pm 0.1$ MJ kg$^{-1}$ |

[a]Norse LT 94, Egersund, Norway. [b]Vitamins/mineral premixes as formulated by Trouw Aquaculture Ltd. (Wincham, U.K.). Supplementary oil (Cod Liver Oil, Seven seas Ltd., Hull, U.K.) was added to basal formulation, providing a carrier for carotenoid (increase final oil level to ca. 26% w/w). Values represent means ($n$=3, $\pm$ SEM).

Table 5.2    Astaxanthin content and composition of the experimental diets

| | Dietary Inclusion (mg kg$^{-1}$) | | | |
|---|---|---|---|---|
| | Diet FR | Diet MONO | Diet DI | Diet HAEM |
| Astaxanthin | 31.65 | 0.55 | _ | 1.37 |
| Astaxanthin monoesters[†] | _ | 31.46 | _ | 17.44 |
| Astaxanthin diesters[‡] | _ | _ | 30.28 | 6.42 |
| Total Astaxanthin | 31.65 ± 1.85 | 32.01 ± 4.32 | 30.28 ± 1.92 | 29.71 ±0.65 |

[†]Monoester extract was 90.51% pure as confirmed by TLC. [‡]Diester extract was 96.62% pure as confirmed by TLC. Lutein and β-carotene were found to constitute these impurities (<1ppm). Relative inclusion of astaxanthin forms determined from percentage contribution as evaluated by TLC (see Materials and Methods). Values represent means ($n$=3, ± SEM).

Table 5.3    Growth performance and feed utilisation of rainbow trout after eight weeks of feeding the dietary treatments

| Growth Data | Diet FR | Diet MONO | Diet DI | Diet HAEM |
|---|---|---|---|---|
| Initial weight | 140.37 ± 1.87 | 141.92 ± 1.98 | 142.03 ± 1.51 | 139.60 ± 1.19 |
| Final weight | 246.70 ± 0.08 | 249.03 ± 0.28 | 252.06 ± 3.78 | 251.32 ± 4.97 |
| FCR | 1.02 ± 0.03 | 1.02 ± 0.03 | 0.99 ± 0.02 | 1.0 ± 0.03 |

Values represent means ($n$=3, ± SEM). No significant differences were recorded between dietary treatments for initial and final weight or FCR.
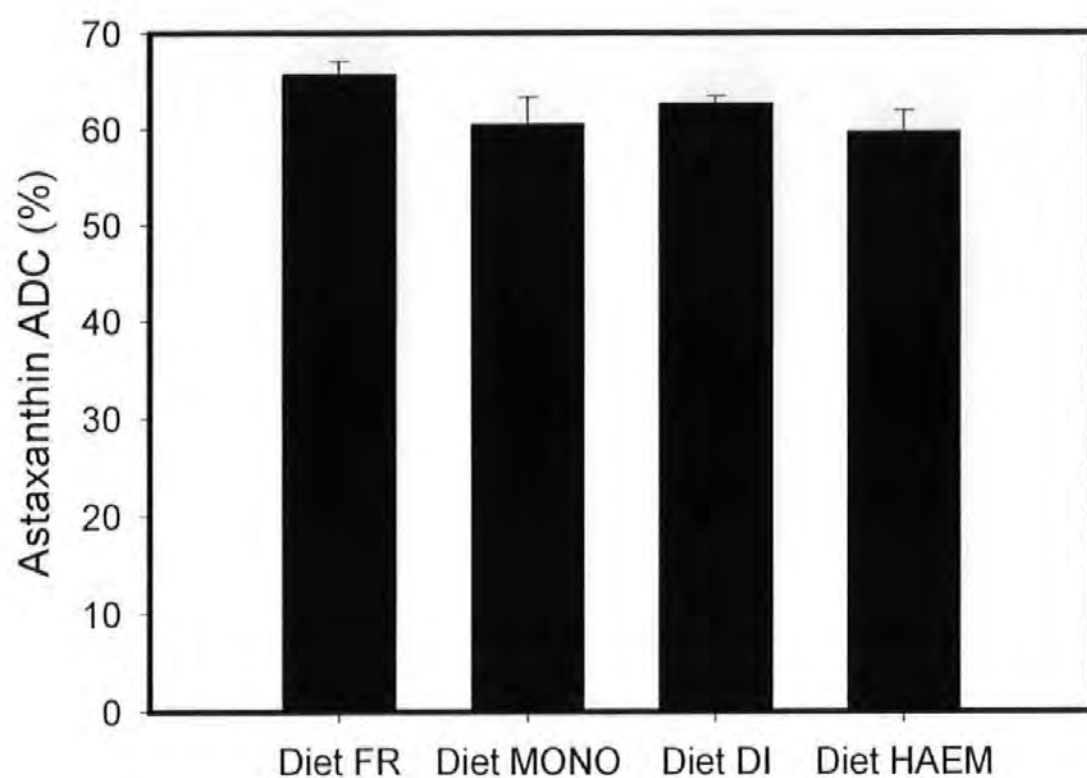
Figure 5.1    Apparent digestibility coefficients (ADC) for astaxanthin for fish fed the experimental diets.    No significant differences (P>0.05) were recorded between dietary treatments.
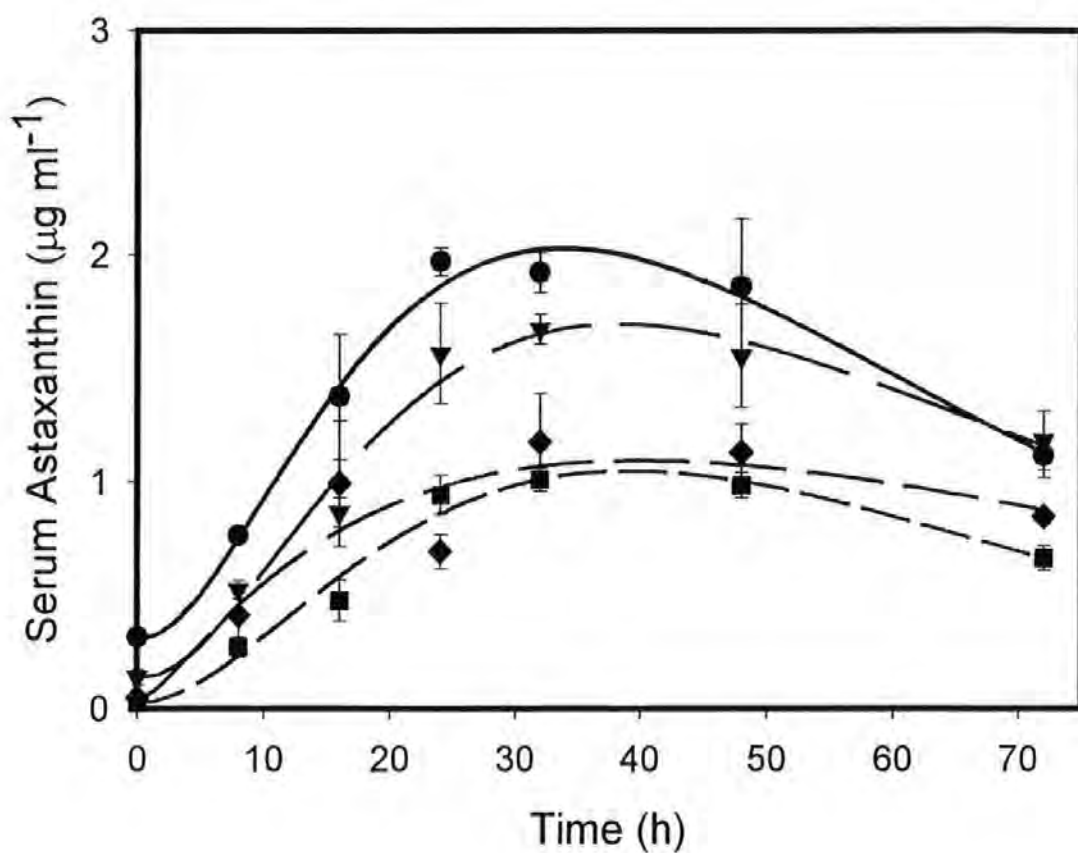
Figure 5.2  Post-prandial astaxanthin serum profiles for trout fed a single meal supplemented with either free astaxanthin (Diet FR ●; solid line); monoester (Diet MONO ▼; long dash); diester (Diet DI ■; short dash) or *Haematococcus pluvialis* extract (Diet HAEM ◆; medium dash).  Values represent the means of replicates ($n=3$, ±SEM).  Maximum absorption rates of astaxanthin determined as described in Materials and Methods (kinetic modelling).  Time 0 represents approx. 1h after fish were fed.
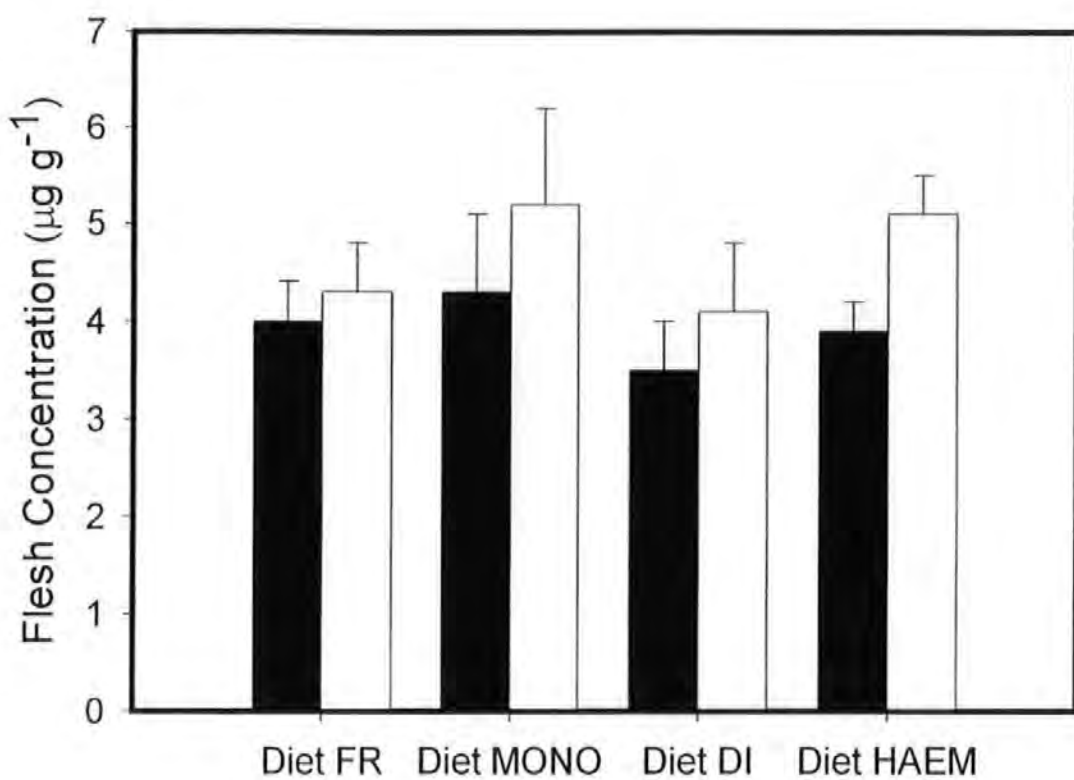
Figure 5.3    Astaxanthin (■) and total carotenoid (□) in white muscle of rainbow trout fed the experimental diets. Values are means ($n$=3, ± SEM). No significant differences (P>0.05) were recorded between dietary treatments.

Table 5.4    Net apparent retention of dietary carotenoid and dietary astaxanthin after 8 weeks of feeding

| Diet | Net Apparent Retention (%) | |
| --- | --- | --- |
| | Total carotenoid[†] | Astaxanthin[‡] |
| Diet CP | 12.3 ± 1.3 | 11.5 ± 1.0 |
| Diet MONO | 14.6 ± 2.9 | 12.2 ± 2.4 |
| Diet DI | 12.3 ± 2.3 | 10.4 ± 1.6 |
| Diet HAEM | 12.6 ± 1.0 | 12.0 ± 0.9 |

[†]Determined spectrophotometrically in n-hexane. [‡]Determined by HPLC analysis. Values represent means ($n$=3, ±SEM). No significant differences were recorded between dietary treatments.

Table 5.5    Chirality of astaxanthin in the feeds, muscle and skin of rainbow trout at the end of the nutrition trial.

| Treatment | Chirality of astaxanthin $(3S,3'S; 3R,3'S; 3R,3'R)$[†] | | |
| | Feeds | White muscle | Skin |
| --- | --- | --- | --- |
| Diet CP | 1.00: 2.00: 1.00 | 0.55: 1.00: 0.54 | 0.53: 1.00: 0.58 |
| Diet MONO | 1.00: 0.08: 0.06 | 1.00: 0.20: 0.10 | 0.66: 1.00: 0.55 |
| Diet DI | 1.00: 0.07: 0.06 | 1.00: 0.40: 0.15 | 0.59: 1.00: 0.49 |
| Diet HAEM | 1.00: 0.09: 0.05 | 1.00: 0.13: 0.06 | 0.85: 1.00: 0.60 |

[†]Ratio of the concentrations of each isomer.  Astaxanthin chirality in the skin was determined following anaerobic saponification (see Materials and Methods).

## 5.4    Discussion

No apparent differences were witnessed in growth of rainbow trout across treatments, indicating that the use of different astaxanthin supplements from *Haematococcus pluvialis* presents no deleterious effects on feed utilisation and growth performance. This confirms the findings of previous trials in this study.

The ADC values recorded for astaxanthin in this trial are in keeping with values previously reported in the literature for rainbow trout of 50-70% (Foss *et al.* 1987; No & Storebakken 1991; Bjerkeng *et al.* 1997). The ADC for free astaxanthin in this study (ca. 65%) was similar to that found by Bjerkeng *et al.* (1997) for trout fed a stereoisomer mixture of free astaxanthin (64%), and for free astaxanthin in diets fed to Atlantic salmon of ca. 59-64% (Storebakken *et al.* 1987; Bjerkeng & Berge 2000). However, the average ADC found for astaxanthin diesters in the current investigation (ca. 63%) was higher than that found for a synthetic astaxanthin dipalmitate fed to Atlantic salmon (47%; Storebakken *et al.* 1987) yet within the range for astaxanthin dipalmitate fed to rainbow trout (42-67%; Foss *et al.* 1987). The lack of significant differences between ADC values in the present study suggests that the hydrolysis of astaxanthin esters and subsequent absorption by intestinal mucosal cells presents no limitation to the use of esterified astaxanthin as a source of pigment in salmonid feeds. These findings are similar to those of Storebakken *et al.* (1987) who found no significant differences in digestibility between astaxanthin and astaxanthin dipalmitate. Furthermore, the degree of esterification i.e. mono- or diesters does not significantly influence the digestibility of astaxanthin, suggesting efficient ester hydrolysis at the gastrointestinal level under the feeding conditions applied. However, although there were no significant differences in ADC values,

there was a trend for fish fed diets supplemented with free astaxanthin to have higher ADC values.

Analysis of post-prandial serum astaxanthin concentrations following ingestion of a single meal show that the absorption rate of free astaxanthin (Diet FR) tended to be higher than that of esterified astaxanthin, although not significantly. This could suggest that the requirement for intestinal ester hydrolysis before absorption into the blood is a limiting factor and may merit further study. This finding agrees in part with the ADC value for Diet FR in this study which was higher than the other treatments but not significantly. The serum absorption rate of astaxanthin in fish fed diets supplemented with diesters was lower than that of fish fed astaxanthin monoesters. This suggests that the degree of esterification of astaxanthin may well limit the absorption of this carotenoid into the blood. Very similar uptake rates for Diet MONO and Diet HAEM may be expected since the cell free carotenoid extract was composed mainly of astaxanthin monoesters. Although the rates of uptake were not significantly different, the differences between them did result in significant differences in serum astaxanthin concentration between treatments at the peak serum time of 24 to 32 hours. Clearly, the extent of esterification of the astaxanthin molecule was negatively related to absorption of astaxanthin into trout serum.

A lack of significant differences in digestibility coupled with significant differences in serum astaxanthin concentrations may suggest that intestinal cells absorb astaxanthin esters intact followed by intracellular hydrolysis prior to blood transport. Indeed, non-specific esterases have been found along the intestinal brush border membrane and within the cytoplasm of enterocytes in Nile tilapia, *Oreochromis niloticus* (Tengjaroenkul *et al.* 2000). Alternatively,

129

variations in sensitivity between the two assessments may account for the differences. Digestibility values may be overestimated due to carotenoid degradation in faecal samples (Meyers 1994). Absorption, as well as removal from the serum for storage; bioconversion and excretion influence blood carotenoid concentrations (Castenmiller & West 1998; Van het Hof *et al.* 2000).

This study revealed that isolated monoesters and diesters of astaxanthin as well as a mixture of both in a total carotenoid extract, were utilised as efficiently as the free synthetic form of astaxanthin in pigmenting rainbow trout under these trial conditions. These results are contradictory to those of other groups who have showed that synthetic astaxanthin dipalmitate was largely ineffective in pigmenting salmonids in comparison to the free, unesterified form of this carotenoid (Schiedt & Leuenberger 1981; Schiedt *et al.* 1985; Foss *et al.* 1987; Storebakken *et al.* 1987). Comparison between these studies and the current investigation are confounded by variations in trial duration and feeding conditions; dietary carotenoid levels and choice of salmonid species. Nonetheless, moderate levels of pigmentation and relatively short trial duration in the current study may have negated development of significant differences between treatments. However, Torrissen *et al.* (1989) indicated that farmed salmonids should have at least 3-4$\mu$g g$^{-1}$ of total carotenoid to be marketable, a level that was achieved for all treatments. Net apparent retention values for astaxanthin and total carotenoid in the current study (10-12%) were lower than the 18% reported by Torrissen & Braekkan (1979) but in keeping with the 7-11% reported by Foss *et al.* (1987). Choubert & Storebakken (1989) obtained a retention coefficient of 12.5% for rainbow trout fed for six weeks on diets supplemented with astaxanthin. Direct comparison is however complicated by differences in calculation and carotenoid content of feeds.

130

Pigmentation results from this study are consistent with those findings of Mori *et al.* (1989) who found no difference between the deposition of a krill astaxanthin diester and synthetic free astaxanthin in juvenile Coho salmon (*Oncorhynchus kisutch*). Moreover, Simpson and Kamata (1979) reported higher levels of pigmentation when using an esterified source of astaxanthin in comparison to the free form. Choubert & Heinrich (1993) reported very low carotenoid retention levels (1.5%) in rainbow trout fed diets supplemented with *Haematococcus* and suggested that the requirement for ester hydrolysis may be a limiting step in the deposition of astaxanthin. Results from the present study suggest that this is not the case and that the cell wall of *Haematococcus* may be the main limiting factor (Johnson & An 1991). Indeed, algal cells used in the study by Choubert & Heinrich (1993) were not homogenised/disrupted prior to addition to the feed thus limiting carotenoid bioavailability.

Enantiomers of astaxanthin in wild *Salmo* and *Oncorhynchus* species are typically found in the ratio 78-85: 2-6: 12-17 (3*S*,3'*S*): (3*R*,3'*S*): (3*R*,3'*R*) (Schiedt 1998). However, synthetic astaxanthin (CAROPHYLL® Pink) has a ratio of 1:2:1 (i.e. a ratio of 1:1 for the racemate forms: Bernhard 1990). When salmonids deposit astaxanthin in their muscle, they do not discriminate between the three enantiomers of unesterified astaxanthin (Foss *et al.* 1984). This is in agreement with findings in the current study since both racemic (synthetic astaxanthin) and (3*S*,3'*S*)-astaxanthin were deposited unchanged. However, it has been reported that astaxanthin esters are utilised differently from the unesterified carotenoid (Schiedt *et al.* 1985) where astaxanthin dipalmitate was utilised in the order racemic > (3*R*,3'*R*) > (3*S*,3'*S*). Indeed, rainbow trout and sea trout (*Salmo trutta.* L.) fed a synthetic mixture of astaxanthin dipalmitate enantiomers deposited (3*R*,3'*R*) astaxanthin in their flesh to

131

a greater extent than (3*S*,3'*S*)-astaxanthin (Foss *et al.* 1987). This may be due to stereo-selective attack by ester hydrolases in the intestinal lumen of the fish and requires further study.

When astaxanthin is deposited in the skin of salmonids it is re-esterified with fatty acids (namely 20:5 and 18:1) although this re-esterification has been found to be unspecific in relation to the enantiomeric form of the carotenoid molecule (Schiedt *et al.* 1985). In the present investigation, whilst no epimerization of astaxanthin in the muscle was recorded, deposition of astaxanthin from algal sources in the skin was accompanied with epimerization of (3*S*,3'*S*)-astaxanthin, with both the (3*R*,3'*S*) and (3*R*,3'*R*) forms being deposited. Specific enrichment of the (3*R*,3'*R*) form in the skin of salmonids has been recorded when dipalmitates of these enantiomers have been used. Racemic astaxanthin dipalmitate was deposited in the ratio 0.4:1.0:1.0 (3*S*,3'*S*): (3*R*,3'*S*): (3*R*,3'*R*) suggesting discrimination against the (3*S*,3'*S*) form (Schiedt *et al.* 1985, Schiedt 1998). *In vivo* racemisation of 3H-(3*S*,3'*S*)-astaxanthin has also been observed in the shrimp *Penaeus japonicus* although it could not be determined whether this occurred during or after absorption (Schiedt *et al.* 1991, 1993).

The presence of geometrical isomers of astaxanthin in the white muscle of salmonids is in agreement with previous findings. The 9-*cis*, 13-*cis* and 15-*cis* forms of astaxanthin have been detected in the white muscle of rainbow trout fed with synthetic astaxanthin (Bjerkeng *et al.* 1997). Furthermore these authors demonstrated that the ADC's were significantly higher for all-*trans* astaxanthin compared to a mixture of *cis/trans* isomers. More recently, selective distribution of geometrical isomers of astaxanthin in the faeces, blood, liver and muscle of rainbow trout has been demonstrated (Østerlie *et al.* 1999). Since isomerisation of all-*trans*

132

astaxanthin may take place in the gastrointestinal tract and also following uptake (Bjerkeng et al. 1997) it is plausible that intestinal hydrolysis of astaxanthin esters might promote *trans-cis* isomerisation. However, the presence of *cis* isomers was minimal and did not seem to have any affect on deposition or retention.

The presence of idoxanthin in the muscle of fish fed unesterified astaxanthin, astaxanthin esters or total carotenoid extract confirms other observations in rainbow trout (Bjerkeng *et al.* 1997) and Atlantic salmon (Schiedt *et al.* 1988a). Since idoxanthin was not found in the feed it is plausible that this carotenoid may represent a reductive metabolite of astaxanthin. Indeed, in Arctic charr (*Salvelinus alpinus*) the recorded major reductive metabolite of astaxanthin was idoxanthin, comprising 47-75% of total fillet carotenoids (Aas *et al.* 1997; Hatlen *et al.* 1995a).

Data from the present investigation demonstrates that natural fatty acid astaxanthin esters (extracted from *Haematococcus*) are as efficiently utilised as a free synthetic form in the pigmentation of rainbow trout. These results have implications for the use of other natural sources of astaxanthin esters e.g. shrimps and krill wastes that contain esterified astaxanthin. In addition, the degree of astaxanthin esterification has no significant influence on the apparent digestibility of carotenoid. However, this study does provide evidence that the extent of esterification of astaxanthin does influence the rate of astaxanthin absorption into the blood that results in significant differences in peak serum concentrations of astaxanthin. This may suggest that hydrolysis may occur intracellularly within the enterocyte post absorption by the intestine and requires further study. The use of younger cultures of this algal may be

recommended since the ratio of monoesters: diesters have been shown to decrease with time (Harker *et al.* 1996b).

Clearly, a more refined understanding concerning the intraluminal digestive and absorptive processes within the salmonid intestine in relation to astaxanthin and its various forms is required. Although *in vivo* feed trials are an excellent indicator of the limitations associated with absorption, complex combinations of physiological events that occur in the living animal and which as yet are not understood, confound detailed assessment. Furthermore, the relative significance of each event e.g. absorption as opposed to metabolism, can not be strictly determined in live animal investigations. Consequently, it is essential to develop *in vitro* models that isolate and enable examination of carotenoid absorption at the gastrointestinal level.

CHAPTER 6.0


EVALUATION OF AN *IN VITRO* PERFUSION SYSTEM TO ASSESS THE UPTAKE OF ASTAXANTHIN IN ISOLATED INTESTINE FROM RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM).


6.1 Introduction


Astaxanthin is an important carotenoid that is commonly used as a feed supplement to pigment cultured salmonid fish (Torrissen 1989a; Nickell & Bromage 1998a) and due to its wide distribution in the animal kingdom must constitute part of the human diet (Østerlie *et al.* 2000). Astaxanthin has been documented as a potential antioxidant in salmonids (Christiansen *et al.* 1995a). In addition, when used as a feed supplement improvements in growth of Atlantic salmon (*Salmo salar*) fry (Torrissen 1984) and growth and survival during the start-feeding period (Christiansen *et al.* 1994) have been recorded. Subsequently, there is great interest in the absorption and metabolism of this carotenoid in the context of promoting fish health and production.


The mechanism of carotenoid absorption in humans and mammals at the gastrointestinal level has been extensively reviewed (Erdman *et al.* 1993; Parker 1996; Furr & Clark 1997; Van den Berg 1999) yet there is an apparent lack of knowledge relating to similar processes in salmonids. In mammals, once released from the food matrix (gastric hydrolysis), carotenoids dissolve into a fine emulsion that passes into the duodenum region of the gastrointestinal tract. Carotenoids are then incorporated into lipid micelles (formation is dependent on bile flow

from the gall bladder) which act as a vehicle for carotenoid transfer across the unstirred water layer. Carotenoids are then passively absorbed by mucosal cells before being packaged into chylomicron like structures, secreted into lymph and subsequently into the systemic blood circulation. There is a disparity of information concerning those intracellular events that result in the formation of these chylomicrons.

A majority of carotenoid absorption studies in both humans and in fish have been assessed primarily through monitoring rise and decline of the carotenoid in the blood of subjects following acute or chronic administration (White *et al.* 1994; Gobantes *et al.* 1997; Van den Berg 1998; Østerlie *et al.* 2000). However, although this approach is informative it does not quantify the amount of carotenoid that is absorbed (Garrett *et al.* 1999a). Furthermore, carotenoid levels in the blood are affected by metabolism and excretion as well as absorption (Castenmiller & West 1998; Van het Hof *et al.* 2000). Digestibility studies have been utilised to indicate the absorption of carotenoid at the intestinal level in salmonids (Torrissen *et al.* 1990; Choubert & Storebakken 1996; Bjerkeng & Berge 2000). However, such measurements are often subject to overestimation due to oxidation of carotenoids in faecal samples (Meyers 1994).

*In vitro* assessment of intestinal nutrient absorption has been carried out following a number of approaches each with their own advantages and disadvantages. One of the earliest *in vitro* preparations designed to monitor intestinal solute uptake was a section of intestine, circularly perfused on its luminal side, with samples being drawn from both the luminal and serosal reservoirs (Fisher & Parsons 1949). This method was later replaced with the established everted sac technique (Wilson & Wiseman 1954) which incorporated an everted segment of

intestine filled with media, sealed at both ends (serosal compartment) and suspended in a luminal reservoir containing the solute of interest. However, this preparation is limited by the inability to oxygenate and sample the serosal compartment throughout the experimental period. Studies where anaesthetised (Sawchuk & Awni 1986; Péres del Castillo *et al.* 1997; Wang *et al.* 1997) and non anaesthetised animals (Saunders & Dawson 1963; Simmonds *et al.* 1968; Thompson *et al.* 1969; Clark *et al.* 1998) are chronically catheterised are believed to be most physiologically representative. Such methods involve surgical procedures that are difficult and often impractical where fish are the subject of investigation, although *in situ* catfish intestinal preparations have been recently described (Kleinow *et al.* 1998; Doi *et al.* 2000). A modern system described by Carmona (1998) used non-recirculating serosally perfused everted segments of intestine that were placed in large volume incubation chambers which avoid large changes in luminal concentration. The continuous flow of perfusate (serosal compartment) avoids accumulation of solutes in the tissue phase and tissue viability is kept optimal by oxygen delivery to both the luminal and serosal media. In addition continuous collection of serosal perfusate fractions allows the determination of translocation kinetics and monitoring of a steady state.

The objectives of the current study were to assess the potential of an intestine perfusion system to monitor and describe the uptake kinetics of astaxanthin in two potential vectors/solubilising agents. Secondly, to assess the viability of intestinal tissue when used in an *in vitro* perfusion system.

## 6.2    Materials and Methods

### 6.2.1    Experimental System

The isolated gut perfusion preparation used throughout the investigations was based on methodology described by Carmona (1998) and Ando *et al.* (1986a).   A diagramatic representation of the system can be seen in Figure 6.1.   Everted intestines were mounted in a gassed (95% $O_2$; 5% $CO_2$) luminal reservoir (15cm x 11cm) containing physiological saline (450ml) of composition (g $l^{-1}$): NaCl 7.37g; KCL 0.31g; $CaCL_2$ 0.17g; $MgSO_4$ 0.14g; $KH_2PO_4$ 0.46g; $Na_2H_2PO_4$ 2.02g; 0.3% Tween 20; 0.1% glucose (pH 7.3) (Al-Khalifa & Simpson 1988).   The preparation was perfused with saline using a peristaltic pump (Gilson Miniplus 3, Villiers le Bel, France) at a set flow rate of 1 ml $min^{-1}$ to mimic the blood flow of fish *in vivo* (Barron *et al.* 1987).   A 250ml glass Erlenmeyer flask was used as a perfusion reservoir and was also gassed (95 % $O_2$; 5% $CO_2$) to ensure preparation viability.   The intestines were mounted on a polythene mesh support (15cm x 8cm) above a magnetic flea, rotated using a magnetic stirrer (H1 190M, HANNA Instruments, UK).   This ensured efficient flow of the saline across the intestinal mucosa.   Both the luminal and perfusate reservoirs were chilled with circulating water (Maxicool 14, IMI Cornelius, U.K.) to ensure the preparation was maintained at a desired temperature below that of ambient, ca. 20°C (typically at 15°C ± 1°C). No inserts were necessary to sustain luminal space along the intestine owing to inherent musculature of the organ.

### 6.2.2 Preparation of the intestine

Rainbow trout (250-400g) were taken from the aquarium system (see section 2.2.2) and immediately culled via a blow to the cranium followed by destruction of the brain. Fish weight was recorded. The gastrointestinal tract was promptly dissected from the fish and the stomach and pyloric region (from the pyloric sphincter to the last pyloric diverticular) separated from that of the remaining intestine, and discarded. The remaining intestine was immediately placed in 100ml glass beaker containing both chilled (5°C) and gassed (95% $O_2$) physiological saline for an initial period of recovery. Any fat deposits adhering to the intestine were gently removed from the organ using tweezers and a scalpel taking care not to tear or puncture the organ. The intestine section was then everted on a stainless steel everting rod (300mm x 2mm) and placed in the luminal reservoir containing 450ml of the physiological saline and dissolved solute (carotenoid). The anterior end of the intestine was supplied with saline from the perfusate reservoir, via the peristaltic pump and PVC tubing (0.63 mm internal diameter) containing a 1.5cm stainless steel insert at its out flow (1ml min$^{-1}$). The intestine was attached to the tubing with surgical suture. The distal end of the preparation was attached directly to similar tubing leading to a rack containing six 20ml pre-weighed glass vials that acted as fraction collectors (10min fractions) throughout the experimental period (60min). The preparation was then back-filled with chilled physiological saline and observed for leaks. An initial period (5 min) of peristaltic flow (1ml min$^{-1}$) was then sustained (elute discarded) to observe preparation viability, and ensure residual carotenoid that may have entered the lumen of the organ during initial transfer would not influence uptake data.

### 6.2.3 Solubilisation of carotenoid

Two different methods of solubilisation were employed in the current study. In Experiment MIC, astaxanthin was dispersed in a non-ionic surfactant (Tween 20, BDH Supplies, Poole, England) which was added to the luminal reservoir saline at 0.3% (v/v) (Al-Khalifa & Simpson 1988). The surfactant was included at an amount higher than its micellar concentration (59 µM) and it was subsequently assumed that carotenoid was incorporated into these micelles. The saline was filtered (Whatman No.1) before use to remove crystalline carotenoid that was not dispersed. Due to the limited solubility of astaxanthin in this solution, a second experiment (Experiment EM) was carried out using water miscible beadlets containing 4.6% astaxanthin (CAROPHYLL® Pink; Roche Ltd., Basel). These were dispersed into saline (in addition to 0.3% v/v Tween 20) by mixing at 40°C. In both experiments Tween 20 was also added (0.3% v/v) to the serosal perfusate to act as a solubilising agent for translocated carotenoid (Loran & Althausen 1969).

### 6.2.4 Astaxanthin analysis

At the end of the experimental period 2ml of methanol (+500ppm BHT) was added to each perfusate fraction followed by rapid vortexing. Dichloromethane (6ml) was then added to each fraction, followed again by vortexing and a 10min extraction period in darkness. Each fraction was then given a final vortex and then centrifuged at 3000 x $g$ for 10 min at 15°C. An aliquot of the resulting hypophase (3ml) was extracted (5ml glass syringe; Hamilton, Reno, Nevada), filtered (0.2µm), blown down under a gentle stream of nitrogen and stored at –20°C

until analysis. Samples were re-suspended in 100μl of dichloromethane, before injection into the HPLC system for carotenoid quantification (see section 2.4.10)

### 6.2.5 Viability experiments

A number of parameters were assessed throughout the investigations to ensure the viability of the preparation and the subsequent value of the data obtained. During the perfusion period the observation of normal peristaltic contractions and constant perfusate out flow were the most efficient indicators of viability. Those preparations that conformed to these parameters were subjected to histological examination and were compared against tissues taken from normal healthy animals.

#### 6.2.5.1 *Perfusate out flow*

This was measured by subtracting the initial weight of the fraction collector (pre-weighed vial) from the final weight of the vial with perfusate. If results varied considerably from one fraction to another e.g. a dramatic drop in serosal perfusate volume, the preparation was deemed non-viable and the data dismissed from further analysis. Tears, leakage and blockages were factors that may have been responsible for variation in flow.

#### 6.2.5.2 *Peristaltic contractions*

Observations of sudden and spontaneous contractions are normal for intestine preparations and were taken as an indicator of physiological viability. Only when contracture was severe and

lasted over long periods was this considered as an indicator of decreased viability and the data was duly disregarded.

### 6.2.5.3 *Histological examination*

Approximately 2cm of the everted intestine sample was typically used for ultrastructure examination and confirmation of preparation viability. The sample was placed in a 20ml glass vial filled with 5% buffered formal saline and left for a minimum of 24 hours. The formal saline was then discarded and the sample washed and stored in 70% alcohol for 3 hours, followed by washing and storage in 90% alcohol for 2 hours. The sample was then washed and kept in absolute alcohol (100%) for a minimum of 12 hours. The alcohol was then decantered and the sample washed and placed in xylene for 2 hours with an intermittent transfer into fresh xylene after 1 hour. Following removal of the xylene the sample was set into a wax block mould.

Sectioning was carried out on a rotary microtome with a typical section thickness of 8μm. Samples were then stained using the Mallory's Trichrome procedure. The slide with specimen was placed in haematoxylin for 20min, followed by washing with water. The specimen was briefly placed in a lithium carbonate solution; differentiated in 1% acid alcohol, and then placed back into the lithium carbonate solution for a few seconds. The specimen was then washed thoroughly in distilled water. The slide was then placed in acid fuchin solution for 10 seconds and again washed in distilled water. Following washing, the slide was placed in Mallory's stain for 10 seconds; washed with distilled water; placed in 90% alcohol for 5

seconds and 100% alcohol 2 min. The sample was then placed in xylene for 2 min and then mounted with a coverslip using DPX.

### 6.2.5.4 *Uptake vs. perfusate volume plots*

Regression analysis and comparison was carried out on cumulative uptake of astaxanthin and cumulative perfusate volume data. Differences in the slopes of regressions between plots suggest uptake is independent of perfusate flow and that uptake is not a factor of solvent drag. Comparisons between total perfusate volume (total perfusate in each time fractions at the end of the perfusion period) at each astaxanthin dose were carried out to determine differences in apparent net water flux.

### 6.2.6 Experiments

Experiment MIC and Experiment EM were carried out as preliminary dose response investigations. Doses of carotenoid were chosen according to representation of physiological concentrations, solubility restrictions and detection limits (see discussion). Uptake rates of astaxanthin were measured as a function of the amount of astaxanthin in the perfusate fractions and normalised for wet weight of the intestinal preparation. Initial uptake rates of astaxanthin were based on the amount of astaxanthin absorbed in the first 10min fraction where overall uptake rates were calculated according to the total amount of astaxanthin taken up over the 60min perfusion period. Absorption coefficients were calculated according to:

100    x    <u>total amount of astaxanthin in perfusate after 60min (µg)</u>

total amount of astaxanthin in luminal reservoir (µg)

Those doses of astaxanthin which generated cumulative uptake data showing typical saturation over time was subjected to Michaelis–Menten kinetic fittings to identify Vmax which represented a theoretical maximum tissue astaxanthin concentration.

### 6.2.7   Statistical analyses

All statistical comparisons were carried out on StatGraphics Plus (2) for Windows. All data sets were subjected to tests for normal distribution and variance checks before comparison with parametric or non-parametric (Kruskall Wallis) tests. Curve fitting and regression analysis of cumulative uptake, initial uptake rates and cumulative volume was performed using SigmaPlot 4.0. Uptake rates, absorption coefficients and Vmax values were compared across treatments using a one-way analysis of variance at the 95% confidence level. Regression analysis of cumulative uptake and volume data generated slope values that were compared using the Kruskal Wallis test at the 95% confidence limit.

## 6.3    Results

### 6.3.1   Viability

Viability assessments demonstrated that greater than 95% of the intestinal preparations were viable over the 60min perfusion period. Preparations mainly failed (<7%) due to tears in the

144

tissue that occurred during excision from the fish. These were quickly noticed due to erratic variations in perfusate volume. Histological examination of the tissue (Figure 6.2) revealed a thinning of the lamina propria within the epithelial tissue cells which occurred in greater than 95% of the samples (as compared to controls).

Comparison of regression line slopes for cumulative uptake and perfusate volume revealed that significant differences (P<0.05) existed between plots and that astaxanthin absorption was not influenced by "solvent drag" (see Figures 6.3-6.8). However, the total perfusate volume (Table 6.1) at the end of the perfusion period did vary between astaxanthin doses in both experiments where a general trend of decreasing volume with increasing dose was recorded. In Experiment MIC, the total perfusate volume at astaxanthin dose $1mg \, l^{-1}$ was significantly higher (P=0.0247) than the perfusate volumes at doses of 2 and $3mg \, l^{-1}$. Furthermore, the total perfusate volume at dose $6mg \, l^{-1}$ in Experiment EM was significantly lower (P=0.0162) than the perfusate volume at doses 2 and 3 mg $l^{-1}$.

### 6.3.2   Astaxanthin uptake

In both experiments there was a significant (P<0.01) positive relationship between initial uptake rate (based on the first 10min fraction) and dose of astaxanthin (Figures 6.9-6.10) with P-values of 0.0021 and 0.0064 for Experiment MIC and Experiment EM, respectively. However, variability in the raw data resulted in moderate $R^2$ and correlation coefficients for both Experiment MIC ($R^2$=32.1%; correlation coefficient=0.56644) and Experiment EM ($R^2$=50.6%; correlation coefficient=0.711024). In Experiment MIC, the initial uptake rate at doses of $2mg \, l^{-1}$ ($0.30 \pm 0.04 \, \mu g \, g^{-1}h^{-1}$; $n$=10, $\pm$SEM) and $3mg \, l^{-1}$ ($0.40 \pm 0.12 \, \mu g \, g^{-1}h^{-1}$; $n$=4,

145

±SEM) were significantly higher (P=0.0156) than that at 1mg $l^{-1}$ (0.15 ± 0.04 µg $g^{-1}h^{-1}$; $n$=10, ±SEM). In Experiment EM, the initial uptake rate at a dose of 6mg $l^{-1}$ (0.31 ± 0.08 µg $g^{-1}h^{-1}$) was significantly (P=0.0270) higher than that at 2mg $l^{-1}$ (0.04 ± 0.01 µg $g^{-1}h^{-1}$) but not significantly different from that at 3mg $l^{-1}$ (0.17 ± 0.07 µg $g^{-1}h^{-1}$)($n$=4, ± SEM). Interestingly, the mean uptake rates of astaxanthin in Experiment MIC (Tween 20) were higher at equivalent astaxanthin doses than those in Experiment EM (dispersible beadlets) although this difference was only significant (P=0.0019) at the 2mg $l^{-1}$ dose.

The overall uptake rate of astaxanthin in both experiments was not significantly different (P>0.05) between doses in each experiment with mean values ranging from ca. 0.1 to 0.13 µg $g^{-1}h^{-1}$ in Experiment MIC and ca. 0.02 to 0.1 µg $g^{-1}h^{-1}$ in Experiment EM. In both experiments the overall uptake rates were lower than the initial uptake rates of astaxanthin and did not show a positive increase with increasing doses of astaxanthin.

Absorption coefficients were generally very small across both experiments (0.002-0.3%) but this may expected since these values are a function of the ratio of total volume of the luminal reservoir (450ml) to the tissue weight ca. 0.5-2g. As the volume of the luminal reservoir decreases the absorption coefficient would increase. In Experiment EM there were no significant differences (P>0.05) in absorption coefficients. However, in Experiment MIC the absorption coefficient generally decreased with increasing dose. Furthermore the absorption coefficient at 1mg $l^{-1}$ (0.025 ± 0.0005%) was significantly higher (P=0.0238) than that at doses of 2mg $l^{-1}$ (0.012 ± 0.002%) and 3mg $l^{-1}$ (0.01 ± 0.003%).

The Vmax values for those doses that generated typical time saturation kinetics (see Figures 6.11-6.12) in the cumulative uptake data (Doses 2 and 3mg $l^{-1}$ in Experiment MIC and 3 and 6mg $l^{-1}$ in Experiment EM) were not significantly different and typically ranged from ca. 0.1 to 0.3 $\mu g\ g^{-1}$.
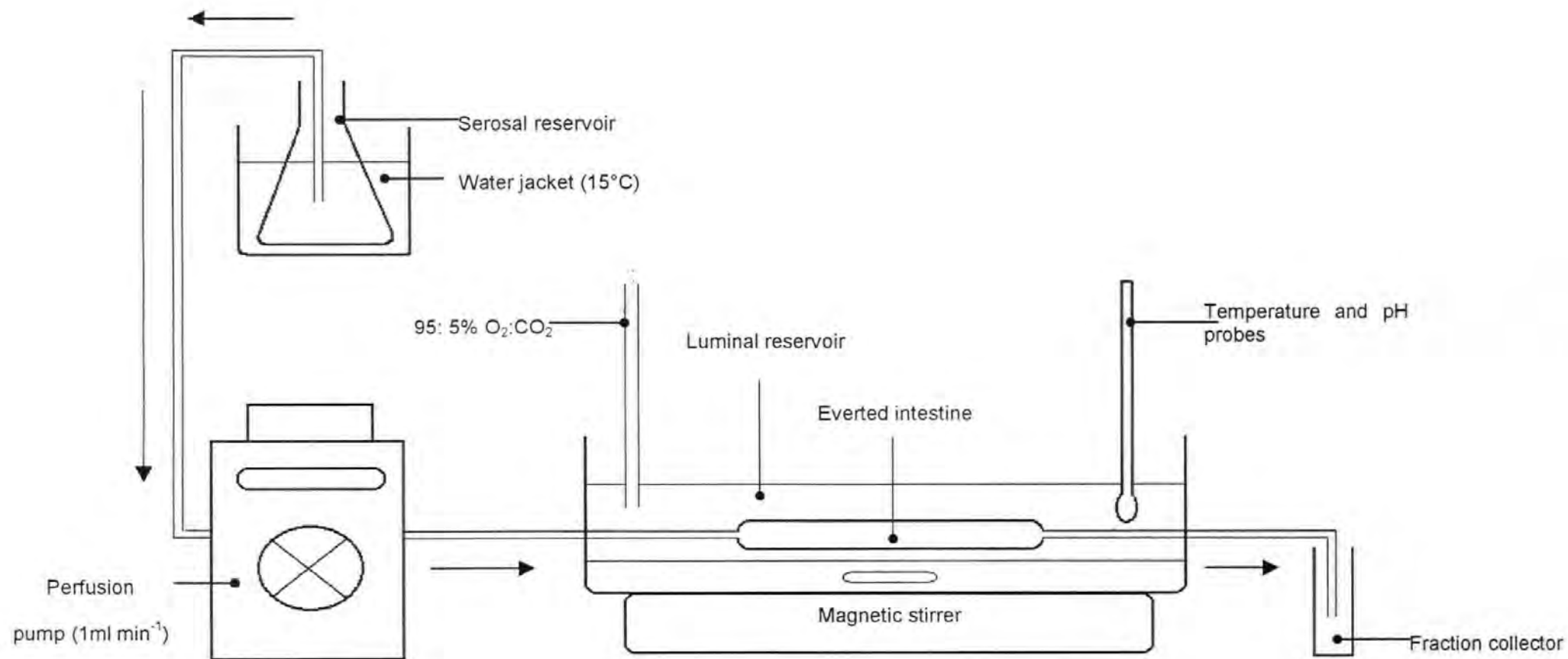
Figure 6.1    Everted intestine perfusion system used in the current study
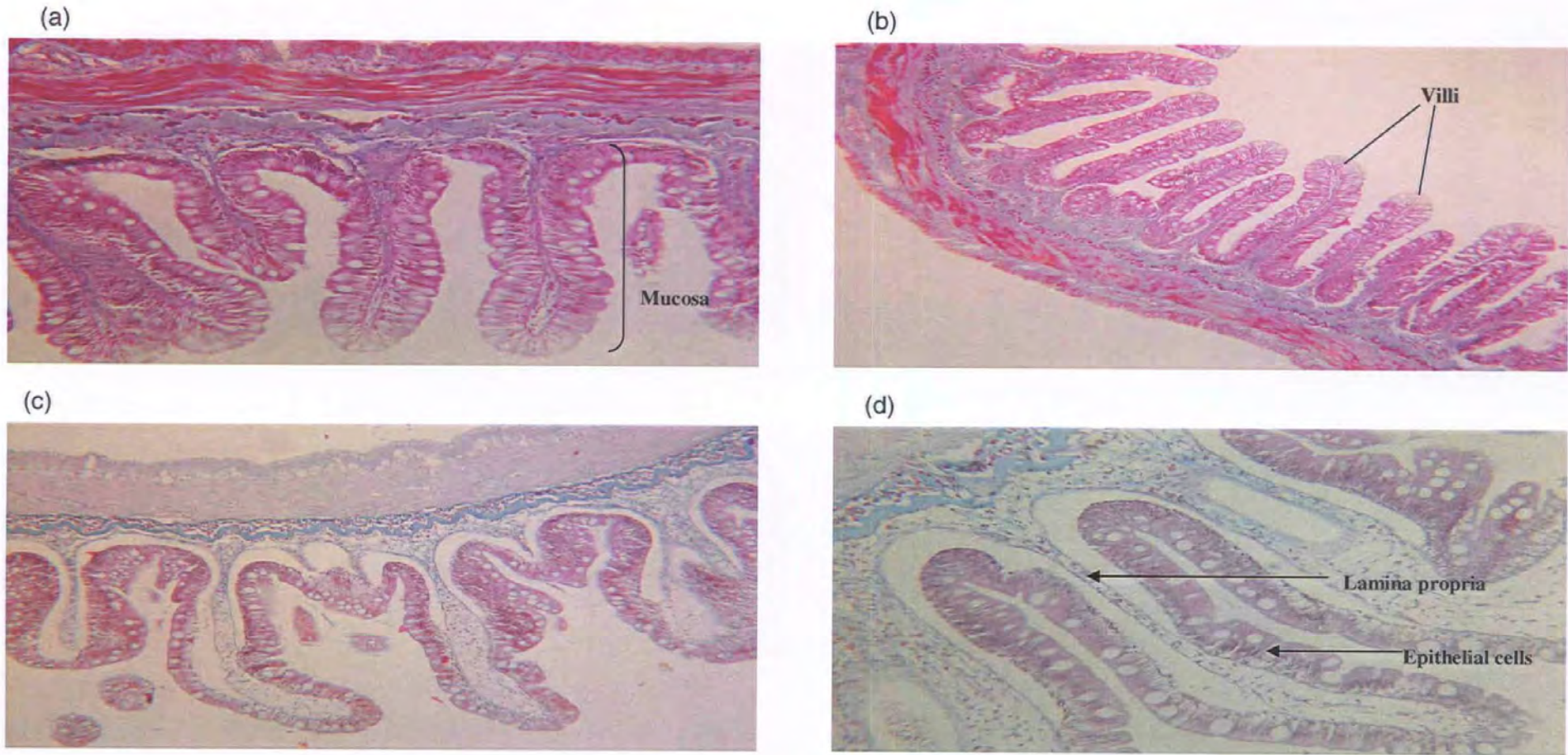
Figure 6.2     Histological pictures of (a) control intestine ($\times$ 20 magnification); (b) control intestine ($\times$ 10 magnification); (c) everted intestine sample ($\times$ 20 magnification) and (d) everted intestine ($\times$ 40 magnification).
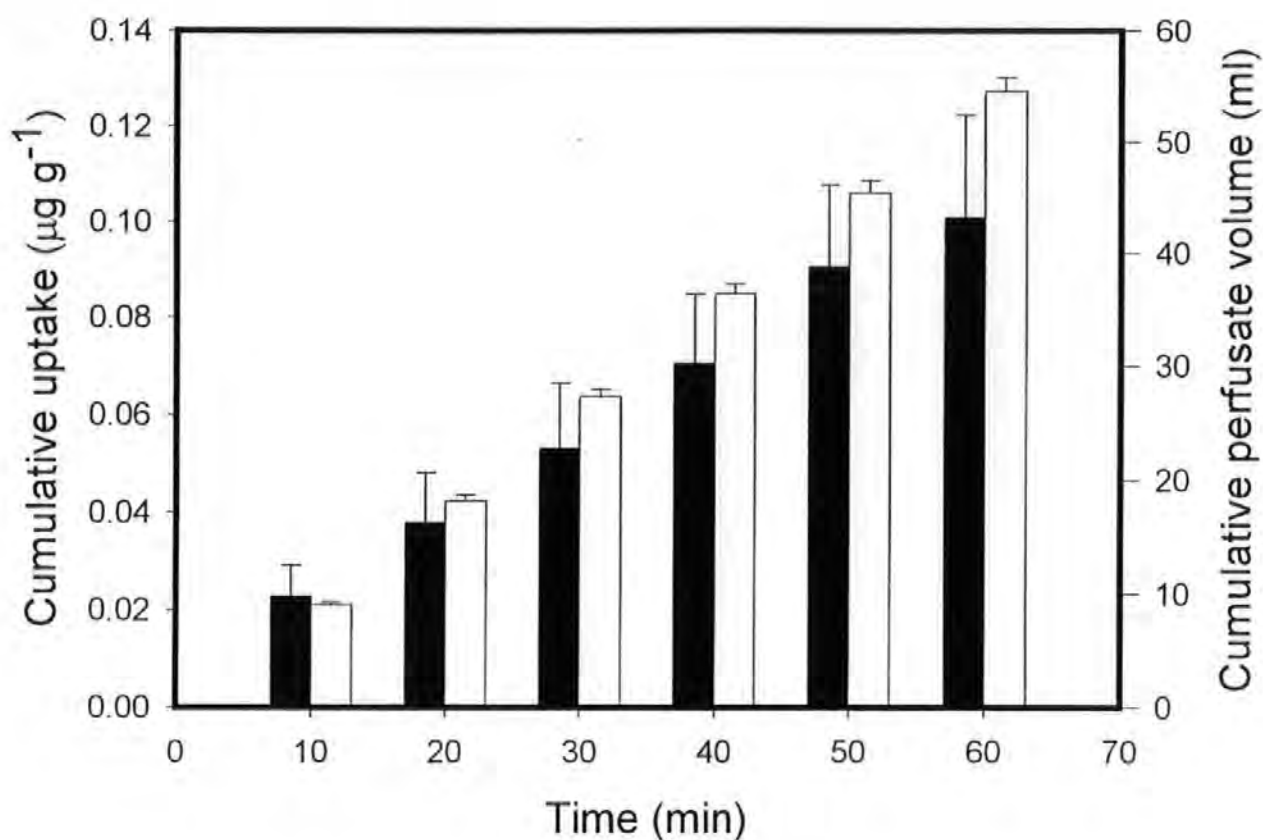
Figure 6.3    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 1mg l$^{-1}$ in Experiment MIC. Comparison of slopes (linear regression) based on raw data ($n$=10) revealed significant (P=0.0090) differences. Values represent means ($n$=10, ±SEM).
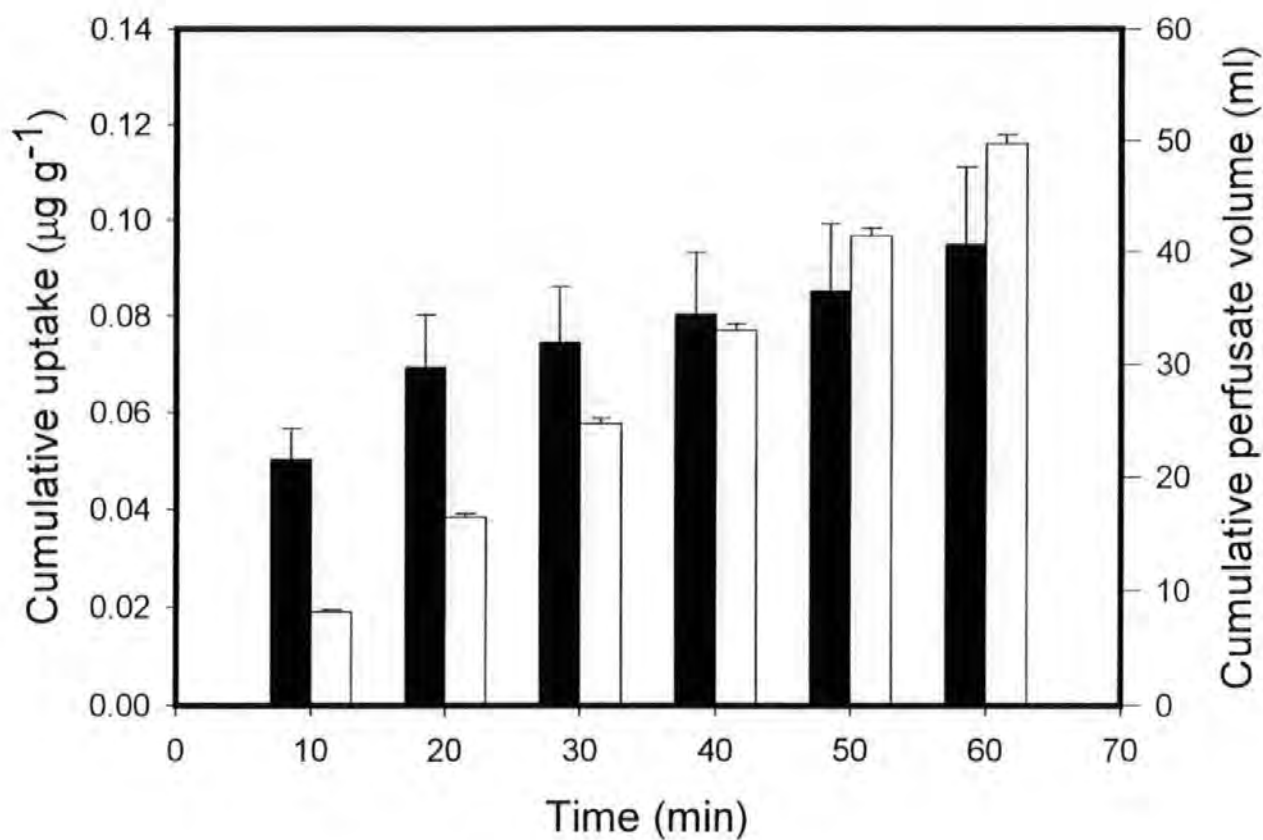
Figure 6.4    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 2mg $l^{-1}$ in Experiment MIC. Comparison of slopes (linear regression) based on raw data ($n$=10) revealed significant (P=0.0088) differences. Values represent means ($n$=10, ±SEM).
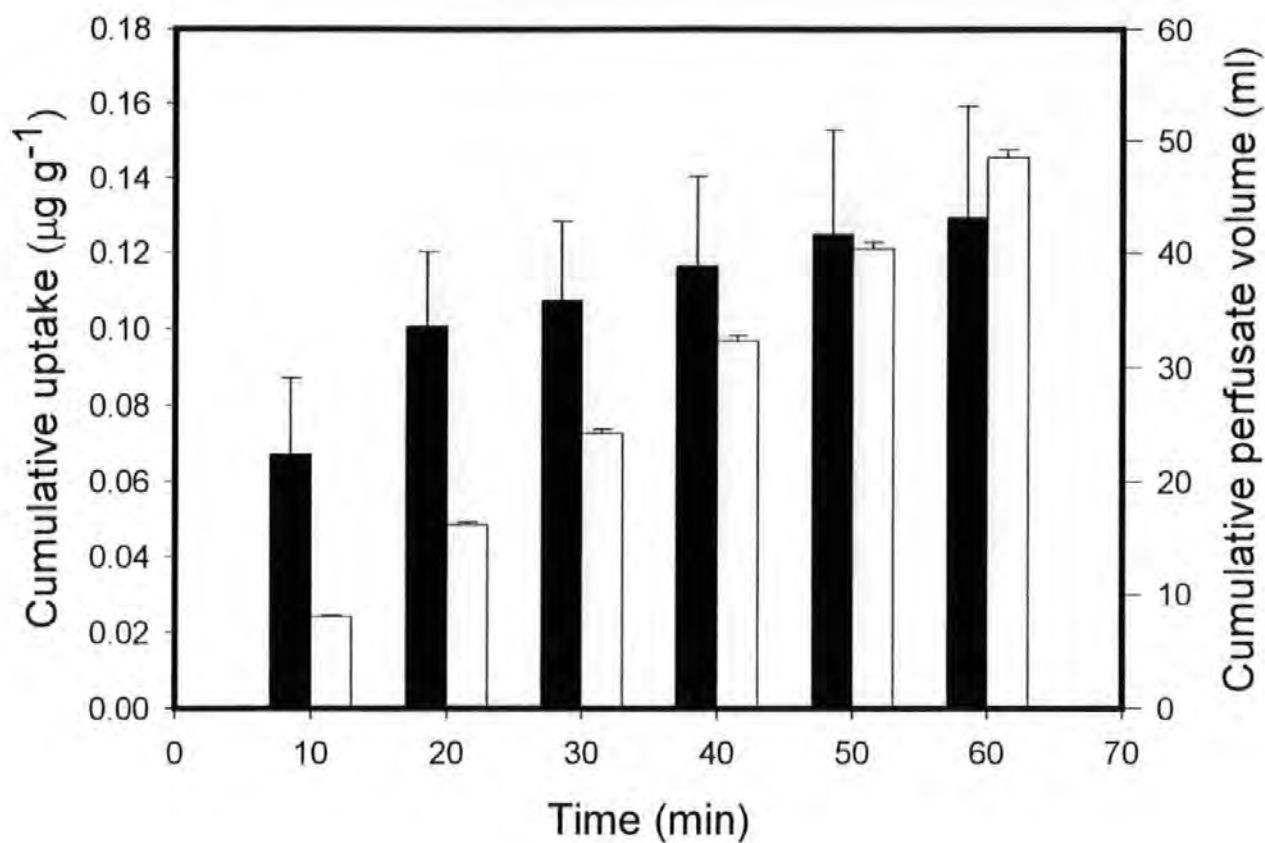
Figure 6.5    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 3mg l$^{-1}$ in Experiment MIC. Comparison of slopes (linear regression) based on raw data (*n*=4) revealed significant (P=0.0292) differences. Values represent means (*n*=4, ±SEM).
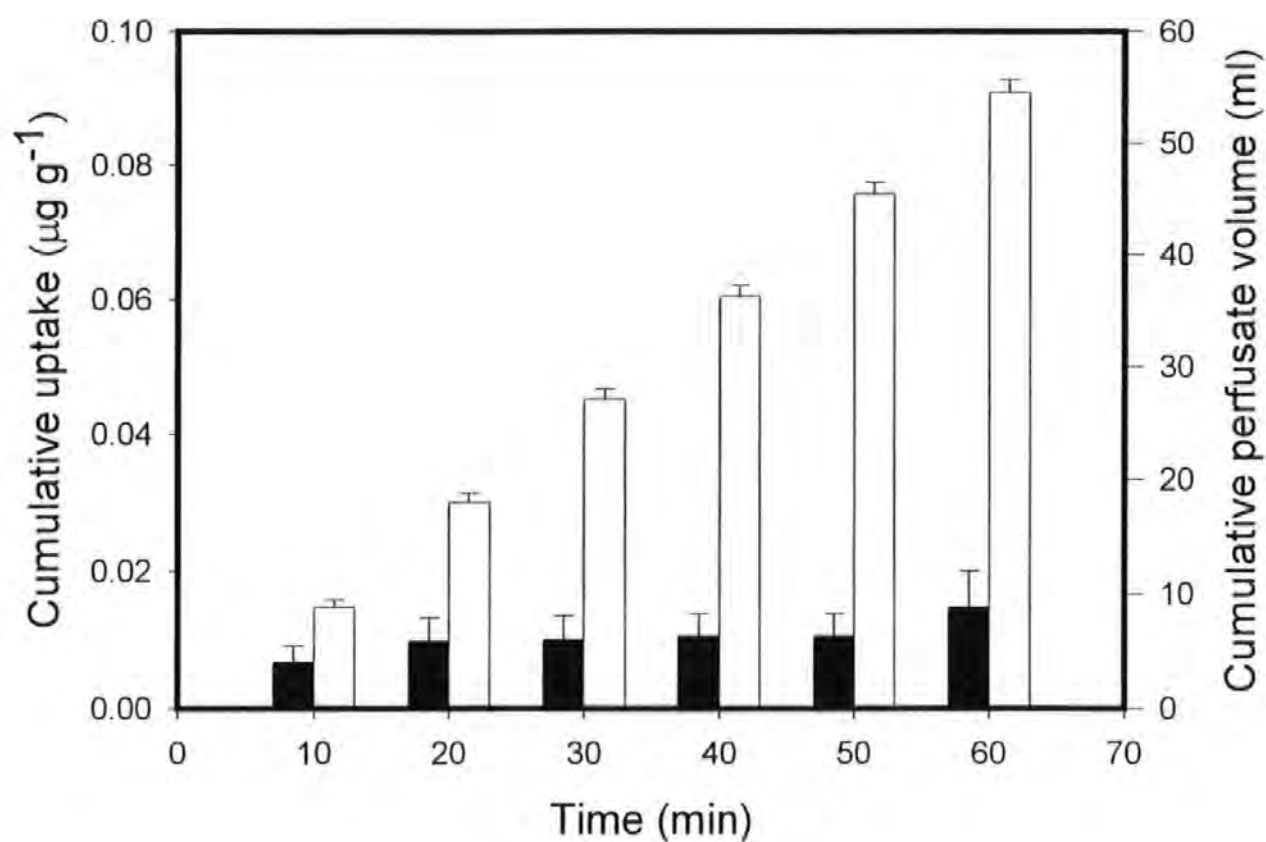
Figure 6.6    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 2mg l⁻¹ in Experiment EM.   Comparison of slopes (linear regression) based on raw data (*n*=4) revealed significant (P=0.0202) differences.  Values represent means (*n*=4, ±SEM).
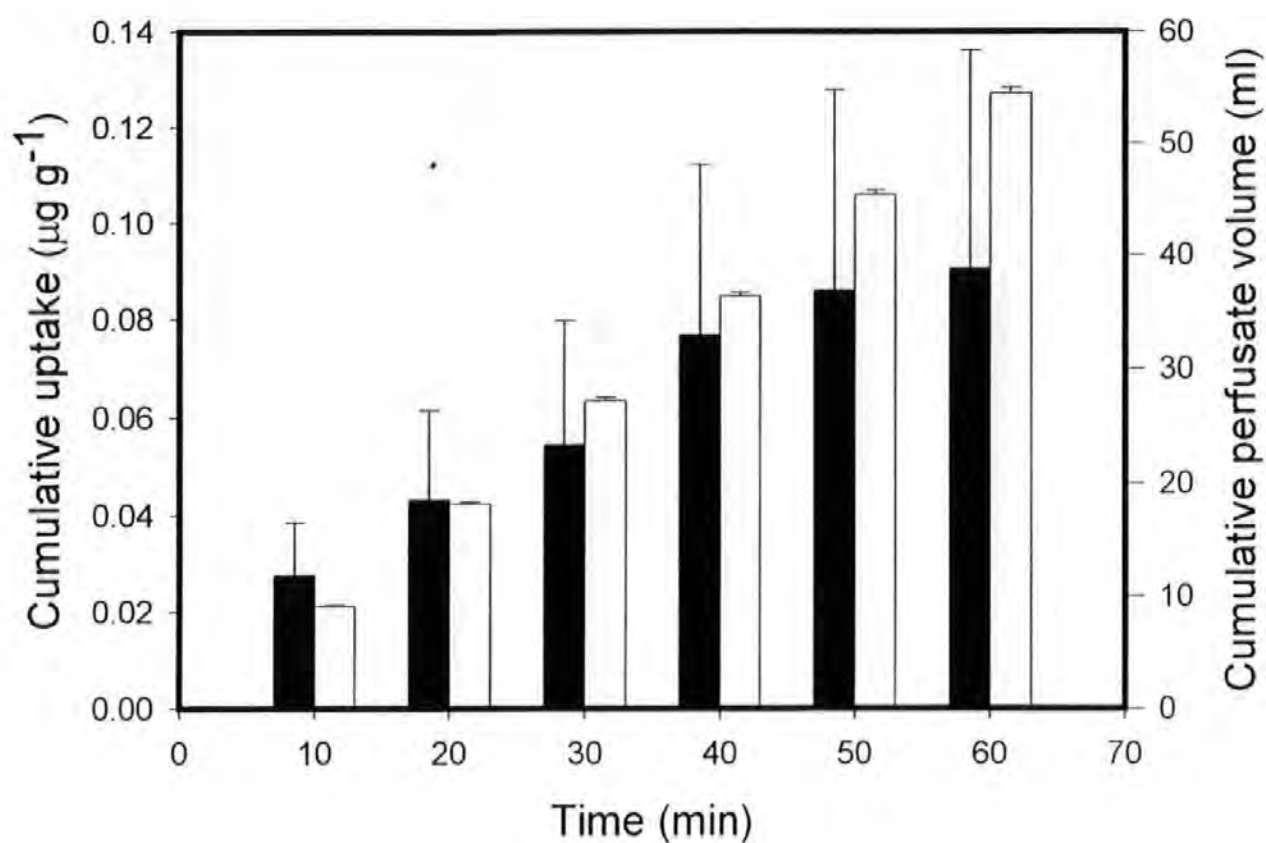
Figure 6.7    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 3mg $l^{-1}$ in Experiment EM. Comparison of slopes (linear regression) based on raw data ($n$=4) revealed significant (P=0.0209) differences. Values represent means ($n$=4, ±SEM).
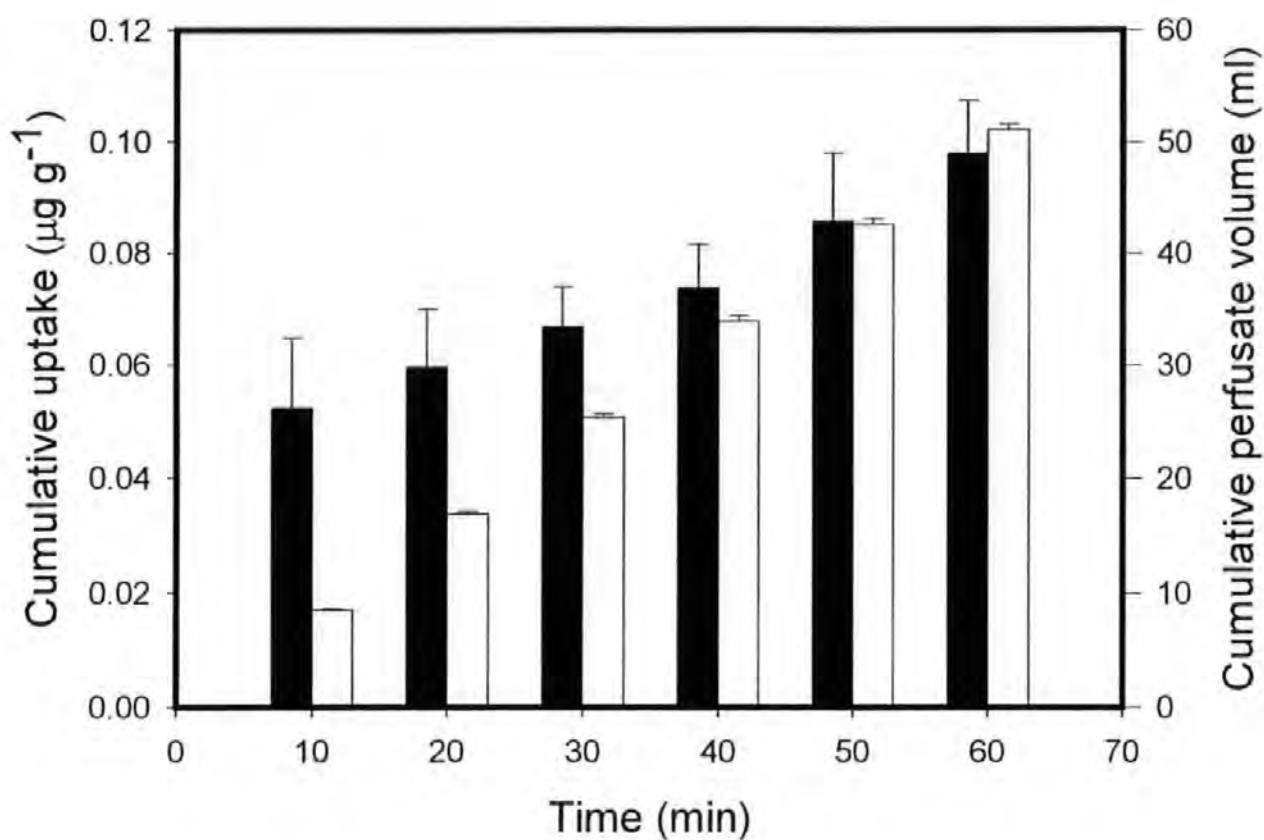
Figure 6.8    Cumulative astaxanthin uptake (solid bars) versus cumulative perfusate volume (open bars) at dose 6mg l⁻¹ in Experiment EM. Comparison of slopes (linear regression) based on raw data ($n$=4) revealed significant (P=0.0202) differences. Values represent means ($n$=4, ±SEM).

Table 6.1    Absorption parameters for perfused everted intestines exposed to different doses of astaxanthin

| Experiment | Dose (mg l$^{-1}$) | Initial uptake rate (µg g$^{-1}$h$^{-1}$) | Overall uptake rate (µg g$^{-1}$h$^{-1}$) | Absorption Coefficient[¶] (%) | Vmax[‡] | Total perfusate volume (ml) |
|---|---|---|---|---|---|---|
| *Experiment MIC | 1 | 0.15 ± 0.04$^A$ | 0.10 ± 0.02 | 0.025 ± 0.005$^A$ | - | 54.49 ± 1.28$^A$ |
| (Tween micelles) | 2 | 0.30 ± 0.04$^B$ | 0.10 ± 0.02 | 0.012 ± 0.002$^B$ | 0.09 ± 0.02 | 49.70 ± 0.81$^B$ |
| | 3 | 0.40 ± 0.12$^B$ | 0.13 ± 0.03 | 0.010 ± 0.003$^B$ | 0.16 ± 0.04 | 48.49 ± 0.67$^B$ |
| [†]Experiment EM | 2 | 0.04 ± 0.01$^A$ | 0.02 ± 0.01 | 0.002 ± 0.002 | - | 54.50 ± 1.13$^A$ |
| (Dispersible beadlets) | 3 | 0.17 ± 0.07$^{AB}$ | 0.09 ± 0.05 | 0.007 ± 0.003 | 0.26 ± 0.17 | 54.45 ± 0.48$^A$ |
| | 6 | 0.31 ± 0.08$^B$ | 0.10 ± 0.01 | 0.006 ± 0.001 | 0.17 ± 0.06 | 51.04 ± 0.50$^B$ |

*Number of replicates varied between the 1 mg l$^{-1}$ ($n$=10); 2mg l$^{-1}$ ($n$=10) and 3mg l$^{-1}$ ($n$=4) doses due to limited solubility of astaxanthin. [†]$n$=4 for each dose. [‡]Estimate of the maximum astaxanthin concentration in intestinal tissue at each dose. Does not apply to some doses due to linearity in regression plots. [¶]Calculated at the end of the perfusion period according to: [cumulative astaxanthin in perfusate (µg)/astaxanthin in luminal reservoir (µg)] × 100. [AB]Values within a column within an experiment bearing different superscripts are significantly (P<0.05) different. All values are means ± SEM.
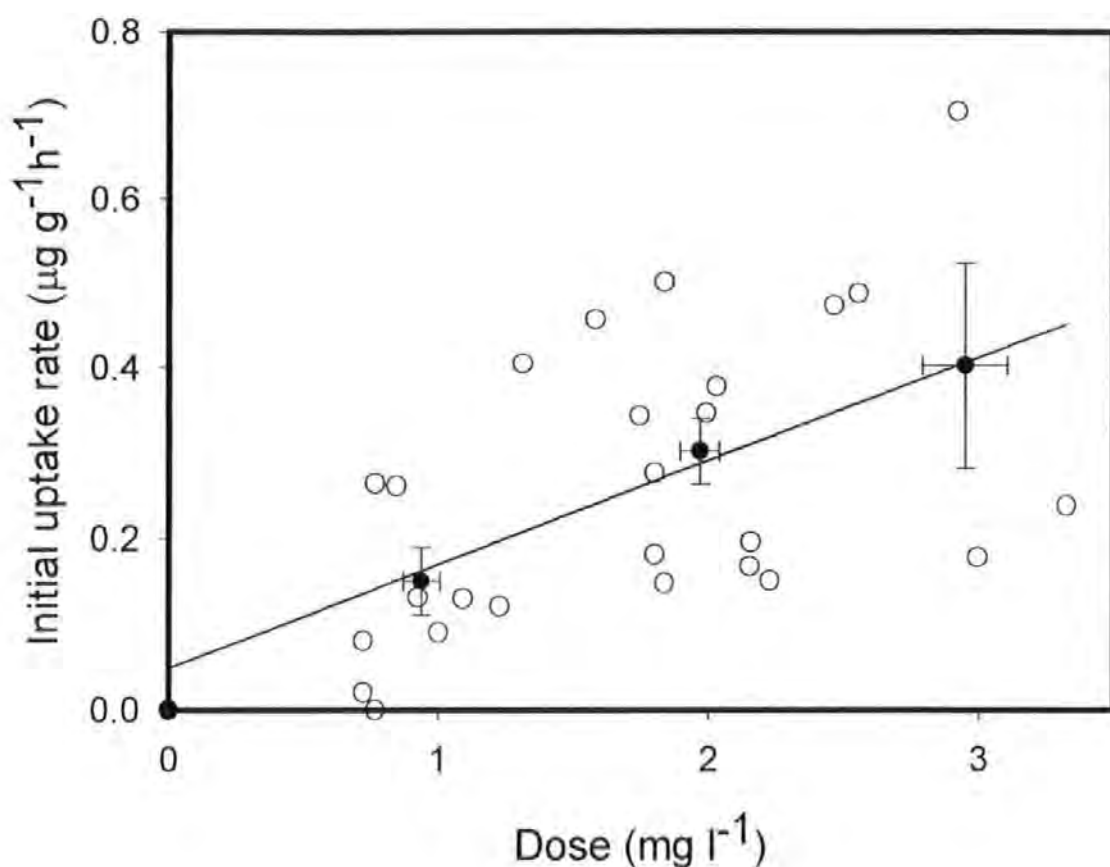
Figure 6.9    Dose response curve for initial uptake rates of astaxanthin by perfused everted intestines in Experiment MIC (astaxanthin solubilise in Tween 20). Open circle symbols represent individual uptake and filled circles represent mean uptake at intended doses (1mg $l^{-1}$ and 2mg $l^{-1}$ $n$=10; 3mg $l^{-1}$ $n$=4, ± SEM). Horizontal error bars indicate variation (SEM; 1mg $l^{-1}$ and 2mg $l^{-1}$ $n$=10; 3mg $l^{-1}$ $n$=4) in dose about the intended mean dose. Regression analysis of raw data gave the equation: uptake rate= 0.0468937+0.122348×dose ($P$=0.0021; $R^2$=32.0854%; correlation coefficient = 0.56644)
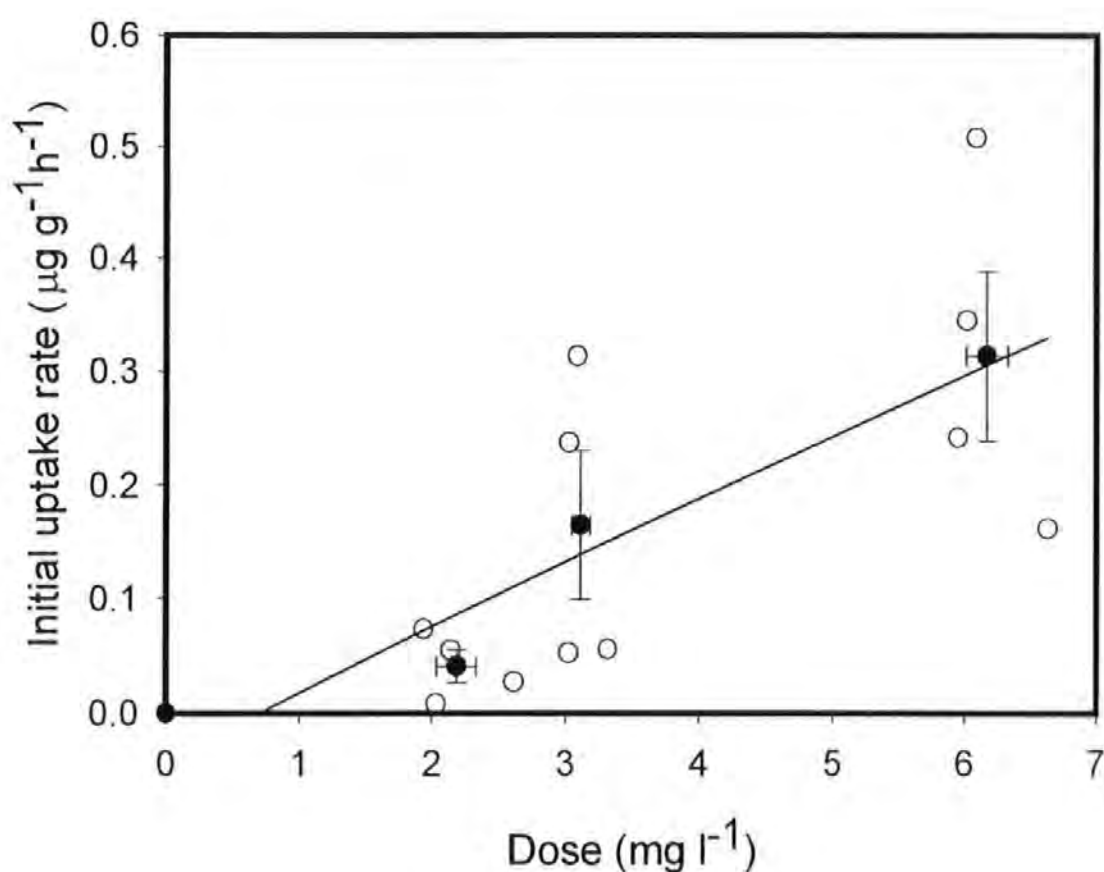
Figure 6.10 Dose response curve for initial uptake rates of astaxanthin by perfused everted intestines in Experiment EM (dispersible beadlets). Open circle symbols represent raw data and filled circles represent means ($n$=4, ±SEM) at intended doses. Regression analysis of raw data gave the equation: uptake rate= -0.0362817+0.0554101×dose ($P$=0.0064; $R^2$=50.5555%; correlation coefficient = 0.711024).
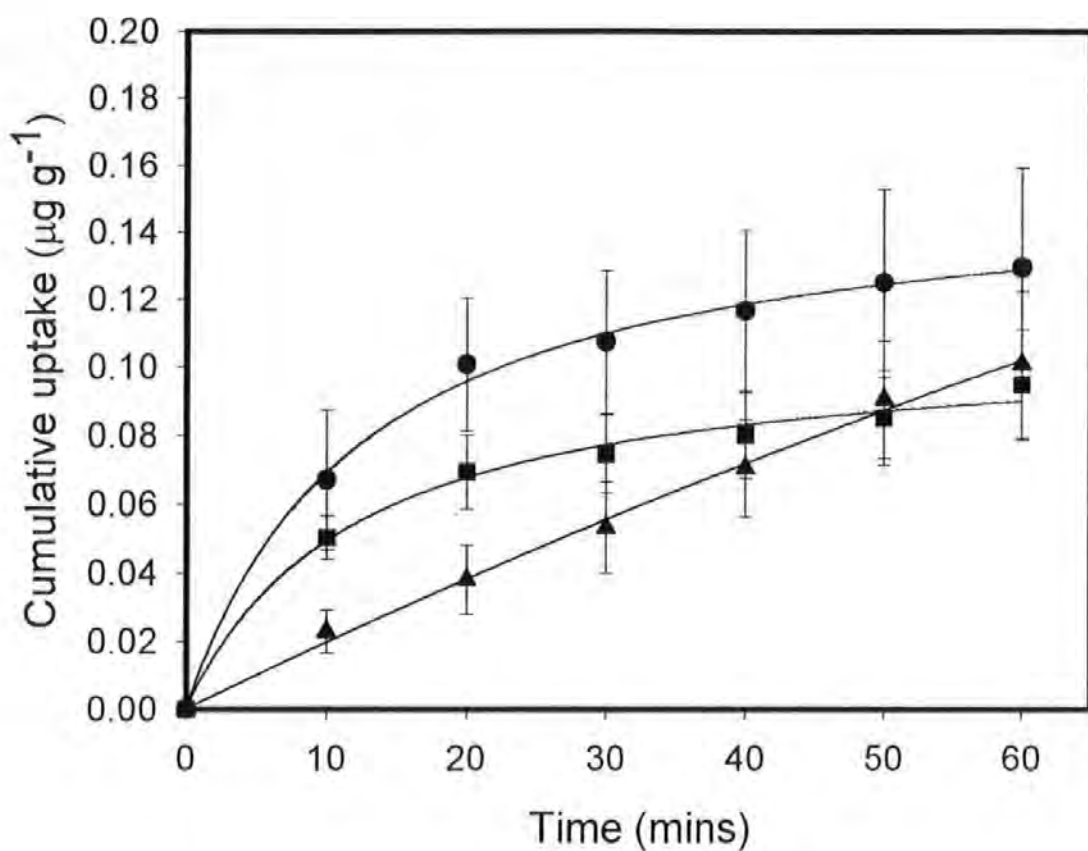
Figure 6.11    Cumulative uptake of astaxanthin in Experiment MIC (Tween 20) by perfused everted intestines at astaxanthin doses of 1mg l$^{-1}$ (-▲-); 2mg l$^{-1}$ (-■-) and 3mg l$^{-1}$ (-●-). Plots for 1mg l$^{-1}$ and 2mg l$^{-1}$ dose represent means of 10 replicates ± SEM. Plots for 3mg l$^{-1}$ dose represent means of 4 replicates ± SEM.
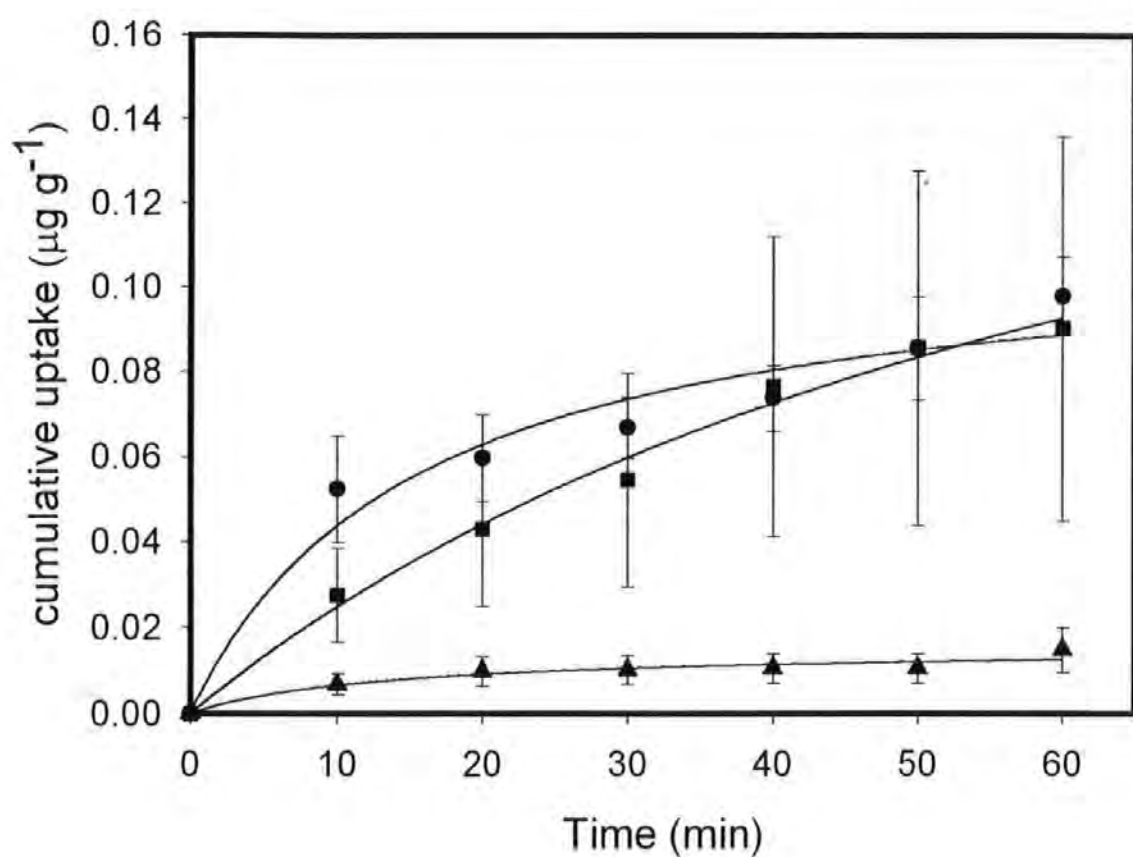
Figure 6.12   Cumulative uptake of astaxanthin in Experiment EM by perfused everted intestines at astaxanthin doses of 2mg l$^{-1}$ (-▲-); 3mg l$^{-1}$ (-■-) and 6mg l$^{-1}$ (-●-). Plots for all doses represent means ($n$=4, ± SEM).

## 6.4 Discussion

Preliminary dose response investigations were carried out in order to evaluate the potential of an everted intestine perfusion system (Carmona 1998) as a useful *in vitro* tool to study the absorption of astaxanthin. This system necessitated the selection of a vehicle or aqueous carrier for carotenoid solubilisation. The current study investigated two such carriers; a non-ionic surfactant (Tween 20) that was included at a quantity above its micellar concentration and a commercial emulsion (CAROPHYLL® Pink, Hoffmann La Roche, Basel) of proprietary composition. Both non-ionic surfactants (El-Gorab *et al.* 1975) and water miscible preparations (Garrett *et al.* 1999) have been used to study the uptake of carotenoid in other *in vitro* models.

### 6.4.1 Viability assessment

Cumulative astaxanthin uptake plots and overall uptake rates seem to show saturation in the mechanism of uptake with increasing astaxanthin dose. For example, in Experiment MIC, translocation of astaxanthin across the intestine at the lowest dose ($1mg\ l^{-1}$) appears to follow a linear trend over the perfusion period but translocation at the higher doses (2 and $3mg\ l^{-1}$) displays saturable kinetics. It is unlikely that saturation was a function of perfusate flow and solvent drag as regression comparisons for cumulative uptake versus cumulative perfusate flow demonstrated significant differences in these two variables (Campbell *et al.* 1999). However, histological examination revealed structural differences concerning the epithelial cells of perfused intestines compared to those from normal trout intestines, namely the separation of the lamina propria from the outer epithelial cells The lamina propria is a layer of loose connective tissue that supports the epithelium and accommodates glands; blood vessels; lymphatic vessels and may contain lymph nodes

(Freeman & Bracegirdle 1967). This separation can often be a function of histological procedures, however intact structure of epithelia and sub-muscular layers suggest that this separation may have been caused by a constituents within the luminal reservoir saline. Detergents or surfactants such as Tween 20 may solubilise tight junction within epithelial cells causing layers to separate on fixation and sectioning during histological procedures. Since this phenomenon was recorded in all cells and to the same extent at each astaxanthin dose (determined from cell counts) it is unlikely that this result was due to astaxanthin movement into lymphatics. Solubilisation of tight junctions would plausibly lead to progressive increases in permeability with exposure time. Schep *et al.* (1998) investigated the permeability of salmon (*Oncorhynchus tshawytscha*) posterior intestine to two hydrophilic markers in the presence and absence of the bile salt sodium deoxycholate (SDA) whose detergent action would mimic that of non-ionic surfactants. Not only did the presence of the bile salt increase the permeability of the markers, but also histological examination seemed to show the same separation of the lamina propria, although the authors made no comment on this. Other authors have found no significant effects of bile salt micelles on the permeability of intestinal preparations (Sallee *et al.* 1972). Indeed, cumulative uptake plots for astaxanthin in the current investigation show rapid initial uptake followed by saturation indicating uptake was independent of this effect. Nevertheless, such changes induced in epithelia raise concerns over the use of surfactants in *in vitro* studies.

### 6.4.2  Solubility of carotenoid

Solubility of astaxanthin was limited when dispersed with the surfactant (Tween 20) with maximal concentration not exceeding 3.5 mg $l^{-1}$. Furthermore, desired carotenoid concentration could not consistently be achieved due to unreliability in solubility.

Combination of these factors resulted in a dose response curve over a narrow range of concentrations with variability in averaged doses. However, based on the size range of fish used in these studies (250-400g) and feed consumption (2% BW feed; 50mg/kg carotenoid inclusion) the maximum intake of carotenoid (ca. 0.4mg) is similar to the range of doses investigated (0.45-1.35mg; based on luminal reservoir volume and astaxanthin concentration). Due to the limited solubility of astaxanthin in Experiment MIC, attempts were made to examine the uptake of astaxanthin at higher doses by using water miscible beadlets containing astaxanthin (Experiment EM). The surfactant (Tween 20) was also included in the medium to prevent bias in tissue viability. However, inconsistency in uptake results between different stock solutions prevented extensive use of this method of solubility (results presented for Experiment EM originate from one batch of stock solution). A reason for inconsistency in uptake between stock solutions in this way may be due to varied incorporation of astaxanthin from the emulsion into the included surfactant (Tween 20) although possible partitioning was not determined. Similarly, Garrett *et al.* (1999) found that almost one third of β-carotene from water miscible beadlets had failed to incorporate into micelles when an attempt was made to make a solution of higher β-carotene concentration than could be achieved with micelles alone.

Discrepancies in uptake rates at similar doses between Experiment MIC (Tween 20) and Experiment EM (water miscible beadlets) may be a result of differences in absorption of astaxanthin from the two different vectors. This phenomenon has been recorded by several groups that have used different vectors to study uptake of carotenoids *in vitro* (El-Gorab *et al.* 1975; Garrett *et al.* 1999b) and *in vivo* (MacMahon & Thompson 1969; Böhm & Bitsch 1999). These findings may suggest that absorption of carotenoid can be influenced by the use of different solubilising agents, yet whether this effect is biochemical and/or physiological remains to be validated. Such variation calls for methods of solubilisation

that are more physiologically representative e.g. bile salt micelles, before extrapolation of findings can take place from *in vitro* to *in vivo* situations.

### 6.4.3 Uptake of astaxanthin

Dose response curves for initial uptake rates of astaxanthin in both experiments showed a significant proportional increase in uptake rate with increasing astaxanthin dose. This would agree with findings *in vivo* where blood carotenoid concentration have been positively related to the carotenoid concentration of feed consumed (Choubert *et al.* 1994a; Storebakken & Goswami 1996) and would tend to agree with the hypothesis that the absorption of carotenoid occurs passively at those doses examined. Indeed, Kiessling *et al.* (1995) stated that the concentration gradient for astaxanthin between intestinal contents and blood of Atlantic salmon (*Salmo salar*) was influential on the uptake of this carotenoid.

The process by which astaxanthin transfers from the mucosal medium to the serosal perfusate is not clear from this model. However, in mammals *in vivo* it is believed that once absorbed by the intestinal enterocyte (believed to be a passive process), carotenoids are incorporated into chylomicrons before being secreted into the lymph for blood transport (Fur & Clark 1997; Van den Berg 1999). The assembly of chylomicrons is dependent on absorption of lipids by the enterocyte; cellular lipid (re)synthesis and translocation of cellular lipid pools; synthesis and post-translational modification of various apolipoproteins and the assembly of lipid and lipoprotein components into a chylomicron (Van Greevenbroeck & de Bruin 1998). Complex intracellular events such as these could plausibly become saturated at various stages resulting in reduced astaxanthin uptake. Hollander (1973) attempted to measure the translocation of a fat-soluble vitamin

164

(Vitamin K) across the intestine into the serosal fluid of mammalian (Sprague-Dawley rats) everted gut sacs. Results from this study suggested that uptake (determined from tissue concentrations) of this vitamin was via an active or facilitated transport system (saturable). Furthermore, translocation of the vitamin into the serosal fluid was minimal which may explain the saturable kinetics witnessed in the cumulative uptake plots from the current study. Csaky (1984) indicated that studies of lipid absorption with *in vitro* intestine systems are of limited usefulness as the epithelium rapidly accumulates the lipid and cannot transport it out of the absorptive cell. Because many lipids of biological importance are metabolised and incorporated into specific lipoproteins within the cell the rate of appearance of these particles at the serosal surface does not reflect the rate of absorption of the constitutive lipids across the intestinal brush border (Sallee *et al.* 1972). However, earlier studies (Loran *et al.* 1961; Loran & Althausen 1969) did demonstrate the transport of a fat-soluble vitamin across the intestine into serosal medium by including a solubilising agent (Tween 80) in the serosal medium. In the current study, Tween 20 was included in the serosal medium at a similar percentage to that in the luminal reservoir (0.3%). Percentage inclusion of this solubilising agent was not increased so as to avoid differences in osmotic gradient and technical difficulties associated with HPLC analysis.

There is evidence in the literature to suggest that lipid absorption in rainbow trout is quite different to that in mammals. Analysis of blood following force feeding with radiolabelled palmitic acid suggested that fish are incapable of delivering triglyceride to its circulation in the usual way and that most of the lipid is absorbed as free fatty acids, probably via the portal system (Robinson & Mead 1973). Indeed other authors have presented evidence that suggests fish do not have true lymphatics (Vogel & Claviez 1981; Vogel 1985). However Sire *et al.* (1981) demonstrated that fatty acids are re-esterified in the trout intestine mucosal cells and transported in very low density lipoprotein like structures.

Furthermore, later studies have isolated chylomicrons from fish serum (Rogie & Skinner 1981; Fremont *et al.* 1981; Sheridan *et al.* 1985). Sheridan *et al.* (1985) suggested that lipid absorption in fish may be a two-step process whereby soluble short chain fatty acids and carrier bound fatty acids are absorbed directly and a slower system where triacylglycerol (TAG) rich lipoprotein particles are formed. Since carotenoids and lipid absorption are associated, this potential route for absorption of carotenoids and its importance requires clarification.

It is interesting to note that the predicted Vmax values for tissue saturation of astaxanthin (0.1-0.3 $\mu$g g$^{-1}$) are similar to findings for intestinal tissue astaxanthin concentrations stated in a previous study by the author (Chapter 3; 0.2-0.9$\mu$g g$^{-1}$). However, data from that study suggested that there were significant differences between the ileal and hind intestine in terms of astaxanthin absorption. The current model used preparations that comprised both these regions and normalisation of data was based on total weight of the two. This may in part explain the large variation between replicates. As such future perfusion investigations should be carried out with isolated regions of intestine i.e. ileal and hind intestine to distinguish potential differences.

The perfusion system used in this investigation has been used successfully to describe the cumulative translocation of astaxanthin across the intestine of rainbow trout and determine initial uptake rates for this carotenoid. Limitations associated with tissue viability; solubility of the carotenoid in aqueous media and issues of variability in uptake between solubilising agents prevented more detailed interpretation of dose response investigations. Those processes that influence translocation of carotenoid across the intestine are obviously complex and difficult to interpret with the current model. As such future developments with similar *in vitro* models should be focussed on the use of preparations

that permit direct and rapid assessment of carotenoid absorption by intestinal tissue e.g.

everted gut sacs, and the use of more physiologically representative means of carotenoid

solubilisation such as bile-salt micelles.

# CHAPTER 7.0

# USE OF EVERTED GUT-SACS TO COMPARE THE ABSORPTION OF ASTAXANTHIN IN SALMONID FISH.

## 7.1    Introduction

The producers of high value salmonid species are in no doubt that the use of salmonids feeds which contain carotenoids and ultimately result in the red colouration of fish flesh is a vital asset to the market value of these fish. Indeed, feeding of pigments is regarded as the most important management practise for marketing of farmed salmon (Moe 1990). Furthermore, Torrissen & Christiansen (1995) have suggested that astaxanthin should be regarded as a vitamin for fish and should be included in feed to ensure the health of the animal. However, astaxanthin may account for 15-20% of the total feed cost or 6-8% of the total production cost of Atlantic salmon (Torrissen 1995). Consequently, knowledge of those factors that may affect the pigmentation is at the forefront of carotenoid research. A variety of biotic and environmental factors may influence the utilisation of carotenoids by salmonids. However, it is not well understood which factors, if not all, influence the absorption of carotenoids at the gastrointestinal level.

Changes in intestinal permeability and properties of the unstirred water layer are known to occur with ageing and will influence absorption of lipid-soluble nutrients (Hollander & Tarnawski 1984). Bjerkeng *et al.* (1992) studied the pigmentation of rainbow trout from start feeding to sexual maturation and found body redistribution of carotenoids coinciding with smoltification and sexual maturation. These authors suggested that these findings could be explained by changes in carotenoid absorption; transport capacity; affinity for carotenoid in various tissues or to catabolism. In mammalian models, size or age has been

shown to be an influential factor in the intestinal absorption of vitamin D (Hollander &

Tarnawski 1984) yet has no influence on the absorption of vitamin A (Hollander &

Morgan 1979). Studies have shown that rainbow trout below an average weight of ca.

150g do not efficiently deposit pigment in their flesh (Abdul-Malak *et al.* 1975; Torrissen

1985a). Similarly, juvenile Coho salmon (*Oncorhynchus kisutch*) of ca. 80g weight are

virtually unable to assimilate astaxanthin from the diet and deposit it in the flesh when

raised in fresh water (Arai *et al.* 1987).


Water temperature significantly influences growth and metabolism in fish (reviewed by

Smith 1989). As fish are poikilotherms, their metabolism and nutrient utilisation are likely

reduced at low temperatures. Commercial salmonid culture in outdoor ponds/lakes is

subject to both regional and temporal fluctuations in ambient temperature. A reduction in

environmental temperature increases the gastrointestinal holding time (Fänge & Grove

1979; Fauconneau *et al.* 1983) leaving more time for efficient digestion and potentially

counteracting reduced digestive enzyme activity. Indeed, the rate of gastric digestion in

fingerling sockeye salmon (*Oncorhynchus nerka*) expresses an optimum at ca 15°C (Brett

& Higgs 1970). However, in salmonids, some studies have reported lower apparent

digestibility coefficients (ADC) of nutrients with decreases in temperature (Atherton &

Aitken 1970; Brauge *et al.* 1995; Olsen & Ringø 1998; Médale *et al.* 1999) while others

have found no influence (Windell *et al.* 1978). A key study by No & Storebakken (1991)

recorded that rainbow trout kept at two different temperatures (15°C and 5°C) and fed

dietary astaxanthin tended to absorb astaxanthin (according to digestibility values) more

efficiently at the higher temperature, although pigmentation was not significantly different.


Genetic differences associated with intestinal uptake of fatty acids have been demonstrated

in mice (Keelan *et al.* 2000). Previous studies have demonstrated interspecific differences

between rainbow trout and Atlantic salmon in their ability to utilise and efficiently deposit ingested pigment (Foss *et al.* 1984; Storebakken *et al.* 1985; Storebakken *et al.* 1986;). Furthermore, compared with rainbow trout, Atlantic salmon have been shown to demonstrate more constant plasma astaxanthin concentrations regardless of dietary astaxanthin levels, indicating that the absorptive capacity of the salmon intestine may limit the amount of astaxanthin that can be advantageously added to their diet (March & MacMillan 1996).

The following study presents a series of experiments carried out to examine the effects of environmental (temperature) and biotic (size and species comparison) factors on uptake of astaxanthin *in vitro* by everted intestine sacs taken from rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*).

## 7.2 Materials and Methods

### 7.2.1 Experimental system

The everted gut-sac preparation used throughout the investigations was based on methodology of Wilson & Wiseman (1954). Everted intestines were mounted in a gassed (95% $O_2$; 5% $CO_2$) bath or luminal reservoir (15cm x 11cm) containing a physiological saline designed for trout (Al-Khalifa and Simpson, 1988) of composition (g $l^{-1}$): NaCl 7.37g; KCL 0.31g; $CaCL_2$ 0.17g; $MgSO_4$ 0.14g; $KH_2PO_4$ 0.46g; $Na_2HPO_4$ 2.02g; 0.1% glucose (pH 7.3). When Atlantic salmon intestine was used, the NaCl content of the saline was increased to 8.77g $l^{-1}$ (Nordrum *et al.* 2000). The everted intestines were laid on a polythene mesh support (15cm x 8cm) above a magnetic flea, rotated using a magnetic stirrer (HI 190M, HANNA Instruments, U.K.) to ensure efficient flow of oxygenated

170

saline across the intestinal mucosa. The preparation was maintained at a desired temperature (commonly $15 \pm 1°C$) below that of ambient (if required) by circulating chilled water around the luminal reservoir.

### 7.2.2 Preparation of a micellar solution

Simple micelles of sodium salt and carotenoid were prepared according to simplified methodology of Canfield *et al.* (1990). Approximately 30mg of crystalline astaxanthin were dissolved in 100ml of dichloromethane containing 25mg of BHT. The solvent was then evaporated in a 250ml round bottom flask almost to dryness (remaining wet film). 100ml of sodium taurocholate solution (0.1 M) were then added to the flask and the contents shaken vigorously. The turbid solution was then placed in a sonicating water bath at 50°C for 2hrs with occasional shaking whilst maintained within a fume hood. On completion of sonication the solution was made to a final volume of 1 litre by adding physiological saline. The mixture was then transferred to ultra centrifuge tubes and centrifuged at 4°C for 4hrs at 50,000 x $g$. Post centrifugation the clear micelle solution was additionally filtered (Whatman no.1).

The micellar solution (filtrate) was then analysed immediately for astaxanthin by adding 2ml of solution to 2ml of methanol and 6ml of dichloromethane in a 20ml glass vial. The contents were then vortexed for 30 seconds and placed in the darkness for a 10min extraction period. The samples were then centrifuged at 2010 x $g$ for 10min and 3mls of the resultant hypophase extracted and blown down under a gentle stream of nitrogen. Samples were either re-suspended in hexane and analysed by HPLC (see section 2.4.10), or, stored at -20°C prior to analysis. All analyses were performed in triplicate. Micelle

solutions were used within 24hrs to ensure stability. Observations to support micellar incorporation were as described by Garrett *et al.* (1999).

### 7.2.3 Preparation of the intestine

Fish were taken from the holding facility (see sections 2.2.2) and immediately culled via a blow to the cranium followed by destruction of the brain (Schedule I, Home Office Procedures). Fish weight was recorded. The gastrointestinal tract was promptly dissected from the fish and the stomach and pyloric region (indicated by the first pyloric diverticular) separated from that of the remaining intestine (ileal and posterior region), and discarded. The remaining intestine was immediately placed in 100ml glass beaker containing both chilled (5°C) and gassed (95% oxygen, 5% $CO_2$) physiological saline for an initial period of recovery. Fat deposits adhering to the intestine were carefully removed from the organ using tweezers and a scalpel. The intestine section was then everted on a stainless steel rod (300mm x 2mm) by securing the ileal end (suture) and rolling the proximal region over it. Once removed from the rod, the proximal end of the intestine was closed with suture and the anterior end secured to a syringe containing chilled physiological saline. The intestine was then filled with saline until distended and removed from the syringe whilst securing tightly. The preparation was then placed in the luminal reservoir containing 450ml of the micellar solution containing astaxanthin for a typical exposure period of 50min.

### 7.2.4 Carotenoid extraction from intestine

Following the exposure period intestinal sacs were removed from the micellar solution and thoroughly washed in a beaker containing saline and sodium taurocholate (10mM). The secured suture was then cut away and the serosal fluid drained and discarded, ensuring the

172

tissue was washed in saline simultaneously to avoid contamination. A section of ileal and posterior intestine were then separated; blotted dry; weighed separately and placed in 20ml glass vials. Methanol (2ml) and distilled water (2ml) was then added to the vials, and the contents homogenised. Dichloromethane (6ml) was then added to each fraction, followed by vortexing and a 10min extraction period in darkness. Vial contents were then given a final vortex and centrifuged at 2010 x $g$ for 10 min at 5°C. An aliquot of the resultant hypophase (3ml) was then blown down under a gentle stream of nitrogen and stored at – 20°C until analysis. Samples were re-suspended in 250μl of n-hexane before injection into the HPLC system for carotenoid quantification (see section 2.4.10).

### 7.2.5 Viability experiments

A number of parameters were assessed throughout the investigations to ensure the viability of the preparation and the subsequent value of the data obtained.

#### 7.2.5.1 *Peristaltic contractions*

Observations of sudden and spontaneous contractions are normal for intestine preparations and are usually taken as an indicator of physiological viability. Only when contracture was severe and prolonged was this considered as an indicator of decreased viability and the data was duly disregarded.

#### 7.2.5.2 *Tissue lactate-dehydrodgenase (LDH), potassium and moisture content*

Measurements of lactate dehydrogenase (LDH) and potassium content of normal (initial) and micelle exposed ileal and posterior intestines were compared ($n$=6). Briefly, ca. 0.5g

of tissue was homogenised in 8ml of distilled water. The homogenates were then centrifuged at 50, 000 X *g* for 30min. The supernatant was then assessed for potassium concentration using a flame photometer (CORNING 480). A 1 in 10 dilution was performed on the supernatant before assessment of LDH was carried out. A significant decrease in this cytosolic enzyme in tissue preparations would indicate tissue damage and reduced viability. The LDH assay was based on the kinetic pyruvate reduction method that detects the disappearance of NADH at 340nm (Sigma diagnostics DG1340-K, Poole, U.K.).

Moisture analysis of tissue samples was carried out by drying tissue samples as previously described (see sections 2.1.5)

### 7.2.6   Statistics

All data was analysed using StatGraphics Plus for windows (version 4.0). Data were subjected to tests for normality before carrying out statistical tests. A one-way ANOVA was used to compare the uptake of astaxanthin at different temperatures, between species and tissue viability measurements between control and exposed intestine. Regression analysis of astaxanthin uptake over time and uptake vs. fish weight was performed using Sigma Plot 4.0.

### 7.2.7   Experiment 1: Tissue saturation

In order to define a suitable experimental exposure period (within linear uptake range) an experiment was carried out to determine the uptake of an above average physiological dose ($5.1 \pm 0.2$mg $l^{-1}$) of dietary astaxanthin over a 1 hour exposure period with tissue uptake

assessment carried out after 30min and 60min. Six individual tissue preparations were analysed at both time points with both the ileal and posterior intestine assessed for astaxanthin content.

### 7.2.8 Experiment 2: Fish size effects

An experiment was carried out to determine whether rainbow trout from two different size classes (204-302g and 802-1689g) displayed differences in intestinal uptake capabilities for astaxanthin. Six preparations from each size class were exposed to a micelle solution of astaxanthin (4.3 ± 0.3mg $l^{-1}$) for a 50min period. Both ileal and posterior intestine were analysed for tissue accumulation of carotenoid

### 7.2.9 Experiment 3: Temperature effect

The effect of temperature on intestinal uptake of carotenoid was examined at three exposure temperatures (8°C, 15°C and 20°C) that may represent typical aquaculture rearing conditions through both temporal and spatial variation. Six intestinal preparations from rainbow trout were examined at each temperature with an exposure dose of 3.9 ± 0.1mg $l^{-1}$ for a period of 50min.

### 7.2.10 Experiment 4: Species comparison

In order to determine whether inter-specific differences in intestinal uptake of carotenoid exist between salmonid species, a comparison was made between rainbow trout and Atlantic salmon (*Salmo salar*). Seven intestinal preparations from each species were exposed to a micelle solution of astaxanthin for a period of 50min of concentration 4.3 ±

0.04mg $l^{-1}$ and 4.3 ± 0.05mg $l^{-1}$ for trout and salmon, respectively, after which they were assessed for tissue accumulation of astaxanthin.

## 7.3 Results

### 7.3.1 Viability

Assessment of viability criteria revealed that the gut-sac preparation remained physiologically viable throughout the 50min exposure period. LDH activity (Figure 7.1) was significantly higher (P<0.05) in the posterior intestine (ca.116-120 U $g^{-1}$) compared to the ileal intestine (ca. 74-79 U $g^{-1}$), regardless of whether the tissue had been exposed to the micelle medium (P=0.0037), or not (P=0.0001). However, no significant differences (P>0.05) were noted between micelle exposed and control intestinal samples from either the ileal (78.47 ± 9.92 and 73.79 ± 5.07 U $g^{-1}$, respectively) or posterior (119.54 ± 4.53 and 116 ± 4.00 U $g^{-1}$, respectively) region ($n$=6, ± SEM).

Interestingly, potassium concentration (Figure 7.2) was also significantly higher in the posterior region (ca. 57-64 µmol $g^{-1}$) compared to the ileal intestine (ca. 51-53 µmol $g^{-1}$) in both micelle exposed (P=0.0001) and control tissues (P=0.0254). Furthermore, potassium concentration in the exposed posterior intestine (63.58 ± 0.64 µmol $g^{-1}$) was significantly higher (P=0.0001) than in the control samples of posterior intestine tissue (57.48 ± 0.67 µmol $g^{-1}$). However, no significant differences (P>0.05) were recorded between the potassium content of the control (53.34 ± 1.43 µmol $g^{-1}$) and exposed (51.74 ± 1.53 µmol $g^{-1}$) ileal intestine.

No significant differences (P>0.05) were recorded for moisture content (Figure 7.3) between the ileal and posterior intestine region. Moreover, no significant differences (P>0.05) in moisture content were noted between control and exposed ileal (80.24 ± 0.30 and 81.33 ± 0.30 %, respectively) and posterior (79.52 ± 0.27 and 78.88 ± 0.27 %, respectively) regions (*n*=6, ±SEM).

### 7.3.2 Time saturation

In experiment 1, tissue astaxanthin concentration in the ileal and posterior intestine increased from time 0 (0.15 ± 0.12 and 0 μg g$^{-1}$, respectively) to 60min (2.64 ± 0.42 and 0.09 ± 0.02 μg g$^{-1}$, respectively; *n*=5, ± SEM) when exposed to astaxanthin micelles (Figure 7.4). The result of fitting linear regressions to the time saturation data suggests that uptake of astaxanthin by the ileal intestinal tissue followed a significant positive relationship (P=0.0045) over the 60 min period at the dose investigated (5.1 ± 0.2 mg l$^{-1}$). Interestingly, uptake of astaxanthin by the posterior intestine showed no similar significant trend (P>0.05). Furthermore, tissue astaxanthin concentrations in the posterior intestine were significantly lower compared to the ileal intestine at 30min (P=0.0001) and at 60min (P=0.001). For experiments 2-4 doses of astaxanthin were kept below 5mg l$^{-1}$ (~4mg l$^{-1}$) and exposure time was limited to 50min (all results recalculated to express uptake over 60min). This ensured astaxanthin uptake would be compared within the linear phase according to the results from experiment 1.

### 7.3.3 Size effects

Comparisons of intestinal uptake by two different size classes of rainbow trout in experiment 2 (Figure 7.5) revealed no significant relationships between fish weight and

uptake at the 90% or higher confidence level, with P-values of 0.1032 and 0.2961 for the ileal and posterior intestine, respectively. The correlation coefficient between fish weight and intestinal uptake for the ileal intestine was –0.52 indicating a moderately strong relationship between the variables. A relatively weak relationship was noted between uptake for the posterior intestine and fish weight with a correlation coefficient of -0.33. Again, astaxanthin uptake by the posterior intestine was significantly (P=0.039) lower than the ileal intestine across the size range of trout used.

### 7.3.4   Effect of temperature

In experiment 3, uptake of astaxanthin by the ileal intestine (Figure 7.6) was significantly higher (P=0.0122) at 20°C (2.12 ± 0.56 μg g$^{-1}$) and 15°C (2.04 ± 0.27 μg g$^{-1}$) compared to that at 8°C (0.63 ± 0.08 μg g$^{-1}$; $n$=6, ± SEM). There was no significant difference (P>0.05) in astaxanthin uptake for the ileal intestine at 15°C compared to 20°C. However, no significant differences (P>0.05) were recorded for astaxanthin uptake for the posterior intestine between the three exposure temperatures. Astaxanthin absorption by the ileal intestine was significantly higher (P=0.0039) than astaxanthin uptake by the posterior intestine at all three temperatures.

### 7.3.5   Species differences

Comparison of intestinal uptake between salmonid species in experiment 4 (Figure 7.7) revealed no significant differences in uptake (P>0.05) for either the ileal or posterior intestine regions. However, uptake by the ileal intestine for Atlantic salmon (0.36 ± 0.09 μg g$^{-1}$) expressed a mean value that was ca. 41% lower than that noted for rainbow trout

$(0.62 \pm 0.09\ \mu g\ g^{-1})$. Uptake by the posterior intestine was again significantly lower than that of the ileal region for both trout $(P=0.0064)$ and salmon $(P=0.0104)$.
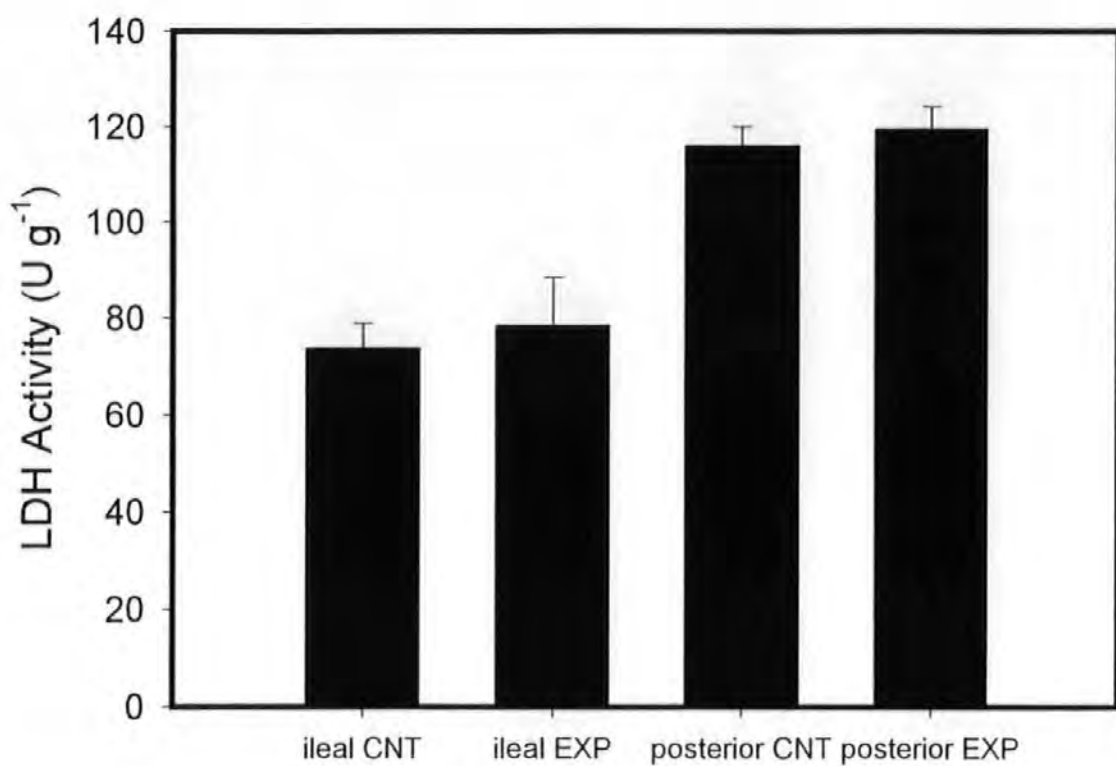
Figure 7.1    Tissue LDH activity of control (CNT) and micelle exposed (EXP) ileal and posterior intestine sections of everted gut sacs from rainbow trout ($n$=6, ± SEM).
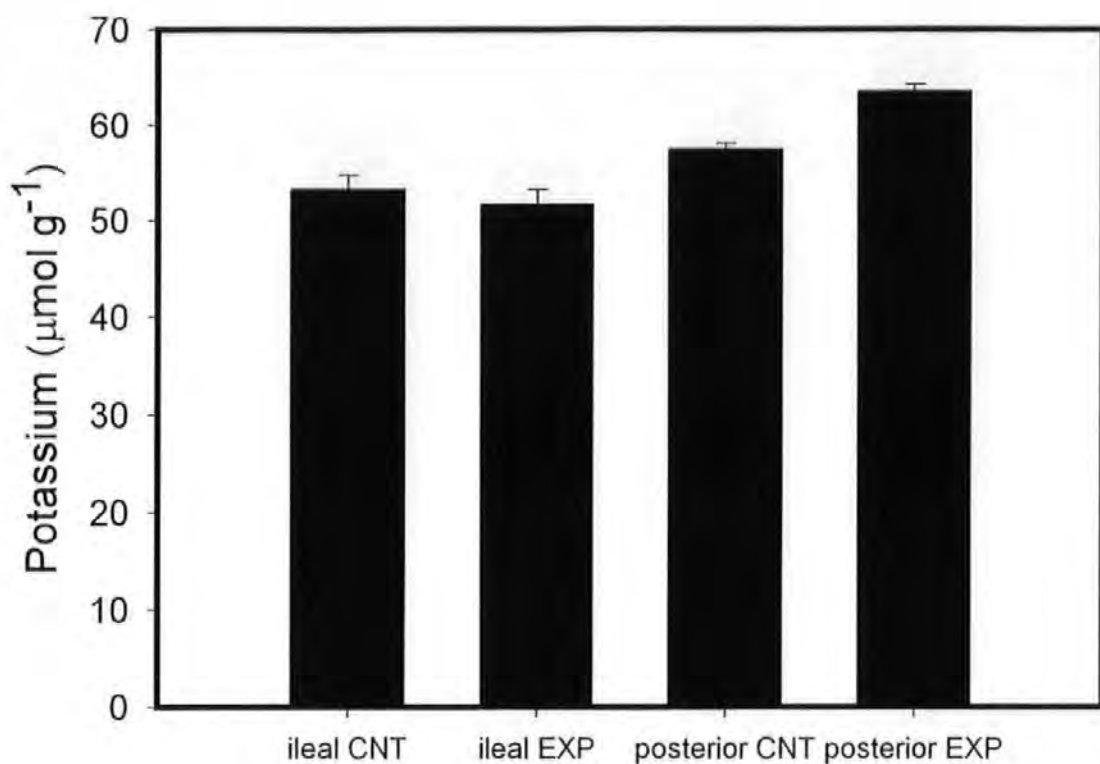
Figure 7.2    Tissue potassium concentration of control (CNT) and micelle exposed (EXP) ileal and posterior intestine sections of everted gut sacs from rainbow trout ($n$=6, ± SEM)
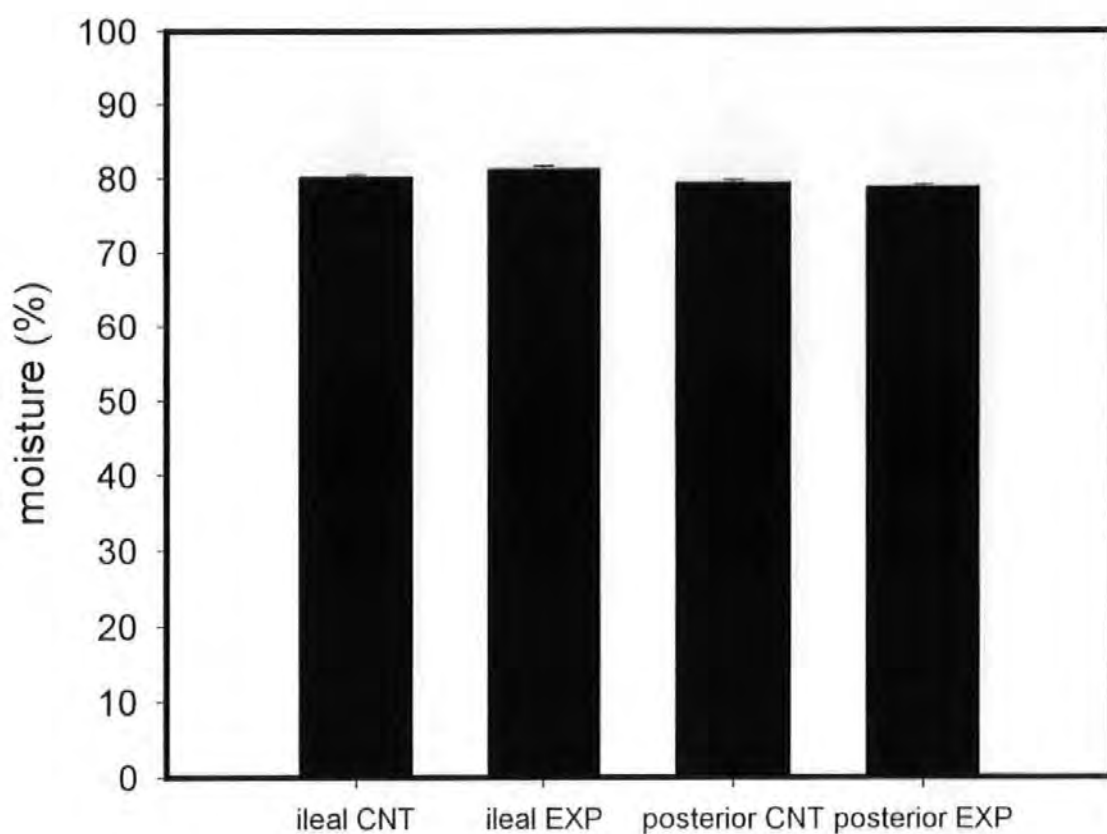
Figure 7.3    Tissue moisture content of control (CNT) and micelle exposed (EXP) ileal

and posterior intestine sections of everted gut sacs from rainbow trout ($n$=6, ± SEM).
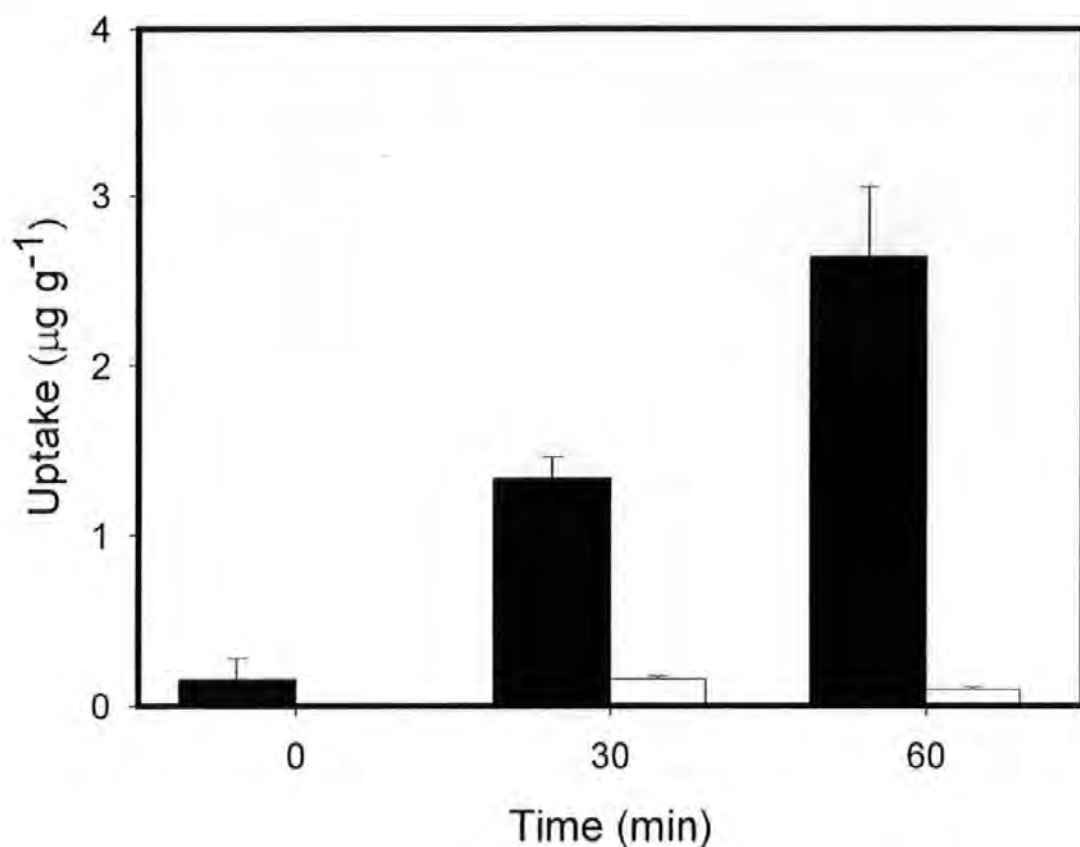
Figure 7.4    Uptake of astaxanthin by everted gut sacs of rainbow trout over time. Open bars represent astaxanthin uptake by the posterior intestine and filled bars represent uptake by the ileal intestine ($n$=6, ± SEM). Astaxanthin dose was 5.1 ± 0.2 mg l$^{-1}$ at 15 ± 1°C. Uptake at time 0 represents the astaxanthin concentration in intestinal tissue of control fish ($n$=3, ± SEM). Linear regression analysis of raw data gave a significant relationship (P=0.0045) between time and uptake ($R^2$=66%; correlation coefficient=0.812006; uptake=0.100909+0.0421212*time) for the ileal intestine. No such relationship was recorded for the posterior intestine (P=0.9108).
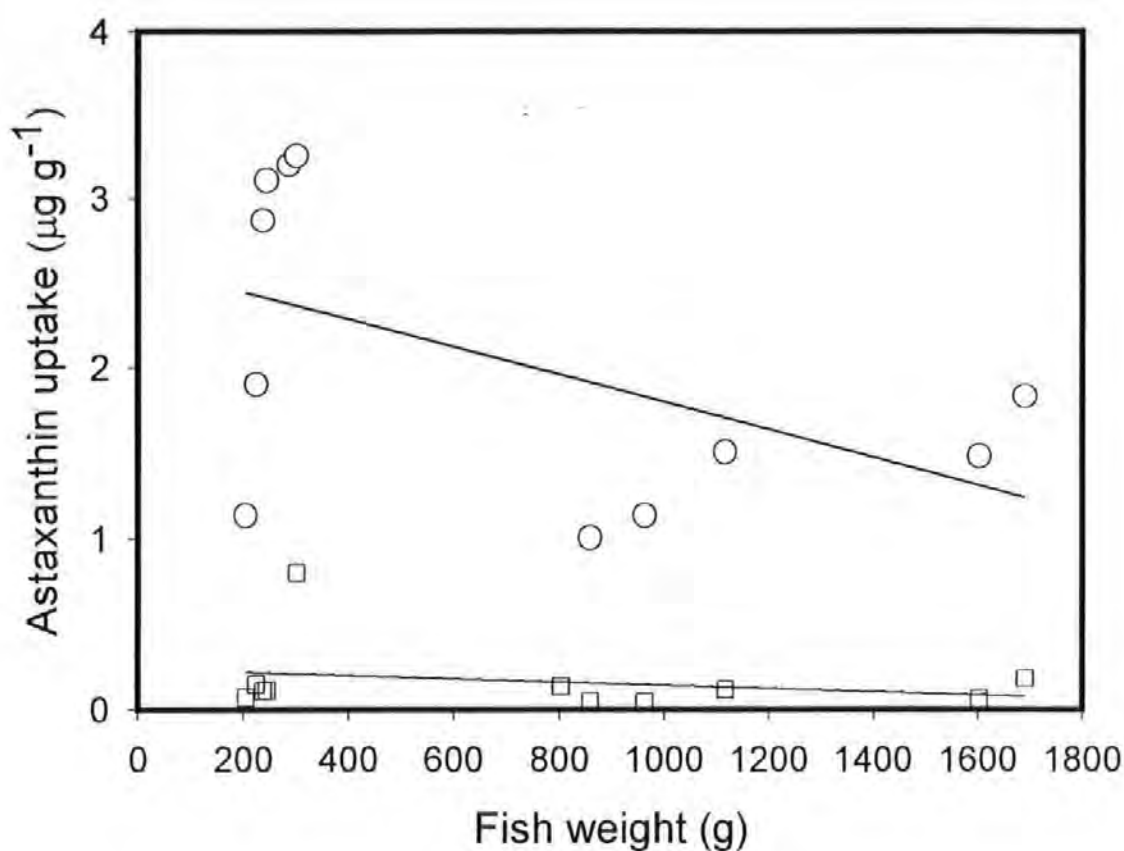
Figure 7.5    Astaxanthin uptake by everted gut-sacs of rainbow trout as a function of fish weight. Open circles and open squares represent astaxanthin uptake by the ileal and posterior intestine, respectively. Astaxanthin dose was $4.3 \pm 0.3$ mg $l^{-1}$ at $15 \pm 1°C$. Regression analysis of uptake vs. fish weight for the ileal and posterior intestine showed non-significant relationships (P>0.05).
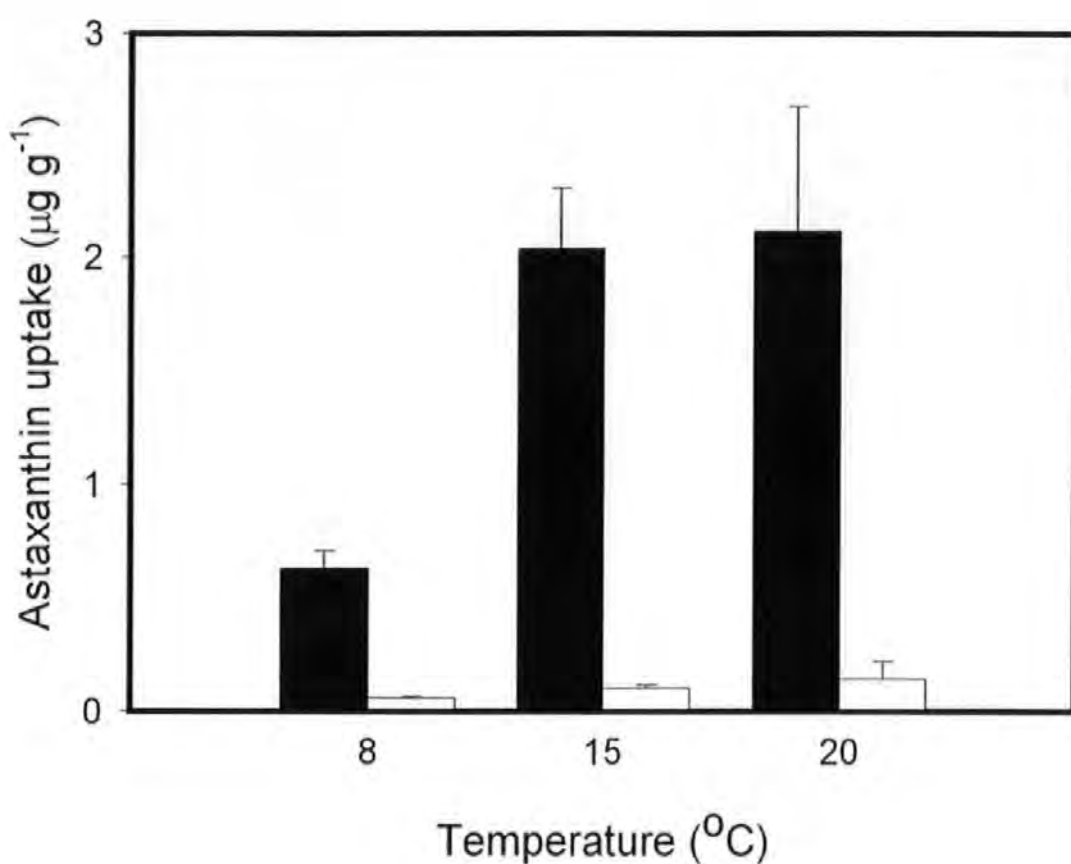
Figure 7.6    Uptake of astaxanthin by everted gut-sacs of rainbow trout under different temperatures. Open and filled bars represent astaxanthin uptake by the posterior and ileal intestine, respectively ($n$=6, ± SEM). Astaxanthin dose was $3.9 \pm 0.1$ mg l$^{-1}$.

Figure 7.7    Astaxanthin uptake by everted gut sacs of rainbow trout and Atlantic salmon. Open and filled bars represent astaxanthin uptake by the posterior and hind intestine, respectively ($n$=7, ± SEM). Astaxanthin dose was 4.3 ± 0.04 mg $l^{-1}$ and 4.3 ± 0.05 mg $l^{-1}$ for trout and salmon respectively at 15 ± 1°C.

## 7.4 Discussion

In the current study a series of experiments was carried out in order to evaluate the potential of an *in vitro* gut sac technique to monitor the absorption of astaxanthin. Previous efforts with an everted gut perfusion system were limited due to an inability to effectively evaluate the translocation of carotenoid across the intestinal barrier. Furthermore, efforts have been made in the current study to solubilise astaxanthin in a near physiologically representative medium, namely bile salt micelles. Micelles were not formulated to incorporate additional fatty acids (mixed micelles) as information relating to typical fatty acid compositions of digesta from salmonids, is lacking.

Measurements of LDH activity have been used previously in other *in vitro* models as an assessment of cellular integrity (Campbell *et al.* 1999; Pfitzner *et al.* 2000). Similarly, damaged intestinal tissue will leak potassium down an electrochemical gradient into the surrounding medium and is a function of anatomical position along the length of the gut (reflecting potassium absorption: Loretz 1995). However, other authors have shown that in African catfish (*Clarias gariepinus*) tissue potassium concentration was not position-dependent and therefore provides a better overall marker of potassium loss (Handy *et al.* 2000). A measurement of water content in the tissue will indicate a net flux of water if the tissue is not in osmotic balance within the medium it is contained. However, these tissue viability assays carried out in this investigation showed no significant deleterious effects of exposing the intestine to the micelle medium within the experimental period (50min) suggesting uptake of astaxanthin into tissue occurred under nominal conditions.

The uptake of astaxanthin over a 60min period (Figure 7.4) seemed to follow a linear trend at a higher than average physiological dose ($\sim$5 mg $l^{-1}$). This may suggest a passive

diffusion process of uptake. However, use of active uptake inhibitors is required before conclusions can be drawn as to the uptake mechanism of carotenoids at the gastrointestinal level. For the purposes of this study, this experiment served to identify an appropriate exposure period and suitable astaxanthin concentration for comparative uptake measurements within the linear range. An exposure period of 50min and dose of 4mg $l^{-1}$ was chosen to ensure linearity in uptake data for comparative purposes.

Tissue concentrations of astaxanthin in this study were recorded above 2µg $g^{-1}$ which are higher than findings *in vivo* (see Chapter 4). This may be partly due to the high "bioavailability" of astaxanthin in micelle media used in the present investigation, compared to *in vivo* where only a fraction of ingested astaxanthin may well be solubilised into micelles (data unavailable). Alternatively, after incubation in the astaxanthin micelle medium, astaxanthin may have been strongly bound to the mucosal cells of the ileal intestine. However, extensive washing with saline containing bile salts did not release significant amounts of astaxanthin. Other authors have found that a percentage of carotenoid presented in this way is surface bound rather than intracellular (El-Gorab *et al.* 1975). Refinements to the current method to distinguish between surface adherent and available substrate include washing with solvents (El-Gorab *et al.* 1975) and use of non-absorbable markers (Salle *et al.* 1972). However, since radiolabelled astaxanthin is difficult to obtain the solvent extraction techniques and forms of analysis (HPLC) used in the current investigation would restrict the choice of an appropriate marker.

Throughout this study a clear discrepancy was recorded between the uptake capacity of the ileal and posterior region of intestine for astaxanthin. Evidence from these *in vitro* experiments suggests that the ileal intestine has a greater affinity for astaxanthin uptake compared to that of the hind or posterior intestine. Indeed, this is supported by the view of

Torrissen (1986) and Al-Khalifa and Simpson (1988) and the current study (see Chapter 4) that absorption of carotenoids occurs mainly along the proximal and mid-intestine of salmonids. This variation in nutrient uptake along intestinal regions is not limited to carotenoids and has been recorded for the amino acids in rainbow trout and Coho salmon, *Oncorhynchus kisutch* (Marcotte & de la Noüe 1984; Collie 1985). Discrepancies in absorptive capacity for astaxanthin along the length of the intestine, necessitates consideration of those factors associated with salmonid feeding practises that influence gut transit time. The feeding level and composition of the diet is a major determinant influencing digestion rate and thereby indirectly affecting absorption efficiency (Jobling 1986).

No significant relationship was recorded between fish weight and uptake of astaxanthin for either the ileal or posterior intestine regions. Uptake values were expressed per gram of intestinal tissue (wet weight) suggesting that there is no physiological change in the intestine associated with astaxanthin absorption, with development and maturation in the rainbow trout. A study that investigated the pigmentation of two size groups of Arctic charr (*Salvelinus alpinus* L.) using dietary canthaxanthin found that those fish which started at a smaller weight (17g), deposited relatively less pigment than those fish starting at a larger weight of 125g (Christiansen & Wallace 1988). Furthermore, the apparent digestibilities of canthaxanthin were 17.9% and 38.7% for the small and large fish, respectively. This suggested that the poorer pigmentation of the smaller size class was a result of reduced digestibility of carotenoid. Similarly, Hollander & Dadufalza (1989) measured the uptake of vitamin E into the lymph and bile of young (4 months), middle-aged (14 months) and old (24 months) Sprague-Dawley rats *in vivo*. These authors recorded increased absorption of this vitamin in old (16,467pmol 5hr$^{-1}$) compared to young (5912pmol 5hr$^{-1}$) rats. However, these findings may be due to a direct increase in

189

intestinal length (and surface area) with increasing size of the animal. In contrast, vitamin A absorption has been shown to increase linearly with age in rats but changes in the absorptive surface area of the intestine were ruled out (Hollander & Morgan 1979). Instead, the authors suggested that a decrease in the unstirred water layer thickness with ageing may have resulted in greater efficiency of absorption associated with age, a phenomenon found with vitamin D (Hollander & Tarnawski 1984). Indeed, Wilson *et al.* (1971) suggested that the unstirred water layer is rate limiting for intestinal absorption of lipids from micellar solutions. Results from the current study suggest that there is no difference in the uptake capacity of the trout intestine for astaxanthin between size classes and it is not clear whether the unstirred water layer is as influential in the absorption of carotenoids, in fish. However, only two size classes of rainbow trout were examined in the current study. Since Torrissen *et al.* (1989) suggest that rainbow trout under 90g pigment poorly, fish within this size range could be examined to define whether the poor pigmentation is associated with an inability to absorb astaxanthin at the gastrointestinal level.

Uptake of astaxanthin was significantly higher for intestines (ileal region) maintained at 20°C and 15°C compared to those at 8°C. These findings are supported from evidence *in vivo* where nutrient absorption has been shown to be greater at higher temperatures. For example, Médale *et al.* (1999) studied the utilisation of a carbohydrate-rich diet by common carp reared at 18 and 25°C. These authors noted that the apparent digestibility coefficients of energy and nutrients including starch were higher at 25°C. Olsen and Ringø (1998) demonstrated that the digestibility of protein, carbohydrate, lipid and dry matter was lower in Arctic charr kept at 0.6°C compared to 10°C suggesting that increased gastrointestinal holding time following low temperature adaptation does not fully compensate for lower digestive/absorptive metabolism. Maintaining the fish at 0.6°C also

significantly reduced the ADC of saturated fatty acids, while the monounsaturated and polyunsaturated fatty acids were not influence by environmental temperature. The absorption of the fat-soluble vitamin K *in vitro* is influenced by temperature (Hollander 1973). However, the latter study demonstrated that this temperature effect was due to the requirement of an energy requiring process for vitamin uptake. Available evidence suggests the absorption of carotenoid is via a passive process (Choubert *et al.* 1994a; Kiessling *et al.* 1995; Storebakken & Goswami 1996). This suggests that the effect noted in the current investigation may be due to the well-established $Q_{10}$ effect that may well affect transporters, much like enzymes. However, speculations concerning the process of astaxanthin uptake are unfounded until *in vitro* experiments, in the presence of oxygen free environments and with active inhibitors, are carried out.

Although no significant differences were found between intestinal uptake of astaxanthin between rainbow trout and Atlantic salmon, mean uptake was notably higher for rainbow trout (41%). Clearly, the use of a larger sample number ($n>7$) is required to define any differences that exist. Indeed, March and MacMillan (1996) suggested that the absorptive capacity of the Atlantic salmon intestine limited the amount of astaxanthin that could be advantageously added to their diet. Similarly, Storebakken *et al.* (1986) found that salmon utilised canthaxanthin less efficiently in comparison to rainbow trout. Torrissen *et al.* (1989) on the basis of reviewed evidence stated that the digestibility of astaxanthin was greater in rainbow trout (91-97%) compared to Atlantic salmon (45-74%). Indeed, findings from our own laboratory suggest that the digestibility of astaxanthin is higher in rainbow trout (ca. 96%) compared to Atlantic salmon (ca. 35%; G. Page pers. comm.). Certainly, the current findings suggest that there is greater scope for the examination of carotenoid uptake between species using the current model and established *in vivo* protocols.

The use of everted gut sacs is favourable for the study of astaxanthin uptake compared to isolated intestinal perfusion studies owing to a number of advantages. The uptake of astaxanthin can be measured directly from tissue accumulation, which presents a significant quantity for reliable HPLC analysis (although samples do have to be concentrated). Samples can be re-suspended in hexane without precipitation from a solubilising agent, plus numerous preparations can be analysed within a comparatively shorter space of time. The advantageous characteristics of this *in vitro* model have allowed the successful examination of astaxanthin uptake at the intestinal level, under varying abiotic (temperature) and biotic (fish size and species) conditions.

# CHAPTER 8.0

## GENERAL DISCUSSION

This study has encompassed a large number of themes related to the absorption and utilisation of astaxanthin forms in artificial feeds for salmonids, as demonstrated for trout and salmon. More specifically, the evaluation of a microalgae source of astaxanthin, namely *Haematococcus pluvialis*, has been undertaken. Scientific examination and assessment of those biological features of the algae and indeed physiological factors related to the gastrointestinal system of the rainbow trout that may limit the efficacy of this pigment source have been addressed. Furthermore, owing to limitations associated with *in vivo* measurements of digestibility and absorption, efforts have been made to develop an *in vitro* model suitable for studying the absorption of carotenoids at the gastrointestinal level in salmonids.

The following discussion begins by individually considering those factors (both biological and physiological) that have been shown to limit the use of *Haematococcus pluvialis* as a source of pigment in salmonid feeds; their relative importance and potential strategies to further improve or validate this carotenoid source. Secondly, the value of *in vitro* models to evaluate the absorption of carotenoids and the current findings from their use are discussed. Possible future experiments and their importance to commercial pigmentation strategies are proposed throughout.

## 8.1 Aplanospore cell wall of *Haematococcus pluvialis*.

There is a general consensus that the aplanospore cell wall of *Haematococcus pluvialis* is a major limiting factor in the utilisation of this algal source of astaxanthin (Johnson & An 1991; Choubert & Heinrich 1993; Sommer *et al.* 1991, 1992; Bowen *et al.* pers. comm.). Subsequently, techniques that can be applied to fracture or crack the cell wall need to be used before the alga is supplemented into animal feeds. Such techniques might include milling, enzyme treatment, homogenisation, or pressure treatment as was examined in Chapter 3. However, a dearth of published information regarding this topic, due to patent and commercial obligations, prevents comparisons being made on the efficiency of these techniques.

Some investigations that have examined the utilisation of carotenoid from *Haematococcus pluvialis* in salmonids have made efforts to crack this cell wall before feed supplementation, where others have not. For example, Sommer *et al.* (1991) used a confidential homogenisation step on part of a concentrated biomass of algal spores and found with microscopic cell counts that ca. 60% of the cells were completely disrupted. Sommer *et al.* (1992) homogenised the total algal spore biomass prior to feed supplementation but failed to report the level of cell disruption with similar evidence from cell counts. However, Choubert & Heinrich (1993), in a feed trial with rainbow trout, used algal spores that had not been homogenised. All of these studies reported poorer levels of pigmentation and flesh retention when using the algae source in comparison to the synthetic source of astaxanthin. However, Barbosa *et al.* (1999) tested an algal biomass that was mechanically ground in a ball grinder for 5min to disrupt the cell wall and assessed grinding efficiency by microscopic observation. These authors noted that the absorption of astaxanthin into serum of rainbow trout was similar for the algal and a

synthetic source at a higher dietary lipid level but was better for the algal source at a lower dietary lipid level.

Data from the current investigations suggest that "efficient" cracking of the cell wall renders contained pigment bioavailable to the fish. Furthermore, the degree of flesh pigmentation when the algal source is used in feed for rainbow trout is comparable to that of the synthetic source of astaxanthin (providing the cell wall is cracked efficiently). Indeed, use of an alga carotenoid extract (removed from the aplanospore) does not result in improved levels of absorption or pigmentation in comparison to the efficiently cracked cell when used in salmonid feeds. Moreover, the presence of a cell wall, although cracked prevents oxidation of contained pigment once supplemented into the feed. This ensures that salmonid feeds exhibit a stable astaxanthin profile over prolonged storage periods prior to use. Furthermore, methodology utilised in the current study to extract the carotenoid from the aplanospores (solvent extraction) would be impractical and uneconomic on a commercial scale.

In the current study, the commercial source of *Haematococcus pluvialis* (NatuRose, Cyanotech, Hawaii; 95% of cells cracked) proved a more efficient pigment source than the algal cells disrupted by pressure treatment (confidential process), in comparison to the commonly used synthetic source of astaxanthin. This was despite electron microscopic examination having been utilised to assess efficacy of cell wall cracking. Although, microscopic examination can reveal the extent to which cells have been ruptured, it is not a real measure of bioavailability. Feeding trials and measurements of pigmentation, such as the ones employed in the current investigations, are costly, time consuming and confounded by physiological and metabolic processes that as yet are not understood. Subsequently, a rapid screening approach is required that measures the relative

bioavailability of pigment from a biomass of cracked cells and that would give greater biological meaning to how "efficient" a cracking technique is. Leaching measurements into polar solvents such as acetone have been used previously to assess the relative bioavailability of carotenoid from aplanospores (Mendes-Pinto *et al.* 2001). This technique has been used to quantitatively estimate the amount of pigment that is potentially bioavailable. However, whether such an approach is physiologically representative is highly questionable. Other authors have assessed carotenoid bioavailability from meals by using *in vitro* digestion methods (Garrett *et al.* 1999a; Garrett *et al.* 2000; Ferruzzi *et al.* 2001). In these studies, natural food items containing carotenoids e.g. carrots and tomato pastes are incubated under those conditions experienced in the stomach and small intestine of adult humans, consecutively. This involves sequential treatments with different enzymes and varying pH that simulate the digestion process. The aqueous fraction that contains micelles is then separated from the digesta by ultracentrifugation and the amount of micellar incorporated carotenoid quantified by HPLC. Further to this, the carotenoid containing micelles can then be exposed to human intestinal cell lines for a relative measurement of absorption. Such techniques could be readily adapted to mimic the digestive processes in fish. Indeed, previous efforts have been made to simulate, *in vitro*, the conditions found in the alimentary tracts of rainbow trout and carp to study the digestibility of proteins of broad bean and soya bean feed supplements (Grabner & Hofer 1985). It is envisaged that these *in vitro* digestion techniques could be used to measure the bioavailability of carotenoid from *Haematococcus pluvialis* aplanospores, thus presenting a physiologically representative technique to evaluate the efficiency of a cell cracking procedure.

## 8.2 Astaxanthin esters

The current investigations have generally shown that the use of dietary astaxanthin esters does limit astaxanthin absorption into the serum of rainbow trout compared to the unesterified form. This is supported by findings from both steady state serum astaxanthin concentrations and rates of absorption. This supports the suggestion that the requirement for intestinal hydrolysis of astaxanthin esters probably is a limiting factor in the absorption of astaxanthin. Moreover, the degree of esterification is negatively related to the serum absorption of astaxanthin so that astaxanthin from monoesters is absorbed more efficiently than from diesters. This may be an important observation when attempting to compare findings from different studies and when evaluating natural sources of astaxanthin. Although it has been reported that astaxanthin in *Haematococcus pluvialis* is primarily monoesterified (Renstrom & Liaaen-Jensen 1981) Choubert & Heinrich (1993) reported a higher astaxanthin diester (ca. 29%) compared to monoester (ca. 12%) content in the algal biomass they used. Moreover, crustacean sources of astaxanthin have been shown to predominantly contain astaxanthin diesters (Torrissen *et al.* 1981; Arai *et al.* 1987; Mori *et al.* 1989). Other authors have examined the utilisation of astaxanthin dipalmitate (diester) and have drawn conclusions regarding the efficacy of astaxanthin esters *per se* in the pigmentation of salmonids (Storebakken *et al.* 1987; Foss *et al.* 1987). Certainly there is potential, at least for *Haematococcus pluvialis*, to be manipulated at the culture stage to increase the astaxanthin monoester content in comparison to the diesterified form. For example, other authors have stated that the monoester: diester ratio in *Haematococcus pluvialis* is a function of the age of the culture (Harker *et al.* 1996b) whereby younger cultures have a relatively higher monoester content.

Apparent digestibility coefficients (ADC) for astaxanthin esters and unesterified astaxanthin in the current investigation also seem to suggest that the absorption of unesterified astaxanthin is more efficient. Furthermore, generally the trends in ADC seemed to correlate with the findings in the serum. However, evidence suggests that a combination of both measurements is required to avoid misleading conclusions regarding absorption.

These combined findings would generally suggest that the hydrolysis of astaxanthin esters likely occurs in the lumen of the intestine prior to absorption into the blood. The pancreas and or the intestinal mucosa are the likely sources of this hydrolytic activity (Mathias *et al.* 1981; Rigtrup & Ong 1992; Tengjaroenkul *et al.* 2000). Erlanson & Borgström (1968) demonstrated through gel filtration of rat pancreatic juice that pancreatic lipase was active in hydrolysing vitamin A palmitate. Similarly, fractions that contained esterolytic activity against a variety of water soluble vitamins also hydrolysed bile salt solutions of vitamin A palmitate. The latter enzyme was a carboxyl ester hydrolase of broad specificity which encompasses the activity of "cholesterol esterase", otherwise known as "non-specific lipase". Indeed, non-specific esterase is used in some methodologies to hydrolyse carotenoid esters prior to analysis (Jacobs *et al.* 1982). This raises the potential to use a non-specific esterase as a feed additive when using esterified forms of astaxanthin in order to facilitate the intraluminal hydrolysis of astaxanthin esters. However, the cost and viability of such a strategy might out-way the benefits of using the natural source of esterified astaxanthin compared to the synthetic unesterified form. Therefore, future scientific assessment of this strategy is required.

Added to the requirement for intestinal hydrolysis of astaxanthin esters prior to absorption into the blood the current investigations have shown a clear regional disparity in esterolytic

198

activity along the gastrointestinal tract of the rainbow trout. Evidence suggests that the majority of astaxanthin ester hydrolysis take place in the pyloric region of the trout gastrointestinal tract. This physiological finding may in part limit further the absorption of astaxanthin from an esterified source depending on gut transit rate of the feed bolus as has been suggested for other nutrients (Staples & Nomura 1976; Pedersen 1987; Cho & Kaushik 1990). Consequently, efforts need to be made to determine the relative kinetics of ester hydrolysis for both monoester and diester forms within the salmonid intestine to establish optimum residence times for the carotenoid supplemented feed bolus. Practically, an *in vitro* approach incorporating intestinal homogenates from various regions of the gastrointestinal tract would be the first step (Koven *et al.* 1994, 1997). Additionally this would confirm the role and comparative importance of the various gastrointestinal regions in ester hydrolysis.

Various factors are known to influence the gut transit time of ingested food including temperature (Brett & Higgs 1970); dietary lipid (Jobling 1986) and feeding frequency (Cho & Kaushik 1990). Plausibly, such factors could be manipulated in feed trials to prolong the residence time of any given feed bolus and subsequently maximise the time available for carotenoid ester hydrolysis. However, it is important not to maximise carotenoid ester hydrolysis at the expense of salmonid growth or final carcass composition. For example, reduced temperatures result in poorer growth rates in salmonids (Azevedo *et al.* 1998) and dietary lipid has obvious consequences in relation to consumer acceptance. However, manipulations of feeding frequency might offer a suitable strategy to maximise the absorption of astaxanthin when supplied in an esterified form. Rather than feeding little and often, one larger single meal may be more effective in this respect. This approach requires evaluation in a series of feeding studies with suitably sized fish.

## 8.3    Chirality of astaxanthin

*Haematococcus pluvialis* contains optically pure astaxanthin (3*S*,3'*S*; Renstrøm *et al.* 1981) unlike synthetic astaxanthin which is a racemic mixture of optical isomers. The current study has noted that the optical purity of astaxanthin esters does not significantly influence the deposition of astaxanthin in the muscle of rainbow trout in comparison to the synthetic unesterified product (CAROPHYLL© Pink; F. Hoffmann La Roche, Basel, Switzerland). This suggests that evaluation of a novel astaxanthin source need not require analysis of enantiomer composition since optical isomers are deposited in the flesh in approximately the same ratio as they are found in the feed. However, some authors have suggested possible stereo-selectivity of ester hydrolysing enzymes within the intestine of the salmonid which do not favour the (3*S*,3'*S*) form (Foss *et al.* 1987). Data from the current study does not support this suggestion although direct examination of such selectivity was not carried out. Either way, this suggestion is an interesting concept that could be ultimately examined in the future using the *in vitro* digestion model discussed previously.

## 8.4    Discrepancy between astaxanthin absorption and deposition

Despite differences in measurements of absorption between astaxanthin supplied as dietary esters or in its free form, no obvious differences were recorded between flesh pigmentation in trout fed diets supplemented with *Haematococcus pluvialis* and synthetic unesterified astaxanthin. The one exception to this was fish fed diets supplemented with cells cracked under different pressures (see Chapter 3) although this is likely due to an inefficiency in the cracking technique as the commercial source of *Haematococcus pluvialis* proved

effective. This raises obvious questions regarding measurements of absorption and their value in predicting the efficacy of a source of pigment.

Choubert *et al.* (1994a) has shown positive linear correlations between plasma astaxanthin concentration and dietary astaxanthin concentration up to 200mg kg$^{-1}$ in rainbow trout. Indeed, Storebakken & Goswami (1996) have demonstrated that measurements of plasma carotenoid (astaxanthin) concentrations were highly correlated with dietary astaxanthin concentration (r = 0.90) in Atlantic salmon. This suggests that plasma concentrations are good indicators of dietary astaxanthin content. Indeed, Storebakken & Goswami (1996) also demonstrated that plasma astaxanthin concentrations were highly correlated with the amount of astaxanthin retained in the flesh per kg of body weight (r = 0.92) and flesh carotenoid level (r = 0.95). This suggested that plasma astaxanthin concentration can be used as a useful indicator of astaxanthin availability and flesh deposition in salmon and thus can serve as a rapid screening test for different carotenoid sources. However, Wathne *et al.* (1998) found that the total carotenoid content in the blood plasma was not a useful indicator of final muscle astaxanthin concentration. However, these authors fed alternate meals with and without astaxanthin that may have resulted in fluctuating levels of blood plasma astaxanthin.

Several authors have suggested that there is a limit or saturation level for the deposition of astaxanthin in trout muscle (Torrissen 1989b; Storebakken *et al.* 1987; Nickell & Bromage 1998b). Moreover, several groups have reported a dose response effect with dietary carotenoid and deposition whereby there is a plateau reached when feed inclusion levels are ca. 60mg kg$^{-1}$ (Choubert & Storebakken 1989; Bjerkeng *et al.* 1990; Torrissen 1995; March & MacMillan 1996).

There are large variations among salmonid fish species in the capability to deposit carotenoids in the muscle. For example muscle astaxanthin concentrations as high as 60mg kg$^{-1}$ have been reported in sockeye salmon, *Oncorhynchus nerka* (Turujman *et al.* 1997) compared to the more modest levels (ca.1-6mg kg$^{-1}$) reported for rainbow trout in the current investigation. However, the amount of dietary astaxanthin that is retained in the flesh rarely exceeds 18% in rainbow trout (Storebakken & No 1992) and 15% in Atlantic salmon with respect to dietary intake (Torrissen *et al.* 1989). The physiological and biochemical constraints to deposition of pigment in the flesh are not well understood. It has been suggested that variations in the number and size distribution of muscle fibres may influence astaxanthin deposition (variability in availability of binding sites) thus limiting flesh retention and causing the plateau effect (Nickell & Bromage 1988b). Certainly data from the current studies would suggest that the deposition of astaxanthin is positively related to growth and muscle tissue accretion. However, Henmi *et al.* (1989) demonstrated that carotenoids form hydrogen bonds with the surface of actomyosin proteins in salmon muscle and found that the binding capacity appears to be higher than observed maximum carotenoid levels. Indeed, Johnston *et al.* (2000) found no significant correlation between muscle fibre density and flesh astaxanthin concentration in Atlantic salmon.

It may be too simplistic to assume that a physiological limitation exists in the muscle tissue specifically. Although the transport capacity of blood proteins for carotenoids appears to be approximately 100-fold higher than levels observed after feeding (Chavez *et al.* 1998) the dynamics of carotenoid transfer from the serum to the muscle tissue (probably associated with lipoprotein transfer) has not been investigated in fish. It is quite possible that discrepancies between high serum carotenoid concentrations and final muscle

concentrations are influenced by this factor. Limitations at the stage of carotenoid deposition in the flesh warrants further study.

Metabolic transformation may also be influential in the final deposition of astaxanthin in muscle of fish. Canthaxanthin is more rapidly cleared from the plasma of rainbow trout than astaxanthin indicating that the metabolic turnover of canthaxanthin is greater in comparison (Gobantes *et al.* 1997). This may in part explain the lower levels of pigmentation encountered in salmonids when using canthaxanthin compared to astaxanthin. However, it does not explain the lower levels of apparent digestibility that are recorded for canthaxanthin in comparison to astaxanthin (Choubert & Storebakken 1996). Bjerkeng & Berge (2000) compared the apparent digestibility coefficients, carotenoid composition in muscle, liver and whole kidney of Atlantic salmon and the white-fleshed Atlantic halibut (*Hippoglossus hippoglossus*). These authors found that the ADC of astaxanthin was significantly higher (10-30%) in Atlantic halibut than in Atlantic salmon yet no astaxanthin was found in the muscle of the halibut and only low levels of carotenoid were detected in the plasma, liver and kidneys. Furthermore, these authors found that halibut appeared to be able to transform astaxanthin reductively to idoxanthin suggesting that the ability to metabolically transform ingested carotenoids is highly influential in carotenoid accumulation and flesh colouration between fish species.

Plausibly, once a threshold capacity is reached in the flesh in terms of astaxanthin deposition, differences between circulating blood levels as a result of consumption of different carotenoid sources becomes less important. Certainly, in the current investigations as the level of carotenoid deposition in flesh became higher the difference between fish fed different astaxanthin sources became less apparent both visually and through chemical analysis. Ultimately, salmonid production and consumer acceptance is

judged solely on the criteria of flesh pigmentation. However, a rapid screening technique is required to primarily assess the absorption of carotenoids in the evaluation of any novel source. This can be carried out *in vivo* with measurements of circulating blood levels. However, excretion, metabolism and absorption affect blood carotenoid concentrations (Castenmiller & West 1998; Van het Hof *et al.* 2000). Furthermore, the use of live animals is wasteful and can be questioned on ethical grounds. Subsequently there is a need for an *in vitro* approach to evaluate the absorption of carotenoids at the gastrointestinal level. The current study has developed such an approach with interesting results.

## 8.4    Evidence from *in vitro* studies on carotenoid absorption.

Based on the information gathered from the *in vitro* gut perfusion and gut-sac experiments carried out in the current study the absorption process of astaxanthin into intestinal tissue is likely passive in nature. This is supported by the linear nature of initial uptake rates in the dose response and the time saturation experiments. This is supported by findings *in vivo* where positive linear correlation between plasma astaxanthin concentration and dietary astaxanthin concentration have been demonstrated in rainbow trout (Choubert *et al.* 1994a) and Atlantic salmon (Kiessling *et al.* 1995). Furthermore, Garrett *et al.* (1999) demonstrated using human Caco-2 intestinal cell lines that the cellular uptake of β-carotene and lutein in mixed micelles was proportional to the concentration of carotenoid in the medium. Other authors have demonstrated linear uptake as a function of substrate concentration for β-carotene in rat gut sacs (El-Gorab *et al.* 1975) and non anaesthetised rats using intestinal loops (Hollander & Ruble 1978).

In contrast, differential absorption of different carotenoids (zeacarotene and lutein) along the intestinal tract of chickens has been demonstrated, suggesting regulatory mechanisms

204

for carotenoid absorption (Tyczkowski & Hamilton 1986b). However, the absorption of carotenoids in chickens is thought to be independent of lipid absorption (Osborne *et al.* 1982) unlike in fish where positive correlations have been recorded between dietary lipid and carotenoid absorption (Torrissen *et al.* 1990; Choubert *et al.* 1991; Gouveia *et al.* 1998). Contrasting data such as this suggests distinct differences between species in relation to the mechanisms of carotenoid absorption. Indeed, the metabolic routes of carotenoids differ quite markedly between fish species (Hata & Hata 1973). Interspecies differences cannot be ruled out since the utilisation of different carotenoids e.g. astaxanthin and canthaxanthin has been demonstrated to be different in Atlantic salmon and rainbow trout. Buttle *et al.* (2001) showed greater utilisation of canthaxanthin in comparison to astaxanthin in the pigmentation of Atlantic salmon. However, other groups have consistently confirmed more effective absorption and deposition of astaxanthin in comparison to canthaxanthin in rainbow trout (Foss *et al.* 1984; Torrissen 1986, 1989; Bjerkeng *et al.* 1990; Choubert & Storebakken 1989; No & Storebakken 1992; Choubert *et al.* 1994a; Gobantes *et al.* 1997). These differences may well be due to discrepancies in absorption of carotenoids between the two species. This is supported by evidence from *in vivo* studies where higher ADC values for astaxanthin in rainbow trout compared to Atlantic salmon have been found (G. Page, pers. comm.). Indeed findings in the current study (using everted gut-sacs) suggest that rainbow trout intestine absorbs astaxanthin to a greater extent than Atlantic salmon intestine although further dose response experiments are required to confirm this finding. In addition, the potential for differences in gastrointestinal hydrolysis of carotenoid esters should not be ignored as differences in the utilisation of astaxanthin esters between Atlantic salmon and rainbow trout have been recorded between different investigation (Storebakken *et al.* 1987; Bowen *et al.* 2001).

A passive carotenoid absorption process might suggest that the pigmentation of salmonids could be achieved more rapidly by simply increasing the dietary carotenoid content. However, as discussed earlier, dose response flesh pigmentation investigations have shown a non-linear response (Torrissen 1989b; Storebakken *et al*. 1987). Furthermore, Apparent Digestibility Coefficients (ADC) for astaxanthin in the current study (ca. 60-75%) suggest that the absorption process is not highly efficient, especially when considering that ADC values are likely elevated due to the oxidation of carotenoids in faeces. Regional variation in carotenoid absorption along the gastrointestinal tract (as demonstrated in Chapter 4) coupled with the effects of gut transit rate may in part account for a reduced digestibility. Alternatively, there may be other stages in the intestinal absorption process that limit the absorption of astaxanthin.

According to the current study, once a carotenoid has been suitably solubilised the absorption of carotenoid by the intestine is a passive process and is not limiting when a concentration gradient is maintained. Subsequently, it seems that the solubilisation stage is more likely influential in the absorption of carotenoid.

In mammals, once released from the feed matrix carotenoids are solubilised in mixed bile-salt micelles (Erdman *et al*. 1993; Parker 1996; Furr & Clark 1997; Van den Berg 1999). It is interesting to note that the formation of micelles is dependent on the production of bile that is itself stimulated by dietary fat. It remains to be shown whether the increased absorption of carotenoids with increasing dietary fat in salmonids (Torrissen *et al*. 1990; Choubert *et al*. 1991; Gouveia *et al*. 1998) is a result of this phenomenon or simply a coincidence. Formation of these micelles is critical to ensure solubilisation and high concentrations of the highly hydrophobic carotenoids in aqueous solution that allows them to cross the unstirred water layer adjacent to the intestinal mucosa. Similarly, in rats it has

been demonstrated that the absorption of the lipophillic vitamin E requires bile salts in the intestine (Pearson & Legge 1972; MacMahon & Thompson 1970). The latter authors showed that although a polar lipid (oleic acid) was absorbed equally from an emulsion or micellar solution, α-tocopherol was absorbed to a lesser extent from the emulsion compared to the micellar solution. This indicates the greater importance of micellar solubilisation of less-polar lipids. In the current study the uptake rates of astaxanthin have been shown to be different when using alternative solubilising strategies. Since the use of non-ionic surfactants above their CMC resulted in the formation of micelles and higher uptake rates compared to an emulsion (dispersible beadlets), the current study supports the contention that micelle incorporation is important in the absorption of hydrophobic carotenoids in salmonids.

It is possible that differences in micellar incorporation owing to differences in polarity may account for the more efficient absorption of astaxanthin compared to canthaxanthin (Choubert et al. 1994a). Astaxanthin is more polar than canthaxanthin and may well partition into bile salt micelles more readily. In support of this, El-Gorab & Underwood (1973) have shown that retinol is almost ten times more readily dissolved in micelles than β-carotene and exhibits a greater polarity. Furthermore, retinol may partition into the more polar peripheral region of the bile salt-micelle where β-carotene is probably solubilised in the hydrophobic core and its solubility may be enhanced by expansion of the micelle with polar lipids (there was no competition between the two for micellar solubilisation). An enhancement of solubilisation such as this could account for the synergistic affects witnessed when both astaxanthin and canthaxanthin are included in salmonid feeds in terms of flesh pigmentation (Foss et al. 1987; Torrissen 1989b). The techniques used in the current study to form micelles could be readily utilised to determine potential differences of micelle incorporation for these two commercially important carotenoids.

Further to their role in the solubilisation of unesterified carotenoids, bile salts are likely necessary for the hydrolysis of astaxanthin esters. Lombardo & Guy (1980) have stated that bile-salts are also involved in providing a suitable substrate (micellar solution of the fat-soluble vitamin esters) for the hydrolytic activity of the carboxyl ester hydrolase of pancreatic juice with the activity of this enzyme enhanced by the trihydroxy bile acids. However, the hydrolysis of vitamin A esters by rat intestine *in vitro* has been demonstrated in the presence of other synthetic solubilising agents (Mahadaven *et al.* 1963).

Other studies have shown that the bile salts are necessary for the metabolism of β-carotene within the intestinal mucosa (El-Gorab *et al.* 1975). The formation of metabolites of astaxanthin in the intestine has not been assessed in the current investigation due to a lack of radiolabelled compound, appropriate standards and analytical equipment. However, it is plausible that measurements of astaxanthin absorption in everted gut-sacs may have been underestimated due to the rapid metabolism and formation of unidentifiable compounds. Indeed other authors have used everted gut sacs of trout intestine exposed to aqueous solutions of astaxanthin ($H^3$-radiolabelled) and have demonstrated the formation of retinol (Al-Khalifa & Simpson 1988). These authors noted that after 60min exposure 3% of astaxanthin absorbed was converted to retinol in the ileum where up to 12% was converted in the pyloric caeca, although this was probably a function of surface area. Since the ileal portion of the intestine was used in the current study it may be assumed that conversion was minimal (ca. 3%). Furthermore, findings presented by Al-Khalifa & Simpson (1988) suggest that bile salts are not necessary for the metabolism of astaxanthin to retinol since they used a synthetic detergent to solubilise the carotenoid. Nevertheless, it is envisaged that the everted-gut sac system could be used to study the metabolism of astaxanthin at the

intestinal level and the factors that influence this process. For example, the system could be used to compare the metabolism of astaxanthin esters, geometric and optical isomers.

## 8.5    Additional future experiments

Due to the focussed nature of the current study not all of the influential factors that have been published concerning the absorption of astaxanthin have been evaluated. However, the everted gut sac methodology used in the current investigations could be applied to assess some of these factors and consequently they require brief discussion. For example, the comparative absorption of astaxanthin stereoisomers (*trans*- and *cis*-astaxanthin) has not been evaluated. Bjerkeng *et al.* (1997) recorded significantly higher apparent digestibility coefficients for astaxanthin for rainbow trout fed all-*E*-astaxanthin (79%) compared to trout fed a stereoisomer mixture (64%) and indicated a competitive mechanism of uptake of the different stereoisomers. It is envisaged that the current methodology could be utilised to compare micellar incorporation and intestinal absorption for the different isomers thus confirming a potential competitive mechanism at the intestinal level. In addition there is some evidence that the fatty acid composition of dietary oils may influence the absorption of astaxanthin (Bjerkeng *et al.* 1999b). Micelle preparations used in the current study could be formulated to incorporate an array of different fatty acid classes representative of the profile of various sources of dietary oil and examine their effects on micellar incorporation and intestinal absorption of astaxanthin.

## 8.6    Conclusions

Several general conclusions can be put forward based on findings from the current study that are relevant to the salmonid feed industry and pigmentation strategies that are utilised in salmonid production.

The microalgae *Haematococcus pluvialis* can be used as a viable source of pigment in salmonid feeds for the production of rainbow trout providing the cell wall structure is efficiently fractured to ensure bioavailability of pigment. Furthermore the presence of a cell wall in a fractured state ensures minimum oxidation of pigment and stable feed astaxanthin profiles.

The extent of esterification does negatively influence the absorption of astaxanthin. However, these differences do not significantly influence the deposition of esterified astaxanthin in the muscle of rainbow trout. This has several implications. Firstly, the extent of esterification should be used as a consideration when choosing and evaluating other novel sources of carotenoid. Secondly, future assessment of natural carotenoid sources that contain esterified astaxanthin should make efforts to report the monoester: diester ratio as this may ultimately affect the findings and conclusions drawn from these studies. Thirdly, with reference to *Haematococcus pluvialis*, efforts should be made to maintain a higher monoester: diester ratio to ensure maximum absorption of astaxanthin (Harker *et al*. 1996b).

The gastrointestinal absorption of astaxanthin and hydrolysis of astaxanthin esters takes place mainly in the pyloric and ileal region of the intestinal system as demonstrated *in vivo* and *in vitro* for rainbow trout and *in vitro* for Atlantic salmon. Such regional variation in

210

carotenoid-ester hydrolysis and astaxanthin absorption has implications related to feeding practises and their influence on gut transit rate.

The absorption of astaxanthin by the intestine appears to be a passive linear process. The size of the fish does not appear to influence the uptake capacity of intestinal tissue in rainbow trout. However, increases in temperature (within a range found under typical culture conditions) result in positive increases in absorption of astaxanthin by trout intestine.

The intestinal absorption of astaxanthin is not significantly different in rainbow trout compared to Atlantic salmon, although it appears to be higher. Subsequently, differences in intestinal absorption between these two salmonid species cannot be ruled out.

Clearly, the role of pigmentation in the intensive culture of salmonid species in today's aquaculture industry is an important one. Consequently, there is still a demand for scientific research into this field in a continuing drive to increase the utilisation of different carotenoid sources from feeds, and subsequently reduce the substantial cost of this micro supplement. Ongoing changes in the manufacture of feeds with respect to macro ingredients and the demand for alternatives, especially with respect to lipid sources, will undoubtedly necessitate further studies in relation to pigmentation efficiency. The increasing production of a larger array of salmonid species will necessitate an understanding of those genetic strains and potential selection programmes of those fish groups that have a higher capacity for the absorption and utilisation of carotenoids. Future research into carotenoids should as a whole encompass factors relating to husbandry and feeding practises in addition to dietary composition. It is essential that this research is

carried out with a marriage of well defined *in vitro* and *in vivo* protocols that will significantly benefit the aquafeed industry in relation to feed pigment supplementation.

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232

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