PHYSIOLOGICAL AND BIOCHEMICAL FACTORS AFFECTING CAROTENOID UTILIZATION IN SALMONID FISH.

By

GREGORY IAN PAGE

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

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
Faculty of Science

In Collaboration with EWOS Innovation AS, Dirdal, Norway.

NOVEMBER 2001
ABSTRACT

Physiological and biochemical factors affecting carotenoid utilization in salmonid fish.

Gregory Ian Page

Carotenoid utilization in rainbow trout (Oncorhynchus mykiss Walbaum) and Atlantic salmon (Salmo salar L.) has been investigated with respect to tissue distribution of carotenoids and the role of the liver on the bioavailability of the lipid soluble carotenoids, astaxanthin and canthaxanthin.

Species-specific and tissue-specific accumulations were noted for astaxanthin and canthaxanthin in the rainbow trout and Atlantic salmon, possibly indicating fundamental differences in their utilization in these species. The liver and the kidney were revealed to be the major tissues involved in carotenoid metabolism in both rainbow trout and Atlantic salmon. Apparent digestibilities (≈96% and ≈30% for rainbow trout and Atlantic salmon, respectively) and flesh carotenoid retentions (≈12% and ≈5.4% for rainbow trout and Atlantic salmon, respectively) differed significantly between species, suggesting that rainbow trout are more efficient depositors of carotenoids within the flesh.

Isolated rainbow trout liver perfusion experiments revealed small differences in the uptake of astaxanthin and canthaxanthin. Uptake of astaxanthin in both synthetically-derived and serum-derived models showed saturable uptake mechanism that occurred earlier than for canthaxanthin. These results can potentially offer an explanation for the better utilization of astaxanthin in rainbow trout, where the liver reduces the bioavailability of canthaxanthin through continued uptake. Results show a low hepatic extraction ratio (0.03-0.07), in line with published post-prandial elimination rates.

Neither astaxanthin nor canthaxanthin significantly induced hepatic or renal xenobiotic-metabolizing enzymes in the rainbow trout, contrary to published reports in rats and mice. This may imply fundamental species-specific differences in the metabolic pathways for these carotenoids.

Histochemical investigations revealed that both carotenoids significantly impacted liver structure, resulting in higher levels of total lipids and mucopolysaccharides. This is thought to be due to their antioxidant functions and their provitamin A activity. Carotenoid-treated fish also had higher levels of glycogen phosphorylase in liver sections, providing the first evidence in fish for the possibility of glucuronidation of their metabolites.

The present investigations demonstrate the liver to be a major organ in carotenoid metabolism, and consequently affects carotenoid distribution and availability. In addition, carotenoid supplementation significantly affects liver structure and may potentially enhance its function. Furthermore, these investigations have provided new avenues of investigation into the use of isolated organ perfusions for biochemical nutrition research, and expanded the knowledge of liver physiology and biochemistry.
CHAPTER 1

GENERAL INTRODUCTION

1.1 Socioeconomic importance of salmonid aquaculture 1

1.2 Overview of carotenoids in salmonid fish 1

1.3 Economic importance of carotenoids in salmonid aquaculture 2

1.4 Functions of carotenoids in salmonid fish 7

1.5 Pro-vitamin A function of carotenoids 9

1.6 Factors influencing carotenoid utilization 10

1.6.1 Dietary feeding regime impacts 12

1.6.2 Intestinal absorption and digestibility 13

1.7 Tissue carotenoid distribution 17

1.7.1 In vivo carotenoid transport 18

1.7.2 Skin 26

1.7.3 Flesh 26
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7.3.1 Flesh pigment retention</td>
<td>29</td>
</tr>
<tr>
<td>1.7.3.2 Isomers</td>
<td>30</td>
</tr>
<tr>
<td>1.7.4 Kidney</td>
<td>31</td>
</tr>
<tr>
<td>1.7.5 Liver</td>
<td>31</td>
</tr>
<tr>
<td>1.8 Metabolism of carotenoids</td>
<td>36</td>
</tr>
<tr>
<td>1.9 Age effects</td>
<td>38</td>
</tr>
<tr>
<td>1.10 Reproductive transport of carotenoids</td>
<td>39</td>
</tr>
<tr>
<td>1.11 Species differences in carotenoid utilization</td>
<td>40</td>
</tr>
<tr>
<td>1.12 Differences between astaxanthin and canthaxanthin utilization</td>
<td>41</td>
</tr>
<tr>
<td>1.13 Research aims</td>
<td>42</td>
</tr>
</tbody>
</table>

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Experimental Animals

2.1.1 Rainbow trout

2.1.2 Atlantic salmon

2.2 Experimental culture facilities

2.2.1 Plymouth University

2.2.2 Feeding

2.3 Growth performance

2.4 Feed Conversion Ratio

2.5 Tissue somatic indices

2.6 Diet preparation

2.6.1 Commercially manufactured diets
2.6.2 In-house top-coating of basal diets

2.7 Proximate chemical composition

2.7.1 Moisture determination

2.7.2 Determination of crude protein

2.7.3 Determination of total lipid

2.7.4 Determination of ash content

2.8 Determination of carotenoid content in feeds and faeces

2.8.1 Extraction procedure for feeds

2.8.2 Faecal astaxanthin analysis

2.9 Determination of tissue carotenoid concentration

2.9.1 Serum

2.9.2 Flesh

2.9.3 Liver, kidney, spleen, heart, and gonads

2.9.4 Gastrointestinal tract

2.10 HPLC conditions

2.11 Sample carotenoid quantification

2.12 Digestibility Determination

2.12.1 Determination of Yttrium oxide content (inert reference marker)

2.12.2 ICP analysis

2.12.3 Determination of apparent digestibility coefficients

2.13 Determination of tissue carotenoid retention

2.14 Statistics
CHAPTER 3

TISSUE CAROTENOID DISTRIBUTION IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM) AND ATLANTIC SALMON (SALMO SALAR L.): A COMPARATIVE INVESTIGATION.

3.1 Introduction

3.2 Materials and Methods

3.2.1 Rainbow trout trial

3.2.2 Atlantic salmon trial

3.2.3 Dietary Analysis

3.2.4 Statistical analysis

3.3 Results

3.4 Discussion

3.4.1 Digestibility

3.4.2 Flesh carotenoid retention

3.4.3 Tissue carotenoid accumulation

3.4.4 Peak proportionality

3.4.5 Liver and kidney as metabolic tissues: A comparison

3.4.6 Other tissues as metabolic organs

3.4.7 Conclusions

CHAPTER 4

THE APPLICATION OF AN IN VITRO ISOLATED PERFUSED LIVER PREPARATION TO EXAMINE HEPATIC CAROTENOID UPTAKE KINETICS IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM).

4.1 Introduction

4.2 Materials and Methods
4.2.1 Perfusion methodology

4.2.2 Perfusate preparation

4.2.3 Viability Measurements

4.2.4 Experimental outline

4.2.5 Calculations

4.2.6 Histology

4.2.7 Statistical analysis

4.3 Results

4.3.1 Viability

4.3.2 Dose effect of carotenoid perfusion

4.3.3 Simultaneous perfusion with beadlet-derived astaxanthin and canthaxanthin

4.3.4 Rainbow trout serum perfusions

4.3.5 Atlantic salmon serum perfusions

4.3.6 EDTA perfusions

4.3.7 Summary

4.4 Discussion

4.4.1 Rationale and assessment of liver perfusion model viability

4.4.2 Dose-effects of carotenoid perfusate concentration

4.4.3 Hepatic extraction ratios

4.4.4 Carotenoid uptake interactions

4.4.5 Piscine serum perfusion studies

4.4.6 Uptake mechanism

4.4.6 General discussion of uptake kinetics
CHAPTER 5

THE EFFECTS OF ASTAXANTHIN AND CANTHAXANTHIN ON LIVER XENOBIOTIC METABOLIZING ENZYMES IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM).

5.1 Introduction 152

5.2 Materials and Methods 155

5.2.1 Diet Preparation 155

5.2.2 Diet analysis 155

5.2.3 Fish and experimental conditions 157

5.2.4 Sampling 157

5.2.5 Cytochrome P450 isozyme activity in microsomal fractions 158

5.2.5.1 Preparation of microsomes 158

5.2.5.2 Dual protein and P450 isoenzyme assays 159

5.2.5.3 Assay for Glucuronosyl Transferase activity 160

5.2.5.4 Assay for glutathione-S-transferase activity 162

5.2.6 Statistical analysis 163

5.3 Results 163

5.4 Discussion 167

CHAPTER 6

THE EFFECTS OF DIETARY ASTAXANTHIN AND CANTHAXANTHIN SUPPLEMENTATION ON LIVER HISTOCHEMISTRY IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM).

6.1 Introduction 178

6.2 Materials and Methods 180

6.2.1 Fish and experimental conditions 180

6.2.2 Diet Preparation 181
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2.3 Diet analysis</td>
<td>181</td>
</tr>
<tr>
<td>6.2.4 Tissue sampling</td>
<td>182</td>
</tr>
<tr>
<td>6.2.5 Tissue sectioning and histochemical staining</td>
<td>182</td>
</tr>
<tr>
<td>6.2.6 Image analysis</td>
<td>185</td>
</tr>
<tr>
<td>6.2.7 Statistical analysis</td>
<td>186</td>
</tr>
<tr>
<td>6.3 Results</td>
<td>186</td>
</tr>
<tr>
<td>6.4 Discussion</td>
<td>189</td>
</tr>
<tr>
<td>CHAPTER 7</td>
<td></td>
</tr>
<tr>
<td>GENERAL DISCUSSION</td>
<td></td>
</tr>
<tr>
<td>7.0 Introduction</td>
<td>197</td>
</tr>
<tr>
<td>7.1 Whole body distribution</td>
<td>198</td>
</tr>
<tr>
<td>7.2 Perfusion model rationale</td>
<td>201</td>
</tr>
<tr>
<td>7.3 Lipoprotein uptake</td>
<td>203</td>
</tr>
<tr>
<td>7.4 Liver perfusion studies</td>
<td>203</td>
</tr>
<tr>
<td>7.5 Liver and introduction to P450</td>
<td>206</td>
</tr>
<tr>
<td>7.6 Enzyme induction/metabolism</td>
<td>208</td>
</tr>
<tr>
<td>7.7 Histochemistry</td>
<td>211</td>
</tr>
<tr>
<td>7.8 Antioxidant roles of carotenoids</td>
<td>212</td>
</tr>
<tr>
<td>7.9 Vitamin E effects on carotenoid retention</td>
<td>213</td>
</tr>
<tr>
<td>7.10 Future investigations</td>
<td>214</td>
</tr>
<tr>
<td>7.11 Conclusions</td>
<td>215</td>
</tr>
<tr>
<td>APPENDIX 1</td>
<td>219</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>221</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Chapter 1

Table 1.1. Proposed biological activities of carotenoids ................................................................. 9

Table 1.2. Apparent digestibility coefficients (ADC), flesh carotenoid concentrations and retention efficiencies in Atlantic salmon, and Rainbow trout by different pigments and diet levels of carotenoids and dietary fat level .......................................................................................................................... 19

Table 1.3. Serum/plasma astaxanthin and canthaxanthin concentrations in rainbow trout and Atlantic salmon as a function of dietary carotenoid concentration and lipid level .................................................................................. 23

Chapter 2

Table 2.1. Basal diet formulation for feeds (top-coated) used in cytochrome P450 (Chapter 5) and liver histology studies (Chapter 6). .......................................................... 48

Chapter 3

Table 3.1. Proximate compositions of commercially manufactured experimental diets fed to rainbow trout, Oncorhynchus mykiss Walbaum, and Atlantic salmon, Salmo salar L .......................................................... 69

Table 3.2. Growth and feed performances of rainbow trout fed two dietary treatments for 10 weeks in freshwater, and of Atlantic salmon fed similar diets for 12 weeks in seawater. Values are expressed as means ± SEM*. (n = 3 tanks Rainbow trout; n = 2 tanks Atlantic salmon). ................................................. 71

Table 3.3. Apparent digestibility coefficients (ADC) and flesh carotenoid retention efficiencies of rainbow trout fed two dietary carotenoid treatments for 10 weeks in freshwater, and of Atlantic salmon fed similar diets for 12 weeks in seawater. Values are expressed as means ± SEM. (n = 3 Rainbow trout; n = 2 Atlantic salmon). .......................................................... 71

Table 3.4. Mean tissue somatic indices in Atlantic salmon and rainbow trout fed dietary carotenoids. Values are expressed as means ± S.D. (n = 200 fish rainbow trout, n = 80 fish salmon) ............................................................. 78

Table 3.5. Relative tissue contributions to total recovered carotenoid in Atlantic salmon and rainbow trout fed diets containing astaxanthin or canthaxanthin. Values are expressed as means ± SEM. (n = 3 rainbow trout, n = 2 Atlantic salmon) ........................................................................ 79

Table 4.1. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin and/or canthaxanthin (derived from synthetic beadlets) at various concentrations. ................................................................................... xi
Values expressed as means ± SEM. (n = 5 livers per carotenoid; n = 6 livers with astaxanthin and canthaxanthin perfused simultaneously).

Table 4.2. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both simultaneously, and derived from rainbow trout serum. Values expressed as means ± SEM. (n = 5 livers astaxanthin; n = 4 livers canthaxanthin; n = 2 livers mixed astaxanthin:canthaxanthin).

Table 4.3. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both simultaneously, and derived from Atlantic salmon serum. Values expressed as means ± SEM. (n = 4 livers per treatment).

Table 4.4. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin alone or in combination with 5 mM EDTA. Values expressed as means ± SEM. (n = 5 livers astaxanthin alone; n = 6 livers astaxanthin with 5 mM EDTA).

Table 4.5. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin (derived from synthetic beadlets) a various concentrations. Values are expressed as means ± SEM. (n = 5 livers per dose per carotenoid).

Table 4.6. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin and canthaxanthin (derived from synthetic beadlets) simultaneously. Values are expressed as means ± SEM. (n = 6 livers).

Table 4.7. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both carotenoids simultaneously and derived from rainbow trout serum, or from synthetic beadlets (Carophyll® Pink or Carophyll® Red: astaxanthin and canthaxanthin, respectively). Values are expressed as means ± SEM. (n = 5 livers astaxanthin, n = 4 livers canthaxanthin, n = 2 livers mixed astaxanthin:canthaxanthin).

Table 4.8. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both carotenoids simultaneously and derived from Atlantic salmon serum, or from synthetic beadlets (Carophyll® Pink or Carophyll® Red: astaxanthin and canthaxanthin, respectively). Values are expressed as means ± SEM. (n = 4 livers per treatment – salmon serum perfusions; n = 5 livers per treatment – beadlet perfusions).

Table 4.9. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin alone or in combination with 5 mM EDTA. Values are
expressed as means ± SEM. (n = 5 livers astaxanthin alone; n = 6 livers astaxanthin with 5 mM EDTA). ................................................................. 126

Chapter 5

Table 5.1. Proximate analysis of experimental diets* used in a pilot study. ............... 156

Table 5.2. Proximate analysis of experimental diets* used in a replicated experiment. ........................................................................................................... 156

Table 5.3. Specific growth rate (SGR) and tissue somatic indices (TSI) of rainbow trout fed diets supplemented or unsupplemented with carotenoids in a pilot study. ....................................................................................................... 163

Table 5.4. Feed conversion ratios (FCR), specific growth rate (SGR), and tissue somatic indices (TSI) of rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone in a replicated experimental trial. Values are expressed as means ± SEM (n = 3). ....................... 164

Table 5.5. Serum carotenoid concentrations (μg·ml⁻¹; mean ± SEM) of rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone in a replicated experimental trial (n = 3) ..................................... 164

Table 5.6. Liver monooxygenase enzyme activities (mean±SD) of a pilot study in rainbow trout fed astaxanthin, canthaxanthin, or control diet (n = 10 pools·treatment⁻¹). ........................................................................................................ 165

Table 5.7. Kidney monooxygenase enzyme activities (mean±SD) of a pilot study in rainbow trout fed astaxanthin, canthaxanthin, or control diet (n = 10 pools·treatment⁻¹). ........................................................................................................ 165

Table 5.8. Liver monooxygenase and conjugation enzyme activities (mean±SEM) in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone (n = 3). .................................................. 166

Table 5.9. Kidney monooxygenase and conjugation enzyme activities (mean±SEM) in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone (n = 3). .................................................. 167

Chapter 6

Table 6.1. Basal diet formulation* and proximate analysis of experimental diets used in the pilot study. Values are expressed as means ± S.D. of triplicate determinations................................................................. 181

Table 6.2. Basal diet formulation* and proximate analysis of experimental diets used in the replicated experiment. Values are expressed as means ± S.D. of triplicate determinations. .................................................. 183

Table 6.3. Mean grey-scale values for differential hepatic histochemical staining for glycogen, total mucopolysaccharides, total lipids, and unsaturated fatty
acids in rainbow trout fed diets supplemented or unsupplemented with astaxanthin or canthaxanthin. Values are expressed as means ± S.D. from 15 fish per tank (n = 1).

Table 6.4. Mean grey-scale values for differential hepatic histochemical staining for glycogen, total mucopolysaccharides, total lipids, unsaturated fatty acids, glycogen phosphohyrase activity, and aspartate aminotransferase activity in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone. Values are expressed as means ± SEM. (n = 3).
LIST OF FIGURES

Chapter 1

Fig. 1.1. Representative carotenoids commonly found in salmonid fish tissues. 3

Fig. 1.2. Structures of all-£-, 9Z-, 13Z-, and 15Z- astaxanthin geometric and stereo isomers. ................................................................. 5

Fig. 1.3. General structure of canthaxanthin.................................................................................. 6

Fig. 1.4. Possible reductive pathways for astaxanthin and canthaxanthin. Based on data from Kitahara (1983), Schiedt et al. (1985), Ando et al. (1986), Guillou et al. (1989) and Bjerkeng et al. (2000)............................................................... 11

Fig. 1.5 Schematic representation of intestinal absorption of carotenoids. .................... 21

Fig. 1.6. Schematic representation for uptake, metabolism and transport of carotenoids. ............................................................ 22

Fig. 1.7 Prominent functions of the fish liver................................................................. 33

Chapter 2

Fig. 2.1 Schematic diagram of an Atlantic salmon (Salmo salar L.), showing the Norwegian Quality Cut (NQC) region of the fish, where flesh samples were taken for carotenoid analysis. The NQC area is that delineated by the posterior edge of the dorsal fin to the pectoral girdle, and ventrally from the anus to the dorsum. Only the left flank of the NQC section was used, after careful removal of the skin and red muscle. .................................................. 54

Fig. 2.2. Diagrammatic representation of individual sections of the gastrointestinal tract of rainbow trout (Oncorhynchus mykiss Walbaum) and Atlantic salmon (Salmo salar L.) used in tissue analyses (Section 1 – stomach; Section 2 – pyloric caeca; Section 3 – hindgut). Note the muscular ridges of the stomach, the ‘pits’ in Section 2, which indicate the openings to the pyloric caeca, and the ridges in the posterior section of the hindgut for increased surface area for absorption..................................................... 56

Fig. 2.3 Representative HPLC chromatogram showing two main peaks corresponding to astaxanthin and canthaxanthin........................................... 59

Fig. 2.4. Validation of HPLC peak area against samples of known concentration in a linear dilution series of an astaxanthin standard. ....................... 61

Chapter 3

Fig. 3.1. Mean wet-tissue carotenoid concentrations of rainbow trout fed astaxanthin or canthaxanthin for 10 weeks............................................... 73
Fig. 3.2. Mean proportionality of carotenoid peak area to total chromatogram peak areas of individual tissues in rainbow trout fed astaxanthin or canthaxanthin for 10 weeks. ................................................................. 74

Fig. 3.3. Mean wet-tissue carotenoid concentrations of Atlantic salmon fed astaxanthin or canthaxanthin for 12 weeks. .................................................................................... 75

Fig. 3.4. Mean proportionality of carotenoid peak area to total chromatogram peak areas of individual tissues in Atlantic salmon fed astaxanthin or canthaxanthin for 12 weeks. .................................................................................... 77

Chapter 4

Plate 4.1 Histology sections (20 μm) of perfused and unperfused control rainbow trout livers. ............................................................................................... 104

Fig. 4.1. Schematic representation of the cannulated liver. ............................................. 96

Fig. 4.2. Schematic representation of perfusion apparatus used in isolated organ perfusions. ................................................................................................. 97

Fig. 4.3. Mean normalized LDH activity profile of isolated rainbow trout livers perfused with synthetic astaxanthin derived from Carophyll® Pink (T is the time of perfusate switch from carotenoid-free to carotenoid-laden perfusate; S - is the time of first sample used for uptake parameter calculation). Values are presented as means of all doses at each time point (n = 20) +/- SEM. Steady state values (from 80-190 min) not significantly different (P<0.05). ........................................................................ 106

Fig. 4.4. Representative cumulative uptake and cumulative flow profiles of isolated perfused rainbow trout livers at different astaxanthin concentrations (A) 2 mg ml\(^{-1}\) (HSI 1.35%) and (B) 32 mg ml\(^{-1}\) (HSI 1.37%), derived from Carophyll® Pink beadlets. .................................................................................................. 109

Fig. 4.5. Mean cumulative hepatic uptake profiles of (A) astaxanthin and (B) canthaxanthin at different doses derived from beadlets. Values are expressed as means +/- SEM (n = 5 livers). Cumulative uptake for astaxanthin saturable only at highest perfusate dose, while canthaxanthin does not show saturable uptake within the doses investigated. ........................................... 113

Fig. 4.6. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers at varying concentrations of (A) astaxanthin and (B) canthaxanthin, derived from synthetic sources (Carophyll® Pink and Carophyll® Red, respectively). Values are expressed as means +/- SEM (n = 5 livers). No appreciable change in hepatic extraction ratio over the course of the perfusions was observed. ........................................... 114
Fig. 4.7. Regression plots for initial hepatic carotenoid uptake rates as a function of perfusate carotenoid concentration in isolated perfused rainbow trout livers with astaxanthin (y = 0.762 + 0.230 x perfusate astaxanthin concentration, \( r^2 = 0.652, p<0.01 \)) and canthaxanthin (y = -0.371 + 0.305 \* perfusate canthaxanthin concentration, \( r^2 = 0.578, p<0.01 \)) derived from synthetic beadlets (Carophyll® Pink and Carophyll® Red, respectively). Points are expressed as means +/- SEM (n = 5 livers). ........ 117

Fig. 4.8. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers simultaneously perfused with astaxanthin (1.61 mg ml\(^{-1}\)) and canthaxanthin (2.00 mg ml\(^{-1}\)) derived from synthetic beadlets (Carophyll® Pink and Carophyll® Red, respectively). Values are expressed as means +/- SEM (n = 6 livers). No selective uptake of either carotenoid was observed ....................................................................................... 119

Fig. 4.9. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers simultaneously perfused with astaxanthin (1.61 mg ml\(^{-1}\)) and canthaxanthin (2.00 mg ml\(^{-1}\)) derived from synthetic beadlets (Carophyll® Pink and Carophyll® Red, respectively). Values are expressed as means +/- SEM (n = 6 livers). No appreciable change in hepatic extraction ratios were observed over the course of the perfusions .................................................. 121

Fig. 4.10. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers perfused with autologous rainbow trout serum as sources of (A) astaxanthin (3.79 mg ml\(^{-1}\)) or (B) canthaxanthin (3.25 mg ml\(^{-1}\)). Values are expressed as means +/- SEM (n = 5 livers for astaxanthin, n = 4 livers for canthaxanthin)............................................................................ 122

Fig. 4.11. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers perfused with autologous rainbow trout serum as sources of astaxanthin or canthaxanthin. Values are expressed as means +/- SEM (n = 5 livers for astaxanthin, n = 4 livers for canthaxanthin). Steady decline to steady state was observed in livers perfused with astaxanthin, but not with canthaxanthin .................................................................................................................................. 123

Fig. 4.12. Mean cumulative uptake and flow profiles of isolated perfused livers of rainbow trout perfused simultaneously with astaxanthin (1.66 mg ml\(^{-1}\)) and canthaxanthin (1.56 mg ml\(^{-1}\)), and supplied as autologous rainbow trout serum as the source of astaxanthin and canthaxanthin. Values are expressed as means +/- SEM (n = 2 livers). No selective uptake of either carotenoid was observed ............................................................. 124

Fig. 4.13. Mean hepatic extraction ratio profiles of isolated perfused livers of rainbow trout perfused simultaneously with astaxanthin (1.66 mg ml\(^{-1}\)) and canthaxanthin (1.56 mg ml\(^{-1}\)), and supplied as autologous rainbow trout serum as the source of astaxanthin and canthaxanthin. Values are expressed as means +/- SEM (n = 2 livers). No appreciable change in the hepatic extraction ratio over the course of the perfusions was noted .......................... 125

Fig. 4.14. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers perfused with Atlantic salmon serum as the source for astaxanthin (1.31 mg ml\(^{-1}\)) or canthaxanthin (1.31 mg ml\(^{-1}\)). Values are expressed as means +/- SEM (n = 4 livers). No selective uptake between carotenoids was observed ................................................................................................ 128
Fig. 4.15. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers perfused with Atlantic salmon serum as the source for astaxanthin (1.31 mg ml⁻¹) or canthaxanthin (1.31 mg ml⁻¹). Values are expressed as means +/- SEM (n = 4 livers). Profiles suggest a decline to a steady state in the hepatic extraction ratio. ............................................................ 129

Fig. 4.16. Mean cumulative uptake and flow profiles of isolated rainbow trout livers perfused simultaneously with both astaxanthin (0.48 mg ml⁻¹) and canthaxanthin (0.73 mg ml⁻¹) using Atlantic salmon serum as the carotenoid source. Values are expressed as means +/- SEM (n = 4 livers). Astaxanthin uptake appears to saturate earlier than does canthaxanthin. .............. 130

Fig. 4.17. Mean hepatic extraction ratio profiles of isolated rainbow trout livers perfused simultaneously with both astaxanthin (0.48 mg ml⁻¹) and canthaxanthin (0.73 mg ml⁻¹) using Atlantic salmon serum as the carotenoid source. Values are expressed as means +/- SEM (n = 4 livers). Profiles show a decline in the hepatic extraction ratio to a steady state. ............... 131

Fig. 4.18. Mean cumulative uptake and flow profile of isolated rainbow trout livers perfused with astaxanthin (8.60 mg ml⁻¹) derived from Carophyll® Pink and 5 mM EDTA. Values are expressed as means +/- SEM (n = 6 livers). .................................................................................................................... 133

Fig. 4.19. Mean hepatic extraction ratio profile of isolated rainbow trout livers perfused with astaxanthin (8.60 mg ml⁻¹) derived from Carophyll® Pink and 5 mM EDTA. Values are expressed as means +/- SEM (n = 6 livers). .......... 134

Chapter 5

Fig. 5.1. Simplified schematic representation of the putative role of cytosolic and microsomal systems in the hepatic metabolism of retinol. ......................... 173

Chapter 6

Fig. 6.1. Proposed metabolic pathway and conjugates metabolites recovered from rats fed astaxanthin. Adapted from Wolz et al. (1999). ................................. 192

Fig. 6.2. A possible role of vitamin A in the synthesis of glycoproteins. Reproduced from Basu and Dickerson (1996). ................................................................. 196

Chapter 7

Fig. 7.1. A scheme proposed for the carotenoid metabolism in spawning-migrating Chum salmon ..................................................................................... 210
Appendix I

Fig. X. 1. Representative standard curve for BSA adducted fluorescamine in 96 well microplate in the extended range concentration. .................................................. 219

Fig. X.2. Representative standard curve for resorufin in 96 well microplate in the extended range concentration for benzoxyresorufin O-deethylase assays. ................................................................. 220
ACKNOWLEDGEMENTS

It is interesting to look back on our lives and examine what our goals have been and how we have come to accomplish those goals. I have come to realize that they are never achieved without the help, support and encouragement of many people.

I would first like to thank my director of studies, Dr. Simon J. Davies for his encouragement and support over the past three years. I would also like to thank Dr. Richard Handy for his guidance and advice.

I would like to extend my sincere and heartfelt thanks to my sponsors, The EWOS Technology Centre, Scotland, for their generous financial, technical, and logistical support of this research, and specifically to Dr. Louise Buttle and Dr. Paul Williams.

Special thanks are also due to the staff and research personnel at the Leningdal Research Station, Norway, and in particular Mr. Tom Olsen and Mrs. Liv Oma, for their invaluable help in conducting the Atlantic salmon research trial of this PhD. Invaluable in this research were Mr. Paul Russell and Mr. Mike Hockings, without whose knowledge and help, this would not have been possible.

There are still many other people who, in some way or other, have helped contribute to the success of this PhD. I wish to thank, in no particular order, Mr. Robert Serwata, Ms. Julie Soane, Dr. Chris Ricketts, Mrs. Lynne Cooper, Mr. Nick Crocker, Mrs. Jo Carter, Ms. Suzie Bloomfield, Mr. Stan MacMahon, and Mr. Chris Coleman.

In addition to these people, I would like to thank the Overseas Research Students Awards Scheme, offered by the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom 1999 (reference ORS/99129001), who have alleviated many of the financial problems associated with these studies.

Despite the many people who have helped me achieve my academic goals there are a multitude of friends, family and colleagues that have supported me in various ways throughout my academic life. First and foremost are my father, Brian, and stepmum Suzanne, who have bee there for me through thick and thin. My brother and sister-in-law Trev and Kathy, friends and colleagues Dan White, Ash Patel and Gavin Stewart, who have helped alleviate many a stressful day with a phone call, email or a pint at the local pub(s). Still further I would like to thank Richard Moccia, without whose help and mentor-like guidance, I may never have attempted this.

I also wish to thank a very special friend, Clare Hawkins, who has been my emotional support in so many ways. We may have only just rediscovered our friendship, but it has been one of the most rewarding outcomes of this whole endeavour.

Finally, and to whom I dedicate the many years of dedicated work are some people who have helped me in ways they may never know and whom I have never been able to adequately thank. They are my godparents, Roger and Anne Page, who provided me and my family a sanctuary over many years in my childhood and who continue to have an open door whenever I come to visit. I thank you for everything you have done.
AUTHOR’S DECLARATION

I hereby declare that this thesis is the culmination of personal research and has been composed solely by me. At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. All the sources of information within this volume have been duly acknowledged.

The husbandry and experimental procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 under the following Home Office license arrangements:

Project License No. PPL 30/1583
Personal License No. PIL 30/4908

This project was financially supported by sponsorship from the EWOS Technology Centre, Livingston Scotland, and from the Overseas Research Students Awards Scheme, Award Number ORS/99129001.

Papers submitted to peer-reviewed journals


Relevant scientific conferences and seminars were attended at which work was presented; and external institutions were visited for consultation purposed and several papers prepared for publication.

Presentations and Conferences Attended

The Ninth International Conference on Nutrition and Feeding in Fish, Miyazaki, Japan, May 2000. Poster presented: The efficacy of Haematococcus pluvialis as a pigment source for rainbow trout (Oncorhynchus mykiss Walbaum).
External Contacts:

Dr. John Leatherland, University of Guelph, Ontario, Canada.
Dr. Santosh Lall, Institute for Marine Biosciences, NRC, Halifax, Canada.
Dr. Patrick Flammarion, Laboratoires D'Ecotoxicologie, Cémagref, Lyon, France.
Dr. Viviane Verlhac, F. Hoffman-La Roche, CRNA, France.
Mr. Robin Ørnsrud, Institute of Nutrition, Bergen, Norway.
Dr. Louise Buttle, EWOS Technology Centre, Scotland.
Dr. Paul Williams, EWOS Technology Centre, Scotland.
Dr. Bjarne Hatlen, EWOS Innovation AS, Dirdal, Norway.

Signed

Gregory J. Page

Date 16/11/2001

Signed

Simon J. Davies

Date 30/11/2001

Simon J. Davies
Director of Studies

xxiii
CHAPTER 1

GENERAL INTRODUCTION

1.1 Socioeconomic importance of salmonid aquaculture

Global aquaculture production exceeded 30 million metric tonnes (MT) in 1999, comprising over 25% of the world's seafood production (FAO 2000). Salmonid fish production has increased from 4,000 MT in 1979, to over 150,000 MT in the early part of the 1990s (Meyers 1994). This rapid expansion of the aquaculture industry over the past two decades, and the anticipated increase expected in the future (Meyers 1994), has lead to increased research into all aspects of production, including disease management, species diversification, production methodology, as well as nutrition.

1.2 Overview of carotenoids in salmonid fish

Salmonid fish such as rainbow trout (Oncorhynchus mykiss Walbaum) and Atlantic salmon (Salmo salar L.) represent some of the most widely cultured fish worldwide. One of the distinguishing features of salmonid fish is the pink to red colour of their flesh, imparted by carotenoids, which significantly contributes to their elite image (Torrissen 1985).

Carotenoids are the widest distributed pigments in plants, certain yeasts and animals with over 600 currently identified. These organic polyene compounds are not synthesized de novo by salmonid fish, and fish must, consequently, obtain these pigments through dietary sources. Carotenoids are lipophilic compounds classified by their structure as carotenes and xanthophylls, which both share a common C₄₀ polyisoprenoid structure containing a series of centrally located, conjugated double bonds (Deming and Erdman 1999).

Carotenes (e.g. β-carotene, Fig. 1.1) are relatively non-polar hydrocarbons, while xanthophylls (e.g. zeaxanthin, β,β-carotene-3,3′-diol, Fig. 1.1) are more polar, containing oxygen either as a hydroxyl or keto group contained in the end group. Some of the naturally occurring carotenoids are chiral, bearing from 1 to 5 asymmetric carbon atoms (Pfander 1992). Most carotenoids are colourful yellow, orange, and red pigments, whose conjugated double bond structure is related to their most important function, the absorption
of light during photosynthesis, protecting cells from photosensitization (Demmig-Adams et al. 1996). Their structure may also provide insight into their absorption, metabolism, and complex biological effects (Britton 1995).

Salmonids, unlike other species, primarily deposit ingested carotenoids in muscle tissues, and indeed flesh coloration, which provides an indication of product quality (Torrissen and Naevdal 1984), has become one of the most important marketing criteria used by consumers and the processing industry. The degree or intensity of pigmentation in cultured salmonids affects consumer product acceptance, as well as variations in price (Gobantes et al. 1998b). Moreover, demand by the consumer for consistently and uniformly pigmented products has increased (Nickell 1998), leading to an augmentation in the level of pigmentation over the years from 2-3 mg·kg\(^{-1}\) in farmed salmonid flesh during the 1970s, to over 6 mg·kg\(^{-1}\) more recently (Torrissen 1995).

1.3 Economic importance of carotenoids in salmonid aquaculture

Chemical synthesis remains the primary source of carotenoid pigments in the production of cultured salmonids (Sommer et al. 1991). The primary carotenoid pigments used in the culture of salmonids are astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) (Fig. 1.2) and canthaxanthin (β,β-carotene-4,4'-dione) (Fig. 1.3), the former being the most commonly used synthetic carotenoid pigment in aquaculture. These pigments may have various stereoisomers, depending on the chiral carbon atom (Fig. 1.2 and 1.3). In order to achieve an acceptable pigmentation level in farmed rainbow trout flesh, these carotenoids are generally supplemented in the feed at concentrations ranging from 40 to 80 mg·kg\(^{-1}\) (Nickell 1998). Feed costs can represent as much as 40-60% of the total operating expenses in an intensive aquaculture operation, especially with carnivorous species such as salmonids (Meyers 1994). With the price of synthetically produced carotenoids
Fig. 1.1. Representative carotenoids commonly found in salmonid fish tissues.
(astaxanthin and canthaxanthin) being over $2500 kg^{-1}$ (McCoy 1999), the addition of astaxanthin to formulated feeds for salmonids comprises approximately 15-20% of the total feed costs (Nickell 1998), and may represent between 6-8% of the total production costs (Torrissen 1995). In 1989, it was estimated that the total cost of pigmenting farmed salmonids was over $20 Million (Hardy et al. 1990), and 10 years later represented approximately $175 Million (McCoy 1999). Therefore it is of significant economic importance that salmonids are efficiently pigmented to meet consumer preferences, while optimizing production costs.

The aim of pigmentation strategies is to provide adequate levels of colouration for consumer acceptance. However, reported flesh astaxanthin retention in trout ranges between 3-18%, making pigmentation deposition relatively inefficient (Nickell 1998). Hardy et al. (1990) estimated that a savings of over $13 Million in pigment feed costs could have been achieved by increasing the deposition efficiency from 5% to 10% of intake. Therefore, the potential exists for considerable economic saving if pigment retention is improved through research and development.

Studies have shown that throughout the growth of the fish, pigmentation may be influenced by a variety of environmental and physiological factors, which collectively result in seasonal variations in flesh colour. Because of the requirement for year-round supplies of product of consistent size and quality, seasonal variations in flesh colour have resulted in significant proportions of product being rejected by processors and retailers (Nickell 1998). The high price of pigment supplementation, and the importance of achieving a satisfactory level of flesh pigmentation, has resulted in increased research efforts into the factors affecting flesh pigmentation (Torrissen 1995).
Fig. 1.2. Structures of all-E-, 9Z-, 13Z-, and 15Z- astaxanthin geometric and stereoisomers.
Fig. 1.3. General structure of canthaxanthin.
1.4 Functions of carotenoids in salmonid fish

Beyond their functions as flesh pigmenters, carotenoids have also been shown to have various other functions in the animal kingdom. Both astaxanthin and canthaxanthin may serve as provitamin A (Schiedt et al. 1985; Al-Khalifa and Simpson 1988; Guillou et al. 1989), while other researchers have shown a variety of other essential functions including antioxidants (Christiansen et al. 1995); $^1\text{O}_2$ quenchers free radical scavengers (Miki 1991), and may potentially serve as in vivo inhibitors of oxidative stress (Torrissen 1990).

Other hypothesized functions for salmonids include skin coloration during sexual maturation, and as signal substances in reproduction (Torrissen 1990). Several review articles have been published detailing the possible biological functions of carotenoids in fish (Tacon 1981; Torrissen 1990) and are summarized in Table 1.1.

Carotenoids have been found to enhance both the non-specific and the specific immune systems of mammals (Bendich and Shapiro 1986; Bendich 1989). Indeed, the potential role of carotenoids in the health of mammalian species continues to spur further research in this field (Deming and Erdman 1999).

In addition, Torrissen (1984) also noted that both astaxanthin and canthaxanthin promoted the growth rate of early start-feeding fish. Christiansen and Torrissen (1996) reported that astaxanthin supplementation resulted in a clear and significant improvement in the growth of juvenile Atlantic salmon (1.7 g initial weight) when fed between 5.3-36 mg·kg$^{-1}$. Christiansen et al. (1994) concluded that astaxanthin is essential to alevins during the first-feeding period, and strongly influenced the growth, survival and vitamin A concentration in the flesh of fish. Similar observations led Torrissen and Christiansen (1995) to propose that both astaxanthin and canthaxanthin should be listed among the fat-soluble vitamins, with a minimum of 10 mg·kg$^{-1}$ of carotenoid in the dry diet. Low levels of astaxanthin are commonly found in fish oil, and may be sufficient to supply the proposed requirement.
Carotenoids have been expected to act as potential membrane antioxidants due to their reactivity with singlet molecular oxygen and peroxyl radicals (Burton and Ingold 1984; Palozza and Krinsky 1992). Mortensen and Skibsted (1997) proposed the following order of antioxidant 'stability' (i.e. lower reactivity): \( \alpha \)-tocopherol > lycopene > \( \beta \)-tocopherol > \( \gamma \)-tocopherol > \( \beta \)-carotene > zeaxanthin > \( \delta \)-tocopherol > lutein > echinenone > canthaxanthin ~ \( \beta \)-apo-8'-carotenal > astaxanthin. Research in bacteria by Milon et al. (1986) suggests that carotenoids may act as reinforcers of lipid unilamellar vesicles, as well as lipid bilayers. Miki (1991) found that astaxanthin showed a strong activity as an inhibitor of lipid peroxidation mediated by oxygen free radicals. Cold-water fish, like salmon, have a high level of polyunsaturated fat in their cellular membranes, and protection of lipid tissue from peroxidation seems to be a possible function of astaxanthin and canthaxanthin in salmonids (Torrissen et al. 1989).

From studies in model membranes, it has been concluded that the lengthy hydrocarbon structure of \( \beta \)-carotene lies fully within the hydrophobic acyl region of the bilayer (Johansson et al. 1981). By contrast astaxanthin has two polar oxygen groups at each end of the molecule, so that this polar carotenoid spans the membrane and each polar end associates with the polar phospholipid head groups (Milon et al. 1986). Since the reactive polyene chain of each carotenoid is well within the bilayer it may provide effective protection against radical reactions propagated inside the membrane (Nakagawa et al. 1997).
Table 1.1. Proposed biological activities of carotenoids.

<table>
<thead>
<tr>
<th>Function/Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant</td>
<td>Burton and Ingold 1984; Palozza and Krinsky 1992; Christiansen et al. 1995</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>In vivo inhibitors of oxidative stress</td>
</tr>
<tr>
<td>Signal substances in reproduction</td>
<td>Torrissen 1990.</td>
</tr>
<tr>
<td>Enhance immune function</td>
<td>Bendich and Shapiro 1986; Bendich 1989.</td>
</tr>
<tr>
<td>Growth enhancer in young fish</td>
<td>Torrissen 1984; Christiansen and Torrissen 1996.</td>
</tr>
<tr>
<td>Respiratory pigment (fish)</td>
<td>Balon 1975; Mikulin and Soin 1975; Soin 1977.</td>
</tr>
</tbody>
</table>

1.5 Pro-vitamin A function of carotenoids

Research has revealed that both astaxanthin and canthaxanthin are reduced to vitamin A (provitamin A; retinal; Fig. 1.1)(Storebakken and No 1992). In addition, Guillou et al. (1992) also found that zeaxanthin (β,β-carotene-3,3'-diol; Fig. 1.1) was a preferential source of vitamin A over astaxanthin and canthaxanthin. Other researchers have elucidated a possible metabolic pathway for this reduction: astaxanthin is reduced to zeaxanthin via β-adonixanthin (3,3'-dihydroxy-β,β-carotene-4-one; Fig. 1.1) (Schiedt et al. 1985; Torrissen et al. 1989; Fig. 1.4). Zeaxanthin is further reduced and transformed to vitamin A₁ or A₂ in the intestinal wall of vitamin A-depleted rainbow trout (Schiedt et al. 1985; Al-Khalifa and Simpson 1988; Storebakken and No 1992). In the rat, β-carotene is converted into retinal (vitamin A₁ aldehyde) via 15,15'-β-carotene dioxygenase in the intestinal wall, but may also take place in the liver, the main storage organ of vitamin A (Olson, 1989). Sivtseva (1982) found that the liver and intestine of trout had the highest concentrations of vitamin A of the tissues examined, which corresponds to the presence of the carotene-cleaving enzyme, β-carotene 15,15'-dioxygenase, in various tissues including the intestine, liver and kidney of rats (Roels and Lui 1973). This may reflect the metabolic capacities of these tissues for the conversion of carotenoid pigments to vitamin A, and may indicate that carotenoids are important in initial development of juvenile fish, particularly with respect to vitamin A formation. In support of this, Czeczuga (1979) observed that the liver was the richest organ in carotenoids in rainbow trout fry. Schiedt et al. (1986) found a liver vitamin
A₁:A₂ ratio of 1:3, identical to the hepatic bioconversion of carotenoids observed by Guillou et al. (1992), where a significantly higher preference for the formation of retinol₂ over retinol₁ (R₂/R₁ ratio equal to 3.0) was observed.

1.6 Factors influencing carotenoid utilization

Despite the many proposed functions of carotenoids in salmonid fish, product marketing in commercial aquaculture relies heavily on flesh pigmentation to meet consumer demands. However, there are several main factors that affect the utilization and retention of carotenoids including: losses during feed processing and storage; feed waste; digestibility or intestinal absorption; and metabolism and excretion (Hardy et al. 1990; Torrissen et al. 1990; Metuslach et al. 1996). However, Torrissen (1985) suggests that when considering only the market demand for pigmented flesh, only biological factors should determine the pigmentation regime: (1) absorption (2) deposition of ingested carotenoids, and (3) metabolic loss of deposited carotenoids. While much research has been devoted to investigations into the absorptive process (namely digestibility) and retention in flesh, few researchers have investigated the metabolic fate of absorbed carotenoids in fish.

To more effectively pigment salmon and trout, further information is necessary to understand the fundamental physiological principles underlying the complex process of pigmentation. Major gaps still remain in our knowledge of the following fundamental pathways (1) the dissociation of carotenoids from the feed bolus; (2) the inclusion of carotenoids into mixed micelles in the intestinal lumen; (3) the uptake of carotenoids into intestinal enterocytes; (4) the assimilation of carotenoids into chylomicrons; (5) the release and transport into the systemic circulation; and (6) the distribution, metabolism, and recycling of carotenoids among tissues (Deming and Erdman 1999).
Fig. 1.4. Possible reductive pathways for astaxanthin and canthaxanthin. Based on data from Kitahara (1983), Schiedt et al. (1985), Ando et al. (1986), Guillou et al. (1989) and Bjerkeng et al. (2000).
1.6.1 Dietary feeding regime impacts

Feeding regimes represent one of the non-biological aspects of the pigmentation process that may play a significant role in the ultimate colouration of salmonid flesh. This area is not well documented in the literature, however, and remains unclear as to the most cost-effective method of supplying carotenoids in diets. This process incorporates both the feeding ration and frequency of feeding, as well as the dietary carotenoid concentration.

Carotenoid retention differences between various feeding regimes have been ascribed to feed losses (uneaten feed) (Storebakken and Choubert 1991), which could potentially be due to the feeding strategy (ration level and frequency of feeding). Feed losses were estimated to be negligible at feeding rations of 0.5%·day$^{-1}$, less than 15% at a ration level of 1.0%·day$^{-1}$, and more than 40% at a ration level of 1.5%·day$^{-1}$ (Austreng 1979, Storebakken and Austreng 1987). However, frequency of feeding has not been well studied to date as related directly to pigmentation efficiency. Wathne et al. (1998) found that maximum pigment deposition can be obtained by frequent feeding of diets containing carotenoids. Despite these considerations, current commercial feeding practices reduce these losses due to mechanized/computerized feed delivery systems which continually monitor feeding patterns and recover feed waste.

Evidence from Foss et al. (1984) and Choubert (1985) suggest that flesh carotenoid concentrations increase during starvation and has been attributed to a reduced catabolic (turnover) rate of carotenoids in comparison with fat or protein. However, Torrissen (1985) found that when pigmented fish are fed a diet without supplemented pigment, a depletion of the deposited carotenoids occurs, and suggests that most of the ‘loss’ can be accounted for by the growth of the fish, but observed that there may also be metabolism of deposited carotenoids.
In addition, Kiessling et al. (1995) noted that pigmented *S. salar* fed diets without pigment supplementation showed more rapid blood pigment clearance than starved fish. These authors suggest that the feed remaining in the alimentary tract of the starved fish is potentially acting as a reservoir of pigment for the blood until the intestinal tract was empty. Therefore, gut retention (transit) time may have a significant effect on pigment uptake, i.e. a longer period for more complete extraction of pigment from the gut lumen. Therefore the efficiency of uptake may be due to the time allowed for complete digestion of feed prior to supplemental diet being administered. Kiessling et al. (1995) also noted that feeding the same diet containing 75 mg astaxanthin·kg⁻¹ diet once daily versus twice daily did not result in suppression of blood pigment levels in Atlantic salmon.

While feeding strategy may offer a tool in the aquaculturists’ repertoire to more effectively pigment fish, ultimately biological factors control the final level of pigmentation. The first step in the pigmentation process is the digestion (digestibility) of carotenoids: the liberation of carotenoids from the food matrix and their subsequent absorption across the gastrointestinal tract.

1.6.2 Intestinal absorption and digestibility

Carotenoids are lipid-soluble compounds that follow the absorption pathway of dietary fat (Deeming and Erdman 1999) and is schematically represented in Fig. 1.5. They are partially released from the food matrix by gastric action, and digestive enzymes. The extent of release is variable and may depend on the level of complexion with other components in the food such as protein, or whether they are in the crystallized state or dissolved in dietary oils (Deeming and Erdman 1999). In addition, digestive enzyme activity may also influence digestibility as observed by Torrissen and Torrissen (1985) where maturing fish had
significantly higher activities of digestive proteases than immature fish, although differences in carotenoid digestibility were not directly measured in this study.

Once released from the food matrix, carotenoids solubilize into lipid globules of various sizes in the stomach. This solubilization of individual carotenoid molecules into lipid emulsions *in vitro* is thought to be selective and related to the specific polarity of each molecule (Borel *et al.* 1996). Non-polar carotenes are thought to migrate to the triacylglycerol-rich core of the mixed micelle (Borel *et al.* 1996), while more polar xanthophylls orient at the surface monolayer along with proteins, phospholipids and partially ionized fatty acids (Deeming and Erdman 1999). This selective orientation in membranes, micelles, and lipoproteins is likely to be similar to that of other lipid molecules and based upon the polarity, length, and structure of the carotenoid molecule (Britton 1995). Astaxanthin possesses two hydroxy-groups in addition to two keto-groups in the ring, while canthaxanthin is only a keto-hydrocarbon, and these differences may affect their solubility into mixed micelles prior to absorption in the gastro-intestinal tract. Gobantes *et al.* (1997) suggest that the differential absorption between astaxanthin and canthaxanthin may be due to the ability of animals to form suitable micellar solutions in the intestinal lumen with one carotenoid but not with the other (Bauernfeind *et al.* 1981). In support of this, Rengel *et al.* (2000) found that *in vitro* astaxanthin incorporation into liposomes was 2.5 times higher than that of canthaxanthin when prepared in equivalent molar concentrations.

The presence of dietary fat in the gastrointestinal tract triggers the release of bile acids from the gall bladder, which aid in the reduction of lipid particle size and stabilization into mixed micelles (Deeming and Erdman 1999). Carotenoids are solubilized into mixed micelles along with other dietary components such as triacylglycerols and their hydrolysis products, phospholipids, cholesterol esters, and bile acids. As carotenoids are lipophilic
compounds, it is likely that dietary fat enhances their absorption (Baurenfiend et al. 1981). Several researchers have reported that carotenoid solubility in lipid is an important physical characteristic, and that dietary lipid promotes carotenoid absorption (Torrissen 1985; Hardy et al. 1990; Torrissen et al. 1990; Choubert et al. 1991). In fact, it has been found that increasing the lipid content of the diet assists the passage of pigment across the gut wall (Abdui-Malak et al. 1975; Spinelli and Mahnken 1978). Torrissen et al. (1990) report that increases in dietary lipid inclusion from 4-24% had a positive influence on the apparent digestibility and retention of carotenoids in rainbow trout.

The extent of carotenoid solubilization into mixed micelles may be affected by structural features of the carotenoid molecule and/or micellar fatty acid composition, as well as the extent of saturation (Borel et al. 1996). In addition, other components within the food can disrupt micelle formation and/or bind carotenoids, thereby inhibiting mucosal uptake of carotenoids. Soluble fibers found in dietary ingredients such as alginates, plant gums and oat bran, are viscous polysaccharides that can solubilize in the gastric contents of the intestinal lumen where partitioning of bile acids into the gel phase of gastric contents could hinder micelle formation, resulting in increased fat and bile acid excretion in the feces (Deeming and Erdman 1999).

In addition to these factors, it has been shown that carotenoids compete with each other for uptake and absorption, possibly for incorporation into mixed micelles. Carotenoids may also interact during exchanges between lipoproteins during the post-prandial state (Deeming and Erdman 1999), and this is extensively reviewed by van den Berg (1999). Torrissen (1986; 1989) found that the plasma astaxanthin:canthaxanthin ratio in rainbow trout was significantly lower than the ratio in the diet, indicating that rainbow trout have a preferred absorption of astaxanthin compared to canthaxanthin. Hata and Hata (1972) suggest that the introduction of hydroxyl groups on the carotene skeleton results in better
absorption and accumulation of astaxanthin. Similar results were also observed by Tidemann et al. (1983), Foss et al. (1984) and Choubert and Storebakken (1989) where flesh concentration of astaxanthin were higher than that of canthaxanthin in rainbow trout. The difference is thought to be due to a higher absorption of astaxanthin than canthaxanthin in the digestive tract (Torrissen 1986, 1989; Foss et al. 1987; Storebakken et al. 1987). This is likely to be due to the inherent structural differences between these carotenoids, which dictate their solubility in mixed micelles prior to absorption in the gastrointestinal tract.

While uptake of carotenoids into the intestinal mucosa in trout and in humans follow a similar path to other lipid components within the mixed micelle, intestinal absorption of carotenoids is thought to occur by passive diffusion (Choubert et al. 1994b; Castenmiller and West 1998), and may be modulated by intraluminal factors (Hollander and Ruble 1978). The diffusion process requires the solubilization of the micelle in the unstirred water layer surrounding the microvillus cell membrane of the enterocyte, where micelles collide and diffuse into the membrane, releasing carotenoids and other lipid components into the cytosol of the cell (Deeming and Erdman 1999). A concentration gradient between the micelle and the cell membrane is thought to determine the rate of diffusion, where high doses of carotenoid may saturate uptake (Parker 1996).

Blood pigment levels were correlated to total gut pigment levels in Atlantic salmon, suggesting that the concentration gradient between feed and blood is an important factor governing pigment uptake rather than the total amount of pigment in the gut (Kiessling et al. 1995). In addition Choubert et al. (1994) observed a linear response in blood plasma astaxanthin by dietary astaxanthin concentrations in the range from 12.5-200 mg·kg⁻¹, indicating passive diffusion along the concentration gradient in rainbow trout.
Digestibility studies by Choubert and Storebakken (1996) revealed that neither feeding rate, nor water salinity affected digestibility of either astaxanthin or canthaxanthin in rainbow trout. However, their results did indicate that maximum apparent digestibilities in rainbow trout were obtained at 25 mg·kg⁻¹ and 50 mg·kg⁻¹ for astaxanthin and canthaxanthin, respectively, and decreased with higher inclusion levels. These authors also found that the digestibility of astaxanthin in rainbow trout was consistently higher than that of canthaxanthin and suggest that the difference may be due to their chemical structures.

Carotenoid uptake into the enterocyte does not however ensure that carotenoids will be absorbed into the body, and these may be lost in the lumen of the gastrointestinal tract due to normal physiological turnover of the mucosal cells (Erdman et al. 1993).

Once carotenoids and other lipid molecules are released into enterocytes, it is thought that they assembled into nascent chylomicrons in the Golgi apparatus, and released into the lymphatics, although the mechanism of carotenoid translocation within the cell is not known (Deeming and Erdman 1999). It should be noted that while xanthophylls may be present as esters, as in the alga Haematococcus pluvialis or yeast Phaffia rhodozyma, only free xanthophylls have been found in serum of trout and salmon, suggesting a requirement for hydrolysis of carotenoid esters prior to uptake (Choubert and Heinrich 1993) and incorporation into lipoproteins.

A representative summary of digestibility estimates from rainbow trout and Atlantic salmon is presented in Table 1.2.

1.7 Tissue carotenoid distribution

While the factors controlling tissue uptake, recycling back to the liver, and excretion are not fully understood (Deeming and Erdman 1999), studies have been performed in an
attempt to identify the carotenoids present in the various tissues of salmonids. Some tissues exhibit specific patterns of carotenoid accumulation, which may suggest that certain carotenoids exert a biological effect in one tissue over another (Deeming and Erdman 1999).

The flesh is thought to be the main organ/tissue for storing carotenoids, followed by the skin, liver and gonads (Metusalach et al. 1996), which is in agreement with the conclusions from the studies of No and Storebakken (1992).

Similar radioactive tracer studies by Choubert et al. (1987) have shown that labelled canthaxanthin is absorbed in the blood, reaching a peak 24 hours after being force-fed a single meal, which is followed by a slow decrease. After 24 hours, most of the radioactivity was found in the pyloric caeca, ovary and skin, while after 72 hours, radioactivity in these tissues decreased with a corresponding increase in the muscle, liver and kidney. This may indicate the pattern of absorption in the gastro-intestinal tract, and release into the blood, followed by clearance of the carotenoids through metabolism (liver) and excretion (kidney). A more detailed examination of individual tissues in terms of their concentrations and their relative contribution to carotenoid distribution follows.

1.7.1 In vivo carotenoid transport

Once carotenoids are absorbed into the gastrointestinal tract they are transported throughout the body to various tissues where they may be deposited and/or metabolized/excreted (Fig. 1.6). The exact mechanism by which carotenoids are distributed and interact with various tissues (i.e. uptake and deposition) is poorly understood.
Table 1.2. Apparent digestibility coefficients (ADC), flesh carotenoid concentrations and retention efficiencies in Atlantic salmon, and Rainbow trout by different pigments and diet levels of carotenoids and dietary fat level.

<table>
<thead>
<tr>
<th>Species</th>
<th>Carotenoid</th>
<th>Dietary conc. (mg·kg⁻¹)</th>
<th>Dietary lipid (%)</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>ADC (%)</th>
<th>Flesh conc. (μg·g⁻¹)</th>
<th>Retention (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBT</td>
<td>A</td>
<td>110</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-RM</td>
<td>71</td>
<td>-17.8</td>
<td>350</td>
<td>540</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-RR</td>
<td>83</td>
<td>-17.8</td>
<td>350</td>
<td>540</td>
<td>7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-RS</td>
<td>74</td>
<td>-17.8</td>
<td>350</td>
<td>540</td>
<td>8.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-SS</td>
<td>88</td>
<td>-17.8</td>
<td>350</td>
<td>540</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-E</td>
<td>36.9</td>
<td>19.2</td>
<td>1000</td>
<td>998</td>
<td>78.9</td>
<td>9.9</td>
<td>34.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-E + Z</td>
<td>35.4</td>
<td>19.5</td>
<td>400</td>
<td>998</td>
<td>51-70</td>
<td>6.4</td>
<td>30-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25-50</td>
<td>17.1</td>
<td>111</td>
<td>163.1</td>
<td>2.9</td>
<td>11.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>18-174</td>
<td>17.1</td>
<td>135</td>
<td>235</td>
<td>61.1-79.1</td>
<td>1.0-3.8</td>
<td>2.8-12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>165</td>
<td>19.0</td>
<td>63</td>
<td>123</td>
<td>1.4-19.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25-100</td>
<td>-7.8</td>
<td>460</td>
<td></td>
<td>3.6</td>
<td>11.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>-13</td>
<td>730</td>
<td></td>
<td>6.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>52</td>
<td>22.7</td>
<td>507</td>
<td>950</td>
<td>51-70</td>
<td>6.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>100</td>
<td>20.6</td>
<td>0.37</td>
<td>350</td>
<td>1.4-19.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-E/Z</td>
<td>35</td>
<td>-19</td>
<td>400</td>
<td></td>
<td>3.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>101</td>
<td>12.8</td>
<td>28</td>
<td>57</td>
<td>4.8</td>
<td>5.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>-9.6</td>
<td>27</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>31</td>
<td>18</td>
<td>6.5-120</td>
<td>400</td>
<td>5.5-8.0</td>
<td>9.4-17.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>45</td>
<td>8-27</td>
<td>54</td>
<td>307-372</td>
<td>6-13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>100</td>
<td>158</td>
<td>634</td>
<td></td>
<td>11.8-15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>40-100</td>
<td>12</td>
<td>191</td>
<td>1157</td>
<td>4.2-5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C (1:1)</td>
<td>60</td>
<td>-22.6</td>
<td>275</td>
<td>350</td>
<td>10.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C (75:25)</td>
<td>186</td>
<td>19.0</td>
<td>63</td>
<td>123</td>
<td>3.2-0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C (50:50)</td>
<td>187</td>
<td>19.2</td>
<td>63</td>
<td>123</td>
<td>2.0-0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C (35:65)</td>
<td>194</td>
<td>19.3</td>
<td>63</td>
<td>123</td>
<td>1.5-1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C (16:84)</td>
<td>192</td>
<td>19.4</td>
<td>63</td>
<td>123</td>
<td>0.6-1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+C</td>
<td>48+27</td>
<td>34</td>
<td></td>
<td></td>
<td>2.6-1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>20.6</td>
<td>0.37</td>
<td>350</td>
<td>3.9-20.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>115</td>
<td>12.3</td>
<td>28</td>
<td></td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>207</td>
<td>19.8</td>
<td>63</td>
<td>123</td>
<td>0.1-1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>26-100</td>
<td>-7.8</td>
<td>460</td>
<td></td>
<td>3.6-9.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>-13</td>
<td>730</td>
<td></td>
<td>6.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Carotenoid</td>
<td>Dietary conc. (mg·kg(^{-1}))</td>
<td>Dietary lipid (%)</td>
<td>Initial weight (g)</td>
<td>Final weight (g)</td>
<td>ADC (%)</td>
<td>Flesh conc. (µg·g(^{-1}))</td>
<td>Retention Efficiency (%)</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>---------------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>--------</td>
<td>---------------------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>14-170</td>
<td>17.1</td>
<td>135</td>
<td>235</td>
<td>54.7-68.6</td>
<td>1.0-2.7</td>
<td>2.3-7.0</td>
<td>Choubert and Storebakken (1989, 1996)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>25-50</td>
<td>17.1</td>
<td>111</td>
<td>163.1</td>
<td>2.0</td>
<td></td>
<td>7.3</td>
<td>Storebakken and Choubert (1991)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>111</td>
<td>17.8</td>
<td>350</td>
<td>540</td>
<td>7.5</td>
<td></td>
<td></td>
<td>Foss et al. (1987)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>43</td>
<td>22.2</td>
<td>176</td>
<td>2900</td>
<td>13.7</td>
<td></td>
<td></td>
<td>Storebakken et al. (1986)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>54-61</td>
<td>4.1-23.0</td>
<td>100-200</td>
<td></td>
<td>1.7-16.0</td>
<td></td>
<td></td>
<td>Torrissen et al. (1990)</td>
</tr>
<tr>
<td>ATS</td>
<td>A</td>
<td>6</td>
<td>26</td>
<td>220</td>
<td>810</td>
<td>0-2.48</td>
<td>7.57</td>
<td></td>
<td>Buttle et al. (2001)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>30-50</td>
<td>35</td>
<td>732</td>
<td>1700</td>
<td>2.5-3.2</td>
<td>6-7.4</td>
<td></td>
<td>Bjerkeng et al. (1999)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>4</td>
<td>23.7</td>
<td>510</td>
<td>1240</td>
<td>2.1-3.1</td>
<td>7.5-12.5</td>
<td></td>
<td>Watne et al. (1998)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>30-90</td>
<td>22.6</td>
<td>62</td>
<td>406</td>
<td>64</td>
<td>2.0</td>
<td></td>
<td>Storebakken et al. (1987)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>30</td>
<td>28</td>
<td>569</td>
<td>1090</td>
<td>1.9-2.1</td>
<td>6-7.4</td>
<td></td>
<td>Bjerkeng et al. (1999)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>0-200</td>
<td>23</td>
<td>115</td>
<td>3200</td>
<td>4</td>
<td>2.2</td>
<td></td>
<td>Torrissen et al. (1995)</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>90</td>
<td>-400</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Schiedt et al. (1988)</td>
</tr>
<tr>
<td>A-RM</td>
<td>A</td>
<td>100</td>
<td>17.8</td>
<td>1230</td>
<td>1570</td>
<td>0.5</td>
<td></td>
<td></td>
<td>Storebakken et al. (1985)</td>
</tr>
<tr>
<td>A-RR</td>
<td>A</td>
<td>100</td>
<td>17.8</td>
<td>1230</td>
<td>1570</td>
<td>0.7</td>
<td></td>
<td></td>
<td>Storebakken et al. (1985)</td>
</tr>
<tr>
<td>A-RS</td>
<td>A</td>
<td>100</td>
<td>17.8</td>
<td>1230</td>
<td>1570</td>
<td>0.3</td>
<td></td>
<td></td>
<td>Storebakken et al. (1985)</td>
</tr>
<tr>
<td>A-SS</td>
<td>A</td>
<td>100</td>
<td>17.8</td>
<td>1230</td>
<td>1570</td>
<td>0.4</td>
<td></td>
<td></td>
<td>Storebakken et al. (1985)</td>
</tr>
<tr>
<td>A-E/Z</td>
<td>A</td>
<td>66</td>
<td>25.6</td>
<td>144</td>
<td>740</td>
<td>59</td>
<td>2.5</td>
<td>3.9</td>
<td>Bjerkeng and Berge (2000)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>60</td>
<td>26</td>
<td>220</td>
<td>810</td>
<td>0-2.72</td>
<td>5.71</td>
<td></td>
<td>Buttle et al. (2001)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>30-90</td>
<td>22.6</td>
<td>62</td>
<td>406</td>
<td>63</td>
<td>1.8</td>
<td></td>
<td>Storebakken et al. (1987)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>43</td>
<td>-22.2</td>
<td>53</td>
<td>1200</td>
<td>6.1</td>
<td></td>
<td></td>
<td>Storebakken et al. (1986)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>100</td>
<td>17.8</td>
<td>1230</td>
<td>1570</td>
<td>0.8</td>
<td></td>
<td></td>
<td>Storebakken et al. (1985)</td>
</tr>
</tbody>
</table>

Species name abbreviations: RBT, rainbow trout (Oncorhynchus mykiss); ATS, Atlantic salmon (Salmo salar).

Fig. 1.5. Schematic representation of intestinal absorption of carotenoids: Transfer sequence of carotenoids from lipid emulsion in the small intestine to bile salt micelles, through the unstirred water layer (UWL) and enterocyte, with incorporation into lymphatic (blood) chylomicrons. The nonpolar carotenes (c) are thought to be located in the hydrophobic core of the lipid emulsions, bile salt micelles, and chylomicrons, while the polar xanthophylls (x) are more likely surface components. Retinol and retinyl esters (RE) are thought to be produced in enterocytes. Adapted from Furr and Clark (1997).
Fig. 1.6. Schematic representation for uptake, metabolism and transport of carotenoids. βC, β-carotene; X-OH, xanthophylls; RE, retinyl esters; RBP, retinol-binding protein; ROH, retinol; n-VLDL, nascent VLDL; LPL, lipoprotein lipase; HL, hepatic lipase. Adapted from Parker et al. (1999).
However, several studies were undertaken to elucidate the transport mechanism of carotenoids in vivo. Ando et al. (1985; 1986a,b) observed that carotenoids were bound non-covalently to serum proteins (carotenoid-carrying-lipoproteins), specifically to high-density lipoprotein (HDL), very high-density lipoprotein (VHDL), and low-density lipoprotein (LDL) fractions in different proportions. Choubert et al. (1992) reported the distribution of canthaxanthin among the various lipoprotein fractions to be 13.9% VLDL, 15.2% LDL, 60.4% HDL, and 10.5% VHDL in the serum of immature rainbow trout, similar to the results of Choubert et al. (1991). Similarly, the distribution of astaxanthin in immature rainbow trout was 0.7% VLDL, 16.8% LDL, 66.3% HDL, and 16.1% VHDL (Choubert et al. 1994). Data by Aas et al. (1999) show a slightly different pattern in large (800-1200g) Atlantic salmon, where the majority of carotenoid (58%) was present in a high density protein fraction (HDPF), in addition to 36% in HDL and 6% in LDL. The HDPF banded parallel to BSA and salmon albumin on a non-denaturing PAGE. Serum concentrations vary with dietary lipid level and dietary carotenoid concentration, and summary examples are shown in Table 1.3.

Table 1.3. Serum/plasma astaxanthin and canthaxanthin concentrations in rainbow trout and Atlantic salmon as a function of dietary carotenoid concentration and lipid level.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pigment</th>
<th>Dietary Conc. (mg·kg⁻¹)</th>
<th>Dietary lipid (%)</th>
<th>Serum Conc. (µg·mL⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBT</td>
<td>A</td>
<td>35</td>
<td>19</td>
<td>4.1-5.7</td>
<td>Østerlie et al. 1999</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>40-100</td>
<td>10</td>
<td>3.1-9.0</td>
<td>March and MacMillan 1996</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>80</td>
<td>8.4</td>
<td>8.4</td>
<td>Choubert et al. 1994b</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>80</td>
<td>9.6</td>
<td>5.3</td>
<td>Choubert et al. 1994b</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>110</td>
<td>24.4</td>
<td>5.5</td>
<td>Barbosa et al. 1999</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>120</td>
<td>9.6</td>
<td>8.4</td>
<td>Barbosa et al. 1999</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>80</td>
<td>2.2</td>
<td>5.5</td>
<td>Choubert et al. 1992</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>80</td>
<td>3.6</td>
<td>2.2</td>
<td>Choubert et al. 1994b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>116</td>
<td>2</td>
<td>3.6</td>
<td>Choubert et al. 1994b</td>
</tr>
<tr>
<td>ATS</td>
<td>A</td>
<td>30-50</td>
<td>35</td>
<td>2.3-2.9</td>
<td>Bjerkeng et al. 1999</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>41</td>
<td>23.7</td>
<td>1.2-1.9</td>
<td>Wathne et al. 1998</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0-200</td>
<td>23</td>
<td>3</td>
<td>Torrissen et al. 1995</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>66</td>
<td>25.6</td>
<td>3.5</td>
<td>Bjerkeng and Berge 2000</td>
</tr>
</tbody>
</table>

* Denotes species. RBT, rainbow trout; ATS, Atlantic salmon.
* Denotes carotenoid. A, astaxanthin; C, canthaxanthin.
While the triacylglycerol-rich, chylomicron fraction of plasma is considered to be more indicative of carotenoid uptake than serum/plasma levels because newly absorbed carotenoids are found primarily in chylomicrons, they only represent a small fraction of total plasma carotenoids due to the rapid rate of catabolism of chylomicrons and uptake by the liver in mammals (Deeming and Erdman 1999). Thus, blood values reported in the literature in fish may underestimate the absorption of carotenoids due to the rapid clearance of chylomicrons from circulation.

March et al. (1990) reported that plasma astaxanthin concentrations in rainbow trout reached a peak 24 h after administration of a single dose of astaxanthin, ranging from 1 to 7 μg·ml⁻¹. These results are in agreement with those Choubert et al. (1987) and Guillou et al. (1987), while somewhat higher serum values of 9.04 μg·ml⁻¹ were found by Choubert et al. (1994 b). Whole plasma pharmacokinetics may not be the most practical method to assess carotenoid status, as plasma (or serum) concentrations are not only a measure of the absorption of carotenoids, but also a measure of the exchange from tissue storage, bioconversion, and excretion (Castenmiller and West 1998).

Chavez et al. (1998) suggested that the high amounts of triglycerides and cholesterol in very lipoproteins in rainbow trout make them the natural carriers of canthaxanthin. These authors propose that the high amount of phospholipid in lipoproteins is responsible for enhanced binding of canthaxanthin, with the amphipilic nature stabilizing the lipoprotein for carotenoid incorporation. From their in vitro carotenoid/lipoprotein saturation studies, they found that the phospholipid content of individual lipoproteins correlated highly to the level of carotenoid in the saturated lipoprotein, and could be used to predict the total amount of carotenoid in the saturated lipoprotein. However, no data is available on the potential astaxanthin saturability in individual lipoprotein fractions, but should provide similar values.
The observed plateau in plasma carotenoid concentration over time with continuous feeding observed by Choubert et al. (1994b) was suggested to be due to an inability to absorb more carotenoid, or to a saturation of the lipoprotein binding sites. However, research in human subjects has shown that lipoprotein fractions that carry carotenoids in the blood evidently have ample binding capacity for carotene, even when carotene intake is high (Matthews-Roth and Gulbrandsen 1974). In support of this, Chavez et al. (1998) revealed that the *in vitro* binding capacity of lipoproteins is not a limiting factor for carotenoid transport in trout. Canthaxanthin in the lipoprotein fractions amounted to 1.2, 1.8, 0.7 μg·ml⁻¹ of serum for VLDL, LDL, and HDL, respectively, *in vivo*, but could be increased 100 fold after saturation experiments to levels of 80.6, 152.8, 145.7 μg·ml⁻¹ serum for VLDL, LDL, and HDL, respectively. Despite the potential capacity, the limiting factors regulating serum carotenoid levels are not known, but could be due to factors affecting gastrointestinal formation of chylomicrons and other lipoproteins.

Both Storebakken and Goswami (1996) and Kiessling et al. (1995) found a strong correlation between dietary astaxanthin and plasma astaxanthin concentration. Storebakken and Goswami (1996) also found a high correlation between plasma astaxanthin concentration and retention in flesh (mg astaxanthin per kg body weight increase), as was the correlation between plasma astaxanthin concentration and flesh carotenoid level. Storebakken and Goswami (1996) concluded that plasma astaxanthin concentrations may be used as an indicator of astaxanthin availability in salmon.

In recent research by Gobantes et al. (1998a), female rainbow trout were found to have higher serum carotenoid concentrations than males, ranging from 2.80 to 3.33 μg·ml⁻¹ for astaxanthin and from 0.67 to 2.32 μg·ml⁻¹ for canthaxanthin, compared to 0.99 to 3.28 μg·ml⁻¹ for staxanthin and 0.78 to 1.28 μg·ml⁻¹ for canthaxanthin in the serum of males.
These authors conclude that serious error can occur when single measurements are used to categorize individuals due to the large inter-individual variations.

1.7.2 Skin

Although there are many studies showing a large variability in the amount of carotenoids (of different types) in the integument (skin) of fish, preliminary studies by Ando (1986) found that no carotenoid metabolism occurred in the integument of Chum salmon. However, skin carotenoid concentrations of Atlantic salmon fed astaxanthin show that only 20% of the carotenoids were present as astaxanthin, 10% as other basic xanthophylls, and the other 70% must therefore be considered as metabolites (Schiedt et al. 1988b). It is claimed that oxycarotenoids are deposited in the flesh in their free form, while in the skin, esters are predominantly found (Schiedt et al. 1985). Katsuyama et al. (1987) confirmed that astaxanthin diesters are mostly accumulated in the integument of rainbow trout, and kept the same native configurations as those fed, but were also partly reduced to (3R,3’R)-(major) (3R,3’S)-(medium), and (3S,3’S)-zeaxanthin (minor) in the skin, but not in the flesh. Confirmation of this was observed in radioactive astaxanthin tracer studies in rainbow trout by Schiedt et al. (1985), where a large proportion of the recovered radioactivity was found unchanged in the flesh (40-80%), and 12-60% was recovered in the skin, largely esterified and partly metabolized.

1.7.3 Flesh

Although Hardy et al. (1990) found higher levels of astaxanthin in plasma, liver, skin, kidney, and gonads than in the muscle on a wet weight basis, they state that muscle is the most important tissue regarding total body contents of astaxanthin due to the large organ to body weight ratio of this tissue. A conclusion shared by Torrissen and Ingebrigsten (1992). A representative summary of flesh values obtained in various studies in both rainbow trout and Atlantic salmon is presented in Table 1.2. The flesh is acknowledged to be the main
organ/tissue for storing carotenoids, followed by the skin, liver and gonads (No and Storebakken 1992; Metusalach et al. 1996). According to some research, salmonids are unable to oxygenate carotenoids, and deposit ingested xanthophylls (astaxanthin and canthaxanthin) without modification in the flesh (Steven 1949; Hata and Hata 1973).

Henmi et al. (1987; 1989) suggest that both astaxanthin and canthaxanthin bind to actomyosin in the muscle by means of weak hydrophobic bonds. In contrast, Choubert (1985) noted that canthaxanthin is stored essentially in the sarcoplasmic membrane of the muscular fibre, which contains phospholipids. The association of pigment to phospholipids was also noted by Chavez et al. (1998) where pigments were bound to the phospholipid moieties of serum lipoproteins. From the studies by Henmi et al. (1987; 1989), it was considered that astaxanthin was primarily associated with the myotomes in muscle tissue. Visual observations by Torrissen and Ingebrigsten (1992) support the pigment-muscle interaction by the observation that muscle myotomes appear pink and the connective tissue is pale. However, the results from the autoradiography study by Torrissen and Ingebrigsten (1992) show that most of the recovered radioactivity is deposited mainly in the connective tissue between the myotomes. These authors concluded that because the connective tissue is pale (i.e. low in pigment), the compounds responsible for the high radioactivity are likely uncoloured metabolites of astaxanthin.

Interestingly, some researchers have reported a regional discrepancy in flesh pigmentation, decreasing in concentration from distal to anterior sections in rainbow trout (March and MacMillan 1996; Nickell and Bromage 1998). However, no plausible explanation for this regional difference has been proposed. Johnston et al. (2000) did not find a correlation between muscle astaxanthin concentration and muscle fiber density. Therefore, another mechanism must be present to account for the regional carotenoid deposition.
Christiansen and Wallace (1988) observed a positive correlation between canthaxanthin and lipid contents deposited in the muscle tissue of small Arctic charr. This seems to agree with observations made by Torrissen and Ingebrigsten (1992) following the administration of radiolabelled carotenoids, and the recovery of high levels of activity in the lipid between the myotomes of the muscle.

Bjerkeng et al. (1990) found a plateau in the flesh pigment levels in rainbow trout in a dose-response to dietary carotenoid concentrations above 50 mg·kg\(^{-1}\), which they suggest cannot be explained by simple saturation of carotenoids in the flesh since the response to canthaxanthin paralleled that of astaxanthin, despite the lower flesh concentrations. Therefore, these authors suggest that the response is due to the capacity for absorption or transport to the various tissues, and the rate of carotenoid catabolism, as reviewed by Torrissen et al. (1989).

Bjerkeng et al. (1992) hypothesize that the flesh has a relatively higher capacity to store carotenoids than the maximum values observed. The hypothesis is based on the assumption put forth by Henmi et al. (1989), who claim that 0.9 mg astaxanthin is able to combine with 1.0 g of the actomyosin complex. Bjerkeng et al. (1992) expanded on this by postulating that if 17% of the flesh is protein, which contains ~50% actin, a theoretical flesh astaxanthin saturation level of nearly 100 mg·kg\(^{-1}\) could potentially be obtained. In addition, some carotenoids are deposited within the fatty intracellular tissues (Torrissen and Ingebrigsten 1992). Therefore, Bjerkeng et al. (1992) concluded that flesh saturation levels are not the limiting factor in pigmentation regimes, and that other factors are causing the low pigment retention rates.
1.7.3.1 Flesh pigment retention

Flesh retention is a measure of the efficacy of pigment utilization throughout the course of an experiment and is based on the amount of recovered carotenoid in the flesh and the amount of dietary carotenoids fed. Pigment retention is a useful comparative method by which efficiency of pigmentation can be assessed between various studies. However, several methods (calculations) have been proposed to estimate pigment retention accounting for the large variation observed (Table 1.2). Torrissen (1995) suggested a new method for the calculation of pigment retention in farmed fish as:

\[
\text{Tissue retention (\%)} = \left( \frac{(C_{xf} \times W_f - C_{xi} \times W_i)}{((W_f - W_i) \times C \times C_{xd}} \right) \times F \times \left( \frac{100}{C_{xf}} \right)
\]

Where: 
- \(C_{xf}\) = astaxanthin concentration in the flesh at termination, 
- \(W_f\) = final fish weight, 
- \(C_{xi}\) = initial astaxanthin concentration, 
- \(W_i\) = initial weight, 
- \(F\) = ratio of flesh to body weight, 
- \(C\) = Feed Conversion Ratio and 
- \(C_{xd}\) = Astaxanthin concentration in the diet.

Results from Torrissen (1995) suggest that in rainbow trout, flesh astaxanthin concentration is linearly related to growth, since growth is related to the amount of astaxanthin consumed, and therefore has a primary influence on the availability for absorption. As small fish (80-100g) tend to deposit lower levels of carotenoids, larger initial weights resulted in significantly higher flesh astaxanthin concentrations, as observed by Abdul-Malak et al. (1975) and Torrissen (1989). In addition, Torrissen (1985) found that while increasing levels of dietary astaxanthin resulted in higher total flesh carotenoid levels, it resulted in a decreased retention ratio of astaxanthin (astaxanthin in flesh to astaxanthin in diet). This may indicate a threshold for the optimum utilization of dietary astaxanthin, either due to reduced digestibility at higher dietary inclusion rates or to excess pigment being degraded and excreted.
1.7.3.2 Isomers

The most important commercial source of astaxanthin (Carophyll® Pink, Hoffman-La Roche, Basel, Switzerland) contains approximately 25% Z-isomers (Bjerkeng et al. 1997). However, during feed processing, carotenoids may be structurally transformed by oxidative and thermal processes, which may degrade these to colourless compounds (Villota and Hawkes 1992). Interest in the optimal presentation form of astaxanthin has prompted investigations into the utilization of the various isomers (Fig. 1.2) in salmonid fish.

Recent research by Bjerkeng et al. (1997) has shown that the isomeric ratios of all-\(E\) and Z-astaxanthin may have an impact on apparent digestibility, indicating competitive uptake mechanisms for the different isomers. Epimerization studies on fish fed astaxanthin suggest that salmonids (e.g. rainbow trout, Atlantic salmon) are unable to isomerize the various astaxanthin isomers in flesh. Instead Storebakken et al. (1985) suggest that the observed isomeric ratios of astaxanthin isomers are derived from dietary sources rather than a metabolic epimerization. Schiedt et al. (1988) also noted that the stereoisomeric composition of idoxanthin consisted of only four of the eight possible isomers upon degradation from the three astaxanthin isomers. The idoxanthin metabolites were identical to the astaxanthin enantiomers from which they were derived, leading to the conclusion that all three astaxanthin stereoisomers were equally reduced. Conversely, Bjerkeng et al. (1997) suggested that the lack of differences found in the isomeric ratios of carotenoids found in flesh tissues between the two dietary treatments used indicates selectivity in metabolism of the different astaxanthin stereoisomers, with substantial isomerization of astaxanthin Z-isomers, thought to occur in the liver (Bjerkeng et al. 1997).

Schiedt et al. (1988 a) found an accumulation of idoxanthin in the intestine and pyloric caeca of Atlantic salmon, leading these authors to conclude that this is the site of reduction
during absorption. These authors also noted a slight decrease of (3S,3'S)-astaxanthin in the liver in favour of the (3R,3'R)-isomer, indicating a possible preference for metabolism of the (3S,3'S)-isomer to zeaxanthin, or a deposition preference of the (3R,3'R)-isomer.

1.7.4 Kidney

Torrissen and Ingebrigsten (1992) observed a high degree of radioactivity in the excretory kidney of fish fed $^{14}$C-astaxanthin, and suggest the possibility of urinary excretion of astaxanthin metabolites. In support of this observation are the results of Hardy et al. (1990) who found high levels of radioactivity in the drain water after feeding $^{14}$C-astaxanthin to rainbow trout. Although no attempts were made to quantify the level of loss of carotenoids from this tissue, it offers an alternate/additional explanation for the discrepancy between the amounts of carotenoids absorbed and the amounts retained in tissues.

As cited by Sivtseva and Dubrovin (1982), there was a high concentration of carotenoids in the kidneys of the rainbow trout, which they suggest is possibly due to their participation in water-salt exchange (Petrunyaka 1979). Despite the coincidental observations of high carotenoid levels in the kidney, no effects of carotenoids on water-salt exchange has been noted in the literature, nor was a mechanism by which this might occur proposed.

1.7.5 Liver

The liver is a key organ that controls many important functions, playing a prominent role in fish physiology (Bruslé and González I Anadon 1996; Fig 1.7). The liver in salmonid fish is a dense unlobed organ, located ventrally in the abdominal cavity. It is a highly vasucalarized tissue receiving a dual blood supply, from the hepatic portal vein, draining the gastrointestinal tract and visceral tissues, and the other delivering freshly oxygenated blood from the celiac artery (from the gills). The liver drains directly into the heart via the
hepatic vein. The liver is also the organ responsible for bile production, which is stored in the gall bladder, which empties into the gastrointestinal tract.

The liver is the main organ in intermediary metabolism and is the primary site of xenobiotic detoxification (Ozaki 1978). Indeed, the liver is thought to be the major organ for the biotransformation of carotenoids to their respective reductive metabolites, due to the absence of canthaxanthin and the presence of its metabolites in the liver of fish fed canthaxanthin (Hardy et al. 1990; Metusalach et al. 1996). Schiedt et al. (1986) suggest that it would not be uncommon to expect varying quantities of precursors and intermediates in the liver, due to a particular metabolic equilibrium. In support of this, No and Storebakken (1992) found a minor amount (0.7-4.6%) of echinenone in liver and gut of trout fed canthaxanthin-supplemented diets, similar to results of Guillou et al. (1989), suggesting reductive metabolism of canthaxanthin (Scheidt et al. 1985).

Results obtained by Hardy et al. (1990) suggest that a significant amount of absorbed $^{14}$C-labelled canthaxanthin was metabolized by the liver due to the high specific activity found in the bile, which was eight times that found in blood serum. Torrissen and Ingebrigsten (1992) also observed a high degree of radioactivity in the bile of fish fed labelled carotenoids, indicating biliary excretion of astaxanthin metabolites. Schiedt et al. (1985) found the highest concentrations of recovered radioactivity in the bile (20-40 µg·g$^{-1}$) of salmon fed radiolabelled carotenoids. Astaxanthin was found to be reductively degraded to idoxanthin and crustaxanthin relatively quickly and eliminated from the liver in salmon (Scheidt et al. 1985). The liver may, therefore, be degrading/metabolizing carotenoids to other pigments that no longer carry a chromophore. Storebakken and No (1992) detected unidentified catabolites of carotenoids in the bile of trout, indicating that the liver is a major metabolic organ for carotenoids.
Digestion
- Bile

Storage
- Lipids
- Carbohydrates
- Vitamin A
- Iron

Reproduction
- Vitellogenin

Immune Defence
- Reticulo-endothelial system
- Ig synthesis

Detoxification
- Toxins
- Pollutants

Blood turn-over

Nitrogen catabolism

Fig. 1.7. Prominent functions of the fish liver. Reproduced from Bruslé and González i Anadon (1996).
Christiansen et al. (1995) observed that the liver was the major site of vitamin A storage in Atlantic salmon. Liver retinol levels in fish fed astaxanthin-supplemented diets were 10 times higher than those fed unsupplemented diets, with both groups receiving diets supplemented with vitamin A levels above normal requirements. Typical dietary inclusion levels of vitamin A in commercial feeds is ~ 12 000 IU·kg⁻¹, while the minimum required inclusion level is 2 500 IU·kg⁻¹ (NRC 1993). Therefore, the increase in vitamin A concentration was due solely to the bioconversion of astaxanthin to vitamin A, which has previously only been described in vitamin A deficient fish (Al-Khalifa and Simpson 1988). Similar results were obtained in tilapia by Katsuyama and Matsuno (1988).

The high level of radioactivity in the connective tissue of the muscle was ascribed to colourless derivatives of astaxanthin metabolites (Torrissen and Ingebrigsten 1992). Similarly, other studies have determined the presence of a yellow non-carotenoid pigment in the flesh of rainbow trout, which displayed no absorption maxima within the visible range (Metusalach et al. 1996; No and Storebakken 1992). No and Storebakken (1992) found a peak with an HPLC retention time between 3.2-3.6 minutes (between cryptoxanthin 3.0 mins and canthaxanthin 4.2-4.5 mins) in liver tissues of rainbow trout, and upon further analysis revealed an absorption maximum at 290 nm, and that the compound was not a carotenoid. The possibility that this compound is a reductive metabolite was not discussed, but is probable as the liver is considered the main metabolic organ in fish.

The presence of radioactivity in the intestinal mucosa may imply reabsorption of the biliary excreted metabolites because no radioactivity was seen in the stomach tissues (Torrissen and Ingebrigsten 1992). These authors conclude that this observation offers evidence in support of biliary excretion of astaxanthin-derived metabolites and for the likelihood of enterohepatic circulation. Indeed, due to the role of the liver in the secretion
of emulsifying agents for lipid absorption (e.g. bile acids and salts), it’s role in mediating the uptake of carotenoids from the gastro-intestinal tract should not be dismissed. However, as no studies have recovered the parent carotenoid from bile, it seems unlikely that enterohepatic circulation is important in carotenoid uptake and retention.

Histological techniques are considered to be an important determinant for the evaluation of the effects of food additives (Johnson and Bergmann 1984). The liver, being the central organ of intermediary metabolism (including the biotransformation of carotenoids), should therefore reflect the effects of carotenoids on fish metabolism (Segner et al. 1989). Segner et al. (1989) examined the effects of dietary astaxanthin on liver histology in tilapia, and found an improvement in the liver structure with increasing levels of astaxanthin (0-132 mg·kg⁻¹), exhibiting a well-organized liver parenchyma, with well-compartmented hepatocytes without pathological alterations in Oreochromis niloticus and Colisa labiosa. These observations lead these authors to conclude that astaxanthin supplementation had a positive effect on liver structure and thus on intermediary metabolism.

1.8 Metabolism of carotenoids

The large difference (approximately 30-50%) between the retention and apparent digestibility of carotenoids (No and Storebakken 1991b), implies that higher levels of carotenoids are absorbed compared to that deposited by salmonids (Torrissen et al. 1989; No and Storebakken 1991b). Hardy et al. (1990) suggest that the observed discrepancy may be due to the metabolism and excretion of absorbed carotenoids. Indeed, the study by Hardy et al. (1990) failed to detect canthaxanthin in the bile when fed ¹⁴C-labelled canthaxanthin, despite the large specific activity found, suggesting that absorbed canthaxanthin was metabolized in the liver and that metabolic product(s) were excreted in the bile. Their results provide the first evidence that biliary excretion is a major excretory
route of metabolized carotenoids, and may be sufficient to explain the discrepancy between absorption and retention of carotenoids in salmonid tissues.

One hypothesis to explain the discrepancy is that carotenoids are enzymatically cleaved to produce vitamin A in the gastrointestinal tissue. Although this has been shown to occur in fish, the extent to which this occurs is thought to be quantitatively insignificant (Schiedt et al. 1985). Alternatively, carotenoids may be oxidatively degenerated in the digestive tract. However, the expected diversity/complexity of carotenoids was not evidenced, indicating that oxidative degeneration in the gastro-intestinal tract was not significant, leading Torrissen et al. (1990) to conclude that the differences observed between absorption and retention may only be explained by metabolism and excretion of absorbed carotenoids.

From post-prandial carotenoid elimination studies an estimate of the clearance, and thus the elimination of absorbed carotenoids, may provide additional information on the potential for catabolism of absorbed carotenoids. Guillou et al. (1993) showed that the blood clearance rates in rainbow trout, calculated from the mean plasma linear regression slopes for astaxanthin and canthaxanthin, were 2.07 %/hour and 1.98 %/hour, respectively. Similarly, these authors calculated the half-lives of astaxanthin and canthaxanthin, and revealed similar half-lives of 24.1 (astaxanthin) and 22.2 hours (canthaxanthin).

Ando (1986) found that significant amounts of carotenoids were decomposed during spawning migration in Chum salmon (*Oncorhynchus keta*), which he attributed to the activity of hepatic heme proteins of the liver. Crozier (1970) also found that a large proportion (more than 60%) of muscle carotenoids were decomposed during spawning in salmon. Ando (1986) noted that the total astaxanthin bleaching activity was highly correlated to the weight of the liver in Chum salmon.
Although fish are unable to synthesize carotenoids \textit{de novo}, recent investigations propose that oxidative pathways may be present in rainbow trout. The results observed by Guillou \textit{et al.} (1992) provide the first evidence that trout may have the ability to oxidize $^3$H-canthaxanthin and $^3$H-zeaxanthin into $^3$H-astaxanthin. This is in contrast to observations by Hata and Hata (1973) who concluded that rainbow trout were unable to convert $^{14}$C-labelled $\beta$-carotene, lutein, or zeaxanthin into astaxanthin. The results observed by Hata and Hata (1973) support the role of the liver in these metabolic pathways, with the liver displaying the highest level of radioactivity when administered $^{14}$C-labelled $\beta$-carotene. These results may offer support for the bioconversion of $\beta$-carotene into vitamin A. The size of the fish used (130-180 g) by Hata and Hata (1973) may also explain the inability of larger fish (1100-1600 g) to convert these compounds into astaxanthin (Guillou \textit{et al.} 1992). This may indicate metabolic pathway preferences between fish of different age groups.

1.9 Age effects

Despite the evidence indicating the role of the liver in carotenoid metabolism and whole body distribution, other factors also influence pigmentation. According to Torrissen and Naevdal (1984) fish size, sex, and degree of sexual maturation may influence carotenoid deposition in fish. These authors found that in addition to these factors, there is also a significant effect of genetics on carotenoid deposition.

In a commercial scale growth trial, the pigment distribution varied in different tissues throughout the complete growth cycle from immature juveniles to large market size rainbow trout (Bjerkeng \textit{et al.} 1992). These authors were unable to detect carotenoids in liver tissue until 58 weeks of post-feeding of dietary carotenoids. In addition, the total carotenoid concents of sexually mature females were 73-79% of immature females, while sexually mature males had 18-19% of the total carotenoid content of immature males.
(Bjerkeng et al. 1992), suggesting better carotenoid retentions in female fish compared to male fish at maturity.

Aas et al. (1997) recovered idoxanthin in the flesh of Arctic charr (Salvelinus alpinus) fed astaxanthin, but in addition, the proportion of idoxanthin to total carotenoid recovered was size-dependent, with the smallest fish containing the highest percentages of idoxanthin when fed astaxanthin. Similar results were obtained by Schiedt et al. (1989) who observed a higher concentration of idoxanthin in the muscle of small (177 g) or medium-sized (406 g) Atlantic salmon compared to large fish (~1200 g), and speculated that the high content of astaxanthin metabolites was due to a higher metabolic activity (i.e. reduction of astaxanthin to idoxanthin). In addition, Arctic charr fed astaxanthin for 3 months contained more idoxanthin relative to astaxanthin, suggesting that younger fish (1+ year class) have a higher turnover of astaxanthin than older fish (3+ year class) (Aas et al. 1997). Indeed, Hatlen et al. (1995) observed that the estimates of astaxanthin deposition in relation to body weight increase indicated that younger Arctic charr (1+ year class) were less efficient at utilizing dietary carotenoids for flesh pigmentation than older fish.

Bjerkeng et al. (1992) observed a slight preference for the deposition of (3R, 3’R) and (3R, 3’S)- isomers relative to the (3S,3’S)- isomer in the liver of immature fish fed astaxanthin, while this preference was not observed in mature trout. They propose that due to the increased transport of carotenoids from the flesh to skin via blood in mature fish (Kitahara 1983), the increased throughput of carotenoids in the liver may outweigh preferred isomers from astaxanthin metabolism.

1.10 Reproductive transport of carotenoids

Steven (1949) and Sivtseva (1982) observed that muscle carotenoids are used as reserves (storage) and are mobilized to developing oocytes during gonadal maturation in female
rainbow trout and brown trout, respectively. Indeed, Ando et al. (1986a) observed that muscle carotenoid levels decreased, while serum carotenoid levels increased during the spawning migration of Chum salmon.

The mobilization of pigments from the muscle to gonadal tissue during sexual maturation has resulted in economic losses due to reduced market acceptability of pigment-depleted products (Torrissen and Torrissen 1985). Crozier (1970) observed that over 60% of the flesh pigments in salmon were metabolized in spawning fish and suggested that these pigments are deposited in different tissues with males preferentially depositing these in the skin, and females in the ovaries.

1.11 Species differences in carotenoid utilization

It has been suggested that the differences in the absorptive process in the small intestine may be responsible for the different concentrations of carotenoids observed in different species of animals (Yang and Tume 1993). According to Furr and Clark (1997), carotenoids move into the enterocytes through passive diffusion in mammals. Slifka et al. (1999) suggest that if this is true, species differences in absorption/accumulation may arise due to species-specific differences in gut luminal events such as pH diversity, gut motility and liposome and micelle formation, in addition to the type and amount of dietary fat. To compound the problem, little is known concerning the mechanism of carotenoid incorporation into lipoprotein fractions and how these enter the circulation (Furr and Clark 1997). Species variations in lipoprotein handling are also likely to be large contributors to variations in the accumulation of carotenoids (Slifka et al. 1999).

In a study by Sigurgisladottir et al. (1994) it was noted that the rate of carotenoid depletion differed between canthaxanthin and astaxanthin in Atlantic salmon. These authors noted that the rate of canthaxanthin depletion was very slow, a reduction of only 0.34 μg·g⁻¹ flesh
over 15 weeks and concluded that this depletion was primarily due to the weight gain of
the fish (doubled). They found that astaxanthin was depleted at a significantly faster rate
than canthaxanthin, and suggest that this may be due to its excretion by the glucuronic acid
pathway, or due to the chemical instability of astaxanthin.

In addition, Atlantic salmon appear to utilize/catabolize absorbed carotenoids differently.
Astaxanthin is reductively catabolized (Schiedt et al. 1985), and accumulated as
idoxanthin in the blood and muscle of Atlantic salmon (Schiedt et al. 1989), as well as one
genus of Salvelinus (Ando et al., 1989, Aas et al. 1997). Only trace amounts of this
carotenoid have been recovered from tissues of rainbow trout (Bjerkeng et al. 1997). While
astaxanthin has been shown to be better utilized in rainbow trout than canthaxanthin
(Torrissen 1986; 1989) the opposite has been shown in Atlantic salmon by Buttle et al.

1.12 Differences between astaxanthin and canthaxanthin utilization
Several studies have found that astaxanthin is better utilized by rainbow trout than
canthaxanthin. Torrissen (1986; 1989) found that the flesh canthaxanthin:astaxanthin ratio
was significantly lower than the ratio in the diet, indicating that rainbow trout have a
preferred absorption of astaxanthin compared to canthaxanthin, and a preferred deposition
in the flesh. Hata and Hata (1972) suggest that the introduction of hydroxyl groups on the
carotene skeleton results in better absorption and accumulation of astaxanthin. Similar
results were also observed by Tidemann et al. (1984), Foss et al. (1984) and Choubert and
Storebakken (1989) where flesh concentration of astaxanthin were higher than that of
canthaxanthin in rainbow trout. The difference is thought to be due to a higher absorption
of astaxanthin than canthaxanthin in the digestive tract (Choubert and Luquet 1979;
Torrissen 1986, 1989; Foss et al. 1987; Storebakken et al. 1987). This is likely due to the
inherent structural differences between these carotenoids which dictate their solubility in mixed micelles prior to absorption in the gastrointestinal tract.

Torrissen (1986) suggests that the high canthaxanthin to astaxanthin ratio in the plasma of rainbow trout indicates organ specificity in carotenoid preference. This author postulates that the discrepancy may be due to a more efficient deposition of astaxanthin in the flesh, or that astaxanthin is preferentially cleared from the plasma at a faster rate than canthaxanthin. This contradicts published half-lives of these carotenoids, where canthaxanthin is cleared at a faster rate than astaxanthin (Guillou et al. 1992; Gobantes et al. 1997).

Choubert and Storebakken (1989) found that rainbow trout pigmented at a faster rate with astaxanthin than with canthaxanthin, where the retention efficiency for astaxanthin was 1.3 times higher than for canthaxanthin, and may indicate a preferential metabolism of canthaxanthin over astaxanthin. In contrast to rainbow trout, Buttle et al. (2001) found that canthaxanthin was preferentially accumulated over astaxanthin in Atlantic salmon.

The study by Gobantes et al. (1997) found a difference in the ability of fish to accumulate keto-carotenoids in the liver of rainbow trout, with astaxanthin being preferentially accumulated over canthaxanthin. This is in contrast for studies in the rat where canthaxanthin accumulated 100 times more than astaxanthin in the liver (Gradelet et al. 1996).

1.13 Research aims

Despite the wealth of information pertaining to pigmentation strategies and the relative differences in carotenoid utilization within species, little direct comparative information is available concerning species differences in carotenoid accumulation. In addition, there still
exist major gaps within the literature concerning the relative importance of various organs in carotenoid utilization. More specifically, the liver is thought to play a major role in carotenoid bioavailability, but no studies have directly quantified the potential catabolic effect of this organ, and is the main focus of the present research programme. With these areas requiring further investigation, the following general aims were developed:

1. To establish any differences with respect to tissue carotenoid distribution and accumulation between Atlantic salmon and rainbow trout.

2. Identify those tissues that demonstrate a metabolically active role in the representative salmonids.

3. Clarify the role of the liver in terms of overall carotenoid bioavailability to the fish:
   • Differences between astaxanthin and canthaxanthin hepatic uptake and clearance rates.
   • Identify the specific transport/uptake mechanism of carotenoids into the liver.

4. Determine the biochemical effects of carotenoid supplementation:
   • Are xenobiotic-metabolizing enzyme systems induced by carotenoids?
   • Determine the effects of carotenoids on liver histochemistry.
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Experimental Animals

2.1.1 Rainbow trout

Unpigmented rainbow trout, *Oncorhynchus mykiss* Walbaum, used in each of the experiments were obtained from local trout farms (Mill Leat Trout, Devon, U.K.; or Hatchlands Trout Farm, Devon, U.K.). Fish were allowed to acclimatize to the experimental conditions at the University of Plymouth for a period of at least two weeks prior to their use in nutritional and experimental trials.

2.1.2 Atlantic salmon

Atlantic salmon (approximately 20 g), *Salmo salar* L., used in nutritional studies were obtained from Lovatt Fisheries (Inverness, Scotland, U.K.). Prior to smoltification fish were maintained in the fresh-water systems outlined below, and then transferred to saltwater at approximately 90 g.

Atlantic salmon utilized for whole body astaxanthin distribution studies were grown in experimental trial facilities at the EWOS Research Station (Lønningdal, Norway). Approximately 800 (200 fish per tank, average initial weight 315 g) non-pigmented post-transfer *S. salar* were individually weighed and stocked into 12 x 3 m cylindrical pump-ashore tanks supplied with flow-through sea water at a flow rate providing 1.5 turnovers hour⁻¹. Temperature and oxygen levels were monitored daily and temperatures for the trial period ranged between 8.6-14.1°C (average 12.0°C), with 24 hour light photoperiod. The trial was run from August 18, 2000 – November 16, 2000.
2.2 Experimental culture facilities

2.2.1 Plymouth University

Rainbow trout trials were conducted in 400 l self-cleaning 1m semi-square tanks supplied with recirculating fresh water at the West Aquarium, Fish Nutrition Laboratories, University of Plymouth. Water quality was maintained via biofiltration units.

Each experimental tank supplied with a parallel input of water at a flow rate of \( \sim 10 \text{ l}\cdot\text{min}^{-1} \). Temperature was constant at 15 ± 0.2 °C. Aeration was achieved by means of single airstones. Lighting was set to operate on a 12 hours light:12 hours dark cycle using artificial illumination from fluorescent tubes simulating natural photoperiod. Pre-filter chambers (following immediate discharge from tanks) were cleaned every two days to remove faecal material and any uneaten feed particles in order to prevent excess nitrogen build-up in the water. Water was then passed through a high surface area biofiltration system where established denitrifying bacteria ensured efficient degradation of toxic ammonia waste products into nitrites and nitrates. Weekly partial water replacement was performed in the system to ensure that all water quality parameters were within the known tolerance limits for rainbow trout.

Post-transfer Atlantic salmon were kept in a recirculating salt-water system with a biofiltration unit. Fish were maintained in rectangular stock tanks (1 x 1.5 x 1.2 m) of 800 litres capacity in the East Aquarium facilities of the Fish Nutrition Laboratories, Davy Building, University of Plymouth, Plymouth, U.K. The flow rate was approximately 9000 l hr\(^{-1}\), and temperature was maintained at 12 ± 0.2 °C, and photoperiod set as a 16 hour light:8 hour dark.
2.2.2 Feeding

Prior to feeding trials, the fish were acclimated for two weeks during which time they were fed *ad libitum* a ration of commercial unpigmented feed (Trouw Aquaculture, standard trout pellet, 4mm)(Trouw Aquaculture, U.K., Wincham, Cheshire, England, U.K.).

During nutritional trials, fish were fed experimental diets, at a restricted ration level, calculated as a percentage of the live weight twice, daily according to the experimental protocols. The restricted ration size was determined on the basis of bi-monthly weighing and the details of feed intake are given where appropriate.

2.3 Growth performance

Specific growth rate (SGR) was used to compare growth in individual dietary treatments on a relative daily basis and is expressed as the percentage increase in live weight over a specified time interval.

\[
SGR (\% \, d^{-1}) = \left( \frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{Time (days)}} \right) \times 100
\]

2.4 Feed Conversion Ratio

Feed conversion ratio (FCR) is the efficacy index of the fish to convert the amount of feed fed into live weight gain and measured as follows:

\[
FCR = \frac{\text{Amount of dry food fed (g)}}{\text{Live weight gain (g)}}
\]

2.5 Tissue somatic indices

Tissue somatic indices (TSI; e.g. hepatosomatic index) are an index of the respective tissue weights to the total body weight of the fish and calculated as follows:
2.6 Diet preparation

Experimental diets used in the present investigations were either prepared by EWOS Technology Centre, Livingston, Scotland, or by a top-coating method developed at the Fish Nutrition Unit, University of Plymouth.

2.6.1 Commercially manufactured diets

Diets produced by the EWOS Technology Centre, Scotland were manufactured using a Wenger extruder (twin screw, TX57) with oil added via a Forberg vacuum coater (F60VC). The diets, formulated to rainbow trout or Atlantic salmon nutritional requirements, were supplemented with either astaxanthin or canthaxanthin at a target inclusion of approximately 50 mg·kg⁻¹. The free astaxanthin (Carophyll® Pink, F. Hoffmann-La Roche, Basel, Switzerland) and canthaxanthin (Carophyll® Red, F. Hoffmann-La Roches, Basel, Switzerland) were added to dry ingredients as water dispersible beadlets (8% w/w astaxanthin; 10% w/w canthaxanthin, respectively). Measured dietary carotenoid contents and proximate compositions are presented in individual chapters.

2.6.2 In-house top-coating of basal diets

Diets prepared by the top-coating method at the University of Plymouth Fish Nutrition Unit were based on a commercially prepared basal diet (4-6 mm pellet, Trouw Aquaculture, Cheshire, U.K.; Formulation Table 2.1), and top-coated with purified cod liver oil (Seven Seas, Hull, U.K.), which had been supplemented with either Carophyll® Pink (8% astaxanthin w/w), Carophyll® Red (10% canthaxanthin w/w), or placebo powder (added to control diet to equalize the dietary composition of all groups; kindly donated by Hoffman La-Roche, Basel, Switzerland), to achieve ~100 mg·kg⁻¹ of carotenoid. The carotenoid beadlets were first dissolved in warmed water (~20 ml at 35°C) and dispersed in cod liver.
oil (heated to 35°C, to provide a final oil percentage of 26% in the feed, and containing 0.09 g·kg⁻¹ ethoxyquin as antioxidant), and added gradually in a continuous stream to pre-heated feed (4 kg batches; heated at 60°C for 1 hour) in a Hobart A120 food processor (Hobart manufacturing Company Ltd., London, U.K.) for 30 min. Measured dietary carotenoid contents and proximate compositions are presented in individual chapters.

Table 2.1. Basal diet formulation for feeds (top-coated) used in cytochrome P450 (Chapter 6) and liver histology studies (Chapter 7).

<table>
<thead>
<tr>
<th>Raw ingredients as percent of diet</th>
<th>Inclusion (% dry diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT fish meal (Norse LT 94, Egersund, Norway)</td>
<td>61.3</td>
</tr>
<tr>
<td>Wheat Gluten</td>
<td>5.6</td>
</tr>
<tr>
<td>Wheat</td>
<td>21.6</td>
</tr>
<tr>
<td>Fish oil</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.8</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>0.7</td>
</tr>
</tbody>
</table>

2.7 Proximate chemical composition

2.7.1 Moisture determination

Moisture determinations in the feeds were carried out according to AOAC (1990). Briefly, feed samples were weighed and fully dried to a final constant weight at 105°C in a fan assisted/exhaust extracted Pickstone E 70F oven (R.E. Pickstone Ltd., Thetford, Norfolk, U.K.). The percentage moisture content in the sample was calculated using the following equation:

\[
\text{Moisture (\%)} = \frac{\text{Change in weight (g)}}{\text{Initial sample weight (g)}} \times 100
\]

2.7.2 Determination of crude protein

The protein content of fish feeds were determined by the Kjeldahl method. One hundred mg of the dried feed was accurately weighed and transferred to a borosilicate digestion tube, to which one Kjeldahl catalyst tablet (3g K₂SO₄, 105mg CuSO₄·5H₂O and 105mg TiO₂; Thomas and Capper Ltd., Runcorn, Cheshire, U.K.) and 10 ml of concentrated H₂SO₄ (BDH Ltd., U.K.) were added prior to the digestion process.
Digestions were performed on a 40 position Gerhardt Kjeldatherm digestion block (C.Gerhardt Laboratory Instruments, Bonn, Germany) for 30 minutes at 220°C, followed by a further 60 min at 380°C. Acid fumes produced were collected and neutralized by 15% NaOH in a Gerhardt Turbosog unit. Samples were removed from the heating block and then allowed to cool to room temperature and diluted with distilled water and neutralized with 37% NaOH using a Gerhardt Vapodest 3S distillation unit.

The ammonia in the sample was collected in a conical flask containing 50 ml orthoboric acid (H₃BO₃; 0.65 M) with ‘4.5’ BDH indicator, by steam distillation. The distillate was then titrated against 0.1 M HCl and the percentage protein in the dry sample calculated by this equation:

\[
\text{Crude Protein (\%)} = \frac{(\text{Sample titre (ml)} - \text{blank titre (ml)}) \times 0.1 \times 14 \times 6.25}{\text{Sample weight (mg)}} \times 100
\]

where:

0.1 = [HCl] in moles per litre.
14 = Relative atomic mass of nitrogen
6.25 = Constant describing relationship between nitrogen and protein content of sample.

2.7.3 Determination of total lipid

Lipid determinations in dry feed were determined gravimetrically by a modification of the Folch et al. (1957) method using an initial acid hydrolysis. Accordingly, 2 g of feed were placed in a 50ml polypropylene centrifuge tube, to which 10 ml of 6M HCl and 10ml of methanol were added. Samples were capped, shaken and then placed in the oven for 30 minutes at 70°C to digest.
Following digestion, samples were allowed to cool to room temperature and the crude fat in the feed was extracted with 18ml of dichloromethane (DCM). Tubes were recapped and shaken vigorously and left to stand for an hour. Following solvent extraction, samples were vigorously shaken and centrifuged for 10 min at 3000 rpm (2000 X g) in a Mistral 3000 refrigerated centrifuge (MSE, U.K.). After centrifugation the upper phase was carefully decanted and 2 ml of the lower phase was carefully drawn into a 5ml Hamilton gas tight syringe (being careful not to displace the feed phase) and 1ml of this sample dispensed into an empty pre-weighed 4ml vial and the syringe washed with a further 1ml of clean DCM into the vial to ensure all lipid from the sample is collected. Samples in the vials are blown down using a gentle stream of nitrogen until all solvent is evaporated. Vials are finally placed into a drying oven at 105°C for 1 hour. The lipid content in the sample is then determined gravimetrically by the increase in weight of the vial and calculated according to the following formula:

\[
\text{Lipid (\%) = \frac{\text{Weight gain of vial (g)} \times 18}{\text{Sample weight (g)}} \times 100}
\]

where:

18 = volume (ml) of solvent used for extraction

Alternatively, lipid content was determined according to a modified Folch et al. (1957) method. Briefly, 0.5 g of dry feed was extracted with 10 ml of 2:1 v/v chloroform:methanol for 48 hours, followed by Buchner filtration using no. 1 Whatman filters. Samples were subsequently washed twice more with 5 ml of solvent (chloroform: methanol 2:1 v/v). Combined filtrates were placed in pre-weighed glass boiling tubes and evaporated to dryness and the lipid percentage of the sample determined gravimetrically using the following equation:
Lipid (%) = \frac{\text{Weight gain of vial (g)}}{\text{Sample weight (g)}} \times 100

2.7.4 Determination of ash content

Ash content of the dried feed samples was determined in accordance with AOAC (1990). Approximately 500 mg of sample was accurately weighed into a pre-weighed ceramic crucible and heated to 550°C for 12 hours in a Carbolite GLM 11/7 muffle furnace (Carbolite Furnaces Ltd., Bamford, Sheffield, U.K.). The resulting ash residue was then weighed (including crucible weight) and the difference in weight from the starting weight of the pre-combustion sample was determined as the ash content of the sample calculated as follows:

Ash (%) = \frac{(\text{Weight of crucible + residue (g)} - \text{weight of crucible (g)})}{\text{Sample weight (g)}} \times 100

2.8 Determination of carotenoid content in feeds and faeces

Determination of carotenoids by High Performance Liquid Chromatography (HPLC). All solvents used were at least HPLC grade. Positive displacement pipettes (Multipette plus, Eppendorf, Hinz GmbH, Hamburg, Germany) were used to dispense solvents.

2.8.1 Extraction procedure for feeds

The carotenoid content of ground feed samples (approx. 100 g) was determined in triplicate weighed subsamples (~4 g) of diet which were sonicated in a water bath (50°C) with 100 mg Maxatase™ (Hoffman La Roche, Basel, Switzerland) and 15 ml H2O for 30 min. Samples were allowed to cool to room temperature, and 15 ml of methanol and 12 ml of dichloromethane added and thoroughly mixed and allowed to extract for 10 min in the dark. Samples were again mixed and centrifuged at 3000 rpm for 15 min at 15°C to allow
phase separation. One ml of the lower phase was removed and evaporated under a gentle stream of nitrogen and stored at \(-20\, ^\circ \text{C}\) in 4 ml amber glass vials. Samples were resuspended in 3-4 ml hexane (supported by sonication), and centrifuged at 13 000 rpm (Micro-Centaur, Sanyo Gallenkamp PLC, Uxbridge, U.K.) for 5 min to remove polar material. The supernatant was analyzed for carotenoid content by HPLC.

2.8.2 Faecal astaxanthin analysis

Faeces samples were collected and freeze-dried and stored at room temperature in a dessicator until analyzed for carotenoid content. Samples were cold extracted from pooled samples from individual tanks for faeces. Approximately 1 g of sample was accurately weighed and extracted with 2 ml methanol, 2 ml distilled water, and homogenized. Six ml dichloromethane was then added, samples vigorously shaken and left to extract for 10 min in the dark. Samples were then centrifuged at 3600 rpm (Mistral 3000, MSE, U.K.) at 15°C for 10 min, and 1 ml of the lower phase decanted into 4 ml amber glass vials and evaporated under a gentle stream of nitrogen and stored at \(-20\, ^\circ \text{C}\) until analyzed. Samples were resuspended in 3-4 ml hexane (supported by sonication), and centrifuged at 13 000 rpm (Micro-Centaur, Sanyo Gallenkamp PLC, Uxbridge, U.K.) for 5 min to remove polar material. The supernatant was analyzed for carotenoid content by HPLC.

2.9 Determination of tissue carotenoid concentration

2.9.1 Serum

Whole blood was collected via the caudal vein with 2 ml non-heparinized disposable syringes fitted with 23.5 gauge needles (Becton Dickinson) and allowed to clot overnight at +4°C. Carotenoid determinations were performed on serum due to the 10% higher recovery observed relative to plasma (Nierenberg 1984; Stacewics-Sapuntzakis et al. 1987). Blood samples were centrifuged at 13,000 rpm (Micro-Centaur, Sanyo Gallenkamp PLC, Uxbridge, U.K.) for 5 min.
Serum (~400 – 1000 µl) was decanted, to which 1 ml ethanol was added to precipitate protein. Following vortexing, 1 ml of diethyl ether and 1 ml of hexane were added, and the mixture vortexed and allowed to extract in the dark for 10 min. An aliquot (500-1000 µl) of the hyperphase was taken and evaporated under a gentle stream of nitrogen prior to storage at -20°C, or immediately analyzed by HPLC.

2.9.2 Flesh

Flesh tissue was collected from individual fish and white muscle analyzed (being careful to remove red muscle) for carotenoid content. Briefly, either whole fillets (rainbow trout trials) or the Norwegian Quality Cut (NQC; Fig. 2.1) were taken from individual fish and the red muscle and skin carefully removed. Individual fillets or pooled tissue samples were homogenized and a 2-5 g sample (analyzed in triplicate) analyzed for carotenoid content. Weighed flesh samples were homogenized with 10 ml water, and extracted with 10 ml methanol and vortexed for 5 s. Samples were extracted with 12 ml dichloromethane and vortexed for 5 s and allowed to extract in the dark for 10 min. Samples were again vortexed for 5 s and centrifuged at 3000 rpm (Mistral 3000, MSE, U.K.) for 15 min at 15°C. Following extraction, 1 ml of the lower phase was decanted into 4 ml amber glass vials and evaporated under a gentle stream of nitrogen and stored at -20°C until analyzed.
Fig. 2.1 Schematic diagram of an Atlantic salmon (*Salmo salar* L.), showing the Norwegian Quality Cut (NQC) region of the fish, where flesh samples were taken for carotenoid analysis. The NQC area is that delineated by the posterior edge of the dorsal fin to the pectoral girdle, and ventrally from the anus to the dorsum. Only the left flank of the NQC section was used, after careful removal of the skin and red muscle.
2.9.3 Liver, kidney, spleen, heart, and gonads

Tissues were carefully dissected and any adhering adipose tissue removed. Whole tissue weight was recorded. Tissues were homogenized individually whole (Ultra-Turrax T8, IKA Labortechnik, Staufen, Germany) or in pooled samples and ~2-5 g (accurately weighed) of the homogenate was analyzed for carotenoid content. Samples were homogenized with a measured volume of water for 30 s, which was followed by the addition of methanol and dichloromethane in a ratio of 1:1:3 (water:methanol:dichloromethane v/v/v) and vortexed for 10 s, and allowed to extract for 10 min in the dark. Samples were then re-vortexed and centrifuged. One ml of the lower phase was removed and evaporated under a gentle stream of nitrogen and stored at -20°C until analyzed by HPLC.

2.9.4 Gastrointestinal tract

The gastrointestinal tract (GI tract) was dissected out and stored frozen at -20°C prior to analysis. The GI tract was thawed and divided into three separate sections (stomach, pyloric caeca, and hindgut) for carotenoid analysis as shown in Fig. 2.2. Sections from individual fish were prepared by removing adhering adipose tissue and faeces through gentle scraping with the blunt edge of a scalpel blade. Pyloric caeca were cut near the distal end of the blind appendages to allow for digesta to be cleared. Sections were then rinsed in distilled water and blotted dry. Pooled sections were then homogenized (X620 Status homogenizer, Philip Harris Scientific, GB-Lichfield, Germany) for 30 s at 9500 rpm, and 2-5 g subsamples were accurately weighed for carotenoid determinations. Samples were homogenized with a measured volume of water for 30 s, which was followed by the addition of methanol and dichloromethane in a ratio of 1:1:3 (water:methanol:dichloromethane v/v/v) and vortexed for 10 s, and allowed to extract for 10 min in the dark. Samples were then re-vortexed and centrifuged. One ml of the lower
Fig. 2.2. Diagrammatic representation of individual sections of the gastro-intestinal tract of rainbow trout (*Oncorhynchus mykiss* Walbaum) and Atlantic salmon (*Salmo salar* L.) used in tissue analyses (Section 1 – stomach; Section 2 – pyloric caecae; Section 3 – hindgut). Note the muscular ridges of the stomach, the ‘pits’ in Section 2, which indicate the openings to the pyloric caecae, and the ridges in the posterior section of the hindgut for increased surface area for absorption.
phase was extracted evaporated under a gentle stream of nitrogen and stored at -20°C until analyzed by HPLC.

2.10 HPLC conditions

All samples were resuspended in mobile phase (or hexane only) prior to isocratic analysis on a Kontron Instruments P332 single channel detector, equipped with a P325 pump and an HPLC 560 autosampler (Kontron Instruments Ltd., Watford, Hertfordshire, U.K.). All samples were integrated using Kroma 2000 software. Samples were separated on a Spherisorb S5-CN nitrile column (Phenomenex, Cheshire, U.K.; length 250 mm; internal diameter 4.6 mm; particle size 5 μm), using 20% acetone in hexane as mobile phase (flow rate 1.5 ml min⁻¹). Under these conditions, run-time was around 10 minutes. The detection wavelength was set at 470 nm. Mobile phase was not recycled during the course of the procedure.

2.11 Sample carotenoid quantification

An external standard of a known concentration, prepared from crystalline astaxanthin or canthaxanthin (F. Hoffman-La Roche, Basel, Switzerland), and run approximately every 20 samples each day of analysis (Fig. 2.3). The concentration of the standard solution was measured using a V530 UV/Vis spectrophotometer (Jasco (U.K.) Ltd., Great Dunmow, Essex, U.K.), an extinction coefficient of $E_{1\%}\,\lambda_{\text{cm}}=2100$ at an absorbance maximum ($\lambda_{\text{max}}=470$ nm) for astaxanthin (Schierle and Härdi, 1994), and $E_{1\%}\,\lambda_{\text{cm}}=2260$ at an absorbance maximum of ($\lambda_{\text{max}}=466$ nm) for canthaxanthin (Weber 1988).

The standard was injected into the HPLC and the area of duplicate standards were averaged. The area of samples were integrated (using Kroma 2000 software (Bio-Tech Kontron, U.K.)), and the relative area to the standard compared to produce the concentration of the injected sample. The concentration of the sample is then corrected for
extraction volume and sample weight to determine original sample concentration as in the following example:

\[
\text{Carotenoid concentration (}\mu\text{g g}^{-1}\text{)} = \frac{\text{Sample area (mV x min)}}{\text{Area of standard (mV x min) x Concentration of standard (}\mu\text{g ml}^{-1}\text{) x dilution (extraction) volume (ml)}} \times \frac{\text{Sample weight (g)}}{\text{Sample weight (g)}},
\]

Validation of HPLC data with spectrophotometric analysis of known concentrations:
The HPLC conditions used to analyze unknown sample carotenoid concentrations were subjected to validation experiment using a measured concentrations (spectrophotometrically) of a standard. Briefly, the astaxanthin standard was prepared according to methods described above. The standard solution was then linearly diluted with solvent (95.5:4.5 v/v n-hexane:chloroform) to produce a standard curve on a V530 UV/Vis spectrophotometer (Jasco (U.K.) Ltd., Great Dunmow, Essex, U.K.) using the extinction coefficient of \(E_{1\%,1cm} = 2100\) at an absorbance maximum (\(\lambda_{max} = 470\) nm) for astaxanthin. The samples were simultaneously analyzed in duplicate on the HPLC, and the average areas plotted against the measured/known astaxanthin concentrations (Fig. 2.4). The high R-squared value of the regression line demonstrates the linearity of the system and validates the output for calculations of carotenoid content for unknown samples.
Fig. 2.3. Representative HPLC chromatogram showing two main peaks corresponding to canthaxanthin and astaxanthin.
2.12 Digestibility Determination

2.12.1 Determination of Yttrium oxide content (inert reference marker)

The Yttrium oxide (Y$_2$O$_3$) content of both the test diets and the faecal material was determined by the analysis for yttrium in samples using Induction Coupled Plasma Atomic Emission Spectroscopy (ICP AES), similar to the methods of Refstie et al. (1997). Due to the inert nature of the yttrium oxide, this could only be conducted after the samples had undergone a preliminary wet acid digestion.

Triplicate 100 mg samples of the test diets and corresponding faecal materials were accurately weighed out into dry Teflon RPFA bombs (120 ml), to which 10 ml of concentrated nitric acid (HNO$_3$) were added, and the bombs assembled and placed in the CEM microwave (MDS-2000, CEM Corporation, Matthews, N.C., U.S.A.). The digestion phase in the microwave encompassed several phases, (1) 10% power for 2 mins., (2) 20% power for 2 min, (3) 30% power for 2 min., (4) 40% power for 8 min., and (5) 0% power for 6 min (cool-down step). After the digestion phase was complete, the bombs, containing digested organic matter, were allowed to cool in the microwave or fume cupboard. The acid digest, a green/yellow solution containing various amounts of precipitate, was then adjusted to a total volume of 25 ml distilled water using a volumetric flask.

2.12.2 ICP analysis

Using ICP AES (Varian Liberty 200, Australia), the samples were analyzed against yttrium oxide standards of known concentration (using a standard curve 0 ppm, 10 ppm, 20 ppm and 40 ppm in HNO$_3$). The absorbance (measured at 371.03 nm) of the sample was then compared to the standard and the concentration of yttrium in the faeces sample determined from the linear correlation (performed by the analytical software of the ICP AES).
Fig. 2.4. Validation of HPLC peak area against samples of known concentration in a linear dilution series of an astaxanthin standard. \( y = 0.7462 + 26.1966 \times \text{concentration} \), \( r^2 = 0.998, P<0.01 \).
2.12.3 Determination of apparent digestibility coefficients

Percentage apparent digestibility coefficients (ADCs) were calculated using the following formulas:

Apparent dry matter digestibility (%) =

\[ 100 - \left( 100 \times \left( \frac{\% \text{ Y}_3 \text{O}_3 \text{ in feed}}{\% \text{ Y}_3 \text{O}_3 \text{ in faeces}} \right) \times \left( \frac{\% \text{ Nutrient in faeces}}{\% \text{ Nutrient in feed}} \right) \right) \]

2.13 Determination of tissue carotenoid retention

Tissue carotenoid retention is a measurement of the amount of nutrient (carotenoid) retained in a tissue relative to that fed to the animal over a specific period of time. The calculation is adapted from the formula of Torrissen (1995):

\[ \text{Tissue retention (\%)} = \frac{(\text{C}_{\text{x}_t} \times \text{W}_r - \text{C}_{\text{x}_i} \times \text{W}_i) \times \text{F} \times 100}{((\text{W}_r - \text{W}_i) \times \text{C} \times \text{C}_{\text{x}_d})} \]

Where:

- \( \text{C}_{\text{x}_t} \) = Tissue carotenoid concentration at termination,
- \( \text{C}_{\text{x}_i} \) = Initial astaxanthin concentration,
- \( \text{W}_r \) = Final fish weight,
- \( \text{W}_i \) = Initial fish weight,
- \( \text{F} \) = Tissue somatic index
- \( \text{C} \) = Feed Conversion Ratio
- \( \text{C}_{\text{x}_d} \) = Carotenoid concentration in the diet.
2.14 Statistics

All data were tested for normality and equality of variance, with percentages being transformed according to Morris (1999) prior to analysis. Data were analyzed by ANOVA (P<0.05) using the statistical software package Statgraphics® Plus 4.0 (Manugistics, Inc., Rockville, Maryland, USA), and regressions determined using SigmaPlot 4 (SPSS Inc., Chicago, Illinois, USA). More specific descriptions of statistical analyses are presented in individual chapters.
CHAPTER 3

TISSUE CAROTENOID DISTRIBUTION IN RAINBOW TROUT
(ONCORHYNCHUS MYKISS WALBAUM) AND ATLANTIC SALMON (SALMO
SALAR L.): A COMPARATIVE INVESTIGATION.

3.1 Introduction

One of the distinguishing features of salmonid fish is their ability to deposit ingested carotenoids in their flesh. Indeed, this ability significantly contributes to their elite image, and the intensity of the flesh colour imparted by these lipophilic compounds, has become an important quality criterion for the consumer (Torrissen 1985).

To date, research has focused primarily on flesh deposition and retention, as affected by the dietary concentrations, individual carotenoids, as well as various sources of pigments used in aquaculture. The available literature highlights a relative lack of information regarding comparative pigmentation efficiencies between Atlantic salmon and rainbow trout. However, despite this relative lack of intensive research, species-specific differences appear to exist (reviewed in Chapter 1).

Notable in these differences is the consistent observation that salmon pigment less efficiently than trout as assessed by flesh deposition (carotenoid concentration), tissue (flesh) retention, and digestibility. Further to this, differences appear to exist in the utilization of astaxanthin and canthaxanthin between these species, where astaxanthin is better utilized in trout than is canthaxanthin (Foss et al. 1984; Torrissen 1989; Gobantes et al. 1997; Choubert and Storebakken 1996), while the reverse has been observed in Atlantic salmon (Buttle et al. 2001). However, no plausible explanation for the observed differences has been proposed and so this remains to be investigated.

64
The observed discrepancy between the levels of absorbed carotenoids and those retained in the flesh (~30-50% No and Storebakken 1992), has not taken into consideration the potential contribution of other organs to total body retention. This is likely due to the small relative tissue sizes of individual organs relative to the total body weight. Estimates show that the flesh accounts for between 55 and 60% of the whole body mass and is therefore the tissue of primary concern in terms of carotenoid retention (Torrissen et al. 1995; Wathne et al. 1998). While the importance of the flesh as a carotenoid storage (pool) depot is not disputed, other tissues may also contribute significantly to explaining some of the “missing” carotenoid in retention studies.

Few studies have comparatively investigated the relative efficiency of carotenoid utilization between species. March and MacMillan (1996) and Storebakken et al. (1986) compared both Atlantic salmon and rainbow trout, one study examining astaxanthin, and the other canthaxanthin. However no studies have directly compared individual species accumulations of these two carotenoids at similar inclusion rates between studies using similar diet formulations. In addition, these studies have focused primarily on flesh pigmentation without regard to the contributions of other tissues.

To this end, only a handful of investigations have studied individual tissue carotenoid concentrations, and most in rainbow trout. Therefore the primary aim of the present study was to identify the relative major tissue carotenoid concentrations in rainbow trout and Atlantic salmon fed similar diets both in proximate composition as well as in carotenoid levels. Further to this, the present study was aimed at identifying differences in carotenoid (astaxanthin vs. canthaxanthin) utilization within a particular species, as well as between species (rainbow trout and Atlantic salmon). In addition, the present study was intended to identify the major tissues and their relative contributions to whole body carotenoid retention.
While metabolism appears to contribute to some of the observed discrepancy between absorbed and retained carotenoid levels, no comparative studies between species have been performed in relation to individual tissue carotenoid profiles. Therefore, an indirect marker (assessed as the relative peak chromatogram area) for relative tissue carotenoid metabolic activity was used to identify specific tissues potentially responsible for carotenoid metabolism.

3.2 Materials and Methods

3.2.1 Rainbow trout trial

Rainbow trout (*O. mykiss*) were obtained from Hatchlands trout farm (Devon, U.K.) and maintained at the Plymouth Aquarium Facilities (Chapter 2.2) for 2 weeks prior to the beginning of the investigation on a pigment-free diet (>1 mg·kg\(^{-1}\) astaxanthin).

Thirty-five individual rainbow trout (initial mean weight 0.135 ± 0.001 kg (SEM)) were allocated to each of three replicate tanks with two treatments; dietary astaxanthin-supplemented vs. canthaxanthin-supplemented feeds. Trout were hand-fed twice daily a total feed ration of 1.2% body weight per day for a total of 10 weeks. Fish were weighed mid-way through the trial to re-assess growth and to adjust feeding rations, as well as to collect faeces for apparent digestibility coefficient estimates.

At the conclusion of the experiment fish were anesthetized (phenoxy-2-ethanol, Sigma, Dorset, U.K.), followed by a blow to the head. Serum samples were collected as described in Chapter 2.9.1. Faeces were then collected according to the methods of Austreng (1979), and stored frozen at −20°C prior to freeze drying. Faeces and feed samples were then analyzed for carotenoid (Chapter 2.8) and Yttrium content for apparent digestibility coefficient calculations as described in Chapter 2.12.
Fillets from 60 fish were carefully dissected out and the skin removed and weighed for flesh tissue somatic index measurements (Chapter 2.5). Red muscle from individual fish fillets was carefully removed and the white muscle homogenized whole and 5 g subsamples analyzed for carotenoid content as described in Chapter 2.9.2. Remaining visceral tissues and kidney samples were carefully dissected, being careful to remove adhering adipose tissue, weighed and stored frozen at -20°C until analyzed for carotenoid content as described in Chapter 2.9.3. The gastrointestinal tract was frozen whole and stored frozen at -20°C until prepared for analysis as described in chapter 2.9.4.

3.2.2 Atlantic salmon trial

Atlantic salmon (S. salar) utilized for whole body carotenoid distribution studies were obtained and maintained at the EWOS Research Station (Lønningdal, Norway). Approximately 800 (200 fish per tank, average initial weight 315 g) non-pigmented post-transfer S. salar were individually weighed and stocked into four 12 x 3 m cylindrical pump-ashore tanks supplied with sea water. Duplicate tanks were assigned to each of two dietary carotenoid treatments; astaxanthin and canthaxanthin. Temperature and oxygen levels were monitored daily and temperatures for the trial period ranged between 8.6 and 14.1°C (average 12.0°C). The trial was run from August 18, 2000 – November 16, 2000. Salmon were fed slightly to excess with a computer-controlled feed-delivery system.

At the conclusion of the experiment, fish were anaesthetized (Aqui-S, Norway), and killed by a blow to the head immediately prior to weighing and collection of whole blood using 5 ml vacutainers. Blood was allowed to clot overnight at +4°C, and serum decanted and stored frozen at -20°C prior to carotenoid analysis as described in Chapter 2.9.1. Faeces were then collected according to the methods of Austreng (1978), and stored frozen at -20°C prior to freeze drying. Faeces and feed samples were then analyzed for carotenoid
(Chapter 2.8) and Yttrium content for apparent digestibility coefficient calculations as described in Chapter 2.12.

Individual tissues and flesh from 80 salmon was carefully dissected and the skin removed for analysis of tissue somatic indices as described in Chapter 2.5. Flesh (area immediately posterior to the dorsal fin and the pelvic fins and from the vent to the adipose fin; Norwegian Quality Cut (NQC), Fig. 2.1.) from 100 fish per tank was carefully dissected free from adhering skin and red muscle and homogenized in pools of 10 fish (using half of each NQC section) using a Waring Blender for 1 min. Triplicate subsamples were analyzed for carotenoid content as described in Chapter 2.9.2.

All other tissues from the same 100 Atlantic salmon were carefully dissected free from adhering adipose tissue and stored frozen at −20°C in pools of 10 fish prior to carotenoid analysis as described in Chapter 2.9.3. Pooled livers were analyzed in triplicate, kidney tissue in duplicate, gastrointestinal tract sections in duplicate, and other tissues using a single pooled sample.

Tissue somatic indices, specific growth rates, feed conversion rates, apparent digestibility coefficients, and pigment retentions were calculated according to the equations previously described in Chapter 2.

Relative carotenoid peak areas were calculated from the total chromatogram peak areas and used as an indirect marker of tissue carotenoid metabolic activity. The values were calculated as follows:

\[
\text{Relative peak area} = \frac{\text{Area of carotenoid peak}}{\text{Total chromatogram peak areas}} \times 100\%
\]
Tissue contribution to body carotenoid retentions were calculated using mean tissue somatic indices for each species, and the measured carotenoid concentrations for each tissue. The total carotenoid contents for each tissue were summed and the relative contribution of each tissue to the total body carotenoid pool determined as follows:

\[
\text{Tissue contribution to whole body pool} = \frac{\text{TSI} \times \text{fish weight (g)} \times \text{tissue carotenoid content (μg g}^{-1})}{\sum \text{(TSI} \times \text{fish weight (g)} \times \text{tissue carotenoid content (μg g}^{-1})}\times 100\%
\]

Where TSI is the individual tissue somatic indices.

And, \(\sum\) is the sum of the individual tissue carotenoid contents.

3.2.3 Dietary Analysis

Four diets were commercially manufactured by EWOS Technology Centre (Livingston, Scotland, UK) as described in Chapter 2.6.1. The diets, designed for rainbow trout, or Atlantic salmon nutritional requirements (relative macro-ingredient ratio and proximate composition shown in Table 3.1), were supplemented with either of two carotenoids, astaxanthin or canthaxanthin, at a target inclusion of 50 mg kg\(^{-1}\).

<table>
<thead>
<tr>
<th>Table 3.1. Proximate compositions of commercially manufactured experimental diets fed to rainbow trout, Oncorhynchus mykiss Walbaum, and Atlantic salmon, Salmo salar L*.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
</tr>
<tr>
<td>Lipid (%)</td>
</tr>
<tr>
<td>Ash (%)</td>
</tr>
<tr>
<td>Moisture (%)</td>
</tr>
<tr>
<td>Carotenoid (mg·kg(^{-1}))</td>
</tr>
</tbody>
</table>

*values are expressed as means ± SD of triplicate analyses.
3.2.4 Statistical analysis

All data were subjected to analysis of variance between individual treatments for rainbow trout and Atlantic salmon. Only data that has been normalized between treatments (i.e. not raw data), were statistically compared between species within treatments. The analyses were carried out using Statgraphics\textsuperscript{\textregistered} Plus 4.0 for Microsoft windows, with a significance level of $P<0.05$ taken unless otherwise stated.

3.3 Results

The present study was undertaken to assess the utilization of astaxanthin and canthaxanthin between rainbow trout and Atlantic salmon by examination of their apparent digestibility coefficients, growth, and tissue carotenoid depositions. Furthermore, the relative peak areas of the fed carotenoids were determined as an indirect marker of metabolic capacity with a lower value for the fed carotenoids indicating a higher level of metabolism.

Both groups of fish (trout and salmon) grew well, without significant differences between the two carotenoid treatments indicating that carotenoid type did not have an effect on the growth parameters measured (Table 3.2). Both trout and salmon more than doubled their weight, with the specific growth rates (SGR) of salmon being slightly higher than those of trout, indicating that salmon performed better, despite similar feed conversion ratios. The mean initial weights of the trout were much lower than those in the salmon trials and as such may have influenced their potential growth (Table 3.2).
Table 3.2. Growth and feed performances of rainbow trout fed two dietary treatments for 10 weeks in freshwater, and of Atlantic salmon fed similar diets for 12 weeks in seawater. Values are expressed as means ± SEM. (n = 3 tanks Rainbow trout; n = 2 tanks Atlantic salmon).

<table>
<thead>
<tr>
<th></th>
<th>Rainbow trout</th>
<th></th>
<th>Atlantic salmon</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Astaxanthin</td>
<td>Canthaxanthin</td>
<td>Astaxanthin</td>
<td>Canthaxanthin</td>
</tr>
<tr>
<td>Mean initial</td>
<td>0.135±0.001</td>
<td>0.135±0.001</td>
<td>0.315±0.001</td>
<td>0.316±0.001</td>
</tr>
<tr>
<td>weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean final</td>
<td>0.351±0.006</td>
<td>0.348±0.001</td>
<td>0.880±0.020</td>
<td>0.895±0.023</td>
</tr>
<tr>
<td>weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGR (%)</td>
<td>1.18±0.02</td>
<td>1.17±0.03</td>
<td>1.36±0.01</td>
<td>1.35±0.01</td>
</tr>
<tr>
<td>FCR</td>
<td>1.06±0.03</td>
<td>1.19±0.11</td>
<td>1.07±0.02</td>
<td>1.03±0.01</td>
</tr>
<tr>
<td>% Mortality‡</td>
<td>0.95±0.95</td>
<td>0.95±0.95</td>
<td>1.50±0.00</td>
<td>8.75±7.75</td>
</tr>
</tbody>
</table>

‡ No significant differences between treatments within species were observed in other parameters.

Mean apparent digestibility coefficients (ADCs) of astaxanthin and canthaxanthin were comparable within a species, but were significantly lower (P<0.05) in Atlantic salmon (Table 3.3). Similarly, flesh retention efficiencies were similar between carotenoids within a species, but were significantly lower (P<0.05) in Atlantic salmon compared to rainbow trout (Table 3.3). Taken together, these data indicate that trout are better able to utilize dietary carotenoids than are Atlantic salmon.

Table 3.3. Apparent digestibility coefficients (ADC) and flesh carotenoid retention efficiencies of rainbow trout fed two dietary carotenoid treatments for 10 weeks in freshwater, and of Atlantic salmon fed similar diets for 12 weeks in seawater. Values are expressed as means ± SEM. (n = 3 Rainbow trout; n = 2 Atlantic salmon).

<table>
<thead>
<tr>
<th></th>
<th>Rainbow trout</th>
<th></th>
<th>Atlantic salmon</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Astaxanthin</td>
<td>Canthaxanthin</td>
<td>Astaxanthin</td>
<td>Canthaxanthin</td>
</tr>
<tr>
<td>ADC (%)</td>
<td>96.51±0.08*</td>
<td>96.49±0.12*</td>
<td>28.20±1.33</td>
<td>31.39±0.68</td>
</tr>
<tr>
<td>Retention (%)</td>
<td>12.58±0.35*</td>
<td>11.55±0.29*</td>
<td>5.47±0.03</td>
<td>5.42±0.46</td>
</tr>
</tbody>
</table>

* indicates that values within a row between species within treatments are significantly different (P<0.05).

Rainbow trout fed diets supplemented with astaxanthin had significantly higher flesh carotenoid levels compared to trout fed diets supplemented with canthaxanthin, despite
similar ADCs between these carotenoids and the slightly higher dietary canthaxanthin content (P<0.05, Fig. 3.1).

In contrast to the flesh, rainbow trout fed canthaxanthin resulted in significantly higher mean carotenoid concentrations in both the kidney and the gonadal (ovaries) tissues, than fish fed astaxanthin (P<0.01, Fig. 3.1). These results appear to indicate selective preferential carotenoid accumulation.

When the peak area of the fed carotenoid was converted as a percentage of the total peak areas of individual chromatograms, as an indirect marker of metabolic potential, the flesh and kidneys of fish fed astaxanthin showed that these peak areas represented significantly higher proportions of the total peak area than of trout fed canthaxanthin (P<0.05 and P<0.01, respectively; Fig. 3.2). However, trout fed canthaxanthin revealed that the peak areas of canthaxanthin in serum and splenic tissues represented significantly higher proportions of the total peak area than did trout fed astaxanthin (P<0.05; Fig 3.2), although the relative differences were less marked than those of the kidney. These data suggest differential accumulation or metabolism of the respective carotenoids.

Atlantic salmon fed diets supplement with canthaxanthin had significantly higher mean kidney carotenoid concentrations than salmon fed similar levels of dietary astaxanthin (P<0.05; Fig. 3.3). No other significant differences were observed between individual tissue carotenoid accumulations, likely due to the low numbers of replicates in this trial.

Peak area proportionalities of salmon fed diets supplemented with astaxanthin revealed that astaxanthin represents a significantly higher proportion of the total peak area than does canthaxanthin. Similarly, astaxanthin represents a larger proportion in the male gonad (P<0.05), while canthaxanthin represents a significantly higher proportion (P<0.01) of the
Fig. 3.1. Mean wet-tissue carotenoid concentrations of rainbow trout fed astaxanthin or Canthaxanthin for 10 weeks. Values are expressed as means +/- SEM. (n = 3). * indicates values are statistically significantly different between treatment means within a tissue (P<0.05).
Fig. 3.2. Mean proportionality of carotenoid peak area to total chromatogram peak areas of individual tissues in rainbow trout fed astaxanthin or canthaxanthin for 10 weeks. Values are expressed as means +/- SEM. (n = 3). * indicates values are statistically significantly different between treatments within a tissue (P<0.05).
Fig. 3.3. Mean wet-tissue carotenoid concentrations of Atlantic salmon fed astaxanthin or canthaxanthin for 12 weeks. Values are expressed as means +/- SEM. (n = 2). * indicates values are statistically significantly different between treatment means within a tissue (P<0.05).
total peak area of ovaries in fish fed canthaxanthin compared to salmon fed astaxanthin (Fig. 3.4).

The relative tissue contributions to total carotenoid retentions provide an indication of the relative importance a tissue has to carotenoid distribution. Therefore, while an individual tissue may have a high carotenoid content on a wet weight basis, their relative tissue somatic index (Table 3.4) may indicate that their contribution to whole body carotenoid content is less important. The present results show similar tissue somatic indices between trout and salmon (Table 3.4). Results show that the flesh contributes to approximately 90% or more of the total body carotenoid pool, regardless of species or carotenoid, although flesh is a significantly more important tissue for astaxanthin retention (as part of the whole body pool) in both species compared to canthaxanthin (Table 3.5). The serum is the second most important tissue in both species for both carotenoids. These tissues accounting for more than 92-98% of the total carotenoids recovered (Table 3.5).

Other differences are also evident. The salmon liver astaxanthin pool contributes significantly less to the whole body carotenoid pool than is the case for canthaxanthin (Table 3.5), and suggests preferential canthaxanthin accumulation in this species. The liver in Atlantic salmon was found to contribute significantly more to the whole body carotenoid pool for both carotenoids, compared to rainbow trout. No differences between astaxanthin and canthaxanthin contribution were observed, consistent with the carotenoid concentrations observed. These results suggest a fundamental difference in liver accumulations between these species.

The kidney canthaxanthin pool in rainbow trout was found to contribute significantly more to the whole body carotenoid pool than does astaxanthin (Table 3.5), and suggests preferential carotenoid accumulation in this tissue in the trout. These observations were not
Fig. 3.4. Mean proportionality of carotenoid peak area to total chromatogram peak areas of individual tissues in Atlantic salmon fed astaxanthin or canthaxanthin for 12 weeks. Values are expressed as means +/- SEM. (n = 2). * indicates values are statistically significantly different between treatments within a tissue (P<0.05).
noted in the salmon. However, the kidney carotenoid pool in rainbow trout appeared to contribute significantly more to the whole body carotenoid pool than in Atlantic salmon (Table 3.5). This suggests species-specific carotenoid accumulations exist.

Table 3.4. Mean tissue somatic indices in Atlantic salmon and rainbow trout fed dietary carotenoids. Values are expressed as means ± S.D. (n = 200 fish rainbow trout, n = 80 fish salmon).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rainbow trout</th>
<th>Atlantic salmon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flesh</td>
<td>49.50±1.96</td>
<td>51.84±3.29</td>
</tr>
<tr>
<td>Serum*</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Liver</td>
<td>1.34±0.37</td>
<td>1.12±0.14</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.66±0.16</td>
<td>0.83±0.13</td>
</tr>
<tr>
<td>Gonads (ovaries)</td>
<td>0.12±0.11</td>
<td>0.11±0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>0.09±0.02</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.10±0.04</td>
<td>0.21±0.07</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>11.00±1.55</td>
<td>7.51±1.06</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.97±0.15</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pyloric ceacae</td>
<td>0.97±0.57</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hindgut</td>
<td>0.21±0.06</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* value taken from Barron et al. (1987).
N.D. indicates not determined as individual tissue section weights not measured.

In addition, the gonads in the rainbow trout also appeared to contribute to a significantly higher proportion of the whole body carotenoid pool than in Atlantic salmon. This may be due to the maturity differences between these two groups of fish, although their relative tissue somatic indices were comparable (Table 3.4). While the heart in Atlantic salmon contributed to a significantly higher proportion of the whole body pool than in rainbow trout (Table 3.5), this is though to be insignificant due to the relative concentrations observed in this tissue, as well as the small tissue somatic index.
Table 3.5. Relative tissue contributions to total recovered carotenoid in Atlantic salmon and rainbow trout fed diets containing astaxanthin or canthaxanthin. Values are expressed as means ± SEM. (n = 3 rainbow trout, n = 2 Atlantic salmon).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rainbow trout Astaxanthin</th>
<th>Rainbow trout Canthaxanthin</th>
<th>Atlantic salmon Astaxanthin</th>
<th>Atlantic salmon Canthaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flesh</td>
<td>95.01±0.78#</td>
<td>90.25±0.92</td>
<td>92.96±0.37#</td>
<td>87.97±0.35</td>
</tr>
<tr>
<td>Serum</td>
<td>3.51±0.77</td>
<td>6.29±1.16</td>
<td>4.45±0.46</td>
<td>4.86±0.25</td>
</tr>
<tr>
<td>Liver</td>
<td>0.14±0.01*</td>
<td>0.19±0.05*</td>
<td>1.96±0.04#</td>
<td>6.68±0.60</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.53±0.03*,•</td>
<td>1.64±0.08</td>
<td>0.21±0.00</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>Gonads</td>
<td>0.74±0.06*,•</td>
<td>1.53±0.21*</td>
<td>0.38±0.03#</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>&gt;0.00*</td>
<td>&gt;0.00*</td>
<td>0.02±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.01±0.00</td>
<td>0.02±0.01</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.01±0.00</td>
<td>0.01±0.00</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pyloric ceacae</td>
<td>0.11±0.01</td>
<td>0.12±0.01</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hindgut</td>
<td>0.02±0.00</td>
<td>0.03±0.00</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Denotes significant differences (P<0.05) between relative tissue contributions between carotenoids within species.

• Denotes significant differences (P<0.05) between species within a tissue within a carotenoid treatment.

N.D. indicates not determined as individual section weights not determined for individual fish.

3.4 Discussion

Results from the rainbow trout growth trial indicate no differences attributable to carotenoid treatment, and growth parameters indicate similar growth potentials. Similarly no differences between carotenoid treatments were observed in the Atlantic salmon growth trial. Although there appeared to be a disproportionate level of mortality in salmon fed canthaxanthin-supplemented diets, the majority of these occurred in a single tank. As the growths between treatments were similar, the mortality rate was deemed to have little effect on the outcome of the trial.

3.4.1 Digestibility

Feed conversion ratios in trout and salmon were comparable, while the apparent digestibility coefficients (ADCs) of either carotenoid were comparable within a species, they were significantly lower in salmon than in trout. The ADCs of astaxanthin (approx. 55%) and canthaxanthin (approx. 10-12%) obtained by Choubert et al. (1995) for rainbow

79
trout, are much lower than those observed in the present study, but are likely due to two factors: firstly the lower dietary lipid content (12%) and the higher carotenoid content (100 ppm) of their diets in comparison to the present study.

Several authors have reported digestibility values for astaxanthin in Atlantic salmon (approx. 96 %) (Torrissen et al. 1981; Choubert and Luquet 1983; Foss et al. 1987; Storebakken et al. 1987), that are approximately three times higher than the present values. These differences could be due to many factors as suggested by Torrissen et al. (1990): differing pigment sources, fish size and species, destruction of carotenoids in the digestive tract, and different analytical methods. Dietary fat levels have been shown to influence carotenoid digestibility and absorption, increasing with increasing dietary fat (Torrissen et al. 1990). The present canthaxanthin ADCs from diets containing 30% lipid are slightly higher than those obtained by Torrissen et al. (1990) who fed diets with 23% fat. These differences may be due to the higher levels of fat used in the present studies, or to losses of carotenoids during the extraction procedure, resulting in an overestimation of digestibility. The present ADCs for astaxanthin in Atlantic salmon were half those obtained by Bjerkeng and Berge (2000) fed diets with a similar dietary fat content (29%). However, the size of fish used for digestibility estimates were different as were the mean temperatures. However, both the present study and that of Bjerkeng and Berge (2000) fed fish slightly in excess, and thus the trials are comparable in most instances, suggesting poorer performances in the present trial.

The digestibility of carotenoids is also affected by dietary concentration. Studies by Choubert and Storebakken (1996) revealed that maximum apparent digestibilities were obtained at 25 mg kg\(^{-1}\) and 50 mg kg\(^{-1}\) for astaxanthin and canthaxanthin, respectively, and decreased with higher inclusion levels. Thus the lower ADCs in Atlantic salmon in the present study may be due to the maximum digestibility of carotenoids being achieved at a
lower dietary inclusion rate. However, the similar dietary inclusions observed in the present experiments suggest that species-differences exist in their ability to absorb carotenoids.

The lower digestibility of carotenoids in salmon compared to trout may be due to lower digestibility of fats. Fat and protein ADCs were not determined in the present trials, however, macro-ingredient digestibilities in Atlantic salmon are generally similar, although slightly lower (8, and 11%, respectively) than those in trout (Refstie et al. 2000). Due to species similarities in fat digestibility, the present results suggest that other factors must be affecting the release of carotenoids from the food matrix, ultimately reducing the availability of carotenoids for absorption in salmon. One possibility is the enzyme systems available for the breakdown of the food matrix. While this is a possibility, it would be expected that digestibility of other dietary nutrients would also be affected, which has not been observed in the literature, as digestibilities for protein and lipid are similar to those of rainbow trout (Refstie et al. 2000). In addition, marked differences in growth performance (e.g. SGR and FCR) would have been expected, but were not observed. Therefore, the formation of suitable mixed micellar fractions in the gastrointestinal tract may be limiting the incorporation of carotenoids into the matrix. Dietary fat stimulates the release of bile salts from the gall bladder (Parker 1996; Williams et al. 1998; Deming and Erdman 1999), and aids in the formation of mixed micelles. Bile salts may be insufficiently released from the liver, as these are considered essential for carotenoid absorption (El-Gorab and Underwood 1973; El-Gorab et al. 1975), or are of a different composition from those in trout, which may negatively impact the solubility of carotenoids for incorporation into the mixed micelle. Further still are intraluminal factors, which may alter the ability of the mixed micelle to transfer through the unstirred water layer in the gastrointestinal tract (Hollander and Ruble 1978; Deming and Erdman 1999).
In addition, other components within the feed can disrupt micelle formation and/or bind carotenoids, thereby inhibiting mucosal uptake of carotenoids. Soluble fibers are viscous polysaccharides that can solubilize in the gastric contents of the intestinal lumen where partitioning of bile acids into the gel phase of gastric contents could hinder micelle formation, resulting in increased fat and bile acid excretion in the feces (Deming and Erdman 1999). Indeed, Krogdahl (1990), noted that plant protein sources contain antinutritional factors that may adversely affect digestion in salmonids. However, the present studies used similar ingredients and diet formulations, which suggests that other factors are playing a role in the variable digestibilities between these species.

3.4.2 Flesh carotenoid retention

Flesh astaxanthin deposition resulted in significantly higher flesh concentrations relative to rainbow trout fed canthaxanthin, and is consistent with previous studies (Henmi et al. 1987; Choubert and Storebakken 1996; Gobantes et al. 1997). Despite the higher flesh carotenoid concentration in trout fed astaxanthin, this did not result in a significantly higher pigment retention relative to trout fed canthaxanthin. These differences are likely due to the relatively small difference in the flesh concentrations and the slightly higher levels of dietary carotenoids in trout fed canthaxanthin than in trout fed astaxanthin.

The higher flesh carotenoid concentration in salmon fed canthaxanthin, compared to those fed astaxanthin, may be due to both a slightly higher apparent digestibility coefficient, combined with a slightly higher dietary canthaxanthin concentration relative to values obtained for salmon fed astaxanthin. The results from the present salmon investigations support the observations of Buttle et al. (2001), where canthaxanthin deposition resulted in higher flesh levels than those of salmon fed astaxanthin. The flesh carotenoid concentrations observed in the present trial were higher than those of Buttle et al. (2001), and Bjerkeng et al. (1999) fed similar levels of dietary fat (30%). The higher mean flesh
canthaxanthin concentrations in salmon may be due to a slower rate of canthaxanthin catabolism than astaxanthin, which was suggested to be due to excretion via the glucuronic acid pathway, or to the chemical instability of astaxanthin compared to canthaxanthin (Sigurgisladottir et al. 1994).

Retention values (Table 3.3) obtained in the present investigations in Atlantic salmon for astaxanthin were comparable to those obtained by Bjerkeng and Berge (2000) of 3.9%, and those of Buttle et al. (2001) where salmon retained 5.7% and 7.6% of astaxanthin and canthaxanthin, respectively.

Retention estimates in rainbow trout range from 1-7% for canthaxanthin and 3-18% for astaxanthin (Torrissen and Braekken 1979; Choubert and Luquet 1983; Foss et al. 1984; Choubert and Storebakken 1989; Choubert et al. 1995). It would appear that trout fed canthaxanthin in the present study pigmented better than in the aforementioned trials as assessed by the higher pigment retention value for canthaxanthin, while trout fed astaxanthin appear to fall within the range of published values. However, as noted by Foss et al. (1984) direct comparisons are complicated by differences in the methods of retention calculation, as well as differences in experimental conditions and dietary treatments.

The observed differences in retention and flesh deposition between these species may be due to differences in their initial starting weights. A positive relationship between initial starting weight and flesh carotenoid concentration has previously been noted by several authors (Abdul-Malak et al. 1975; Torrissen 1989; Torrissen 1995). Alternatively, the limitation to carotenoid utilization for muscle pigmentation may be the rate of metabolic transformation (Bjerkeng and Berge 2000).
Tissue retention efficiencies show that astaxanthin is retained to a lesser degree in Atlantic salmon than is canthaxanthin, possibly due to a higher rate of metabolism of this carotenoid in comparison to canthaxanthin. This is in spite of similar apparent digestibility coefficients for both carotenoids. In contrast to this, the serum astaxanthin concentrations were higher in astaxanthin fed-fish than was canthaxanthin. This is potentially due to a higher astaxanthin clearance rate from circulation compared to canthaxanthin in salmon. However, no comparative data of post-prandial astaxanthin and canthaxanthin clearance have been performed in Atlantic salmon. Results from Aas et al. (1999) show that astaxanthin is cleared from plasma with a half-life of 30 hours, which is slightly longer than the half-life of astaxanthin in rainbow trout (24 hours) observed by Gobantes et al. (1997). No data is available for canthaxanthin clearance in Atlantic salmon, whereas this carotenoid is cleared with a half life ranging from 16-22 hours in rainbow trout (Guillou et al. 1993; Gobantes et al. 1997). If the patterns of carotenoid deposition in flesh hold, one would expect that astaxanthin is cleared from plasma at a faster rate (shorter half-life) than is canthaxanthin in Atlantic salmon. Further research into carotenoid clearance differences is required to understand the apparent species-specific carotenoid utilization.

Despite these minor differences, the present results show equivalent utilization of astaxanthin and canthaxanthin in Atlantic salmon as observed by Storebakken et al. (1987) and Buttle et al. (2001). If carotenoid utilization is assessed by flesh carotenoid concentrations only, it would appear that trout fed astaxanthin pigmented better than those fed canthaxanthin, while the reverse is true in Atlantic salmon. However, based on pigment retention values, both astaxanthin and canthaxanthin appear to be utilized to the same degree within a species, with rainbow trout being more efficient than Atlantic salmon.
3.4.3 Tissue carotenoid accumulation

Lower astaxanthin accumulation in the livers of Atlantic salmon compared to canthaxanthin is consistent with mammalian studies which show that canthaxanthin accumulates to much higher levels than does astaxanthin (Gradelet et al. 1996). This is potentially due to the lipid profile in the liver where the relative lipophilicity and polarity of these carotenoids may influence their degree of solubility. The low concentrations of astaxanthin and canthaxanthin recovered in the liver of rainbow trout, as compared to the levels recovered in salmon, indicate that this is not a storage organ for these carotenoids in rainbow trout, which is in agreement with the observations of Østerlie et al. (1999).

Relative differences between trout and salmon were observed in the deposition of carotenoids in various tissues (Figs. 3.1 and 3.3). Trout deposited higher levels of both astaxanthin and canthaxanthin in the gonads than did salmon. This may be due to the relative stage of maturity of the fish used in both studies, although relative tissue somatic indices were similar between species, suggesting preferential carotenoid deposition in this tissue in the trout.

Serum levels between species were variable, although mean serum canthaxanthin levels were higher than those of fish fed astaxanthin in both trout and salmon, consistent with previous research (Table 1.3).

3.4.4 Peak proportionality

The higher proportionality of astaxanthin peak area in rainbow trout flesh compared to canthaxanthin, suggests that canthaxanthin may be metabolized at a faster rate than is astaxanthin. Autoradiographic observations by Torrissen and Ingebrigsten (1991) demonstrated that recovered $^{14}$C-astaxanthin-derived radioactivity in muscle of Atlantic salmon is mainly in the connective tissue between the myotomes. These authors ascribed this radioactivity to colourless metabolites.
Astaxanthin peak areas in the kidney were found to represent a significantly higher proportion of the total peak areas of chromatograms in rainbow trout compared to canthaxanthin. This suggests that canthaxanthin may be metabolized to a larger extent than is astaxanthin and may account for the significantly higher flesh astaxanthin concentration compared to Atlantic salmon. It follows that astaxanthin peak areas should represent a smaller proportion of the total peak areas compared to canthaxanthin in Atlantic salmon. While this general trend was observed, the proportionalities of either carotenoid were not significantly different in the flesh of Atlantic salmon. However, the low number of replicates in this study may mask any inherent difference, considering that flesh canthaxanthin concentrations were consistently higher than those of astaxanthin.

From the carotenoid peak area proportionality diagrams it is apparent that astaxanthin, in most tissues, represents over 90-95% of the peak areas in rainbow trout, consistent with published reports stating that astaxanthin represents more than 90% of the flesh total carotenoid content of rainbow trout (Bjerkeeng et al. 1997). Although it should be noted that the present data are not based on concentrations, due to the inability to identify and quantitate other compounds, the relative proportions appear to be similar between species and carotenoids. However, the supplemented carotenoids account for only approx. 20-25% of the total peak area in the livers of rainbow trout, suggesting that absorbed carotenoids in the liver are rapidly metabolized. This implies that trout have a high metabolic capacity for the conversion of these carotenoids, and that the liver is not selective in their accumulation or metabolism. Conversely, it appears that salmon are comparatively slower at metabolic conversion of absorbed carotenoids by the liver as evidenced by the fact that the peak areas of these carotenoids are two-fold higher than those in trout. This suggests that salmon have either a slower metabolism, which may account for the higher levels of
absorbed carotenoids in the liver compared to the trout, or a fundamental shift in the tissue responsible for carotenoid catabolism in Atlantic salmon.

The relative contribution of astaxanthin peak area to total chromatogram area, while an indirect measure of the relative concentrations, and thus contribution to the tissue carotenoid profile, can nonetheless provide some information regarding the potential metabolic activity in various tissues. Present results show that in the liver, astaxanthin represents less than 50% of the total peak area in Atlantic salmon. This is consistent with results obtained by Schiedt et al. (1988a) who found that astaxanthin represented only 27% of the total carotenoids recovered. The relative difference between these studies may be due to the inability to directly identify and quantify the other carotenoids present. In addition, the present study used larger fish (approx. 860 g) compared to the study by Schiedt et al. (1988a) of 400 g. Astaxanthin represented approx. 80% of the relative area in the kidney chromatograms, and is higher than the relative contribution of 14% observed by Schiedt et al. (1988 a). These discrepancies are potentially due to other compounds absorbing at the same wavelength, which contribute to the chromatogram areas, confounding interpretation.

Atlantic salmon fed canthaxanthin showed that canthaxanthin represented a significantly lower amount of the total chromatogram area in the female gonads compared to salmon fed astaxanthin, which could be due to preferential metabolism of this carotenoid in the ovaries. Guillou et al. (1989) suggested that mature rainbow trout may have a metabolic pathway for the direct conversion of astaxanthin into vitamin A. A similar pathway may be present in salmon that allows for the conversion of canthaxanthin.
3.4.5 Liver and kidney as metabolic tissues: A comparison

Both salmon and trout had significantly higher mean canthaxanthin concentrations in the kidney compared to astaxanthin, which suggests a tissue-specific accumulation of these carotenoids. Trout appeared to accumulate higher levels than those in the serum indicating selective uptake, while those in salmon kidney were lower than in the serum, and therefore renal tissue carotenoid concentrations in salmon may be a reflection of the serum levels due to the high blood content of this tissue. This may imply a species-specific pattern of tissue accumulation of carotenoids, which may be related to the function of the kidneys in the respective culture environments of salmon and trout; saltwater vs. freshwater. Choubert et al. (1987) and Hardy et al. (1990) both observed the accumulation of carotenoids in the kidney of rainbow trout, supporting the present results.

In contrast, the liver of rainbow trout did not accumulate high levels of carotenoids compared to salmon. It would appear that trout do not show a selective accumulation of either carotenoid, whereas salmon show distinctly higher canthaxanthin concentrations compared to astaxanthin, both of which are higher than serum values. Taken together these data suggest that contrary to kidney tissue accumulations, salmon retain higher levels of either carotenoid (especially canthaxanthin) in the liver, whereas the opposite relationship is observed in rainbow trout. These observations may be related indirectly to salinity, although previous authors have not noted significant differences in digestibility as a result of salinity differences (Choubert and Storebakken 1996), nor in carotenoid retention (Storebakken and No 1992).

In addition to individual tissue concentrations in both salmon and trout, the liver and kidney chromatogram peak areas of the fed carotenoids contribute less to the total chromatogram areas than other tissues, suggesting these tissues are highly active in carotenoid catabolism. These observations are consistent with previous observations by
Hardy et al. (1990) who recovered radioactive water from metabolic chambers in trout fed radiolabeled astaxanthin, suggesting urinary excretion. Torrissen and Ingebrigsten (1991) also recovered a high level of radioactivity in the bile of salmon fed $^{14}$C-astaxanthin.

3.4.6 Other tissues as metabolic organs

Other tissues/organs, namely the spleen, heart, and gastro-intestinal tissues showed similar tissue concentrations between carotenoids and between species. These observations suggest that these tissues play a minor role in total body carotenoid retention, due not only to their low absolute concentrations, but also to their low tissue somatic indices.

However, salmon appear to have higher metabolic capacities in the gastro-intestinal sections as observed by the relatively lower proportionality of the peak areas compared to rainbow trout. This observation may account for some of the differences seen in the serum levels observed between salmon and trout. This data suggests that proportionally more absorbed carotenoids are degraded in the enterocytes in Atlantic salmon than in rainbow trout. This is potentially due to higher levels of catabolic enzymes in salmon than in trout. However, both salmon and trout are known to be able to convert both astaxanthin and canthaxanthin into vitamin A in the gastro-intestinal tract (Schiedt et al. 1985; Al-Khalifa and Simpson 1988; Guillou et al. 1989), although a comparative investigation into the species-specific catabolic efficiencies have not been conducted.

When we compare both the trout and salmon in terms of tissue somatic indices, it becomes apparent that individual organs contribute relatively the same amount to the total body compositions in both species. The relative contribution to the whole body carotenoid pool will therefore be similar, with the flesh contributing the highest amount to total body content of carotenoids, followed by the serum, kidney and livers. These tissues combined account for greater than 95-98% of the total body carotenoids as measured from the tissues.
analyzed in the present investigation. Therefore while differences in carotenoid accumulations in other tissues are of interest, their relative contribution to the whole body carotenoid pool is insignificant.

3.4.7 Conclusions

The present results show species differences as well as tissue differences the contribution to the whole body pools, indicating fundamental differences in tissue accumulations between salmon and trout. More specifically, the liver contributes to a higher degree to the whole body pool in salmon than in trout, while the kidney and gonads are more important carotenoid-accumulating tissues in trout than in salmon. These results, taken together with the relative carotenoid peak areas, suggests tissue-specific patterns in carotenoid catabolism that differ between trout and salmon, where the kidney is more active in the salmon than in trout, and the liver more active in the trout than in salmon.

In conclusion the present results show differential tissue accumulation profiles for astaxanthin and canthaxanthin between tissues both within and between species. Further to this, both the liver and kidney appear to be important organs for the metabolic transformation of carotenoids in both species. However, the flesh is the primary tissue in carotenoid accumulation and retention, accounting for more than 90% of the recovered carotenoids in the tissues examined. The relative importance of the liver in carotenoid distribution requires further elucidation in terms of its effect on carotenoid bioavailability. Therefore, future investigations should examine the role of the liver in carotenoid clearance and elimination.
CHAPTER 4.
THE APPLICATION OF AN IN VITRO ISOLATED PERFUSED LIVER PREPARATION TO EXAMINE HEPATIC CAROTENOID UPTAKE KINETICS IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM).

4.1 Introduction

Dietary carotenoids are absorbed into the plasma and subsequently transported and distributed to various tissues and organs. Ando et al. (1985; 1986a,b) identified that carotenoids were bound non-covalently to serum proteins (carotenoid-carrying-lipoproteins), specifically to HDL, VHDL, and LDL lipoprotein fractions. Choubert et al. (1991) reported the distribution of canthaxanthin among the various lipoprotein fractions to be LDL, 28.3%; HDL, 61.1%; VHDL, 10.6% in the serum of immature rainbow trout. Both the absorption and metabolism of carotenoids are likely to be highly dependent on their structure. However, the mechanisms by which carotenoids are taken up from the plasma by tissues and released back into plasma from specific tissues, have not yet been clarified (Olson, 1993). It has been proposed that carotenoids (e.g. β-carotene) in humans are taken into cells passively along with lipoproteins via the LDL receptors (Kaplan et al. 1990; Schmitz et al. 1991; Stahl et al. 1992). The catabolism of lipoproteins (e.g. LDL) in rainbow trout appears to follow the endocytic-lysosomal pathway described for many macromolecules in mammalian cells (Gjøen and Berg 1992, 1993 a,b). LDL constitutes an important physiological ligand catabolized in the liver, with the main lipoprotein class in salmonid fish, HDL, being catabolized in both the liver and kidney (Gjøen and Berg 1992). As carotenoids are bound strictly to lipoproteins in vivo in fish, as they are in mammals, it would be reasonable to infer that carotenoids are taken into liver cells by the same process (receptor-mediated endocytosis of lipoproteins with bound carotenoid), rather than through a specific cellular carotenoid-receptor protein or through passive diffusion.
The relatively small contribution of the fed carotenoid peak area to total chromatogram area for liver (Chapter 3, Fig. 3.2), would appear to suggest that the liver plays a significant role in carotenoid metabolism. Other in vivo studies have confirmed the role of the liver in carotenoid metabolism but have not provided evidence as to the potential rates or hepatic contribution to total body clearance. Hardy et al. (1990) suggested that a significant amount of absorbed 14C-canthaxanthin was metabolized by the liver due to the high specific activity found in the bile, which was eight times that found in blood serum. Schiedt et al. (1985) found the highest concentrations of recovered radioactivity in the bile (20-40 μg g⁻¹) of salmon fed radiolabeled carotenoids. Similarly, Storebakken and Ingebrigsten (1992) also observed a high degree of radioactivity in the bile of fish fed labeled carotenoids, indicating biliary excretion of astaxanthin metabolites. Astaxanthin was found to be reductively degraded to idoxanthin and crustaxanthin relatively quickly, and eliminated from the liver of Atlantic salmon (Schiedt et al. 1985). The liver may, therefore, be catabolizing carotenoids to other pigments or to metabolites that no longer carry a chromophore. Storebakken and No (1992) detected unidentified catabolites of carotenoids in the bile of trout, indicating that the liver is a major metabolic organ for carotenoids.

While the available evidence points to the role of the liver in carotenoid homeostasis, no studies have attempted to elucidate its role or to quantify its potential in carotenoid clearance. Current hepatic metabolism studies in fish nutrition relate primarily to measurements of steady state levels of the parent compound of interest and/or its metabolite(s) in vivo. These studies provide useful information, but limitations exist relating to sampling for statistical validity, as well as the inability to directly manipulate the amount of an individual carotenoid to which the liver is exposed for quantitative
analysis of its role in clearance in the absence of other confounding organ contributions to the elimination profile \textit{in vivo}.

The use of \textit{in vitro} systems eliminates many of the problems associated with \textit{in vivo} aquatic studies, such as the ability to assess organ function without interference from other body compartments, sampling from large populations, and limited use of radiolabeled chemicals caused by costs of disposal, as well as large volumes of contaminated water (Singh \textit{et al.} 1996). In addition, they allow for more direct control of the carotenoid concentration in extracellular fluids to which the tissue is exposed, which may not be possible \textit{in vivo}.

There are several methods currently in use for \textit{in vitro} metabolic studies relating to hepatic function including isolated hepatocyte cultures, liver slices and whole organ perfusion systems. The less invasive methodology of isolated liver perfusion offers many advantages in examining liver metabolism because the tissue or organ, as a whole, functions differently from isolated cells (Cascales \textit{et al.} 1997). Perfusion allows the study of exact metabolic use of substrates by determining their concentrations pre- and post-hepatically, thus enabling the study of short- to medium-term effects of the substrate on the liver (Cascales \textit{et al.} 1997).

The liver is a key organ that controls many important functions, playing a prominent role in fish physiology (Bruslé and González I Anadon 1996; Fig 1.7). The liver in salmonid fish is a dense unlobed organ, located ventrally in the abdominal cavity, that is highly vascularized, receiving a dual blood supply, from the hepatic portal vein, draining the gastrointestinal tract and visceral tissues, and from the hepatic artery, delivering freshly oxygenated blood from the gills. Normal blood flow through the liver is estimated at between $4.47 \pm 2.69 \text{ ml hr}^{-1} \text{ g}^{-1}$ (Barron \textit{et al.} 1987). The liver drains directly into the heart.
via the hepatic vein. The liver is also the organ responsible for bile production. The stroma of the liver is comprised largely of hepatocytes that surround bile ducts, draining into the gall bladder.

The use of liver perfusion in piscine metabolic studies has been limited to date. Indeed, to this authors’ knowledge there have only been a handful of studies evaluating the function of liver in metabolism, most concentrating on gluconeogenesis and glucose flux (Hayashi and Ooshiro 1975; Ottolenghi et al. 1985 a,b; McKinley and Hazel 1993), with some investigating xenobiotic biotransformation (Andersson et al. 1983), selenium deficiency (Bell et al. 1986), or thyroid hormone metabolism (Brett et al. 1998). The aims of the present investigations were to develop and evaluate the use isolated liver perfusions as a model in examining the role of the liver in carotenoid clearance. The investigations were also aimed at identifying differences between astaxanthin and canthaxanthin uptake patterns, and to provide estimates of hepatic uptake kinetic parameters using liver carotenoid uptake rates as an indirect marker of carotenoid metabolism.

4.2 Materials and Methods

4.2.1 Perfusion methodology

Rainbow trout were maintained on carotenoid-free stock diets at the Plymouth West Aquarium facilities for at least two weeks prior to isolated organ perfusion studies as previously described (Chapter 2.2).

Trout were anaesthetized (2 ml l⁻¹ phenoxy-2-ethanol, Sigma, Poole, U.K.) and inverted on an operating board and 2 ml of 800 IU ml⁻¹ sodium heparin (from ovine mucosa; Sigma, Poole, U.K.) was injected via caudal venipuncture using a tissue culture medium (L-15 Leibovitz medium, Sigma, Poole, U.K.) vehicle. After two/three mins, the abdominal cavity was opened by ventral and midline longitudinal incisions, which extended to the
anterior zone of the visceral cavity. A transversal incision was made as far as the operculum in order to remove the muscle wall, exposing the whole liver.

The hepatic portal vein was cannulated using surgical tubing (0.8 mm inner diameter, 1.6 mm outer diameter) and secured with surgical suture (3-0 braided silk). Approximately 5 ml of L-15 (Leibovitz) tissue culture medium supplemented with 2 mM EDTA (flushing solution) was then perfused through the liver to flush blood from the preparation using a 10 ml syringe. A ligature was then placed on the hepatic artery to block cardiac circulation. The liver was kept moist with flushing solution.

The gall bladder and bile duct were ligated to prevent leakage into the perfusate. Preparations (Fig. 4.1) that exhibited evidence of leakage from regions other than the sites of perfusate sample collection were discarded.

The liver was then excised and placed in a sterilized (washed in 70% ethanol) plastic mesh sieve on a retort stand, over a funnel. Modified culture medium (L-15 supplemented with 50% bovine calf serum (FCS)) was then perfused through the preparation using a variable flow peristaltic pump (Gilson Minipuls 3, Villiers-le-bal, France). Flow rates were adjusted to provide approximately 4.5 ml hr$^{-1}$ g$^{-1}$ (Barron et al. 1987) using a liver weight estimated to be 1.3% of body weight. Tissue culture medium has been shown to be successful in prolonging isolated organ perfusion preparations (Campbell et al. 1999). Total surgery time, until re-establishment of flow, took approximately 15-20 min from the time of anticoagulant injection.

The liver was then connected to the perfusion circuit (Fig. 4.2). Perfusions were carried out in a Perspex cabinet with an aerated water reservoir to create a humid atmosphere (as
Fig. 4.1. Schematic representation of the cannulated liver, showing the inflow at the hepatic portal vein (cannula with luer fitting to attach to perfusion apparatus), and the ligated hepatic artery. Perfusate was allowed to flow freely from the hepatic vein, which drained into a funnel and receptacle.
Fig. 4.2. Schematic representation of perfusion apparatus used in isolated organ perfusions. Perfusate flows from a chilled reservoir through the adjustable peristaltic pump to the bubble trap, where trapped gas bubbles escape for the perfusate. The bubble trap is chilled via a water jacket connected to a water chiller. Perfusate flowed gravimetrically through the liver and collected in glass vials as 10 min fractions. Perfusate temperature was monitored via a temperature probe placed before the liver. The environment was kept moist (as assessed by hygrometer) by bubbling air through a water bath inside the sealed Perspex cabinet.
assessed by a hygrometer inside the cabinet; all perfusions were maintained above 70% relative humidity). A water cooled bubble trap attached to the inflow prevented gas embolisms in the isolated organ and also maintained perfusate temperature at 16.0 ± 0.5°C. Organs perfused with beadlet-derived astaxanthin (see below) were stabilized for 60 min, and the eluent fractions collected (5 min fractions) discarded after viability assessment (lactate dehydrogenase (LDH), Sigma kit #DG1340-K, Poole, U.K.).

Following the recovery/stabilization period, carotenoid-supplemented perfusate (see below) was perfused through the liver and eluent perfusate samples (10 min fractions) collected in glass vials, with aliquots immediately taken for viability analysis (LDH activity). One ml of the remaining eluent perfusate was analyzed for carotenoid content.

Aliquots of pre- and post-hepatic perfusates were analyzed for carotenoid content by HPLC. One ml samples were extracted in the following ratios sample:ethanol:diethyl ether:hexane (1:1:1:1; v/v/v/v), or diluted 1:1 (v/v) with water and extracted, the mixture vortexed for 5 s, and allowed to extract in the dark for 10 min. Samples were again vortexed for 5 s, and centrifuged at 3000 rpm (2000 X g) in a Mistral 3000 refrigerated centrifuge (MSE, U.K.). Aliquots of the supernatant extracts were directly analyzed by HPLC according to the methods described in Chapter 2.11.

4.2.2 Perfusate preparation

Carotenoid-supplemented serum was first prepared by dissolving the commercial water dispersible beadlets of either Carophyll® Pink (astaxanthin), or Carophyll® Red (canthaxanthin), from Hoffman-La Roche (Basel, Switzerland), into warmed (35°C) bovine calf serum (Sigma, Poole, U.K.; FCS) on a stirring hot plate. Once cooled to room temperature, serum was centrifuged at 2000 X g (Mistral 3000; MSE, Cheshire, UK) for 30 min, to pellet undissolved pigment, and stored frozen (-20°C) in fractions for later use.
Piscine sera used in the autologous fish serum experiments were collected from rainbow trout and Atlantic salmon used in the carotenoid distribution studies in Chapter 3. Blood was collected as described in Chapter 2.9.1 and left to clot overnight at +4°C, and the serum decanted and stored frozen at -20°C until used in the in the perfusion studies. Perfusate was prepared as described below.

Perfusate(s) were prepared by dissolving serum fractions (beadlet-FCS, or piscine) into L-15 tissue culture medium at a ratio of 1:1 (v/v). Appropriate concentrations of pigments were prepared by mixing unpigmented serum with carotenoid-dosed FCS to achieve the desired concentration of carotenoid. Piscine sera were used undiluted.

4.2.3 Viability Measurements

Isolated liver perfusion preparations were subject to a number of viability checks before, during and after experiments to ensure healthy tissue and haemodynamic characteristics. These included:

1. Perfusion leakage: Preparations exhibiting leakage from sites other than the site of collection were discarded. Livers exhibiting uneven perfusion throughout the organ (as assessed visually by colour, and absence of localized swelling) were discarded.

2. LDH concentration of perfusates: The elevation of this cytosolic enzyme in organ perfusates during experiments is used to indicate tissue damage and reduced viability (Campbell et al. 1999). The LDH activity of eluted perfusates were determined immediately at the end of each collection period. The LDH assay, which is a modification of the kinetic pyruvate reduction method, detects the oxidation of NADH at 340 nm (Plummer 1971). The rate of change in absorbance of a reaction mixture containing: (a) 2.8 ml of 0.6 mmol l⁻¹ pyruvate in 50 mmol l⁻¹ phosphate buffer (pH 7.5); (b) 0.1 ml of 0.6 mmol l⁻¹ NADH solution; (c) 0.1 ml of sample was determined
over 1 min. Data from preparations which produce consistently increasing perfusate LDH activities were discarded.

4.2.4 Experimental outline

Perfusion experiments were designed to investigate dose-response effects on hepatic uptake parameters for both astaxanthin and canthaxanthin derived from commercial beadlet sources. The range of concentrations were chosen to reflect normal in vivo concentrations of carotenoids, as well as supra-physiological concentrations to examine uptake patterns. Both carotenoids were also perfused simultaneously at similar concentrations to determine if interactions existed during uptake. In an attempt to identify the method of carotenoid uptake, livers were perfused with beadlet-derived astaxanthin in addition to EDTA (5 mM). A cation chelating agent which would theoretically prevent active uptake. In order to more closely approximate the in vivo condition, piscine sera (rainbow trout and Atlantic salmon) were perfused to deliver carotenoids bound to individual lipoprotein fractions, their natural in vivo carriers (Ando et al. 1985, 1986 a,b).

4.2.5 Calculations

Carotenoid uptake rates were determined from the eluent (post-hepatic fraction) concentrations relative to the initial perfusate (pre-hepatic fraction) concentrations. Carotenoid uptake rates for each 10 min fraction were calculated as follows:

\[
\text{Uptake rate (µg g}^{-1} \text{ hr}^{-1}) = \frac{\text{Absolute amount of carotenoid removed (µg) x 60 min hr}^{-1}}{10 \text{ (min)} \times \text{liver weight (g)}}
\]

The initial and final carotenoid uptake rates were determined as those uptake rates from the 100 and 190 min fractions, respectively. The overall uptake rate was determined as the
cumulative absolute uptake (µg), divided by the total perfusion time and corrected by liver weight.

The mean hepatic extraction ratio was calculated from the entire 90 min perfusion period. The hepatic extraction ratio, which provides a direct measurement of chemical removal from the liver (Shargel and Yu 1999) was calculated as follows:

\[
\text{Hepatic extraction ratio} = \frac{(\text{Pre-perfusate } C_x (\mu g \text{ ml}^{-1}) - \text{Post-perfusate } C_x (\mu g \text{ ml}^{-1}))}{\text{Pre-perfusate } C_x (\mu g \text{ ml}^{-1})}
\]

Where \( C_x \) is the carotenoid concentration in the perfusate.

Hepatic clearance (\( C_{lh} \)) is defined as the unit of serum that is cleared of carotenoid per unit of time (Shargel and Yu 1999) and was calculated as follows:

\[
\text{Hepatic clearance (ml hr}^{-1} \text{)} = \text{Hepatic extraction ratio} \times \text{perfusate flow rate (ml hr}^{-1} \text{)}
\]

The rate of drug removal by the liver usually approximates the rate of drug metabolism (Shargel and Yu, 1999), and may be expressed in terms of hepatic clearance and drug concentration entering the liver (\( C_a \))

\[
\text{Liver metabolic rate (µg hr}^{-1} \text{)} = \text{Hepatic clearance (ml hr}^{-1} \text{)} \times \text{Pre-perfusate } C_x (\mu g \text{ ml}^{-1})
\]

Where \( C_x \) is the carotenoid concentration.
The cumulative carotenoid removed from perfusate (in absolute amounts (μg)) was then used to calculate the cumulative uptake over the entire perfusion and normalized for individual liver weight as follows:

\[
\text{Cumulative uptake (μg g}^{-1}\text{)} = \left(\frac{\text{Cumulative absolute amount of carotenoid removed (μg)}}{\text{Liver weight (g)}}\right)
\]

4.2.6 Histology

Post-perfused or unperfused control livers (from same pool of fish used for perfusions), were dissected free and immediately frozen in liquid N\textsubscript{2} prior to sectioning on a Leica 2700 Frigocut cryostat. Liver sections (20 μg) were mounted on glass slides and stained with 1% (w/v) Methylene Blue to 10 s, and rinsed in tap water. Slides were dried and mounted with DPX. Liver sections from both control and perfused livers were compared under light microscopy for general morphology using an Olympus VANOX-T microscope (Olympus Optical Co., Ltd., Tokyo, Japan), with an attached digital camera. Representative histological sections are presented in Plate 4.1.

4.2.7 Statistical analysis

Descriptive statistics were determined for HSI, flow rates, and LDH activity prior to statistical analysis. All data were submitted to a one-way analysis of variance, followed by LSD multiple range tests, Student's t-test, or Kruskal-Wallis followed by Box-and-Whisker plots (for non-parametric data). Data were analyzed using Statgraphics\textsuperscript{®} Plus 4.0 for Windows (Manugistics, Inc., Rockville, Maryland, USA) and differences were determined at the P<0.05 level of significance unless otherwise stated. Statistical comparisons were made between doses within a treatment, and between similar concentrations with different carotenoid delivery vectors. Correlations between uptake variables and perfusate carotenoid concentrations were made using SigmaPlot 4.0 for
Windows (SPSS Inc., Chicago, Illinois, USA) and significance level and correlation coefficients or R-squared values are reported where appropriate.
Plate 4.1. Histology sections (20 µm) of perfused and unperfused control rainbow trout (*Oncorhynchus mykiss* Walbaum) livers. A- perfused (X 400); B- unperfused control (X 400); C- perfused (X 200); and D- unperfused control (X 200). Frozen liver were sectioned using a Leica 2700 Frigocut cryostat and stained with 1% Methylene Blue for 10s. Note the similarities in structure, blood vessels, and the presence of erythrocytes in the stroma of blood vessels of unperfused control livers.
4.3 Results

4.3.1 Viability

Results from lactate dehydrogenase activity viability assessments revealed that isolated liver perfusions were stable for the duration of the data collection periods used in the present investigations (Fig. 4.3). In addition, histological examination of perfused livers, compared to unperfused control livers, did not reveal any damage to the liver ultrastructure (Plate 4.1). Cascales et al. (1997) used 20 min to stabilize the liver, which is in contrast to the present results which indicate that the organ may require 40 min to establish a stable LDH activity. Therefore, it was decided that experiments should to start at 80 min to ensure stability. In the dose-response studies LDH activities were not significantly different between doses before carotenoid administration (Table 4.1). However, in the astaxanthin dose-response investigation, after carotenoid administration, livers perfused with 32 μg ml$^{-1}$ showed significantly lower activities than livers perfused at 2, and 8 μg ml$^{-1}$ (Table 4.1). This may be related to the antioxidant properties of astaxanthin at this dose, or to differences in perfusate "batches", and requires further investigation.

In order to account for the mixing of the carotenoid-unsupplemented versus supplemented perfusates in the bubble trap (perfusate exchange time of apparatus of approx. 7 min), as well as the transition time in the liver tissue post-switch (T, at 80 min; Fig 4.3), the first two 10 min fractions (80 and 90 min fractions) were not used in estimation of carotenoid uptake or other data. Therefore the 100 min fraction was used as the fraction for initial uptake rate determinations (Fig. 4.3), and the 190 min fraction for the final uptake rate (Fig. 4.3).
Fig. 4.3. Mean normalized LDH activity profile of isolated rainbow trout livers perfused with synthetic astaxanthin derived from Carophyll Pink (T is the time of perfusate switch from carotenoid-free to carotenoid-laden perfusate; S - is the time of first sample used for uptake parameter calculation). Values are presented as means of all doses at each time point ($n = 20$) +/- SEM. Steady state values (from 80-190 min) not significantly different ($P<0.05$).
Table 4.1. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin and/or canthaxanthin (derived from synthetic beadlets) at various concentrations. Values expressed as means ± SEM. (n = 5 livers per carotenoid; n = 6 livers with astaxanthin and canthaxanthin perfused simultaneously).

<table>
<thead>
<tr>
<th>Carotenoids (beadlets)</th>
<th>Perfusate concentration (μg ml⁻¹)</th>
<th>HSI (%)*</th>
<th>Flow rate (ml g hr⁻¹)</th>
<th>Mean LDH activity – before (U L⁻¹ g⁻¹ liver)</th>
<th>Mean LDH activity – after (U L⁻¹ g⁻¹ liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin</td>
<td>2.17±0.03</td>
<td>1.39±0.09</td>
<td>4.86±0.28</td>
<td>26.4±5.14</td>
<td>24.3±4.3^b</td>
</tr>
<tr>
<td></td>
<td>4.10±0.04</td>
<td>1.46±0.08</td>
<td>4.63±0.20</td>
<td>25.7±3.2</td>
<td>19.5±3.2^b</td>
</tr>
<tr>
<td></td>
<td>8.59±0.19</td>
<td>1.29±0.07</td>
<td>5.25±0.14</td>
<td>40.3±10.6</td>
<td>31.6±7.6^b</td>
</tr>
<tr>
<td></td>
<td>31.95±0.15</td>
<td>1.51±0.08</td>
<td>4.15±0.24</td>
<td>15.5±3.7</td>
<td>7.9±2.3^a</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>1.95±0.03</td>
<td>1.62±0.09</td>
<td>4.45±0.23</td>
<td>10.4±2.4</td>
<td>14.1±2.1</td>
</tr>
<tr>
<td></td>
<td>4.04±0.05</td>
<td>1.55±0.20</td>
<td>4.77±0.44</td>
<td>17.9±6.7</td>
<td>18.1±6.4</td>
</tr>
<tr>
<td></td>
<td>7.63±0.21</td>
<td>1.30±0.16</td>
<td>4.87±0.35</td>
<td>23.7±5.3</td>
<td>26.2±9.1</td>
</tr>
<tr>
<td></td>
<td>34.95±0.56</td>
<td>1.54±0.21</td>
<td>4.09±0.36</td>
<td>29.6±8.7</td>
<td>30.6±4.4</td>
</tr>
<tr>
<td>Simultaneously</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>perfused</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>1.61±0.06</td>
<td>1.99±0.16</td>
<td>3.78±0.19</td>
<td>20.1±6.6</td>
<td>18.4±3.7</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>2.00±0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values within a column within a treatment sharing similar superscripts are not significantly different (P>0.05).

*HSI similar to unperfused livers from rainbow trout of the same size (Chapter 7).
Cumulative flow and cumulative uptake do not show a similar linear relationship (Fig 4.4 a and b), and suggest that the uptake mechanism for carotenoids is not due to net solvent drag between perfusate and liver tissue. The combined data regarding the viability and the lack of perfusate solvent drag effects, show that the in vitro isolated liver perfusion system is capable of removing carotenoid and is viable for the duration of the perfusion period, and is therefore a suitable model for investigations into carotenoid clearance.

Comparisons of flow rates and hepatosomatic indices between perfusate astaxanthin and canthaxanthin dose-response relationships failed to detect any significant differences in haemodynamics (Table 4.1). These data suggest that differences observed between any uptake parameters measured are solely due to hepatic metabolic effects and inflow perfusate carotenoid concentration, rather than artifacts of organ pathology or change in organ extracellular volume during the experiment.

Isolated rainbow trout livers perfused simultaneously with both astaxanthin and canthaxanthin showed similar LDH activities to those reported for individual perfusions, and suggests that there is no interactive effect on liver viability (Table 4.1).

The similar mean hepatosomatic indices, flow rates and LDH activities in livers perfused with rainbow trout serum, in comparison to those of the beadlet model, suggest that perfusion viability and hepatic function are not affected by the source of carotenoid (Table 4.2).

Viability assessment of livers perfused with Atlantic salmon serum show no detrimental effects relative to livers perfused with trout serum or beadlets (Table 4.3).
Fig. 4.4. Representative cumulative uptake and cumulative flow profiles of isolated perfused rainbow trout livers at different astaxanthin concentration (A) 2 µg ml⁻¹ (HSI 1.35%) and (B) 32 µg ml⁻¹ (HSI 1.37%), derived from Carophyll Pink beadlets.
Table 4.2. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both simultaneously, and derived from rainbow trout serum. Values expressed as means ± SEM. (n = 5 livers astaxanthin; n = 4 livers canthaxanthin; n = 2 livers mixed astaxanthin:canthaxanthin).

<table>
<thead>
<tr>
<th>Carotenoid Perfusate concentration</th>
<th>HSI (%)*</th>
<th>Flow rate</th>
<th>Mean LDH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Rainbow trout serum)</td>
<td>(µg ml⁻¹)</td>
<td>(ml g hr⁻¹)</td>
<td>(U L⁻¹ g⁻¹ liver)</td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>3.79±0.02</td>
<td>1.36±0.19</td>
<td>4.58±0.35</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>3.25±0.00</td>
<td>1.27±0.02</td>
<td>4.90±0.19</td>
</tr>
<tr>
<td>Astaxanthin (mixed)</td>
<td>1.66±0.01</td>
<td>1.22±0.21</td>
<td>4.71±0.81</td>
</tr>
<tr>
<td>Canthaxanthin (mixed)</td>
<td>1.56±0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No significant differences were observed between treatments (P>0.05).

Table 4.3. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both simultaneously, and derived from Atlantic salmon serum. Values expressed as means ± SEM. (n = 4 livers per treatment).

<table>
<thead>
<tr>
<th>Carotenoid Perfusate concentration</th>
<th>HSI (%)*</th>
<th>Flow rate</th>
<th>Mean LDH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Atlantic salmon serum)</td>
<td>(µg ml⁻¹)</td>
<td>(ml g hr⁻¹)</td>
<td>(U L⁻¹ g⁻¹ liver)</td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>1.31±0.01</td>
<td>1.73±0.11</td>
<td>4.53±0.27</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>1.31±0.00</td>
<td>1.56±0.22</td>
<td>4.48±0.53</td>
</tr>
<tr>
<td>Astaxanthin (mixed)</td>
<td>0.48±0.00</td>
<td>1.56±0.28</td>
<td>4.61±0.40</td>
</tr>
<tr>
<td>Canthaxanthin (mixed)</td>
<td>0.73±0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No significant differences were observed between treatments (P>0.05).
No differences in the mean LDH activities between livers perfused with astaxanthin or astaxanthin with 5 mM EDTA, suggest that EDTA supplementation did not significantly affect liver viability over the course of these perfusions. However, significantly higher perfusion rates were observed in control livers (Table 4.4).

4.3.2 Dose effect of carotenoid perfusion

Dose response investigations for beadlet-derived canthaxanthin did not show a saturable uptake mechanism within the concentration range investigated, while beadlet-derived astaxanthin dose-response showed a slight saturable uptake mechanism at the highest perfusate astaxanthin concentration (Fig. 4.5). Astaxanthin uptake parameters showed slightly higher initial uptake rates than canthaxanthin at lower doses, with canthaxanthin initial uptake being higher than astaxanthin at the highest perfusate carotenoid concentrations (Fig. 4.5).

Comparisons of initial and final uptake rates within a carotenoid treatment at the same dose reveal that livers perfused with astaxanthin at 32 µg ml⁻¹ show a significant (P<0.05) reduction in the uptake rates at the end of perfusions relative to initial uptake rates, and suggest a saturation of the uptake process at this perfusate concentration of astaxanthin. In contrast, similar saturation-like results were not observed for any other concentration or carotenoid. The cumulative uptake profiles of the respective carotenoids at each dose confirm these results (Fig. 4.5).

Initial uptake rates were significantly higher for astaxanthin than canthaxanthin at both 2 and 8 µg ml⁻¹ (Table 4.5). Similarly the mean final astaxanthin uptake rate for livers perfused at 2 µg ml⁻¹ were significantly higher than livers perfused at a similar
Table 4.4. Mean hepatosomatic indices, flow rates and liver viability data of isolated rainbow trout livers perfused with astaxanthin alone or in combination with 5 mM EDTA. Values expressed as means ± SEM. (n = 5 livers astaxanthin alone; n = 6 livers astaxanthin with 5 mM EDTA).

<table>
<thead>
<tr>
<th>Carotenoids (beadlets)</th>
<th>Perfusate concentration (µg ml⁻¹)</th>
<th>HSI (%) *</th>
<th>Flow rate (ml g hr⁻¹)</th>
<th>Mean LDH activity – before (U L⁻¹ g⁻¹ liver)</th>
<th>Mean LDH activity – after (U L⁻¹ g⁻¹ liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin + 5 mM EDTA</td>
<td>8.60±0.06</td>
<td>1.55±0.11</td>
<td>4.36±0.19</td>
<td>24.0±3.0</td>
<td>25.0±6.0</td>
</tr>
<tr>
<td>Astaxanthin control</td>
<td>8.59±0.19</td>
<td>1.29±0.07</td>
<td>5.25±0.14*</td>
<td>40.3±10.6</td>
<td>31.6±7.6</td>
</tr>
</tbody>
</table>

* Indicates significant differences between values within a column (P<0.05).
Fig. 4.5. Mean cumulative hepatic uptake profiles of (A) astaxanthin and (B) canthaxanthin at different doses derived from beadlets. Values are expressed as means +/- SEM (n = 5 livers). Cumulative uptake for astaxanthin saturable only at highest perfusate dose, while canthaxanthin does not show saturable uptake within the doses investigated.
Fig 4.6. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers at varying concentrations of (A) astaxanthin and (B) canthaxanthin, derived from synthetic sources (Carophyll Pink and Carophyll Red, respectively). Values are expressed as means +/- SEM (n = 5 livers). No appreciable change in hepatic extraction ratio over the course of the perfusions was observed.
Table 4.5. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin (derived from synthetic beadlets) at various concentrations. Values are expressed as means ± SEM. (n = 5 livers per dose per carotenoid).

<table>
<thead>
<tr>
<th>Carotenoids (beadlets)</th>
<th>Perfusate concentration (μg ml⁻¹)</th>
<th>Mean initial uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean final uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean hepatic extraction ratio</th>
<th>Mean hepatic clearance rate (ml hr⁻¹)</th>
<th>Mean metabolic Rate (μg hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin</td>
<td>2.17±0.03</td>
<td>1.177±0.364</td>
<td>1.188±0.447</td>
<td>0.109±0.036</td>
<td>2.026±0.681</td>
<td>4.401±1.491</td>
</tr>
<tr>
<td></td>
<td>4.10±0.04</td>
<td>1.363±0.0541</td>
<td>1.317±0.727</td>
<td>0.069±0.037</td>
<td>1.206±0.623</td>
<td>4.942±2.535</td>
</tr>
<tr>
<td></td>
<td>8.59±0.19</td>
<td>3.500±0.214</td>
<td>2.223±0.606</td>
<td>0.058±0.005</td>
<td>0.947±0.045</td>
<td>8.157±0.511</td>
</tr>
<tr>
<td></td>
<td>31.95±0.15</td>
<td>7.944±1.094</td>
<td>2.027±0.818</td>
<td>0.026±0.010</td>
<td>0.604±0.253</td>
<td>19.378±8.157</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>1.95±0.03</td>
<td>0.285±0.109</td>
<td>0.111±0.087</td>
<td>0.020±0.009</td>
<td>0.552±0.247</td>
<td>1.095±0.490</td>
</tr>
<tr>
<td></td>
<td>4.04±0.05</td>
<td>0.841±0.179</td>
<td>0.921±0.318</td>
<td>0.054±0.025</td>
<td>1.396±0.630</td>
<td>5.556±2.454</td>
</tr>
<tr>
<td></td>
<td>7.63±0.21</td>
<td>1.844±0.398</td>
<td>1.542±0.341</td>
<td>0.040±0.008</td>
<td>0.937±0.249</td>
<td>7.066±1.866</td>
</tr>
<tr>
<td></td>
<td>34.95±0.56</td>
<td>10.304±3.537</td>
<td>9.407±3.149</td>
<td>0.056±0.020</td>
<td>1.098±0.483</td>
<td>38.166±16.321</td>
</tr>
</tbody>
</table>

* Indicates significant differences between initial and final uptake rates within a dose within a treatment (P<0.05, denoted by *, P<0.01, denoted by **).

a,b,c Indicates values with different superscripts within a column are significantly different between doses within a carotenoid treatment (P<0.05).

* Indicates values within a column are significantly different between carotenoids at similar concentrations (P<0.05, denoted by *, P<0.01, denoted by **).

* Hepatic extraction ratios determined from the mean of the hepatic ratios of each collection period.
canthaxanthin concentration. The mean hepatic extraction ratio profiles of livers perfused with astaxanthin or canthaxanthin at various concentrations (Fig. 4.6), show no distinct pattern (i.e. stable extraction ratios over the perfusion period) for both carotenoids at each perfusate concentration except livers perfused with astaxanthin at 32 \mu g ml^{-1}, which appear to show a reduction in the hepatic extraction ratio over the duration of the perfusion. This observation is also suggestive of uptake saturation at this dose of astaxanthin. In contrast, livers perfused with canthaxanthin did not show any significant differences between mean initial and mean final uptake rates within any of the concentrations investigated, suggesting that the uptake process is not saturating over the course of the perfusions at these concentrations. However, there appeared to be significant (P<0.01) linear concentration effects on the initial uptake rates for both astaxanthin and canthaxanthin, suggesting a direct relationship between perfusate carotenoid concentration and the initial uptake rate (Fig. 4.7). However, the initial carotenoid uptake rate regression lines were not significantly different between astaxanthin and canthaxanthin (P>0.05).

4.3.3 Simultaneous perfusion with beadlet-derived astaxanthin and canthaxanthin

No significant differences between any parameters were observed in livers perfused simultaneously with both astaxanthin and canthaxanthin (Table 4.6) during the course of the perfusion experiments, suggesting similar uptake potential for these carotenoids. Mean cumulative uptake plots for these carotenoids confirm these results (Fig. 4.8), showing a linear response, consistent with the cumulative plots for both carotenoids perfused individually at this concentration (Fig. 4.5). The lack of a significant difference in mean initial uptake rates between these carotenoids when perfused simultaneously is in contrast to the results obtained in livers perfused with each carotenoid individually at these concentrations, where mean initial astaxanthin uptake rates were significantly higher than those for canthaxanthin. This discrepancy is likely due to the potential difficulty in
Fig. 4.7. Regression plots for initial hepatic carotenoid uptake rates as a function of perfusate carotenoid concentration in isolated perfused rainbow trout livers with astaxanthin ($y = 0.762 + 0.230 \times$ perfusate astaxanthin concentration, $r^2 = 0.652$, $p<0.01$) and canthaxanthin ($y = -0.371 + 0.305 \times$ perfusate canthaxanthin concentration, $r^2 = 0.578$, $p<0.01$) derived from synthetic beadlets (Carophyll Pink and Carophyll Red, respectively). Points are expressed as means +/- SEM ($n = 5$ livers).
Table 4.6. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin and canthaxanthin (derived from synthetic beadlets) simultaneously. Values are expressed as means ± SEM. (n = 6 livers).

<table>
<thead>
<tr>
<th>Carotenoids (beadlets)</th>
<th>Perfusate concentration (μg ml⁻¹)</th>
<th>Mean initial uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean final uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean hepatic extraction ratio</th>
<th>Mean hepatic clearance rate (ml hr⁻¹)</th>
<th>Mean metabolic Rate (μg hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin</td>
<td>1.61±0.06</td>
<td>0.806±0.214</td>
<td>0.992±0.296</td>
<td>0.112±0.030</td>
<td>2.164±0.578</td>
<td>4.314±1.151</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>2.00±0.03</td>
<td>0.961±0.231</td>
<td>1.036±0.281</td>
<td>0.100±0.025</td>
<td>1.951±0.507</td>
<td>3.917±1.041</td>
</tr>
</tbody>
</table>

No significant differences were observed between carotenoids (P>0.05).

Table 4.7. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both carotenoids simultaneously and derived from rainbow trout serum, or from synthetic beadlets (Carophyll® Pink or Carophyll® Red: astaxanthin and canthaxanthin, respectively). Values are expressed as means ± SEM. (n = 5 livers astaxanthin, n = 4 livers canthaxanthin, n = 2 livers mixed astaxanthin:canthaxanthin).

<table>
<thead>
<tr>
<th>Carotenoid/source</th>
<th>Perfusate concentration (μg ml⁻¹)</th>
<th>Mean initial uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean final uptake rate (μg g⁻¹ hr⁻¹)</th>
<th>Mean hepatic Extraction ratio</th>
<th>Mean hepatic clearance rate (ml hr⁻¹)</th>
<th>Mean metabolic Rate (μg hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin (serum)</td>
<td>3.79±0.02</td>
<td>1.672±0.461</td>
<td>0.127±0.320</td>
<td>0.042±0.016</td>
<td>0.718±0.295</td>
<td>2.741±1.121</td>
</tr>
<tr>
<td>Astaxanthin (beadlet)</td>
<td>4.10±0.04</td>
<td>1.363±0.541</td>
<td>1.317±0.727</td>
<td>0.069±0.037</td>
<td>1.206±0.623</td>
<td>4.942±2.535</td>
</tr>
<tr>
<td>Canthaxanthin (serum)</td>
<td>3.25±0.00</td>
<td>1.405±0.663</td>
<td>1.245±0.577</td>
<td>0.046±0.012</td>
<td>0.814±0.216</td>
<td>2.650±0.702</td>
</tr>
<tr>
<td>Canthaxanthin (beadlet)</td>
<td>4.04±0.00</td>
<td>0.841±0.179</td>
<td>0.921±0.318</td>
<td>0.054±0.025</td>
<td>1.396±0.630</td>
<td>5.566±2.454</td>
</tr>
<tr>
<td>Serum astaxanthin (mixed)</td>
<td>1.66±0.01</td>
<td>3.524±1.126</td>
<td>1.316±0.660</td>
<td>0.070±0.047</td>
<td>1.007±0.696</td>
<td>1.671±1.158</td>
</tr>
<tr>
<td>Serum canthaxanthin (mixed)</td>
<td>1.56±0.00</td>
<td>0.286±0.002</td>
<td>1.111±0.789</td>
<td>0.076±0.073</td>
<td>1.099±1.061</td>
<td>1.709±1.649</td>
</tr>
</tbody>
</table>

No significant differences were observed between carotenoids (P>0.05).
Fig. 4.8. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers simultaneously perfused with astaxanthin (1.61 μg ml⁻¹) and canthaxanthin (2.00 μg ml⁻¹) derived from synthetic beadlets (Carophyll Pink and Carophyll Red, respectively). Values are expressed as means ± SEM (n = 6 livers). No selective uptake of either carotenoid was observed.
detecting such small differences between pre-and post-hepatic perfusates where initial concentrations are low (i.e. nearing the detection limit (Fig. 2.3)). The hepatic extraction ratio profiles (Fig. 4.9) of these carotenoids show similar responses, suggesting indiscriminate uptake between astaxanthin and canthaxanthin.

4.3.4 Rainbow trout serum perfusions

No significant differences in any uptake and clearance parameters measured were observed between livers perfused with rainbow trout serum as the source for astaxanthin or canthaxanthin, implying that the liver does not treat these two carotenoids differently (Table 4.7). Despite these observations, livers perfused with astaxanthin (derived from trout serum) appear to show a saturation phenomenon during the course of the perfusions that was not seen in livers perfused with canthaxanthin (Fig. 4.10). Even with the lack of difference in the mean hepatic extraction ratios between livers perfused with serum-derived astaxanthin or canthaxanthin, their respective mean hepatic extraction ratio profiles (Fig. 4.11), appear to show a saturation profile over the course of the perfusion in livers perfused with astaxanthin.

This is substantiated by the reduction in the extraction ratio from the beginning to the end of the perfusion. In contrast, livers perfused simultaneously with trout serum containing both astaxanthin and canthaxanthin showed no significant differences between any parameters investigated (Table 4.7), suggesting no preferential accumulation of either carotenoid, when perfused at the lower concentration. This observation is confirmed by the cumulative uptake profiles of the carotenoids when perfused simultaneously (Fig. 4.12), as well as by their mean hepatic extraction ratio profiles (Fig. 4.13).
Fig. 4.9. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers simultaneously perfused with astaxanthin (1.61 μg ml⁻¹) and canthaxanthin (2.00 μg ml⁻¹) derived from synthetic beadlets (Carophyll Pink and Carophyll Red, respectively). Values are expressed as means +/- SEM (n = 6 livers). No appreciable change in hepatic extraction ratios were observed over the course of the perfusions.
Fig. 4.10. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers perfused with autologous rainbow trout serum as sources of (A) astaxanthin (3.79 μg ml⁻¹) or (B) canthaxanthin (3.25 μg ml⁻¹). Values are expressed as means +/- SEM (n = 5 livers for astaxanthin, n = 4 livers for canthaxanthin).
Fig. 4.11. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers perfused with autologous rainbow trout serum as sources of astaxanthin or canthaxanthin. Values are expressed as means +/- SEM (n = 5 livers for astaxanthin, n = 4 livers for canthaxanthin). Steady decline to steady state was observed in livers perfused with astaxanthin, but not with canthaxanthin.
Fig. 4.12. Mean cumulative uptake and flow profiles of isolated perfused livers of rainbow trout perfused simultaneously with astaxanthin (1.66 μg ml⁻¹) and canthaxanthin (1.56 μg ml⁻¹), and supplied as autologous rainbow trout serum as the source of astaxanthin and canthaxanthin. Values are expressed as means +/- SEM (n = 2 livers). No selective uptake of either carotenoid was observed.
Fig. 4.13. Mean hepatic extraction ratio profiles of isolated perfused livers of rainbow trout perfused simultaneously with astaxanthin (1.66 μg ml⁻¹) and canthaxanthin (1.56 μg ml⁻¹), and supplied as autologous rainbow trout serum as the source of astaxanthin and canthaxanthin. Values are expressed as means +/- SEM (n = 2 livers). No appreciable change in the hepatic extraction ratio over the course of the perfusions was noted.
Table 4.8. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin or canthaxanthin or both carotenoids simultaneously and derived from Atlantic salmon serum, or from synthetic beadlets (Carophyll® Pink or Carophyll® Red: astaxanthin and canthaxanthin, respectively). Values are expressed as means ± SEM. (n = 4 livers per treatment – salmon serum perfusions; n = 5 livers per treatment – beadlet perfusions).

<table>
<thead>
<tr>
<th>Carotenoid/source</th>
<th>Perfusate concentration (µg ml⁻¹)</th>
<th>Mean initial uptake rate (µg g⁻¹ hr⁻¹)</th>
<th>Mean final uptake rate (µg g⁻¹ hr⁻¹)</th>
<th>Mean hepatic Extraction ratio</th>
<th>Mean hepatic clearance rate (ml hr⁻¹)</th>
<th>Mean metabolic Rate (µg hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin (serum)</td>
<td>1.31±0.01</td>
<td>0.738±0.177</td>
<td>0.193±0.038</td>
<td>0.048±0.006</td>
<td>0.760±0.104</td>
<td>0.993±0.130</td>
</tr>
<tr>
<td>Astaxanthin (beadlet)</td>
<td>2.17±0.03</td>
<td>1.177±0.364</td>
<td>1.187±0.447</td>
<td>0.109±0.036</td>
<td>2.026±0.681</td>
<td>4.401±1.491</td>
</tr>
<tr>
<td>Canthaxanthin (serum)</td>
<td>1.31±0.00</td>
<td>0.649±0.112</td>
<td>0.209±0.051</td>
<td>0.044±0.012</td>
<td>0.646±0.179</td>
<td>1.122±0.235</td>
</tr>
<tr>
<td>Canthaxanthin (beadlet)</td>
<td>1.95±0.03</td>
<td>0.285±0.109</td>
<td>0.111±0.087</td>
<td>0.020±0.009</td>
<td>0.552±0.247</td>
<td>1.095±0.490</td>
</tr>
<tr>
<td>Astaxanthin (mixed)</td>
<td>0.48±0.00</td>
<td>0.221±0.040</td>
<td>0.048±0.017</td>
<td>0.037±0.005</td>
<td>0.492±0.056</td>
<td>0.238±0.028</td>
</tr>
<tr>
<td>Canthaxanthin (mixed)</td>
<td>0.73±0.01</td>
<td>0.381±0.046</td>
<td>0.103±0.029</td>
<td>0.044±0.007</td>
<td>0.612±0.150</td>
<td>0.443±0.110</td>
</tr>
</tbody>
</table>

*Indicates significant differences between initial and final mean uptake rates within a treatment (P<0.05).

Table 4.9. Mean carotenoid uptake rates, hepatic extraction ratios, clearance rates, and metabolic rates of isolated rainbow trout livers perfused with astaxanthin alone or in combination with 5 mM EDTA. Values are expressed as means ± SEM. (n = 5 livers astaxanthin alone; n = 6 livers astaxanthin with 5 mM EDTA).

<table>
<thead>
<tr>
<th>Carotenoid/source</th>
<th>Perfusate concentration (µg ml⁻¹)</th>
<th>Mean initial uptake rate (µg g⁻¹ hr⁻¹)</th>
<th>Mean final uptake rate (µg g⁻¹ hr⁻¹)</th>
<th>Mean hepatic Extraction ratio</th>
<th>Mean hepatic clearance rate (ml hr⁻¹)</th>
<th>Mean metabolic Rate (µg hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin + 5 mM EDTA</td>
<td>8.60±0.06</td>
<td>4.436±0.894</td>
<td>7.057±1.200</td>
<td>0.136±0.023</td>
<td>2.169±0.337</td>
<td>18.734±3.024</td>
</tr>
<tr>
<td>Astaxanthin control</td>
<td>8.59±0.19</td>
<td>3.500±0.214</td>
<td>2.223±0.606²</td>
<td>0.058±0.005*</td>
<td>0.947±0.045*</td>
<td>8.157±0.511*</td>
</tr>
</tbody>
</table>

*Indicates significant differences between values within a column (P<0.05).
4.3.5 Atlantic salmon serum perfusions

Results from comparisons of the various parameters between livers perfused with Atlantic salmon serum did not reveal any significant differences between canthaxanthin and astaxanthin. (Table 4.8). However, livers perfused with salmon serum showed significant differences (P<0.05) between mean initial and mean final uptake rates for both astaxanthin and canthaxanthin in livers perfused with salmon serum (Table 4.8), suggesting that the uptake mechanism is saturable. These results are confirmed by the non-linear mean cumulative uptake profiles of both carotenoids when perfused separately (Fig. 4.14), as well as by their respective mean hepatic extraction ratio profiles (Fig. 4.15), which show a declining trend in the mean hepatic extraction ratio over the course of the perfusion. No significant differences were observed in comparisons between any of the parameters measured when perfused with serum or beadlets as the source of either carotenoid (Table 4.8), which is similar to results observed for livers perfused with trout serum. However, comparisons between the hepatosomatic indices of livers perfused with a beadlet source of astaxanthin were significantly lower than those of livers perfused with salmon serum (P<0.05). Despite this, no significant differences in the normalized flow rates (by organ weight) were observed.

Comparisons between individual carotenoid uptake parameters in livers perfused simultaneously with both astaxanthin and canthaxanthin derived from salmon serum showed no significant differences (Table 4.8), suggesting similar uptake potential for both carotenoids. However, the cumulative uptake profiles of these carotenoids in simultaneous astaxanthin-canthaxanthin perfusions (Fig. 4.16), appear to show that astaxanthin uptake becomes saturated more quickly than does canthaxanthin, despite the lower perfusate astaxanthin concentration (Table 4.8). However, comparisons of normalized percentage increase (approx. 460%) in cumulative uptakes between initial and final values were not significantly different between these carotenoids, and suggests that the liver does not
Fig. 4.14. Mean cumulative uptake and flow profiles of isolated perfused rainbow trout livers perfused with Atlantic salmon serum as the source for astaxanthin (1.31 μg ml⁻¹) or canthaxanthin (1.31 μg ml⁻¹). Values are expressed as means +/- SEM (n = 4 livers). No selective uptake between carotenoids was observed.
Fig. 4.15. Mean hepatic extraction ratio profiles of isolated perfused rainbow trout livers perfused with Atlantic salmon serum as the source for astaxanthin (1.31 µg ml⁻¹) or canthaxanthin (1.31 µg ml⁻¹). Values are expressed as means +/- SEM (n = 4 livers). Profiles suggest a decline to a steady state in the hepatic extraction ratio.
Fig. 4.16. Mean cumulative uptake and flow profiles of isolated rainbow trout livers perfused simultaneously with both astaxanthin (0.48 μg ml⁻¹) and canthaxanthin (0.73 μg ml⁻¹) using Atlantic salmon serum as the carotnoid source. Values are expressed as means +/- SEM (n = 4 livers). Astaxanthin uptake appears to saturate earlier than does canthaxanthin.
Fig. 4.17. Mean hepatic extraction ratio profiles of isolated rainbow trout livers perfused simultaneously with both astaxanthin (0.48 μg ml⁻¹) and canthaxanthin (0.73 μg ml⁻¹) using Atlantic salmon serum as the carotenoid source. Values are expressed as means +/- SEM (n = 4 livers). Profiles show a decline in the hepatic extraction ratio to a steady state.
preferentially accumulate either carotenoid. This is confirmed by the mean hepatic extraction ratio profiles of astaxanthin and canthaxanthin when perfused simultaneously (Fig. 4.17), where both showed similar saturable profiles over the course of the perfusion. This implies that the saturation process is a function of the uptake mechanism and not due to the perfusate carotenoid concentration.

4.3.6 EDTA perfusions

Livers were perfused with 5 mM EDTA as a chelating agent for calcium ions (Ca<sup>2+</sup>), in an attempt to reduce active uptake and thus to identify whether passive diffusion was responsible for carotenoid uptake in the beadlet model. Comparisons in relation to control livers did not show significant differences in the mean initial uptake rates, but did show a significant difference between mean final uptake rates between these two perfusion models (Table 4.9), where livers perfused with 5 mM EDTA had significantly higher mean final uptake rate relative to control livers. This is likely due to the binding of extracellular calcium, which may have increased the volume of distribution of carotenoid, and thus liver uptake. In spite of this, the cumulative uptake profile of livers perfused with 5 mM EDTA show a similar linear response to control livers (Fig. 4.18). However, the significantly higher flow rates in control livers (Table 4.4) may account for any observed differences, as flow rate can potentially affect extraction ratios (Shargel and Yu 1999). Mean extraction ratios were significantly different between test and control livers (Table 4.9), and while potentially due to flow rate, do not show the same pattern as those of control livers. Instead of a steady-state as was observed in control livers, livers perfused with 5 mM EDTA show a steady increase in the hepatic extraction ratio profile (Fig. 4.19), and is more likely to be due to an increase in the volume of distribution as well as increased diffusion into more porous cells within the liver.
Fig. 4.18. Mean cumulative uptake and flow profile of isolated rainbow trout livers perfused with astaxanthin (8.60 μg ml−1) derived from Carophyll Pink and 5 mM EDTA. Values are expressed as means +/- SEM (n = 6 livers).
Fig. 4.19  Mean hepatic extraction ratio profile of isolated rainbow trout livers perfused with astaxanthin (8.60 μg ml⁻¹) derived from Carophyll Pink and 5 mM EDTA. Values are expressed as means +/- SEM (n = 6 livers).
4.3.7 Summary

The major findings in the present experiments show:

- Astaxanthin uptake is faster than canthaxanthin at low doses.
- Uptake parameters show a dose-response effect with perfusate concentration.
- Carotenoid uptake from trout or salmon serum appears saturable and occurs earlier with astaxanthin than with canthaxanthin.
- Carotenoid uptake occurs by a non-selective mechanism.

4.4 Discussion

4.4.1 Rationale and assessment of liver perfusion model viability

The following criteria were used to assess hepatic perfusion viability:

- No leakage from sites other than collection point,
- Uniformity of perfusion and flow,
- Consistent/stable LDH activity throughout perfusion.

The more routine and preferred markers for liver viability for experienced experimenters are the gross appearance of the liver, smoothness of the operative procedure and the stability of liver weight (should remain as a constant percentage of total body weight at the end of the perfusion experiment relative to normal in vivo conditions; Pang 1984). In the case of the present rainbow trout liver perfusions, this range was maintained between 0.8-1.8 % body weight from rainbow trout, and is consistent with the ratio observed in previous experiments (Chapter 3).

In addition, assessment of liver function should be performed to establish good viability and functionality of the liver prior to utilization of the technique in drug metabolism and drug toxicity studies, and include biochemical tests (e.g. LDH, SGOT) in hepatic venous
blood and histological examination of ultrastructure by light and electron microscopy (Pang 1984). Despite the modifications to the perfusate in the present investigations, viability, structure, and thus normal function, did not appear to be negatively impacted.

The rate of bile production has often been used as an indicator of liver viability, but is no longer an acceptable criterion to assess metabolic function of the liver as bile flow fluctuates with perfusion flow rate, temperature, the presence of bile acids (which promote bile flow), and gross deteriorations of the liver often precedes cessation of bile flow (Pang 1984). Because of these considerations, and the low rate of bile production, bile flow was not determined in the present investigations.

While hepatocyte cultures and liver slice methods have been widely used in the past as in vitro techniques for metabolic studies, neither can simulate the function of the whole intact organ, and as such may not adequately reflect the true metabolic potential of the organ. For these reasons the present investigations utilized the isolated liver perfusion system as a model that closely mimics in vivo conditions. The development of a suitable method and model for investigations into hepatic carotenoid uptake requires the validation of the methodology used in the series of experiments.

Perfusion medium varies from laboratory to laboratory, but the common features entail an oxygen carrier such as erythrocytes and an oncotic agent (in the form of protein or a plasma expander such as dextran) which aids in exerting a hydrostatic pressure to prevent perfusate water from entering cells. The fortification with glucose and nutrients aids in functional intermediary metabolism, and thus do not deplete liver stores (Pang 1984).

It has been proposed by Campbell et al. (1999), that improvements in the longevity of a perfused head technique in rainbow trout was achieved through the use of tissue culture
medium (L-15 medium) as a perfusate. These authors suggest that the increased longevity may be due to the perfusate having similar haemodynamic properties to that of blood (Bornancin et al. 1985; Perry et al. 1985). In addition, according to Campbell et al. (1999), the L-15 perfusate, which contains important sources of ‘high energy’ phosphates and antioxidants, aided in the improvement of viability, compounds that were previously absent from salines (e.g. Bornancin et al. 1985). However, no current literature suggests the use of L-15 for use in trout liver perfusion studies (Hayashi and Ooshiro 1975; Ottolenghi et al. 1985; McKinley and Hazel 1993; Cascales et al. 1997; Brett et al. 1998), although Bell et al. (1986) used a modified Hanks medium, and others have modified salines to include some amino acids (Brett et al. 1998). While the use of Ringer solutions (salines), allow for complete control of the experimental medium, pre-existing cell-culture media could be altered or substituted to supply the required nutrients for metabolic studies, as was done in the present investigations.

Bovine calf serum was employed as the oncotic agent in the current experiment, presented as 50% of the perfusate medium (in order to simulate in vivo concentrations), where the osmolarity was measured at approx. 320 mosm (data not shown). The perfusion medium used in previous trout liver perfusion investigations did not adequately simulate in vivo conditions through the supplementation of serum. This is potentially due to the inconsistent nature of serum from different batches, as well as the fact that most compounds investigated were water soluble in nature and thus do not require a carrier. However, DeKoning and Kaattari (1991) concluded that homologous trout serum was required for optimal trout lymphocyte cell responsiveness in normal culture conditions. While the use of homologous fish serum for use in in vitro liver perfusion studies may closely mimic in vivo conditions, the limited availability of the large volumes of serum required for these experiments, may limit the use of homologous serum. As such, the present investigations utilized a stable and known source of cell-culture grade serum. In addition, the use of 50%
bovine calf serum in the perfusate also provided the organ with lipoproteins to ensure normal function of membranes, as well as to resemble the normal haemodynamic properties of whole blood. With the modified perfusate used, liver preparations were viable and stable over the perfusion period. In addition, the linearity of the cumulative flow rates (without back-pressure build-up) indicate that oedema into tissue did not occur. This was further supported by histological examination of perfused livers relative to controls, and displayed no pathology associated with perfusion (Plate 4.1).

The composition of the perfusion medium strongly influences organ functionality; the absence of red blood cells (and perfusate flow), the absence or presence of proteins, the presence or absence of nutrients and of precursors (Pang 1984). For this reason, data obtained from different laboratories may not be strictly comparable because of differences in the composition of the medium (Pang 1984). As the present investigations are markedly different in both their aims and their preparation (e.g. perfusate composition), further studies performed under similar conditions should be conducted in order to corroborate this data.

A single-pass (flow-through) perfusion circuit was chosen for the present experiments as this design permits the flow of perfusate through the liver only once, thereby eliminating subsequent metabolism and the accumulation of potentially deleterious metabolites. Steady-state conditions are attained readily, at which time the binding processes that influence loss of substrate in addition to eliminatory processes are essentially completed; thus the rate of loss at steady state is attributed to rates of metabolism and excretion (Pang 1984).
4.4.2 Dose-effects of carotenoid perfusate concentration

The carotenoid dose-response uptake studies revealed only minor differences between canthaxanthin and astaxanthin uptake parameters within any of the perfusate doses investigated. The dose or concentration of the substrate in perfusate entering the liver, which influences the degree of saturability of metabolic pathways, is a controlling factor in perfusion experiments (Pang 1984). This concept is confirmed by the present results which show a linear relationship between initial uptake rate and perfusate carotenoid concentration (Fig. 4.7).

The cumulative uptake profiles appear to suggest that astaxanthin uptake becomes saturated faster than does canthaxanthin when perfused at higher doses. The apparent saturation observed in the mean cumulative astaxanthin uptake profile of livers perfused with 32 µg·ml⁻¹ astaxanthin, but not in livers perfused with the highest dose of canthaxanthin, may provide evidence that there is an inherent difference between astaxanthin and canthaxanthin uptake. While the apparent relationships between perfusate carotenoid concentration and the many uptake parameters measured in this study appeared to provide varying relationships, the lack of any significant difference in the overall relationship between astaxanthin and canthaxanthin initial uptake rates and their perfusion concentration (due to large variability within a concentration) suggests that the liver does not preferentially accumulate either carotenoid. As carotenoids are highly lipophilic, their degree of lipophilicity and polarity may impact on their relative uptake rates, and consequently their saturability. This may be due to the individual chemical structures of these keto-carotenoids, and may provide some insight into their absorption, metabolism, and complex biological effects (Britton 1995).
4.4.3 Hepatic extraction ratios

The hepatic extraction ratio is a measure of the liver's ability to remove a particular compound from circulation. In direct contrast to the hepatic extraction ratio profiles for astaxanthin, those for canthaxanthin appear to show an increase in the extraction ratio with increasing perfusate carotenoid concentration. However, no apparent trend is observable within any of the doses examined, thereby not showing any saturation profile as is seen in the astaxanthin profiles. It should be noted however that due to the nature of the analysis, measurement by difference in perfusate carotenoid concentrations pre- and post-hepatically, that observing small differences (less than 10%) at low perfusate carotenoid concentrations is nearing the discrimination level of the detection system used in these investigations (Fig. 2.3), and thus should be interpreted with caution. Because of this, future investigations should use a more sensitive detection system such as radiolabeled carotenoids, which would offer the advantage of isolating and identifying specific metabolites from post-hepatic perfusate samples.

The hepatic extraction ratio profiles of livers perfused with salmon serum show a distinct pattern that was not evident in previous perfusions, save that of livers perfused with 32 μg·ml⁻¹ of synthetic beadlet-derived astaxanthin. These profiles show a distinct decline in the extraction ratio over the course of the perfusion, culminating in a steady-state. The apparent initial rapid rate of removal (as assessed by the hepatic extraction ratio) is potentially due to the time required for the particles to fill the sinusoidal space in the liver as was suggested by Yu et al. (1999), where chylomicron remnant removal in the perfused mouse liver was investigated. These authors observed a similar pattern in mouse livers perfused with bovine serum albumin (BSA), where after 10 min of perfusion, approx. 10-20% of the BSA was removed/pass, and appeared to decline further with time. It is possible that the relatively low extraction ratios observed in the beadlet models may be due to the length of time that livers were stabilized prior to carotenoid treatment. However,
livers perfused with piscine serum (from trout or salmon), did not show significantly different mean hepatic extraction ratios from beadlet-perfused livers at similar concentrations, and as such, the hepatic extraction ratios observed are likely to be a good approximation of the \textit{in vivo} condition.

Despite these minor differences the relative hepatic extraction ratio profiles in perfused livers remained relatively stable, being little affected by the carotenoid, or the concentration with which they were perfused. These observations suggest that liver uptake is less governed by the concentration of the carotenoid, but by the mechanism of uptake, and the chemical characteristics of the individual carotenoids.

4.4.4. Carotenoid uptake interactions
In livers perfused with both astaxanthin and canthaxanthin (beadlet-derived) simultaneously at similar concentrations, no differences in uptake, clearance or metabolic rates were observed between these carotenoids, providing further evidence that there is no preferential uptake of either carotenoid in the isolated perfused liver model.

Mixed astaxanthin-canthaxanthin salmon serum perfused livers showed that the uptake of both carotenoids were saturable. Livers perfused with salmon serum astaxanthin appeared to saturate at a faster rate than livers perfused with serum-derived canthaxanthin despite the lower perfusate astaxanthin concentration. However, when normalizing the final cumulative uptake, as a percentage increase from the initial sample uptake, for the initial perfusate carotenoid concentration, results showed that hepatic uptake for both carotenoids is similar. Thus differences in cumulative uptake in these perfusions are a function of their concentration and not to preferential carotenoid accumulation. This is supported by the hepatic extraction ratio profiles, which show similar patterns and levels for both carotenoids.
4.4.5 Piscine serum perfusion studies

The investigations utilizing trout serum, which contain the naturally occurring carotenoid-carrying lipoprotein fractions (e.g. LDL, HDL, and VLDL), should provide more physiologically relevant information to the \textit{in vivo} condition.

Comparisons between livers perfused individually with either astaxanthin or canthaxanthin derived from trout serum did not show significant differences in the uptake or clearance rates measured. These observations are consistent with the free carotenoid models, and offer support for the contention that the liver does not preferentially accumulate either carotenoid. However, the mean cumulative uptake profiles show that livers perfused with trout serum derived astaxanthin appear to saturate during the course of the perfusions, and that this observation is not shared in livers perfused with canthaxanthin. The large degree of variability, in the cumulative canthaxanthin uptake profile, which is consistent with perfused organ models, may mask any saturable effect, and as such more research is required to determine if true differences exist in carotenoid uptake. Despite the variability, the differences are consistent with the observed cumulative profiles of livers perfused with the free carotenoid models, where astaxanthin appeared to show saturation at high perfusate concentrations, while canthaxanthin did not. These observations may be interpreted to mean that as canthaxanthin uptake saturation occurs much later than does astaxanthin uptake, that much more canthaxanthin is soluble in the liver tissue. In support of these observations, \textit{in vivo} results from mammalian studies revealed that dietary canthaxanthin was readily incorporated into the liver 75-100 times more than dietary astaxanthin in the liver of the rat (Gradellet \textit{et al.} 1996). If this relationship is maintained in trout, then the differences observed in the clearance rates (half-lives) between astaxanthin and canthaxanthin (Guillou \textit{et al.} 1993; Gobantes \textit{et al.} 1997) may be due to hepatic uptake differences between these carotenoids in terms of the time required for saturation to occur.
No differences in uptake parameters were observed in livers perfused simultaneously with both astaxanthin and canthaxanthin derived from trout serum, providing further support for the concept that neither astaxanthin nor canthaxanthin is cleared from perfusates preferentially. However, broad conclusions about interactions between astaxanthin and canthaxanthin in simultaneous perfusion experiments cannot be suggested due to the low level of replication in this study (due to small amount of serum available). Comparisons between sources (serum vs. beadlet) of individual carotenoids perfused at similar concentrations did not show any significant differences in any of the parameters examined, suggesting that the liver does not treat the carotenoids differently, regardless of source/presentation form.

Trout livers perfused with salmon serum as the source of carotenoids showed markedly different patterns of uptake to livers perfused with trout serum. Both sources of sera showed saturable uptake mechanisms, despite the lower carotenoid content of the salmon perfusates when compared to livers perfused with trout serum. This discrepancy may be due to the serum lipoprotein profiles of these two species. Salmon serum may potentially have higher concentrations of ligands, and thus saturate hepatocyte receptor sites, resulting in saturation of carotenoid uptake earlier than in livers perfused with trout serum. Yu et al. (1999) noted that uptake saturation of receptor-mediated endocytosis of the LDL-receptor ligand in perfused mouse livers was related to protein content of the chylomicron remnants in the perfusate. The earlier saturation process observed in the salmon serum perfusions may potentially be due to higher serum protein concentrations relative to trout serum or beadlet perfused livers. However, protein content was not measured and is a variable that should be investigated in future. Despite these observations, no significant differences in initial or cumulative uptake were observed between livers perfused with astaxanthin or canthaxanthin (salmon serum), and these observations are consistent with the previous
models, and solidify the concept that preferential carotenoid accumulation does not take place.

4.4.6 Uptake mechanism

The supplementation of perfusates with 5 mM EDTA (ethylenediaminetetraacetic acid; a cation chelating agent), which should have bound extracellular calcium and thus reduced active uptake, were unsuccessful in clarifying the mechanism of carotenoid uptake. Perfusion of livers with EDTA did not show the expected reduction in hepatic extraction ratio, but instead increased (Fig. 4.19). This is likely due to binding of extracellular calcium, thus increasing paracellular space, as well as increasing the porosity of cell membranes, which ultimately resulted in a higher rate of uptake by the end of the perfusions, as well as an increase in the hepatic extraction ratio. Therefore, perfusions supplemented with 5 mM EDTA were unsuccessful in clarifying the uptake mechanism and future investigations should pursue studies utilizing a specific active uptake inhibitor that will not have an impact on the volume of distribution.

The present results show no inherent differences in the respective mechanism of uptake of astaxanthin or canthaxanthin, nor do they show preferential accumulation. Thus observed differences between the carotenoids is most likely due to their chemical structure. Despite these observations, relating these uptake parameters to in vivo situations will provide a better understanding of the potential role of the liver in carotenoid utilization and thus the remainder of the discussion is devoted to that end.

4.4.6 General discussion of uptake kinetics

The relatively low hepatic extraction ratios observed in both serum-perfused and beadlet-perfused rainbow trout livers (Tables 4.5 – 4.8) offers the first direct evidence of liver potential in carotenoid elimination. In pharmacokinetic modeling, a proportionality
constant that relates the rate of change of the concentration of a chemical within a compartment to the driving force is termed a first-order rate constant \((k, \text{ units of time}^{-1})\) (Barron et al. 1990). The rate constant is defined as the fractional rate of removal of chemical per unit of time, and often expressed as:

\[
X_f = X_f^0 \times e^{k_2 t}
\]

Where \(X_f^0\) is the amount of chemical in the organism at the start of the elimination experiment, and assumes that there is no process that inputs chemical into the animal after the beginning of the experiment (Barron et al. 1990). The elimination half-life \((t_{1/2})\) can be calculated from the elimination rate constant as follows:

\[
t_{1/2} = \frac{\ln 2}{k_2}
\]

where \(\ln 2 = 0.693\) (Barron et al. 1990; Shargel and Yu 1999).

From these equations an estimate of the concentration at any given point in time will be a function of the elimination rate constant \((k_2)\), which is estimated from the number of passes of the total blood volume through the liver in an hour, multiplied by the hepatic extraction ratio. Making certain assumptions, an estimate of the elimination rate constant, as well as the half life of carotenoids, due solely to hepatic elimination, can be made.

The mean blood volume of trout of 4.49 ml 100g\(^{-1}\) of body weight (Gingerich and Pityer 1989), would yield an estimated total blood volume of approx. 13.47 ml for a 300 g fish. In addition, the mean hepatosomatic index from the present studies (approx. 1.45 % body weight) would yield a liver of 4.35 g. Taking into account the hepatic blood flow (4.47 ml
g⁻¹ hr⁻¹; Barron et al. 1987), the total blood flow through the liver is approx. 19.44 ml hr⁻¹, which equates to 1.44 passes hr⁻¹. From these values the hepatic elimination rate constants are 0.060 hr⁻¹ and 0.066 hr⁻¹, for astaxanthin and canthaxanthin, respectively (extraction ratios derived from trout serum, Table 4.7), which translate to half-lives of approximately 12 hr and 10.5 hr, respectively. These times are much shorter than those values obtained in vivo for these carotenoids of 24.1 and 22.2 hours, respectively for astaxanthin and canthaxanthin as observed by Guillou et al. (1993). The relative ratios between these values and those of the present investigation are consistent, offering strong support for the present half-life estimations. The differences are likely due to the fact that the present system assumes that absorption of further carotenoid does not occur over the course of the elimination profile. In addition, in vivo absorption is a continuous process, which may lead to the slower elimination profile observed. In addition, the blood/serum pool in vivo is not isolated from other organs which may release carotenoids back into circulation over a prolonged period. Further to this, the liver receives a dual supply of blood (from the hepatic portal vein, as well as the hepatic artery), and as such the estimated elimination rate constant is likely a slight overestimation than the in vivo condition. Despite these observations, the present results are in line with the hypothesis that the liver plays a major role in carotenoid elimination.

Further calculations can also be estimated from the present data, including an estimation of the reduced bioavailability due to liver absorption upon first-pass, which may vary with hepatic blood flow. From the present studies, where blood flow is maintained at a constant rate of approximately 4.47 ml g⁻¹ hr⁻¹, we may estimate the bioavailability factor $F'$ as follows:

\[
F' = 1 - \frac{Cl_h}{Q}
\]
Where $C_{lh}$ is the hepatic clearance and $Q$ is the blood flow through the liver (Shargel and Yu 1999). Based on data obtained from the isolated trout livers perfused with astaxanthin or canthaxanthin derived from trout serum (Table 4.7), and the blood flow rates determined above, both carotenoids appear to be approximately 95% absorbed systemically after initial liver extraction. However, this fraction will decrease with each subsequent pass through the liver, as well as other organs associated with the removal of carotenoids from circulation.

The results from HepG2 cell culture experiments by Martin et al. (1997) show that approximately 13% of the delivered $\beta$-carotene was accumulated by hepatocytes within 24 hours. This is much lower than the present estimations, which suggest that the liver is capable of removing up to 50% in 24 hours in the perfused liver model. However, differences in the level of serum (lipoprotein) supplementation may account for the differences. In addition the present model, being a single pass, would likely over-estimate the removal of carotenoids as it assumes a linear removal relationship. While the hepatic extraction ratio appears to be stable, the in vivo or recirculating perfusion model would be more appropriate as the clearance response would be an exponential decay (as described above), and as such, may be closer to the in vitro conditions described by Martin et al. (1997). Despite these differences, these authors noted that accumulation of carotenoids in confluent cell cultures was positively correlated to carotenoid concentration in the medium, which is consistent with the observed results in the present investigations.

In principle the uptake rate of carotenoids will only reach a maximum when individual lipoprotein capacity for carotenoid transport is saturated. According to Chavez et al. (1998) the binding capacity of lipoproteins in trout have the potential to be 100 fold higher than what is typically found in vivo, as determined by saturation experiments, reaching levels as high as 80.6, 152.8, 145.7 $\mu$g ml$^{-1}$ serum for VLDL, LDL, and HDL, respectively.
According to Pang (1984), compounds with very low extraction ratios are little affected by flow rate, and thus remain stable. Therefore, carotenoid uptake rates will only be limited by the rate of receptor-mediated endocytosis of lipoprotein molecules. In the present experiments, the hepatic extraction ratio is consistent in the steady-state perfused liver, and thus cumulative uptake will be a dynamic curvilinear process, being primarily determined by perfusate carotenoid concentration. In a closed system, this process will show a similar exponential decay elimination profile to that seen in vivo, in fish fed a single meal (Guillou et al. 1993, Gobantes et al. 1997).

The saturation response observed in trout livers perfused with piscine serum is likely due to the saturation response of the lipoprotein uptake mechanism. Normal liver function is maintained in isolated perfused organs, and Yu et al. (1999) found that receptor-mediated endocytosis of chylomicron remnants in perfused mouse livers was responsible for the observed clearance, as it does in vivo. In addition, according to Romanchik et al. (1995), only a few molecules of each lipoprotein fraction contain any carotenoid (e.g. approximately four carotenoid molecules per 1000 are associated with each VLDL and one with each LDL particle, whereas 25 of every 1000 HDL particles contain carotenoid, which include xanthophylls, lycopene, and β-carotene) in human serum. As such, binding of LDL to the LDL-receptor on hepatocytes, which is known to be a saturable process, will ultimately limit the uptake of carotenoids. Similarly in fish, it is likely that not all lipoprotein molecules will contain carotenoid. Because the in vivo circulatory system is a closed system, hepatic clearance of carotenoids will be a function of serum concentration (as evidenced by the linear regression in the beadlet model), and not due to up-regulation of the uptake mechanism. As the uptake process reaches a steady-state, the carotenoid uptake rate will be dependent upon the hepatic extraction ratio, the endocytic rate, as well as the perfusate carotenoid concentration.
The current data suggest that the initial uptake rate of carotenoids in livers perfused with 2 and 4 μg ml⁻¹ carotenoid, ranges between approximately 0.5 and 3 μg g⁻¹ hr⁻¹. However, trout liver carotenoid concentrations rarely achieve very high concentrations in vivo (Chapter 3), and implies that a large proportion of the absorbed carotenoid pool is either redistributed in released lipoprotein molecules, or metabolized. Current literature suggests that metabolism of absorbed carotenoids by the liver is likely the largest source of loss. In a recent study by Wolz et al. (1999), over 50% of the delivered astaxanthin was metabolized to glucuronide conjugates within 24 hours in rat hepatocytes. This rate of metabolism appears to correlate well with the present data which suggests that the extraction ratio (approx. 0.025 at the steady-state condition in serum models, Figs. 4.11 and 4.15) would result in approximately 50% uptake in 24 hours. In humans, LDL fractions circulate for 2-3 days before they are cleared from the liver, whereas HDL circulate for about 5-6 days (Chopra and Thurnham 1999). In addition, the in vivo half life of carotenoids (24.1 and 22.2 hours, for astaxanthin and canthaxanthin, respectively (Guillou et al. 1993)), also appear to correlate well with the observed rate of lipoprotein clearance where approx. 50% would be cleared in 24 hours. These observations are consistent with results reported by Gjøen and Berg (1992) who found that LDL are cleared with a half-life of about 30 hours in vivo and are mainly metabolized by the liver in rainbow trout.

As HDL are longer lived particles (5-6 d) (Chopra and Thurnham 1999), the resulting removal of these molecules from the circulation would thus result in a longer time prior to complete carotenoid clearance from serum (assuming no transfer of carotenoids between lipoprotein moieties). Combined with the data that only 25 of every 1000 particles of HDL contain carotenoid (Romanchik et al. 1995), it follows that total clearance would take longer than the half-lives estimated here for an open system. It should also be noted that
the present investigations are single pass experiments where perfusate carotenoid concentrations are constant, and thus do not reflect the in vivo recirculating blood system.

The observed similarities in the measured parameters between the two models used, namely beadlet-derived and piscine serum-derived carotenoid models may be due to the potential association of the “free” carotenoids with lipoproteins in the bovine calf serum. Anecdotal evidence by Jyonouchi et al. (1995) suggests that carotenoids (astaxanthin and β-carotene) bind to lipoproteins in tissue culture medium supplemented with 100 ml·1⁻¹ FCS.

The present results indicate that the uptake mechanism is not selective for either astaxanthin or canthaxanthin as assessed by their similar mean hepatic extraction ratios. However astaxanthin uptake appears to saturate earlier than does canthaxanthin, which could explain the more efficient utilization of astaxanthin over canthaxanthin in rainbow trout as observed by several authors (Torrissen 1986, 1989; Foss et al. 1987; Storebakken et al. 1987). However, the lack of any significant differences in the uptake parameters measured at any concentration, would suggest that this may play only a small role in the differences in carotenoid utilization. One possibility to explain these differences may lie in the hepatic metabolism of these carotenoids, and is the subject of future experiments.

In conclusion, the present results offer the first reported evidence for the potential role of the liver in carotenoid utilization and confirm that the liver plays a significant role in carotenoid clearance, although a direct relationship to its potential in metabolism could not be established. Uptake of both astaxanthin and canthaxanthin appear to increase in a dose-response manner with increasing perfusate carotenoid concentration. Piscine serum perfusions demonstrate that the carotenoid uptake mechanism is saturable, and non-selective for either astaxanthin or canthaxanthin. The present data appear to correlate well
with previous results in terms of the half-life of both canthaxanthin and astaxanthin observed \emph{in vivo}, and strengthen the potential of the isolated perfused liver to be used as model tool for the investigation of liver metabolism of carotenoids.
CHAPTER 5.

THE EFFECTS OF ASTAXANTHIN AND CANTHAXANTHIN ON LIVER XENOBIOTIC METABOLIZING ENZYMES IN RAINBOW TROUT (ONCORHYNCHUS MYKISS WALBAUM).

5.1 Introduction

Studies in fish indicate that the absorption of lipophilic toxicants is closely linked to and enhanced by dietary fat digestion and absorption (Vetter et al. 1985; Van Veld et al. 1987). Following absorption, the distribution and retention of organic toxicants depends largely on their susceptibility to biotransformation by various drug-metabolizing enzyme systems (Van Veld 1990). The purpose of these enzymes, which include oxidative and conjugative enzyme systems, are to metabolize xenobiotics into more water-soluble and less toxic metabolites, and are primarily catalyzed by the cytochrome P450 mediated monooxygenase system, located in the endoplasmic reticulum (microsomes) of many tissues (Stegeman and Kloepper-Sams 1987; Åström and DePierre 1986). The liver is generally the organ with the highest concentration and highest amount of cytochrome P450 (Armstrong 1987), and is therefore the major site of biotransformation and excretion of xenobiotic compounds (Meyers and Hendricks 1985).

Important dietary antioxidants include tocopherols and tocotrienols (vitamin E), ascorbic acid (vitamin C), carotenoids, and flavonoids, as well as synthetic food additives (i.e., butylated hydroxytoluene) (Kubow 1998). Antioxidants inhibit oxidation by either acting as preventative inhibitors, which retard the initiation phase, or by acting as free radical change-breaking antioxidants, which effectively remove free radicals (Kubow 1998). While many functions of carotenoids have been noted in the literature, few authors have reported their metabolic conversions, including their rates of catabolism and their pathways in salmonid fish.
The conversion of carotenoids to vitamin A in salmonids has been reported to take place in the intestinal wall (Schiedt et al. 1985; Al-Khalifa and Simpson 1988), which suggests that astaxanthin must be given in the feed to function as a provitamin A. However, Schiedt et al. (1985) concluded that this conversion is quantitatively insignificant, and thus inadequate to account for the discrepancy between the absorbed level of carotenoids and those retained in tissues. Therefore, an alternate metabolic pathway must be present to account for the observed discrepancy in carotenoid absorption and retention observed by No and Storebakken (1991).

The cytochrome P450 monooxygenase (phase I) and conjugating enzyme (phase II) systems in fish are known to participate in the biotransformation of a variety of organic xenobiotics and endogenous compounds (Chambers and Yarbrough 1976; Hansson et al. 1978). The mixed function oxidase system (MFO) carries out a series of oxidation reactions which convert nonpolar lipid-soluble compounds into more polar metabolites, which can be readily excreted and detoxified (Stegeman and Hahn 1994). Phase II enzymes conjugate these metabolites with an endogenous compound (primarily by transferases) to form water soluble derivatives which are easily excreted with bile and urine (Vigano et al. 1993). Both enzyme systems are inducible by dietary ingredients. Mulder and Van Doorn (1975) reported that high lipid solubility is a prerequisite for a high rate of glucuronidation. From the toxicokinetic viewpoint, induction of the biotransformation enzymes should result in a faster rate of elimination and decreased toxicity and bioaccumulation (Förlin et al. 1986).

Blom and Förlin (1997) note that in fish, organic lipophilic compounds need to be detoxified and biotransformed to more hydrophilic forms prior to excretion. Both
canthaxanthin and astaxanthin, which are known lipophilic compounds, must therefore be metabolized prior to excretion.

Although the liver is considered the major organ for the detoxification of lipophilic xenobiotics in fish, the kidney also plays an important role in detoxification (Pesonen et al. 1987). High cytochrome P450 activities seem to coincide with the tissues where the majority of astaxanthin metabolism is thought to take place, i.e. in the liver and kidney. For example, high levels of recovered radioactivity are found in these tissues of fish fed radiolabeled carotenoid (Torrissen and Ingebrigsten 1992; Hardy et al. 1990). In addition, due to the fact that astaxanthin induces xenobiotic metabolizing enzymes in rats and mice (Gradelet et al. 1996; Astorg et al. 1994, 1997; Jewell and O'Brien 1999), it is possible that these enzymes may be indirectly responsible for a portion of the metabolism or degradation of these pigments. However no studies have been published on cytochrome P450 isoenzyme induction in salmonids relative to dietary carotenoid intake.

Markers of the P450 phase I xenobiotic metabolizing isoenzymes that are important in metabolism have been measured in liver microsomes, including ethoxyresorufin O-deethylation (EROD), a marker of P4501A1; methoxyresorufin O-demethylation (MROD), a marker of P4501A2; pentoxyresorufin O-dealkylation (PROD), a marker of P4502B1/2; and benzoxyresorufin O-dearylase (BROD) an unspecific activity catalyzed by P4501A, 2B and 3A (Gradelet et al. 1996). Jewell and O'Brien (1999) found that both canthaxanthin and astaxanthin significantly increased EROD and MROD activities in the liver, lungs and kidneys of the rat. Induction of drug metabolizing enzymes in extrahepatic tissues, including the kidney, is of importance as the kidneys are the primary route of excretion of xenobiotics from the body (Jewell and O'Brien 1999). In addition, Gradelet et al. (1996) and Astorg et al. (1994; 1997) also observed a significantly higher specific activity of liver xenobiotic-metabolizing enzymes in rats and mice fed astaxanthin or canthaxanthin.
Accordingly, it seems possible that a potential function of astaxanthin and canthaxanthin is to act as membrane antioxidants, protecting lipids from cytochrome P450 peroxidation, resulting in the partial catabolism of these carotenoids from this phase II enzyme system. Therefore, this study was aimed at determining whether dietary carotenoids induce the cytochrome P450 enzyme system in a model salmonid, the rainbow trout. In addition, the study was aimed at identifying differences between the inductive capacities of astaxanthin and canthaxanthin, and whether the relative differences between carotenoid retentions in rainbow trout are due to preferential metabolism of canthaxanthin over astaxanthin.

5.2 Materials and Methods

5.2.1 Diet Preparation

Experimental diets were prepared according to the top-coating method described in Chapter 2.6.2. Diets were formulated to contain 100 mg kg\(^{-1}\) of carotenoid (astaxanthin (Ax), canthaxanthin (Cx)), Control (0 mg kg\(^{-1}\) (Co); the carrier medium of the commercial astaxanthin and canthaxanthin preparations) or β-naphthoflavone (β-NF).

5.2.2 Diet analysis

Proximate and carotenoid analysis of prepared feeds was determined according to methods described in Chapter 2.7 and 2.8, and results are presented in Tables 5.1 and 5.2.
Table 5.1. Proximate analysis of experimental diets* used in a pilot study.

<table>
<thead>
<tr>
<th>Proximate analysis</th>
<th>Co</th>
<th>Ax</th>
<th>Cx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>48.48 ± 0.35</td>
<td>48.60 ± 1.41</td>
<td>44.92 ± 0.98</td>
</tr>
<tr>
<td>Crude Lipid (%)</td>
<td>27.87 ± 0.84</td>
<td>27.03 ± 0.34</td>
<td>27.24 ± 0.18</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>9.92 ± 0.01</td>
<td>9.96 ± 0.04</td>
<td>9.80 ± 0.17</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>3.67 ± 0.26</td>
<td>4.31 ± 0.03</td>
<td>4.99 ± 0.04</td>
</tr>
<tr>
<td>Ax (mg·kg⁻¹)</td>
<td>N.D.</td>
<td>95.2 ± 2.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cx (mg·kg⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>98.8 ± 0.5</td>
</tr>
</tbody>
</table>

* Basal diet formulation common to all experimental diets (61.3% LT fish meal (Norse LT94, Ergersund, Norway), 21.6% wheat, 5.6% wheat gluten, 10% fish oil, 0.8% vitamin premix, and 0.7% mineral Premix).

Co, control; Ax, astaxanthin; Cx, canthaxanthin.
N.D. indicates not detectable.

Table 5.2. Proximate analysis of experimental diets* used in a replicated experiment.

<table>
<thead>
<tr>
<th>Proximate analysis</th>
<th>Co</th>
<th>Ax</th>
<th>Cx</th>
<th>β-NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>45.00±0.31</td>
<td>45.23±0.45</td>
<td>46.25±0.66</td>
<td>44.87±0.32</td>
</tr>
<tr>
<td>Crude Lipid (%)</td>
<td>30.53±0.18</td>
<td>30.55±0.43</td>
<td>30.77±0.26</td>
<td>30.33±0.45</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>9.81±0.02</td>
<td>9.84±0.03</td>
<td>9.92±0.09</td>
<td>9.98±0.03</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>6.25±0.09</td>
<td>5.68±0.36</td>
<td>6.53±0.10</td>
<td>6.33±0.27</td>
</tr>
<tr>
<td>Ax (mg·kg⁻¹)</td>
<td>N.D.</td>
<td>84.4±1.0</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cx (mg·kg⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>90.8 ± 1.2</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Basal diet formulation common to all experimental diets (61.3% LT fish meal (Norse LT94, Ergersund, Norway), 21.6% wheat, 5.6% wheat gluten, 10% fish oil, 0.8% vitamin premix, and 0.7% mineral Premix).

Co, control; Ax, astaxanthin; Cx, canthaxanthin; β-NF, β-naphthoflavone.
N.D. indicates not detectable.
5.2.3 Fish and experimental conditions

A pilot study was initiated to develop the methodologies to be used as well as to investigate the effects of dietary carotenoid supplementation on cytochrome P450 MFO enzymes and liver histology. Single tanks for each diet contained 40 fish each (all-female rainbow trout obtained from Mill Leat Trout Farm, Devon, U.K.) with an initial starting weight of approx. 140 g ± 16 g, and fed a ration of 1.0% total body weight per day for 28 days. Fish were maintained in the aquarium facilities at the University of Plymouth as described in Chapter 2.2 for 4 weeks from September to October, 2000.

The replicated experimental study used a negative control (Co) and positive control (using β-naphthoflavone (β-NF) as a classical inducer of liver xenobiotic-metabolizing enzymes) treatments in addition to carotenoid treatments (astaxanthin (Ax), canthaxanthin (Cx)). Twelve fish each (mixed-sex trout obtained from Hatchlands Trout Farm, Devon, U.K.), with an initial starting weight of 260 g, were allocated to three replicates for each of four individual treatments (Ax, Cx, Co, β-NF) and maintained in 400 l fibreglass tanks in a recirculating system at 16 ± 1 °C for two weeks prior to the start of the experiment to allow for acclimation to culture conditions. For the experiment, fish were fed a ration of 1.2% BW per day for 21 days prior to tissue sampling. The experiment ran for three weeks in February 2001.

5.2.4 Sampling

Fish were euthanized with phenoxyethanol followed by a blow to the head. Fish were immediately weighed and blood samples collected from the caudal vein using non-heparinized syringes (2.5 ml) with 23 gauge needles (Becton Dickinson) and allowed to clot overnight at 4°C. Fish that showed no sign of growth (emaciation) were removed from the trial as these fish did not feed.
Liver and kidney samples were carefully dissected, removed, blotted dry, and weighed. Livers were sectioned into two subsamples, one for histological analysis and the other for enzymatic determinations. Tissue samples (liver and kidney) were pooled in both the pilot (3-4 fish pool\(^{-1}\); 10 pools tank\(^{-1}\)) and main study (2-3 fish pool\(^{-1}\); 4 pools tank\(^{-1}\)). Liver and kidney samples for enzymatic determinations were immediately frozen in liquid nitrogen and stored at \(-20^\circ\text{C}\) until microsomal fractions were analysed for cytochrome P450, glutathione S-transferase (GST) and UDP glucuronosyl transferase (UDPGT) enzyme activities.

Since it is known that xenobiotics are capable of inducing liver enlargement in fish (Poels \textit{et al.} 1980), the liver somatic index was determined, in addition to the kidney somatic index, according to the equation in Chapter 2.5.

Serum was extracted and analyzed for carotenoid content as described in general materials and methods in Chapter 2.9.1. Serum was used as an indirect measure of tissue exposure to carotenoid.

5.2.5 Cytochrome P450 isozyme activity in microsomal fractions

5.2.5.1 Preparation of microsomes

Liver and kidney microsomal fractions were prepared by a modification of the calcium chloride precipitation method of Gibson and Skett (1999). Frozen tissue samples were weighed and a 20% w/v tissue homogenate in ice-cold 0.25 M sucrose buffer (pH 7.4) was made using an X620 Status homogenizer (Philip Harris Scientific, GB-Lichfield, Germany) (20s at 9500 rpm). Homogenates were centrifuged at 12, 500 \(\times\) g (4 °C) for 15 minutes in a KR22i Jouan (Jouan SA, Saint-Herblain, France) centrifuge using a TX 121.1 rotor. The resulting supernatant was decanted and to each 1 ml aliquot of supernatant, 0.1
ml of an 88 mM CaCl₂ solution was added and swirled occasionally for 5 minutes to precipitate the calcium dependent aggregated endoplasmic reticulum microsomal particles. This mixture was then centrifuged at 27,000 X g (4 °C) for 15 minutes and the supernatant discarded, being careful not to disturb the pellet. The pellet was then resuspended in approximately 5-7.5 ml of ice-cold 20% (v/v), 50 mM Tris-0.25 M sucrose buffer pH 7.4. The microsomal fraction, after thorough mixing was aliquoted into appropriate volumes for duplicate analyses of phase I and phase II xenobiotic metabolizing enzyme activities (EROD, MROD, PROD, BROD, GST, and UDPGT) and stored frozen at -80 °C until enzymatic analysis.

5.2.5.2 Dual protein and P450 isoenzyme assays
The simultaneous determination of protein and phase I xenobiotic-metabolizing enzyme activities were performed according to the methods of Lagueux et al. (1997) with minor modification. To each well of a 96 well microplate (Corning), 100 µl of 50 mM sodium orthophosphate (pH 7.4) buffer was added. For the protein standard curve, bovine serum albumin (BSA) working standards were prepared using a stock BSA solution (prepared in 0.9% (w/v) saline) which was serially diluted with 0.9% (w/v) saline to obtain concentrations of 0, 0.25, 0.5, 1.0, 2.0, 5, 10, 25, 50, 75, 100 or 125 µg/25 µl (representative standard curve shown in Fig. X.1). Duplicate standard curves were prepared by pipetting a fixed volume (i.e. 25 µl). Similarly, the resorufin standard curve was prepared by adding 10 µl of resorufin working standards (1mM stock resorufin solution (in DMSO) was serially diluted with 50 mM sodium orthophosphate (pH 7.4) to obtain final concentrations of 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 200 or 300 pmoles/10 µl), using a fixed volume (i.e. 10 µl)(representative standard curve shown in Fig. X.2). Fixed volumes were adopted to reduce errors in standard curves due to pipetting error. Both standards and samples were analyzed in duplicate on a single plate.
For the dual protein-P450 isozyme assay, 35 μl of each microsomal protein suspension (liver or kidney) were added, followed by 25 μl of 7 mM NADPH to each well, including those of combined standard curve and the plate incubated for 2 min. The enzymatic reaction was initiated with 10 μl well⁻¹ (all wells) of the substrate (e.g. ethoxyresorufin (final concentration: 10 μM)), plates shaken and the incubation allowed to proceed for 30 min at room temperature (ca. 20 °C). The reaction was stopped by adding 50 μl well⁻¹ of acetonitrile containing 300 μg ml⁻¹ of the protein binding fluorochrome, fluorescamine.

After 15 min incubation with the fluorescent dye (to allow for protein binding), the resulting fluorescence was read with an excitation filter 395/25 nm and emission filter 460/40 nm for proteins, and with an Ex 530/25 nm and Em 590/35 nm, for isoenzyme activity on a Cytofluor II (PerSeptive Biosystems GmbH, Freiburg, Germany) spectrofluorometer.

All substrates (ethoxyresorufin; methoxyresorufin; benzoxyresorufin; pentoxyresorufin; Molecular Probes, Leiden, The Netherlands) were dissolved in dimethyl sulfoxide, supported by ultrasonic dispersion, to yield stock solutions of 170 μM. Resorufin (Molecular Probes, Leiden, The Netherlands) for working standards was first dissolved in dimethylformamide, supported by ultrasonic dispersion, to yield a 1 mM stock solution.

Specific activities for each isoenzyme were determined from their respective standard curve fluoresences for both protein (fluorescamine) and isoenzyme (resorufin).

5.2.5.3 Assay for Glucuronosyl Transferase activity

The phase II conjugating enzyme activity of glucuronosyl transferase (which conjugates the hydroxyl, carboxyl or amino groups of a substrate with glucuronic acid) were determined according to the method of Gibson and Skett (1999). The method uses 2-
aminophenol as it readily forms an \( O \)-linked glucuronide conjugate in the presence of microsomal fractions and UDP-glucuronic acid. The assay procedure is based on the colorimetric diazotization method for free primary amino groups, whereby a brightly coloured azo compound (2-aminophenyl glucuronide) is produced and spectrophotometrically analyzed.

A glucuronosyl transferase enzyme cofactor solution was used (Gibson and Skett 1999), containing:

- 0.1 M Tris buffer pH 8.0, 8 ml
- 0.15 M MgCl\(_2\) (hexahydrate), 1 ml
- 1% (w/v) Triton X-100, 0.5 ml
- 0.02 M ascorbic acid, 1 ml
- UDP-glucuronic acid, 10 mg.

One ml of cofactor solution was mixed with 0.5 ml of 1 mM 2-aminophenol and the reaction initiated with 0.5 ml microsomal protein (diluted 1:10 (v/v) with microsomal resuspension buffer) in a shaking water bath. A blank was prepared by adding 0.5 ml H\(_2\)O instead of 2-aminophenol. Reactions were incubated for 30 min, and terminated with the addition of 1 ml of ice-cold 20% trichloroacetic acid in 0.1 M phosphate buffer (pH 7.2), and allowed to stand on ice for 5 min. The supernatant was clarified by centrifugation at 13,000 rpm in a microcentrifuge (MSE, U.K.). To 1 ml of supernatant, 0.5 ml (fresh) 0.1% sodium nitrite was added and thoroughly mixed by vortexing for 5 s and allowed to stand for 2 min. To the solution, 0.5 ml, 0.5% ammonium sulfamate was added and again mixed and allowed to stand for 3 min. Finally, 0.5 ml, 0.1% N-naphthylethylene diamine was added and the solution, mixed and allowed to stand at room temperature in the dark for 60 min. The absorbance of the final solution was read at 540 nm against the substrate blank.
Values for absorbance were then calculated against an aniline standard curve prepared as follows:

A stock solution of 0.1 mM aniline in 6% trichloroacetic acid was made and a linear dilution series of 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of 0.1 ml aniline stock solution and made up to a final volume of 1 ml with appropriate volumes of 6% trichloroacetic acid in 0.1 M phosphate buffer, pH 2.7, was prepared. To each tube, 0.5 ml (fresh) 0.1% sodium nitrite was added and the tubes thoroughly mixed and allowed to stand for 2 min. The final steps are as those for the samples starting from the addition of 0.5 ml of 0.5% ammonium sulfamate. Absorbances were read at 540 nm against the substrate blank and the standard curve prepared by plotting the absorbance values at 540 nm against the aniline concentration.

Specific activities were calculated based on their absorbance relative to the standard curve and corrected for protein content.

5.2.5.4 Assay for glutathione-S-transferase activity

Glutathione S-transferases are a family of isoenzymes that catalyze the conjugation of glutathione with electrophilic xenobiotics or their metabolites. The determination of the specific activity was measured by the enzyme catalyzed condensation of glutathione with the model substrate 2,4-dinitro-1-chlorobenzene. The resulting product absorbs light at 340 nm and the extinction coefficient is known to be 9.6 mM$^{-1}$ cm$^{-1}$. The assay procedure was as described by Gibson and Skett (1999).

The glutathione-S-transferase activity was determined by the change in absorbance over 1 min (linear) of a sample cuvette relative to a blank. Briefly, to each of two 3 ml spectrophotometer cuvettes, 0.1 ml of 30 mM glutathione, 0.1 ml of 30 mM dinitrochlorobenzene (in ethanol) and 2.2 ml of 100 mM potassium phosphate buffer, pH 6.5
were added. Reactions were initiated by the addition of 0.6 ml of microsomal protein and the blank (reference) balanced by the addition of 0.6 ml of the microsomal resuspension buffer. Samples in the cuvettes were mixed with a cuvette paddle and the increase in absorbance at 340 nm with time over 1 min recorded.

The specific activities were determined according to the following equation:

\[
\text{Specific activity} = \frac{\text{Absorbance change (340 nm) per min (linear portion)}}{\text{Extinction coefficient (9.6 mM}^{-1}\text{ cm}^{-1}) \times \text{protein concentration (mg ml}^{-1})}
\]

Where specific activity is in nmol min\(^{-1}\) mg protein\(^{-1}\)

5.2.6 Statistical analysis

All data were submitted to a one-way analysis of variance. All variances were within the expected range for normally distributed populations. Treated group means from each tank were compared with the control group means using ANOVA. Data were analyzed using Statgraphics\textsuperscript{®} Plus (4.0) for Windows and statistical significance determined at P<0.05.

5.3 Results

No significant differences in the SGR or tissue somatic indices were noted in the pilot investigation, with the hepatosomatic indices ranging from 0.75 to 2.79%, and the kidney somatic index ranging from 0.74 to 3.98% (Table 5.3).

Table 5.3. Specific growth rate (SGR) and tissue somatic indices (TSI) of rainbow trout fed diets supplemented or unsupplemented with carotenoids in a pilot study. (n = 30 fish-treatment -1).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGR (%)</td>
<td>1.11</td>
<td>0.98</td>
<td>1.03</td>
</tr>
<tr>
<td>Liver TSI (%)</td>
<td>1.81±0.28</td>
<td>1.81±0.46</td>
<td>1.85±0.34</td>
</tr>
<tr>
<td>Kidney TSI (%)</td>
<td>1.52±0.79</td>
<td>1.55±0.68</td>
<td>1.69±0.82</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means (P>0.05).
The replicated experiment utilized an additional positive control treatment (β-naphthoflavone), as a classical inducer of cytochrome P450 systems. Wet weight gain, SGR, feed conversion ratio (FCR), and tissue somatic indices showed no dietary effect in the replicated experimental trial (Table 5.4). Liver somatic indices ranged from 0.80 to 2.23%, while the kidney somatic index ranged from 0.60 to 1.46% across all dietary treatments. SGR ranged from 0.72 to 1.42% d⁻¹, while FCR ranged from 0.82 to 1.73.

Table 5.4. Feed conversion ratios (FCR), specific growth rate (SGR), and tissue somatic indices (TSI) of rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone in a replicated experimental trial. Values are expressed as means ±SEM (n = 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
<th>β-naphthoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCR</td>
<td>0.92 ± 0.04</td>
<td>0.95 ± 0.10</td>
<td>0.92 ± 0.07</td>
<td>0.95 ± 0.11</td>
</tr>
<tr>
<td>SGR (%)</td>
<td>1.11±0.19</td>
<td>1.26±0.07</td>
<td>1.30±0.07</td>
<td>1.37±0.07</td>
</tr>
<tr>
<td>Liver TSI (%)</td>
<td>1.50±0.05</td>
<td>1.46±0.04</td>
<td>1.55±0.06</td>
<td>1.55±0.02</td>
</tr>
<tr>
<td>Kidney TSI (%)</td>
<td>0.93±0.07</td>
<td>0.94±0.05</td>
<td>0.88±0.01</td>
<td>0.88±0.02</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means (P>0.05).

Serum carotenoid concentrations between astaxanthin and canthaxanthin fed fish in the replicated feed trial revealed no significant differences in levels of exposure (Table 5.5).

There was a low level of carotenoid detected in both the β-naphthoflavone and placebo control groups due to the inherent carotenoid content of the basal feed, but deemed insignificant to affect the current results.

Table 5.5. Serum carotenoid concentrations (µg·ml⁻¹; mean ± SEM) of rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone in a replicated experimental trial (n = 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin</td>
<td>12.5±1.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>0.2±0.0</td>
<td>11.1±0.7</td>
</tr>
<tr>
<td>Control</td>
<td>0.1±0.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>β-naphthoflavone</td>
<td>0.1±0.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Serum concentrations between astaxanthin and canthaxanthin treatments not significantly different (P>0.05).
Liver xenobiotic metabolizing enzyme activities in the pilot study showed no significant differences, although carotenoid treatment appeared to show a slight inhibition of EROD activities relative to those of the control fish (Table 5.6). Similarly, kidney EROD activities were not significantly different between dietary treatments (Table 5.7).

Table 5.6. Liver monooxygenase enzyme activities (mean ± SD) of a pilot study in rainbow trout fed astaxanthin, canthaxanthin, or control diet \((n = 10\ \text{pools}\cdot\text{treatment}-1)\).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>12.74 ± 3.41</td>
<td>11.60 ± 3.42</td>
<td>17.11 ± 6.07</td>
</tr>
<tr>
<td>MROD</td>
<td>5.06 ± 1.23</td>
<td>5.58 ± 1.57</td>
<td>6.12 ± 1.85</td>
</tr>
<tr>
<td>PROD</td>
<td>0.66 ± 0.19</td>
<td>0.36 ± 0.07</td>
<td>0.23 ± 0.19</td>
</tr>
<tr>
<td>BROD</td>
<td>1.66 ± 0.19</td>
<td>1.87 ± 0.47</td>
<td>1.93 ± 0.44</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means \((P>0.05)\).

Note: EROD, ethoxyresorufin 0-deethylase; MROD, methoxyresorufin 0-demethylase; PROD, pentoxyresorufin 0-dealkylase; BROD, benzoxyresorufin O-dearylase. All specific activities expressed as \(\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}\).

Liver and kidney monooxygenase and conjugation enzyme activities of rainbow trout in the replicated experimental trial are shown in Tables 5.8 and 5.9. No significant differences in enzyme induction were observed between any dietary treatments in either tissue. The specific activities from the experimental trial were lower than those of the pilot study. This is thought to be due to strain differences between fish farms as was observed by Koponen et al. 1997).

Table 5.7. Kidney monooxygenase enzyme activities (mean ± SD) of a pilot study in rainbow trout fed astaxanthin, canthaxanthin, or control diet \((n = 10\ \text{pools}\cdot\text{treatment}-1)\).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>2.78 ± 0.76</td>
<td>2.89 ± 1.57</td>
<td>3.61 ± 1.87</td>
</tr>
<tr>
<td>MROD</td>
<td>1.66 ± 0.38</td>
<td>1.34 ± 0.52</td>
<td>1.93 ± 0.82</td>
</tr>
<tr>
<td>PROD</td>
<td>0.91 ± 0.41</td>
<td>0.25 ± 0.14</td>
<td>0.53 ± 0.51</td>
</tr>
<tr>
<td>BROD</td>
<td>0.42 ± 0.25</td>
<td>0.53 ± 0.23</td>
<td>0.80 ± 0.42</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means \((P>0.05)\).

Note: EROD, ethoxyresorufin 0-deethylase; MROD, methoxyresorufin 0-demethylase; PROD, pentoxyresorufin 0-dealkylase; BROD, benzoxyresorufin O-dearylase. All specific activities expressed as \(\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}\).
Table 5.8. Liver monooxygenase and conjugation enzyme activities (mean ± SEM) in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-napthoflavone (n = 3).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
<th>β-napthoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>2.82 ± 0.14</td>
<td>2.57 ± 0.22</td>
<td>3.13 ± 0.27</td>
<td>3.11 ± 0.57</td>
</tr>
<tr>
<td>MROD</td>
<td>1.15 ± 0.25</td>
<td>1.02 ± 0.21</td>
<td>1.19 ± 0.25</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>PROD</td>
<td>1.54 ± 0.33</td>
<td>1.72 ± 0.19</td>
<td>1.72 ± 0.25</td>
<td>1.99 ± 0.15</td>
</tr>
<tr>
<td>BROD</td>
<td>1.67 ± 0.12</td>
<td>1.79 ± 0.22</td>
<td>1.94 ± 0.05</td>
<td>2.31 ± 0.11</td>
</tr>
<tr>
<td>GST</td>
<td>412 ± 59</td>
<td>465 ± 51</td>
<td>401 ± 41</td>
<td>425 ± 25</td>
</tr>
<tr>
<td>UDPGT</td>
<td>1237 ± 70</td>
<td>1326 ± 123</td>
<td>1198 ± 93</td>
<td>1269 ± 124</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means (P>0.05).

Note: EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; PROD, pentoxyresorufin O-dealkylase; BROD, benzoxyresorufin O-dearylase (monooxygenase specific activities expressed as nmol·min⁻¹·mg protein⁻¹); UDPGT, UDP-glucuronosyl transferase (pmol·min⁻¹·mg protein⁻¹); GST, glutathione S-transferase (nmol·min⁻¹·mg protein⁻¹).
Table 5.9. Kidney monooxygenase and conjugation enzyme activities (mean ± SEM) in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
<th>β-naphthoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>2.54±0.31</td>
<td>2.40±0.27</td>
<td>2.21±0.23</td>
<td>2.17±0.19</td>
</tr>
<tr>
<td>MROD</td>
<td>1.72±0.17</td>
<td>1.60±0.17</td>
<td>1.63±0.15</td>
<td>1.59±0.19</td>
</tr>
<tr>
<td>PROD</td>
<td>1.77±0.36</td>
<td>1.60±0.16</td>
<td>1.60±0.20</td>
<td>1.71±0.15</td>
</tr>
<tr>
<td>BROD</td>
<td>0.59±0.11</td>
<td>0.54±0.19</td>
<td>0.79±0.01</td>
<td>0.74±0.11</td>
</tr>
<tr>
<td>GST</td>
<td>75 ± 12</td>
<td>99 ± 24</td>
<td>87 ± 19</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>UDPGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between treatment means (P > 0.05).

Note: EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; PROD, pentoxyresorufin O-dealkylase; BROD, benzoxyresorufin O-dearylase (monooxygenase specific activities expressed as nmol·min⁻¹·mg protein⁻¹); UDPGT, UDP-glucuronosyl transferase (pmol·min⁻¹·mg protein⁻¹); GST, glutathione S-transferase (nmol·min⁻¹·mg protein⁻¹).

5.4 Discussion

The growth indices and/or tissue somatic indices of the respective experiments showed no dietary carotenoid effect. While carotenoid supplementation has been shown to be beneficial in young Atlantic salmon (Christiansen et al. 1995), no growth promoting effects were observed in the present studies, nor did carotenoid supplementation affect tissue somatic indices. The experimental trial appears to show a slight trend toward a lower liver somatic index in carotenoid treated fish relative to placebo fed fish, which was also observed in the pilot study, but due to large inter-individual variation, no significant differences were observed.

The fact that β-naphthoflavone did not significantly induce liver xenobiotic enzymes in rainbow trout is of concern. It is generally accepted that β-NF, which is a potent flavonoid compound, is a classic inducer of MFO enzymes (Elcombe and Lech 1979; Haasch et al. 1993) and it was expected to have produced an elevated response in MFO activity. This may be due to several reasons. Firstly, β-NF is generally provided as an intraperitoneal injection using an oil carrier and the dosage delivered at approximately 20 mg kg⁻¹ of body weight (Koponen et al. 1997). Therefore, for a fish of similar weight to those used in the
present study (~250-300 g), this would represent an exposure of ~5-6 mg per fish. The dietary inclusion rate in the present study (100 mg kg⁻¹) and delivered at a feed ration of 1.2% body weight would have supplied only approximately 0.2 to 0.3 mg per fish per day, fish would only be exposed to approximately 6.3 mg over the entire experimental treatment. Thus the fish may have ample capacity to clear the consumed compound (assuming 100% digestibility) on a daily basis, thus resulting in no induction. Alternatively, the method of delivery in the present study (oral) versus those of other studies (intraperitoneal injection) may provide another reason for the observed discrepancy. It is possible that digestive enzymes significantly degrade the compound in the gastrointestinal tract or that absorption is poor (low digestibility) which would reduce the exposure of the already low daily dose. In addition, it has been reported that repeated dosages of β-NF has resulted in significant inhibition of the normal induction response by its metabolites via enterohepatic circulation (George 1994). Thus the repeated doses given on a daily basis over three weeks may have masked any inductive effect normally seen by intraperitoneal injection. Further still, the levels of xenobiotic-metabolizing enzymes could be deemed pre-stimulated in all treatments, masking any inherent induction due to dietary treatments. This could potentially be due to compounds already present in the recirculating water system used in the present experiments, which may include plasticisers or trace petroleum products. Therefore future experiments should be repeated in a non-recirculating system, with known uninduced fish stocks. Despite these concerns, this treatment was included as a positive control only to ensure the validity of the methods used. The methods used in the present investigations have been well-established in the literature, and given the high correlation observed in the resorufin standard curve (Appendix 1, Fig. X.2), this would suggest methodological considerations are not likely the source of error.
The supplementation of diets with astaxanthin and canthaxanthin at approximately 100 mg·kg⁻¹ resulted in mean serum carotenoid concentrations slightly higher than a previous study supplementing carotenoids at similar dietary concentrations (March and MacMillan 1996). This is likely due to the higher dietary lipid incorporation in the present study (28%, Table 5.5), compared to that in of March and MacMillan (1996)(10-12%). However, rainbow trout fed astaxanthin or canthaxanthin did not show significant differences in mean serum carotenoid concentrations, indicating equivalent exposures to the different carotenoids. As such, any effects on liver xenobiotic metabolizing enzymes would solely be due to the individual carotenoid’s biochemical influence.

The present values obtained for xenobiotic metabolizing enzyme activities are within the published range for basal monoxygenase activities in rainbow trout (Haasch et al. 1993; Koponen et al. 1997). Differences in basal levels of monoxygenase and conjugation enzyme activities observed between the pilot study and those of the experimental trial are likely due to strain differences as has been observed by Pedersen and Hershberger (1976) and Koponen et al. (1997). However, the present study shows no inductive effect of either astaxanthin or canthaxanthin on liver or kidney xenobiotic metabolizing enzyme activities in rainbow trout. In a recent study culturing trout hepatocytes with astaxanthin in vitro, Amcoff et al. (1998) failed to detect the induction of hepatic cytochrome P4501A after exposure to astaxanthin. However, hepatocytes were only cultured for 48 hrs, and as such, the exposure time may have been insufficient to induce the cytochrome P-450 enzyme system. Their in vivo study using yolk-sac fry only used a single injection of a combination of PCB/astaxanthin, measuring the resulting EROD activity 36 days later. The single injection of astaxanthin may have been insufficient to maintain elevated levels of P450 activity to be evident at the time of measurement. It is possible that the cytochrome P450 activity in these fish may have had ample capacity to metabolize the administered carotenoid without the requirement for induction. Despite these considerations, no
induction was observed in the present study, confirming the results of Amcoff et al. (1998).

The present results are in direct contrast to several mammalian studies that revealed significant elevations in P450 isoenzyme activities due to dietary administration of astaxanthin and canthaxanthin. For example, Gradelet et al. (1996) observed that canthaxanthin increased liver content of P450, the activities of NADH and NADPH-cytochrome c reductase, and produced a substantial increase of some P450-dependent activities, especially EROD and MROD. Canthaxanthin also increased PROD and BROD, but did not affect NADPH-cytochrome c reductase and erythromycin N-demethylase (ERDM) activities and decreased nitrosodimethylamine N-demethylase (NDMAD) activity. Phase II p-nitrophenol UDP-glucuronosyl transferase (4NP-UGT) and quinone reductase (QR) activities were also increased by canthaxanthin treatment. Astaxanthin induced the same pattern of enzymes activities as canthaxanthin, but to a lesser extent (Gradelet et al. 1996). The present study was deliberately designed to be within the regulatory limits currently within some sectors of the aquaculture industry for dietary inclusion of astaxanthin and canthaxanthin to 100 mg kg$^{-1}$ and 80 mg kg$^{-1}$, respectively (USFDA 1998). Gradelet et al. (1996) examined the induction of the MFO system in a dose-response study which exposed rats to a similar dietary inclusion rate of 100 mg kg$^{-1}$, where both astaxanthin and canthaxanthin significantly elevated microsomal phase I enzyme activities with 56 and 18-fold higher EROD activities for canthaxanthin and astaxanthin, respectively. Their results are in direct contrast to the present results which did not detect an inductive effect as a result of dietary carotenoid supplementation. While no induction was seen in rainbow trout as a result of these dietary treatments, this may be due to the lower level of dietary carotenoid inclusion in the present experiment (100 mg kg$^{-1}$) in comparison to studies by Astorg et al. (1994, 1997), and Jewell and O’Brien (1999), who supplemented rat diets at an inclusion rate of 300 mg kg$^{-1}$. Thus it remains to be
elucidated whether these enzyme systems in rainbow trout respond in a dose-response relationship to higher dietary inclusion levels than used in the present studies.

Despite the lack of induction (Tables 5.8 and 5.9), these enzymes may be involved in carotenoid metabolism due to the location of the enzymes. An immunocytochemistry study by Lester et al. (1992) was used to localize cytochrome P4501A1 in the liver of rainbow trout, and showed that P4501A1 labeling was sequestered in plasma and endothelial membranes of hepatocytes, as well as over granular endoplasmic reticulum (GER) in perinuclear sites. Indeed, these authors noted that the plasma membrane was labelled heavily at bile canaliculi and in the space of Disse. Bruni and Porter (1965) noted that it is likely across this surface that plasma proteins, lipids and lipoproteins, must be absorbed, and released. Astaxanthin and canthaxanthin, which are known to be incorporated into lipid bilayers (Milon et al. 1986) and in lipoprotein fractions (Ando et al. 1985, 1986 a,b), may therefore gain access to the active site of the enzyme. Lester et al. (1992) suggest that the localization of P4501A1 in these cells, which provide an interface between blood-borne xenobiotics and epithelial cells, may be of importance in toxicity. A similar suggestion has been noted for TBT, which has lipophilic properties, potentially allowing the compound to penetrate the hydrophobic membrane environment (Reader et al. 1996), in which the cytochrome P450 is embedded, and thereby gain access to the active site of the enzyme for catabolism. Furthermore, the observation that phase II enzyme activities such as UDPGT and GST were also elevated upon feeding canthaxanthin in rats (Astorg et al. 1994) may suggest co-operative protein-protein interactions and enzyme functions in the elimination of carotenoids. Taura et al. (2000) observed that CYP1A1 may interact with UDPGT to facilitate a series of multistep drug metabolic conversions. Further support is offered by the locations of these two enzyme systems; P450 is located outside the endoplasmic reticulum (ER), while UDPGT is considered to be present in the lumen of the ER (Meech and Mackensie 1997). Both astaxanthin and canthaxanthin can serve as provitamin A in
salmonid fish (Fig. 1.4; Schiedt et al. 1985; Guillou et al. 1989; Al-Khalifa and Simpson 1988), and with the reductive metabolic pathway for retinol proposed by Leo and Leiber (1999) (Fig. 5.1), it is possible that cytochrome P450 enzymes may be involved indirectly in xanthophylls metabolism.

The observation that these carotenoids do not induce P450 isoenzymes in rainbow trout may be due to fundamental differences between species. It is generally observed that fish are less active than mammalian species in metabolizing xenobiotics (Buhler and Rasmussen 1968). Indeed, there is considerable species-specificity in the level of induction due to dietary astaxanthin and canthaxanthin administration in rats and mice. When comparing the studies of Gradelet et al. (1996) in the rat and that of Astorg et al. (1997) in mice, it can be seen that species differences may exist in the level of induction due to these carotenoids. Both studies investigated canthaxanthin and astaxanthin effects on phase I and phase II enzyme activities, but showed marked differences in the levels of induction. Gradelet et al. (1996) observed a high level of EROD induction (X139) relative to controls in rats fed canthaxanthin for 15 days, while the mice treated in the same fashion by Astorg et al. (1997), only showed a weak induction of threefold higher relative to controls.

Similarly, astaxanthin significantly induced these enzymes in rats (but to a lesser extent than canthaxanthin), but no induction was observed in mice.
Fig. 5.1. Simplified schematic representation of the putative role of cytosolic and microsomal systems in the hepatic metabolism of retinal. CRD, cytosolic retinal dehydrogenase; MRD, microsomal retinal dehydrogenase; ARAT, retinol fatty-acyltransferase; REH, retinyl ester hydrolase. Adapted from Leo and Leiber (1999).
Although no significant differences were observed between the various treatments with respect to the enzyme activities measured, there appeared to be several tank-related differences within replicate tanks within treatments. This may be due to the inherent intraspecies variability within treatments or due to differences in the isolation of microsomal fractions, although the latter is unlikely. No spatial orientation differences were apparent in relation to the tank differences within a treatment, and as such, are likely due to inherent variation among fish populations. Indeed, Koponen et al. (1997) found significant intrastrain differences in UDPGT and GST, as well as significant interstrain differences in EROD activities in rainbow trout. Therefore, even within a given population significant differences may emerge within a treatment as seen in the present study, which may account for the differences in tanks observed. These data were not related to differences in carotenoid content of the serum as no differences were detected in the present study within a treatment and its replicates.

Furthermore significant intrastrain variability also exists in MFO and conjugating enzyme systems in rainbow trout (Koponen et al. 1997), and therefore caution should be exercised in eliminating the MFO system from the contribution to carotenoid catabolism and excretion. It may be that uptake into the tissue is rate limiting in carotenoid metabolism, and as such no induction would be observed. Future studies should investigate a dose dependency above that studied in the present investigations to determine if these systems are inducible by these compounds as they are in mammalian species, as well as temporal and subject size aspects to the responses.

However, if a similar induction relationship exists in rainbow trout at higher inclusion levels, this may offer some potential insight into the metabolic pathways in fish. Should canthaxanthin induce these enzymes to a higher extent than does astaxanthin in rainbow trout, as they do in mammals, it may be speculated that canthaxanthin is degraded to a
larger extent than astaxanthin, which may correlate with the half-life of canthaxanthin clearance observed in vivo post-prandial studies by Guillou et al. (1993). These authors found that blood clearance rates from mean plasma linear regression slopes for astaxanthin and canthaxanthin were 2.07 %/hour and 1.98 %/hour, respectively, correlating to half-lives of 24.1 and 22.2 hours, for astaxanthin and canthaxanthin, respectively. In contrast, calculated half-lives in a similar study revealed much longer times for astaxanthin of 34.8 hrs, compared to 12.6 hrs for canthaxanthin (Gobantes et al. 1997).

In addition to the liver enzyme system induction, Jewell and O’Brien (1999) provided additional evidence to suggest that enzyme system induction may also occur in other tissues. These authors found that both canthaxanthin and astaxanthin significantly increased EROD and MROD activities in the liver, lungs and kidneys of the rat. Induction of drug metabolizing enzymes in extrahepatic tissues, including the kidney is of importance as the kidneys are the primary route of excretion of xenobiotics from the body (Jewell and O’Brien 1999). Indeed, despite the lower catalytic activity for biotransformation systems in the gill, the higher perfusion rates in this tissue, relative to the liver, may influence the amount of chemical that reaches the blood (Barron et al. 1987). The gill is between the arterial and venous circulation and receives nearly all of the cardiac output, while the liver receives only about 10% (Cameron 1975). However, the present study did not investigate the gill with respect to the influence of these dietary treatments on xenobiotic metabolizing enzyme activities. Despite the potential of the gills to influence carotenoid clearance and metabolism, no literature is available to suggest that the gill plays any role in carotenoid metabolism. The proximity of the gill to the environment and its associated pro-oxidant exposure may mean that carotenoids potentially serve as antioxidants in this tissue although this has yet to be investigated.
In mammalian studies, dietary canthaxanthin was readily incorporated into the liver 100 times more than dietary astaxanthin but only five times more than lycopene in the liver of the rat (Gradelet et al. 1986). In hepatic microsomes, canthaxanthin accumulated 75 times more than astaxanthin and six times more than lycopene (Gradelet et al. 1996). Among the molecules tested in this and a previous work (Astorg et al. 1994), the fact that only carotenoids bearing oxo function in 4 and 4' are P450 1A inducers must be underlined (Gradelet et al. 1996). In contrast, carotenoids do not accumulate selectively or to high levels in the liver of rainbow trout as seen in previous experimental work in this laboratory (Chapter 3, Fig. 3.1). Values for carotenoid accumulation in rat liver for canthaxanthin are approximately 0.2 μg g⁻¹ (Gradelet et al. 1996), which is similar to values normally recovered in rainbow trout. Thus the lack of significant induction in the present investigations may not due to different concentrations or exposure to carotenoids, and is likely to be a species-specific pattern, or to the possibility that the enzyme systems were already induced.

Astaxanthin and canthaxanthin are not provitamin A sources in mts and mice (Gradelet et al. 1996; Astorg et al. 1994; 1997), and as such the rainbow trout, which has been shown to be able to metabolize these compounds to vitamin A (Schiedt et al. 1985), shows fundamental differences in their metabolic capacities for these compounds. Therefore in light of the lack of induction it may be that fish are capable of rapidly catabolizing these compounds such that they do not result in induction of these enzymes. Rainbow trout may have a specific metabolic pathway for their degradation which has yet to be elucidated, or alternatively, as carotenoids are lipoprotein bound, the uptake of carotenoids into the tissues may limit the rate of carotenoid catabolism (i.e. uptake into the liver is rate limiting and not the enzymatic catabolism). Therefore the enzymatic catabolic systems in trout may have ample capacity for the degradation of carotenoids, which is capable of much higher levels than are delivered to the liver for catabolism.
Gradelet et al. (1996) conclude that the two 4-oxocarotenoids, canthaxanthin and astaxanthin, are substantial inducers of liver P450 1A1 and 1A2 in the rat. While these carotenoids may induce these enzyme systems, it would appear from the results of Wolz et al. (1999) that cytochrome P450 isoenzymes are not responsible for the metabolism of astaxanthin. This claim is based on the observation that both induced and uninduced rat liver microsomes did not convert astaxanthin and that hepatocytes from both pretreated and not pretreated rats metabolized astaxanthin similarly. However, it should be noted that these studies were based on supplementing astaxanthin at 2 µM, near its solubility point in culture medium, and thus may have been too low to show differences that may have been apparent at higher concentrations where induced microsomes or hepatocytes from pretreated rats may have had higher metabolic capacities.

In conclusion, results indicate that dietary carotenoid incorporation of astaxanthin or canthaxanthin at 100 mg kg⁻¹ did not induce liver or kidney xenobiotic-metabolizing enzyme systems in the rainbow trout. However, the contribution of the cytochrome P450 enzyme system and phase II conjugating enzymes cannot be ruled out. Lack of enzyme induction could be due to pre-stimulated (induced) enzyme systems, effectively masking potential dietary effects. Therefore, further research is required to determine if carotenoid induce these systems, and to elucidate the exact mechanism of carotenoid metabolism in salmonid fish, which may be accomplished via histochemical examination of liver tissue.
CHAPTER 6
THE EFFECTS OF DIETARY ASTAXANTHIN AND CANTHAXANTHIN
SUPPLEMENTATION ON LIVER HISTOCHEMISTRY IN RAINBOW TROUT
(ONCORHYNCHUS MYKISS WALBAUM).

6.1 Introduction

The characteristic pink colour of salmon flesh is caused by the deposition of ingested carotenoids (mainly astaxanthin) in the muscle. The flesh colour is used as an indication of product quality in aquaculture (Torrissen and Naevdal, 1984). However, fish are unable to synthesize pigmenting carotenoids de novo, and must therefore obtain these from dietary sources.

Carotenoids have been ascribed a multitude of functions beyond their involvement in flesh pigmentation. Both astaxanthin and canthaxanthin may serve as provitamin A (Schie dt et al. 1985; Guillou et al. 1989; Al-Khalifa and Simpson 1988). Other researchers have suggested a variety of other essential functions as reviewed in Chapter 1. Christiansen et al. (1994), concluded that astaxanthin is essential to alevins during the first-feeding period, and strongly influenced the growth, survival and vitamin A concentration in the flesh of juvenile Atlantic salmon. Similar observations lead Torrissen and Christiansen (1995) to propose that both astaxanthin and canthaxanthin should be listed among the fat-soluble vitamins, with a minimum of 10 mg kg\(^{-1}\) of carotenoid in the dry diet.

The liver is of key importance for maintaining the internal homeostasis in vertebrates. It adapts to fluctuating environmental conditions by continuously regulating hepatocellular structures and functions such as: metabolism of nutrients; storage of energy (e.g. glycogen and lipid); synthesis and secretion of proteins (e.g. albumin, vitellogenin, lipoproteins); maintenance of plasma glucose levels; elimination of nitrogen components after urea or
ammonia formation; metabolism of hormones; metabolism of xenobiotics; and bile formation (Segner 1998).

According to Metón et al. (1999), somatic factors including body and liver weight, as well as metabolic factors such as metabolite and enzyme activity are currently used to determine the capacity for metabolic adaptation to dietary supply in fish. The liver-somatic index (LSI), and hepatic glycogen content and various liver enzyme activities of intermediary metabolism have been shown to correspond well with the nutritional status and growth rates of fish (Brauge et al. 1994; Pelletier et al. 1994). Glycogen phosphorylase (enzyme involved in glycogenolysis) is proposed to play a role in carotenoid metabolism as an enzyme responsible for the provision of the required substrate (UDP-glucuronid acid) for glucuronidation. Aspartate aminotransferase (glutamine-oxaloacetic transaminase (GOT)), an indicator of liver function in rainbow trout, has previously been shown to be influenced by carotenoid supplementation (Nakano et al. 1995),

The liver, being the central organ of intermediary metabolism (including the biotransformation of carotenoids), should reflect the effects of carotenoids on fish metabolism (Segner et al. 1989). Histological techniques are considered to be an important determinant for the evaluation of the effects of food additives (Johnson and Bergmann 1984). Indeed, Segner et al. (1989) examined the effects of dietary astaxanthin on liver histology, and found that the livers of fish (Oreochromis niloticus and Colisa labiosa) fed higher levels of astaxanthin exhibited a well-organized liver parenchyma, with well-compartmented hepatocytes and without pathological alterations. These observations lead Segner et al. (1989) to conclude that astaxanthin improved the liver structure and had a positive nutritional function in the intermediary metabolism of fish.
Further research by Nakano et al. (1995), concluded that astaxanthin confers positive metabolic effects on fish, e.g. acceleration of digestion and/or absorption. These authors based their conclusion on the observation of serum glutamic-oxaloacetic transaminase activities, a known index for diagnosis of liver function (Ozaki 1978), which were significantly lower in diets supplemented with astaxanthin. In addition, they cite Miki (1993) who claims that astaxanthin is safe to administer to animals without exerting any toxic effect on cellular function. Therefore, Nakano et al. (1995) suggested that any damage or malfunction might not occur in the livers of fish fed a diet supplemented with astaxanthin.

However, no direct observations of the effects of astaxanthin and/or canthaxanthin on hepatic histology have been performed in salmonid fish, despite these fish having intrinsically different physiology relating to carotenoids (i.e. high retention in flesh and gonads). As such, the proposed trial seeks to determine the effects of astaxanthin and canthaxanthin supplementation on liver histochemistry in rainbow trout. More specifically, the influence of these carotenoids on liver glycogen, lipid storage, and GOT, and glycogen phosphorylase will be assessed.

6.2 Materials and Methods

6.2.1 Fish and experimental conditions

A pilot study was initiated to investigate and develop methodologies into the effects of dietary carotenoid supplementation on liver histochemistry. The fish and experimental conditions were as previously described in Chapter 5.2.3.

The replicated experimental study utilized a negative control (Co) and positive control (using β-naphthoflavone (β-NF) as a classical inducer of liver xenobiotic-metabolizing enzymes) treatments in addition to carotenoid treatments. Twelve fish each (obtained from Hatchlands Trout Farm, Devon, UK) were allocated to three replicates for each of four
individual treatments (Carophyll Pink, Carophyll Red, Control, \(\beta\)-naphthoflavone) and maintained for two weeks prior to the start of the experiment to allow for acclimation to culture conditions. The fish and experimental conditions were as previously described in Chapter 5.2.3.

### 6.2.2 Diet Preparation

Experimental diets were prepared according to the top-coating method as described in Chapter 2.6.2. Diets were formulated to contain 100 mg kg\(^{-1}\) of carotenoid (astaxanthin (Ax) or canthaxanthin(Cx)), Control (Co) or \(\beta\)-naphthoflavone (\(\beta\)-NF).

### 6.2.3 Diet analysis

Proximate and carotenoid analysis of prepared feeds was determined according to methodologies in Chapter 2.7. The basal diet formulation and proximate composition of experimental diets are presented in Tables 6.1 and 6.2.

**Table 6.1. Basal diet formulation* and proximate analysis of experimental diets used in the pilot study. Values are expressed as means ± S.D. of triplicate determinations.**

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>48.60 ± 1.41</td>
<td>44.92 ± 0.98</td>
<td>48.48 ± 0.35</td>
</tr>
<tr>
<td>Crude Lipid (%)</td>
<td>27.03 ± .34</td>
<td>27.44 ± .18</td>
<td>27.87 ± .84</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>9.96 ± 0.04</td>
<td>9.80 ± 0.17</td>
<td>9.92 ± 0.01</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>4.31 ± 0.03</td>
<td>4.99 ± 0.04</td>
<td>3.67 ± 0.26</td>
</tr>
<tr>
<td>Astaxanthin (mg/kg)</td>
<td>95.2 ± 2.2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Canthaxanthin (mg/kg)</td>
<td>N.D.</td>
<td>98.8 ± 0.5</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*Basal diet formulation common to all experimental diets comprising of LT fish meal (Norse LT94, Egersund, Norway; 61.3%), wheat gluten (5.6 %), wheat (21.6%), fish oil (10%), vitamin premix (0.7%) and mineral premix (0.8%).
N.D. indicates not detectable.
6.2.4 Tissue sampling

At the termination of the feeding trial, fish were euthanized with phenoxyethanol followed by a blow to the head, and immediately weighed. Fish that showed no sign of growth (emaciation) were omitted from the trial as these were deemed unexposed to dietary treatments. Fifteen fish were randomly taken from the 40 fish in the pilot study for histochemistry. All of the non-emaciated fish were used for histochemical examinations in the replicated feed trial.

Livers were dissected out, blotted dry, and weighed. Livers were sectioned medially, and the left lobe used for histological analysis, and the right lobe for enzymatic determinations (Chapter 6). Liver samples were frozen in liquid nitrogen, stored (-80°C), and sectioned on a Leica 2700-frigocut cryostat at 12μm, prior to histochemical staining, and image analysis by light microscopy.

6.2.5 Tissue sectioning and histochemical staining

Firstly a Methylene Blue stain (1%) was used to examine the general histology of the livers prior to further analysis.

 Unsaturated lipids were stained according to High (1984) by immersing frozen sections in an osmium tetroxide (1% OsO₄ w/v) solution for 60 min. Slides were air dried and mounted using DPX.
Table 6.2. Basal diet formulation* and proximate analysis of experimental diets used in the replicated experiment. Values are expressed as means ± S.D. of triplicate determinations.

<table>
<thead>
<tr>
<th>Proximate analysis</th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
<th>β-NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>45.23±0.45</td>
<td>46.25±0.66</td>
<td>45.00±0.31</td>
<td>44.87±0.32</td>
</tr>
<tr>
<td>Crude Lipid (%)</td>
<td>30.55±0.43</td>
<td>30.77±0.26</td>
<td>30.53±0.18</td>
<td>30.33±0.45</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>9.84±0.03</td>
<td>9.92±0.09</td>
<td>9.81±0.02</td>
<td>9.98±0.454</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>5.68±0.36</td>
<td>6.53±0.10</td>
<td>6.25±0.09</td>
<td>6.33±0.27</td>
</tr>
<tr>
<td>Astaxanthin (mg/kg)</td>
<td>84.34±1.02</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Canthaxanthin (mg/kg)</td>
<td>N.D.</td>
<td>90.79±1.15</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*Basal diet formulation common to all experimental diets comprising of LT fish meal (Norse LT94, Egersund, Norway; 61.3%), wheat gluten (5.6%), wheat (21.6%), fish oil (10%), vitamin premix (0.7%) and mineral premix (0.8%).
N.D. indicates not detectable.
Phospholipids and some fatty acids were stained by placing frozen sections in a 1% sudan black (w/v) solution for 30 min. Sections were then thoroughly washed with 70% alcohol, followed by serial rehydration in 50%, 30% alcohols (v/v), and finally in distilled water at 2 min each. Slides were then covered and mounted using DPX.

Glycogen in frozen sections was stained using the periodic acid-schiff (PAS) method of (High 1984). In summary, matched sections from the same liver were brought to water, and one set were placed in a 3% diastase solution for 15 min at 37°C for the removal of glycogen. The other set were stored in water. All sections were then oxidised for 5-10 min in 1% Periodic Acid, and washed in running water for 5 min, and finally rinsed in distilled water. Sections were then treated with Schiff's reagent for 20 min and washed in running water for 10 minutes. Sections were dehydrated, covered and mounted using DPX. The Periodic Acid oxidises carbohydrates (e.g. glycogen) to an aldehyde, where the 'Schiff's' stains the aldehyde pink. The control sections will be lighter as no glycogen was stained (removed).

Glycogen phosphorylase (enzyme involved in glycogenolysis) was stained using dextran (Pearse 1972). Sections were first incubated for 30 min (at 37 °C) in a solution containing 100 mg disodium glucose-1-phosphate, 50 mg disodium ethylaminediamine tetra acetate, 40 mg sodium fluoride, 25 ml acetate buffer (10 mM, pH 5.6), and 2 g dextran. Sections were shaken to remove excess incubation medium, washed in 40% ethanol and fixed in absolute alcohol for 10 min. Sections were then treated according to the normal PAS technique as described above for glycogen. Sections were dehydrated, covered and mounted using DPX.

Aspartate aminotransferase (glutamine-oxaloacetic transaminase (GOT)), was stained according to Pearse (1972). Frozen liver sections were incubated for 1 hour at 37 °C in a
solution containing: 1 ml of 0.2M Aspartic acid, 1 ml 0.1M 2-Oxoglutaric acid, 3-5 mg Fast violet B salt, 0.5 ml of 0.3% EDTA, 2 ml of 0.1M Phosphate buffer pH 7.4, and adjusted to pH 7.4 with 1 M NaOH. Slides were washed in distilled water, and incubated in 0.1M CuSO₄ for 3-5 min and washed in distilled water. Sections were dehydrated, covered and mounted using DPX.

6.2.6 Image analysis

PAS stained slides were examined with a Zeiss photomicroscope II and the images captured using an Hitachi 3CCD colour camera. The analogue signal was then imported into a Quantimet 570 image analyser. The colour image was detected by thresholding for mainly the red component. Once the binary image was created, it was measured for ‘grey’ by area and intensity. The diastase treated control slides were used to determine the threshold level of detection to ensure that the results indicated glycogen staining rather than all PAS positive components. This was repeated six times for each slide. The frequency distribution of grey levels were saved to disc and imported into a spreadsheet for data manipulation.

Sudan black, GOT, osmium tetroxide, and glycogen phosphorylase slides were examined with a Zeiss photomicroscope II at X160 and images captured and imported as for the PAS slides above. The blue component of the colour image was used as a grey image and a macro program used to detect only the sinusoids in the liver. Areas containing blood vessels and bile ducts were avoided.

Grey-level values of the glycogen phosphorylase slides were corrected for background staining by subtracting the mean grey-levels of the PAS stained slides for each liver. Therefore, there is a possibility of obtaining negative grey-level values. These values represent differences between two grey-levels and are not negative absolute grey-levels.
Grey-level values are based on a range from 0 to 255, where 0 represents a darker slide and therefore more of the measured (stained) parameter, and 255 representing a lighter slide (i.e. less of the stained parameter). Grey-levels are arbitrary units and are not calibrated for white or black as is done for percent transmission or optical density measurements.

6.2.7 Statistical analysis

Statistical evaluation of the results was assessed by an analysis of variance and LSD multiple range tests on the means and a Kruskal-Wallis test of the medians using the Statgraphics® Plus software package (P<0.05).

6.3 Results

Approximately 25% of the rainbow trout in the pilot investigation exhibited signs of pathological renal lesions (white nodules). Therefore extreme caution should be taken in the interpretation of the results.

Results from the pilot investigation appear to show variable glycogen depositions in fish fed astaxanthin (higher glycogen relative to controls), while canthaxanthin fed fish were similar to controls (Table 6.3). However, fish fed either canthaxanthin or astaxanthin had lower mean mucopolysaccharides levels compared to control fish (Table 6.3). Both total lipid and unsaturated lipids showed no distinct pattern with respect to carotenoid treatments, relative to controls (Table 6.3).
Table 6.3. Mean grey-scale values for differential hepatic histochemical staining for glycogen, total mucopolysaccharides, total lipids, and unsaturated fatty acids in rainbow trout fed diets supplemented or unsupplemented with astaxanthin or canthaxanthin. Values are expressed as means ± S.D. from 15 fish per tank (n = 1).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>5.47±21.81</td>
<td>55.24±24.75</td>
<td>51.70±17.78</td>
</tr>
<tr>
<td>Mucopolysaccharides</td>
<td>159.07±20.68</td>
<td>113.04±19.56</td>
<td>101.49±20.68</td>
</tr>
<tr>
<td>Total lipid</td>
<td>124.82±16.18</td>
<td>76.63±17.93</td>
<td>97.05±20.60</td>
</tr>
<tr>
<td>Unsaturated lipids</td>
<td>88.48±30.39</td>
<td>69.35±29.00</td>
<td>86.83±30.73</td>
</tr>
</tbody>
</table>

No statistical tests performed.

No significant differences in glycogen concentrations were observed between the various treatments (P>0.05; Table 6.4), as assessed by frozen liver sections.

Histochemical staining analysis of mean mucopolysaccharides grey-scale values revealed a statistically significant difference in concentrations. Livers of astaxanthin and canthaxanthin fed fish had significantly higher concentrations of mucopolysaccharides compared to the livers of controls (P<0.05; Table 6.4). Comparisons of mean total lipid grey-values revealed a significantly higher concentration (P<0.01) of total lipids in livers of carotenoid treated trout relative to control livers (Table 6.4).
Table 6.4. Mean grey-scale values for differential hepatic histochemical staining for glycogen, total mucopolysaccharides, total lipids, unsaturated fatty acids, glycogen phosphorylase activity, and aspartate aminotransferase activity in rainbow trout fed diets supplemented or unsupplemented with carotenoids or β-naphthoflavone. Values are expressed as means ± SEM. (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Astaxanthin</th>
<th>Canthaxanthin</th>
<th>Control</th>
<th>β-NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>135.60±6.35</td>
<td>132.02±8.23</td>
<td>140.08±5.17</td>
<td>138.94±3.44</td>
</tr>
<tr>
<td>Mucopolysaccharides</td>
<td>175.73±5.81</td>
<td>167.87±7.74</td>
<td>223.81±0.15b</td>
<td>223.17±0.48b</td>
</tr>
<tr>
<td>Total lipid</td>
<td>102.20±6.82</td>
<td>104.54±4.74a</td>
<td>172.47±1.54b</td>
<td>172.49±2.77b</td>
</tr>
<tr>
<td>Unsaturated lipids</td>
<td>45.69±8.46</td>
<td>66.11±13.04</td>
<td>69.49±7.00</td>
<td>77.71±8.32</td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>-5.45±1.42a</td>
<td>0.96±1.05b</td>
<td>2.22±2.67b</td>
<td>10.31±3.17c</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>134.53±8.16</td>
<td>131.08±10.48</td>
<td>158.56±4.61</td>
<td>155.09±7.48</td>
</tr>
</tbody>
</table>

†Values within a row sharing similar superscripts are not significantly different (p<0.05).

No significant differences were observed between the mean hepatic unsaturated lipid grey-scale values of carotenoid treated fish relative to control livers (P>0.05; Table 6.4), suggests that the significant differences in the total lipid profiles of carotenoid treated fish is due to a higher concentration of saturated lipids. However, fish treated with β-naphthoflavone had significantly less unsaturated lipids relative to the livers of astaxanthin fed fish (based on Fisher’s least significant difference, P<0.05).

Analysis of variance between the various treatments in the replicated trial revealed no significant difference in GOT grey-scale levels based on the comparison of means (P = 0.09; Table 6.4).

Results from comparisons of mean grey-scale values for glycogen phosphorylase activity revealed that livers of trout fed diets supplemented with astaxanthin had significantly higher levels of glycogen phosphorylase than those of the control fish (P<0.01; Table 6.4). Levels of glycogen phosphorylase in livers of fish fed canthaxanthin were not significantly
different from those of the control fish, but were intermediate between those of the control and astaxanthin fed fish. Livers in fish fed β-naphthoflavone had significantly lower levels of glycogen phosphorylase relative to all other treatments (P<0.01; Table 6.4).

6.4 Discussion

As the liver is suggested to be the major site for carotenoid metabolism (Hardy et al. 1990; Metusalach et al. 1996), it would seem likely that any physical effects of carotenoid supplementation would be apparent in the histology of the liver (Segner et al. 1989).

The results in the pilot study do not appear to show any trends in any of the parameters measured. In addition, lesions were observed in the kidneys of these fish, and it is possible that metabolic stresses may have impacted the outcomes of the aims of this investigation. Therefore these results were omitted from general interpretation.

As the growth of the fish under the different treatments were unaffected by carotenoid supplementation (Tables 5.3 and 5.4), this would suggest that observed differences are not due to growth or size differences between groups of fish.

Livers of carotenoid treated rainbow trout in the replicated experiment did not reveal any differences in glycogen deposition (Table 6.4). In direct contrast, Segner et al. (1989) observed higher glycogen deposition in the liver compared to their controls in Tilapia fed dietary carotenoids. This may indicate fundamental differences in the utilization of dietary carotenoids in these two species. However, Segner et al. (1989) used an arbitrary scale for the determination of glycogen content, which could have been more rigorously investigated using automated image analysis as performed in the present study. While species differences between the present study and that of Segner et al (1989) may be a true physiological effect, the observed differences are likely due to the relatively short duration
of the present trial (3 weeks) as compared to Segner et al. (1989) who investigated liver tissues after feeding Tilapia for 23 weeks. In addition, there are fundamental differences between the rearing temperatures between these studies, which may also impact on liver physiology and biochemistry, making direct comparisons difficult.

It is known from studies in rats that both astaxanthin and canthaxanthin are metabolized and form conjugates. Wolz et al. (1999) recovered glucuronide conjugates of astaxanthin in the bile from rats fed 300 mg kg\(^{-1}\) astaxanthin (Fig. 6.1), while rats fed a similar dietary concentration of canthaxanthin excreted similar glucuronide conjugates in the urine (Bausch et al. 1999). Biliary metabolites from trout and salmon fed radiolabeled carotenoids (Schiedt et al. 1985; Hardy et al. 1990; Torrissen and Ingebrigsten 1992).

Glucuronidation has been claimed to be quantitatively the most important pathway for xenobiotic elimination in fish (Clarke et al. 1991), and with the suggestion of glucuronidation of carotenoid metabolites by Sigurgisladottir et al. (1994), it is reasonable to infer that this pathway likely plays a role in carotenoid elimination in rainbow trout.

Glucuronidation is catalysed by UDP-glucuronosyltransferases mainly in hepatocytes, and is a reaction of vital importance in the detoxification and elimination of lipophilic compounds. The availability of UDP-glucuronic acid, the cosubstrate of the enzyme system, is rate limiting in glucuronidation. UDP-glucuronic acid is produced from UDP-glucose in the first step of the uronic acid pathway in the liver or kidney of vertebrates (Braun et al. 1997). Glycogen phosphorylase is an enzyme involved in substrate supply of UDP-glucuronic acid consuming processes, such as glucuronidation and ascorbate synthesis, has been shown previously (Bánhegyi et al. 1988, Braun et al. 1994). Therefore higher levels of glycogen phosphorylase, as found in the present investigations, indicates a higher level of glycogen catabolism to supply the required substrate for conjugation of metabolites. As the only dietary differences are due to carotenoid supplementation, it is
reasonable to infer that the increase in hepatic glycogen phosphorylase content is due to a higher requirement for the conjugating substrate for the elimination of carotenoid metabolites.

Thus the slight pattern of glycogen depletion, although not significant, suggests that glycogen may be used in this fashion, with canthaxanthin being degraded to a larger extent, thereby requiring more glycogen to be metabolised. The failure to find a significant difference in glycogen deposition between carotenoid treated and the control-fed fish may be due to the ample supply of substrates for de novo glycogenesis in the basal diet (common to all treatments). Thus the synthesis of glycogen may have been in excess of that required for the metabolism and glucuronidation, resulting in equivalent glycogen deposition. However, the speculative suggestion that glucuronidation plays a role in carotenoid metabolism is further supported by the observed significant differences in the levels of glycogen phosphorylase observed from histochemical staining of frozen liver sections. Glycogen phosphorylase levels of astaxanthin and canthaxanthin fed-fish were significantly higher than the control-fed fish, suggesting that glycogenolysis is occurring at a faster rate than in control fish. The lack of a significant difference between the levels of glycogen phosphorylase between astaxanthin and canthaxanthin-fed fish suggests that there is no difference in the requirement of glucose-6-phosphate in the conjugation and elimination of these two carotenoids. Indeed, the requirement is theoretically equivalent based on the similarities between their structures and the molar ratio of glucuronic acid to substrate of 1:1, assuming similar metabolites are formed in trout as observed in rats by Wolz et al. (1999) and Bausch et al. (1999) (Fig. 6.1). Biochemical analysis of glycogenolysis enzyme activities in combination with LC/MS analyses of bile from carotenoid-fed fish may further elucidate the pathways of carotenoid metabolism.
Fig. 6.1. Proposed metabolic pathway and conjugates metabolites recovered from rats fed astaxanthin. Adapted from Wolz et al. (1999).
Results appeared to show a higher total lipid deposition in canthaxanthin treated fish (Table 6.4). The observed differences in the histochemical staining of liver sections from carotenoid-supplemented trout would appear to indicate an alteration in the lipid composition of the membranes that may arise from the prevention of phospholipid peroxidation.

Carotenoids have been expected to act as potential antioxidants due to their reactivity with singlet molecular oxygen and peroxyl radicals (Burton and Ingold 1984; Palozza and Krinsky 1992). Evidence for the function of carotenoids in the prevention of lipid peroxidation has been shown by the presence of carotenoids in the microsomal membrane, which partially inhibited the loss of α-tocopherol, especially during the later phase of oxidant stress (Nakagawa et al. 1997). When lipid peroxidation is generated by membrane-bound cytochrome P450, the specific measurement of phospholipid hydroxyperoxides (PLOOH) clearly demonstrates that the presence of carotenoids provides antioxidant protection (Nakagawa et al. 1997). These PLOOH can lead to abnormality of membrane functions, and further perturbation of cellular signalling pathways (Nakagawa et al. 1997). Therefore, the potential protection offered by carotenoids may lead to beneficial effects in the liver, relating to these functions.

The higher concentration of saturated lipids stained in the livers of carotenoid-fed fish relative to control livers (Table 6.4), may thus be due to the prevention of lipid peroxidation, resulting in a higher retention of these lipids. The functional significance of this is unknown, but is possibly due to an artefactual preservation of these lipid moieties by the antioxidant role of these carotenoids. Further elucidation of these observations is required, and future investigations should corroborate biochemical analyses of enzyme activities (normalized by tissue protein content which was not possible in the present investigation) with histological data.
The increases noted in mucopolysaccharide concentrations between carotenoid fed fish relative to control fed fish was an unexpected observation (Table 6.4). As yet no literature has observed such a marked effect due to dietary carotenoid administration in fish. The antioxidant role of the carotenoids is potentially responsible for this observation, but it is more likely due to their metabolic reductions to vitamin A (Schiedt et al. 1985; Al-Khalifa and Simpson 1988; Guillou et al. 1989). Retinol functions as a cofactor in the synthesis (and it’s induction) of certain glycoproteins and mucopolysaccharides necessary for mucous production and normal growth regulation. This is accomplished by phosphorylation of retinol to retinyl phosphate, which then functions similarly to dolichol phosphate (DeLuca 1972; Deluca et al. 1977; Fig. 6.2). Induction of increased synthesis of mucopolysaccharides due to retinoic acid was also observed in cultured mouse keratinocytes (DeLuca and Yuspa 1974; Yuspa and Harris 1974). If this is the mechanism of action of astaxanthin and canthaxanthin in rainbow trout, perhaps the extent to which these carotenoids are reduced to vitamin A, has been underestimated. As the general dietary compositions were similar, and therefore supplied similar levels of vitamin A, the dietary treatments should not have produced such a marked difference, especially considering that the bioconversion of xanthophylls to vitamin A is considered to be quantitatively insignificant (Schiedt et al. 1985). Vitamin A levels were not measured in the present study, but should be re-examined in future.

The present study did not detect a significant difference in aspartate aminotransferase activity between the various treatments, both carotenoid treated fish showed higher mean concentrations relative to those of the control fish, suggesting a lower release of this enzyme from the liver. These observations appear to be inconsistent with a previous study Nakano et al. (1995), who observed lower levels of this enzyme in the serum, indicating that this enzyme was not leaking from the liver. This observation would appear to suggest
a beneficial effect of carotenoid supplementation on liver function, as noted by Nakano et al. (1995).

In conclusion, the present results suggest that both astaxanthin and canthaxanthin influence the levels of glycogen phosphorylase activity, providing the first evidence of the potential glucuronidation of their metabolites in rainbow trout. These carotenoids also appear to have significant impacts on the total lipid profiles of the liver, potentially due to their antioxidant functions. Furthermore, the significant increase in mucopolysaccharides as a result of carotenoid intake, is potentially due to provitamin A activity of these carotenoids, suggesting the metabolic reduction of these carotenoids may have been underestimated. These results, taken together suggest positive metabolic influences of carotenoid supplementation in rainbow trout. Further characterization of this response and the full biochemical impacts on liver function is required.

In conclusion, the present results suggest that both astaxanthin and canthaxanthin influence the levels of glycogen phosphorylase activity, providing the first evidence of the potential glucuronidation of their metabolites in rainbow trout. These carotenoids also appear to have significant impacts on the total lipid profiles of the liver, potentially due to their antioxidant functions. Furthermore, the significant increase in mucopolysaccharides as a result of carotenoid intake, is potentially due to the provitamin A activity of these carotenoids, suggesting the metabolic reduction of these carotenoids may have been underestimated. These results, taken together suggest positive metabolic influences of carotenoid supplementation in rainbow trout.
Fig. 6.2. A possible role of vitamin A in the synthesis of glycoproteins. Reproduced from Basu and Dickerson (1996).
CHAPTER 7

GENERAL DISCUSSION

7.0 Introduction

The wealth and breadth of the current investigations into the physiological and biochemical effects of dietary carotenoid supplementation on carotenoid utilization in salmonid fish, and with particular emphasis on the role of the liver, requires a rationale for discussion. In order to do so, the main objectives of the present research programme should be borne in mind. They are summarized as follows:

1. Establish if any differences exist with respect to tissue carotenoid distribution and accumulation between Atlantic salmon and rainbow trout.

2. Identify those tissues that demonstrate a metabolically active role in the representative salmonids.

3. Clarify the role of the liver in terms of overall carotenoid bioavailability to the fish:
   - Differences between astaxanthin and canthaxanthin hepatic uptake and clearance rates.
   - Identify the specific transport/uptake mechanism of carotenoids into the liver.

4. Determine the biochemical effects of carotenoid supplementation:
   - Are xenobiotic-metabolizing enzyme induced by carotenoids?
   - Determine the effects of carotenoids on liver histochemistry.

With the objectives in mind the results of these investigations will each be discussed in turn in relation to their influence on the current knowledge of carotenoid utilization in salmonid aquaculture.
The present results obtained for flesh carotenoid retention in both Atlantic salmon and rainbow trout were similar to previously published results (Henmi et al. 1987; Choubert and Storebakken 1996; Gobantes et al. 1997; Bjerke and Berge 2000). Henmi et al. (1987, 1989) suggested that carotenoids were bound by weak hydrophobic interactions to the actomyosin in the myotomes in the flesh. The binding is considered non-specific, with the hydroxyl and keto groups at the β-end of the carotenoid increasing the binding strength (Henmi et al. 1989). This observation has lead Torrissen and Ingebrigsten (1992) to suggest that the binding strength of astaxanthin out-competes other carotenoids in terms of deposition. However, the observed differences between trout and salmon with regard to the preferential carotenoid accumulated in the flesh, provides evidence for species-specificity in carotenoid utilization that appears to disagree with this proposed model. Astaxanthin is apparently better utilized than canthaxanthin in rainbow trout, in line with previous reports (Henmi et al. 1987; Choubert and Storebakken 1996; Gobantes et al. 1997), while the reverse is true in Atlantic salmon (Buttle et al. 2001). Therefore there must be an alternate explanation for the observed species-specificity in carotenoid accumulation.

However, autoradiographic observations by Torrissen and Ingebrigsten (1991) clearly demonstrate that recovered ¹⁴C-astaxanthin-derived radioactivity in the muscle of Atlantic salmon is mainly in the connective tissue between the myotomes. These authors ascribed this radioactivity to uncoloured metabolites. However, the preferential accumulation of these metabolites in lipid tissue has not been investigated, and should be the subject of future research.

The main observations from the tissue carotenoid accumulation studies are centered not on pigment deposition, but on identifying the underlying reasons for the observed differences in carotenoid utilization, using rainbow trout as a model salmonid fish. The higher
digestibility of both astaxanthin and canthaxanthin in rainbow trout, relative to Atlantic salmon (Chapter 3), may account for the observed differences in carotenoid retention between these species. It is generally thought that carotenoids move into enterocytes by passive diffusion (Furr and Clark 1997), and Slifka et al. (1999) contend that if this standard holds, that species differences in absorption/accumulation may be due to differences in gut luminal events such as pH diversity, gut motility and micelle formation, as well as the type and amount of dietary fat. Further to this, these authors note that species variations in lipoprotein handling are also likely to contribute to differences in the accumulation of carotenoids. From the present investigations it would appear that few differences exist between the absorptive capacities of either carotenoid within a species, as assessed by their similar apparent digestibility coefficients (ADCs, Chapter 3, Table 3.3). The similar feed compositions used in these two studies imply that dietary factors are not contributing to the observed differences, suggesting that other biological factors are responsible for observation that rainbow trout absorb dietary carotenoids more efficiently than Atlantic salmon. Further to this, the differences in the relative retention efficiencies, which take into account the species-specific ADCs, would suggest that other species-specific biological factors are affecting carotenoid utilization between rainbow trout and Atlantic salmon, and may be due to differences in lipoprotein metabolism.

Schiedt et al. (1988 a) examined the ratio of astaxanthin/idoxanthin in tissues of one Atlantic salmon fed astaxanthin and found that in the gastro-intestinal tract (intestine and the pyloric caeca) 78% of total carotenoids were comprised of idoxanthin and 22% astaxanthin. This information, combined with the observed results of the possible bioconversion of astaxanthin to vitamin A (Schiedt et al. 1985, Al-Khalifa and Simpson 1988), would appear to suggest that the intestine may be an important site for carotenoid metabolism. This was indirectly confirmed by the lower relative peak areas of astaxanthin.
and canthaxanthin in the gastrointestinal tract tissue chromatograms in Atlantic salmon (comprising approximately 60-70% of the total chromatogram area only).

The available information from the general tissue carotenoid accumulation profiles between rainbow trout and Atlantic salmon, center on the relative differences between the livers and the kidney of these two species in terms of their respective capacities to metabolize carotenoids. Higher carotenoid concentrations were observed in the livers of salmon, compared to rainbow trout, while the opposite relationship was observed in the kidney (Figs. 3.1 and 3.3). These observations, taken together with the general information garnered from the relatively lower peak chromatogram contributions of the fed carotenoids in these tissues (Figs. 3.2 and 3.4), would suggest that these organs are highly active in carotenoid metabolism. Furthermore, these results suggest fundamental differences in the utilization of carotenoids between Atlantic salmon and rainbow trout, which may explain the differences in flesh carotenoid retentions observed between these species (Chapter 3, Table 3.3).

Although the present results suggest that the liver plays a major role in the metabolism of carotenoids in vivo (Chapter 3), other tissues may also play a role in carotenoid homeostasis. Indeed, the kidney, which is also known to endocytose and metabolize LDL and HDL (Gjøen and Berg 1992, 1993 b), may also have an active involvement, as was suggested by the lower contribution of the fed carotenoid peak areas relative to the total chromatogram peak areas observed in Figs. 3.2 and 3.4. Therefore the presence of carotenoids and/or metabolites in the kidney may be due to the high level of lipoprotein catabolism observed in this tissue (Gjøen and Berg 1992). The kidney is also a very active organ in xenobiotic detoxication (Ingebrigsten 1991), and may be involved in the catabolism of carotenoids as has been observed in rats (Bausch et al. 1999). Thus future
investigations should examine the role of the kidney in carotenoid metabolism in salmonids.

While astaxanthin and canthaxanthin are not retained to a large extent in the liver of rainbow trout (Chapter 3), their lack of accumulation may be due to rapid metabolism. Indeed, the low contribution of both astaxanthin and canthaxanthin peak areas to the total chromatograms (Chapter 3), suggests the liver is highly active in carotenoid metabolism. Previous literature emphasizes the role of the liver in carotenoid metabolism (Hardy et al. 1990; Metusalach et al. 1996), and therefore the present studies were focused on determining the contribution of the liver to carotenoid disposition.

7.2 Perfusion model rationale

The single-pass perfusion model used in the present investigations was chosen because of its specific advantages as outlined by Lindros (1974), which include the provision of sampling large volumes of perfusate (inflow and outflow), a direct assessment of material balance for substrate loss across the liver, and the accrual of metabolic events at steady-state conditions. The perfused liver preparation also allows for xenobiotic removal by the liver, be it metabolic or excretory, and is a preparation that incorporates flow dynamics similar to those in the in vivo situation (Pang 1984). However, recirculating designs conform more closely to the circulation in vivo, but can potentially accumulate endogenous metabolites, which sometimes inhibit or influence organ function (Pang 1984). Because of these potential complications, this model was deemed unsuitable in the present investigations.

A drawback in the single-pass design lies in the possible depletion of cosubstrates or endogenous compounds that are repetitively "washed out" of the liver (Pang 1984). As the present investigations have utilized a tissue culture medium as the carrier vehicle, in
addition to bovine calf or piscine sera, it is unlikely that cosubstrates, essential for normal hepatic function, have been sufficiently depleted during the perfusion. To this author’s knowledge no studies have cultured fish cells with carotenoids in vitro and therefore, there is little data on the usefulness of the various vectors for use in fish cell culture. According to Martin et al. (1997), human and animal cells may be useful models for studying the accumulation and metabolism of carotenoids, however, the extreme hydrophobicity and relative instability of carotenoids in oxygen-containing environments make their delivery to cultured cells problematic. Various studies have added carotenoids to tissue culture media using organic solvents (e.g. dimethyl sulfoxide) (Amcoff et al. 1998), liposomes, water-miscible beadlets, and micelles as vehicles (Martin et al. 1996). Martin et al. (1997) investigated the suitability and stability of lipoproteins as carotenoid vectors to HepG2 human liver cells. These authors’ results indicate that lipoproteins were a stable vehicle for delivery of ß-carotene and α-tocopherol to human liver cells. Confluent cultures of HepG2 liver cells express LDL receptors and accumulate and metabolize human LDL (Dashti and Wolfbauer 1987; Javitt 1990). Martin et al. (1997) suggest that it is likely that ß-carotene accumulation from both LDL and HDL was mediated, at least in part, by receptor-mediated endocytosis. Since carotenoids, are only bound to lipoproteins in vivo in trout, it was hypothesized that fish serum containing various lipoprotein fractions with bound carotenoids would provide an ideal delivery vehicle to isolated perfused organs. In addition, portal tracts, sinusoids, and space of Disse, which are channels that facilitate material exchange in the liver, remain unperturbed in isolated perfused organs (Pang 1984). Since the Space of Disse is likely the site of lipoprotein uptake (Bruni and Porter 1965), the current model using lipoprotein fractions with bound carotenoids is as close to in vivo as possible with this preparation.
7.3 Lipoprotein uptake

An essential function of the vertebrate liver is the removal of macromolecules from the bloodstream by receptor-mediated endocytosis (Gjøen and Berg 1993), which is a process whereby cells internalize macromolecules and involves invagination of the plasma membrane and the formation of endosomes (Røsjø et al. 1994).

Gjøen and Berg (1993 a,b) found that LDL was mainly endocytosed by parenchymal cells of the liver and that the catabolism of lipoproteins (e.g. LDL) in rainbow trout appear to follow the same endocytic-lysosomal pathway described for many macromolecules in mammalian cells (Gjøen and Berg 1993 b). LDL constitutes an important physiological ligand catabolized in the liver, with the main lipoprotein class in salmonid fish, HDL, also being catabolized in both the liver and kidney (Gjøen and Berg 1992). It is hypothesized that carotenoids are taken into the cell using this process, rather than through a specific cellular carotenoid-receptor protein. However, a cellular carotenoid-binding protein has been identified in ferret liver, but does not appear to bind carotenoids with substituted β-ionone rings (e.g. astaxanthin or retinol) (Rao et al. 1997). Despite this observation, the presence of an astaxanthin/canthaxanthin-binding protein in fish should not be discounted.

7.4 Liver perfusion studies

Isolated liver perfusion studies in rainbow trout of both astaxanthin and canthaxanthin show that there are some differences in carotenoid uptake between these compounds. It became apparent that astaxanthin uptake saturates earlier than does canthaxanthin (Chapter 4), despite similar overall mean hepatic extraction ratios, and would appear to suggest that canthaxanthin is preferentially removed from circulation. This observation may provide some insight into the relative utilization of both carotenoids in vivo, whereby canthaxanthin is preferentially removed from the circulation by the liver, thus limiting the
amount available for flesh deposition. Despite these trends, no differences in carotenoid clearance were observed, which may possibly be due to the relatively short duration of the perfusion period (190 min), and the large inter-individual variability, which may mask true differences.

These relatively small differences in the plasma/serum clearance rates between astaxanthin and canthaxanthin have previously been observed in vivo, as determined by the half-lives of 24.1 and 22.2 hours, respectively in rainbow trout (Guillou et al. 1993), may be within the scope of the relative differences in uptakes of these carotenoids at the physiological perfusate concentrations investigated in the present perfused liver model. In contrast, calculated half-lives in a similar rainbow trout study by Gobantes et al. (1997) revealed a much longer time for astaxanthin of 34.8 hrs, while a shorter time of 12.6 hrs for canthaxanthin. The similar uptake rates observed between astaxanthin and canthaxanthin in the perfused liver investigations are unlikely to account for these large differences between their half-lives. More likely differences in their initial digestibility prior to absorption may account for the differences observed in the study by Gobantes et al. (1997).

For pharmacokinetic research, the first-pass effect can produce a most interesting phenomenon, a “secondary depot effect” (Kübler 1989), which can modify (especially delay) the kinetics of invasion into the central circulation very strongly (Kübler 1989) (i.e. lag phase). For restricted absorption, which is typical for higher doses of carotenoids, there is a longer lasting influx of substance carrying chylomicra over 6-8 hours. This corresponds to the passage time of the absorbing (small) intestine. This is thought to be due to the remaining depot of carotenoid in the intestinal (Kiessling et al. 1995), from which absorption occurs as long as the substance passes through the segment of the intestine where nutrients can penetrate the mucosal membrane. As a consequence of slow elimination rates from lipoprotein fractions, carotenes, caratenoids, and tocopherols
accumulate up to very high concentrations in the blood plasma (Kübler 1989). The low hepatic extraction rate observed in the present studies may account for the long retention time of these compounds in serum. Therefore steady-state plasma carotenoid concentrations reflect the intake of the substance over a longer period of time (Kübler 1989). According to Kübler (1989), the application of higher concentrations of carotenoid does not influence the blood concentration because the maximal absorption capacity is (at least nearly) reached. Thus, the only way to enhance the retention of carotenoids is by modification of the feeding regime (i.e. applicate the substance at shorter intervals). Alternatively, the metabolism of the compound of interest could potentially be inhibited via competitive inhibition.

Compounds that show low hepatic extraction ratios and are eliminated primarily by metabolism, demonstrate marked variations in overall elimination half-lives within a given population (Shargel and Yu 1999). This variation in $t_{1/2}$ is thought to be due to genetic differences in intrinsic hepatic enzyme activity. Moreover, the elimination half-lives of compounds are also affected by enzyme induction, enzyme inhibition, age of the individual, nutritional, and pathologic factors (Shargel and Yu 1999). Indeed, previous authors have noted large inter-individual variability in tissue carotenoid concentrations (Gobantes et al. 1998a), which could potentially be accounted for by the inherent genetic differences in ability to absorb and/or metabolize carotenoids. Hence, the MFO system could potentially account for these differences. As both astaxanthin and canthaxanthin have low hepatic extraction ratios (approx. 3-7% as determined by in vitro liver perfusion studies), the uptake of carotenoids into tissues could be rate-limiting in the metabolism of carotenoids, implying that the MFO systems have ample capacity to metabolize absorbed carotenoids.

In an attempt to further clarify the potential differences in carotenoid utilization, liver and kidney xenobiotic-metabolizing enzyme system induction studies were undertaken.
Results from the cytochrome P450 induction trials did not show any significant induction in MFO activity in the liver or kidney, suggesting that rainbow trout have ample capacity to metabolize the absorbed carotenoids at the dietary carotenoid levels supplemented. Alternatively, the fish used in these studies may have already been induced, masking inherent differences due to carotenoid supplementation. Despite of the lack of induction, cytochrome P450 enzyme systems may still be involved in carotenoid metabolism in salmonid fish.

Ando (1986) found that significant amounts of carotenoids were decomposed during spawning migration in Chum salmon, which he attributed to the activity of hepatic heme proteins of the liver. Because of the proximity in absorption spectra of the heme-proteins observed by Ando (1986), it is possible that these represent cytochrome P450 activity. In support of this is the observation that astaxanthin induced cytochrome P450 in rats (Astorg et al. 1994; Gradelet et al. 1996; Jewell and O’Brien 1999), and mice (Astorg et al. 1997). However, recent results by Wolz et al. (1999) indicate that cytochrome P-450 enzymes were not involved in astaxanthin metabolism in rat hepatocytes, despite the fact that recovered metabolites were glucuronide conjugates, products from phase II metabolism. However, their results did indicate that as much as 50% of absorbed carotenoid is degraded within 24 hours, indicating a very active metabolism by the liver by an as yet unidentified metabolic pathway (Fig. 6.1). The low hepatic extraction ratios in the present study led to estimates of hepatic carotenoid half-lives in the range of 10.5 for canthaxanthin and 12 hrs for astaxanthin.

The enzymes responsible for oxidation and reduction of drugs (xenobiotics) and certain natural metabolites, such as steroids, are monooxygenase enzymes known as the mixed-
function oxidases (MFOs). Hepatic parenchymal cells contain the MFOs in association with the endoplasmic reticulum, a network of lipoprotein membranes within the cytoplasm and continuous with the cellular and nuclear membranes (Shargel and Yu 1999).

The enzymes involved in the metabolism of drugs may be altered by diet, and the co-administration of other drugs and chemicals. Enzyme induction is a chemical-stimulated increase in enzyme activity usually due to an increase in the amount of enzyme present (Shargel and Yu 1999). Enzyme induction usually requires some onset time for the synthesis of enzyme protein. Evidence in rats (Gradelet et al. 1996) suggests that both astaxanthin and canthaxanthin are potent inducers of the MFO system. Despite the induction evident in rats, hepatic and renal xenobiotic-metabolizing enzyme systems did not show significant induction between carotenoid-treated (astaxanthin and canthaxanthin) and control (carotenoid-unsupplemented) trout in the present studies. Therefore differences in carotenoid utilization in rainbow trout is likely due to preferential uptake of canthaxanthin over astaxanthin, rather than preferential catabolism in the liver. Alternatively, another enzyme system could be responsible for carotenoid metabolism.

In a study by Leo and Lieber (1999), a metabolic pathway system was proposed for retinol, where β-carotene is cleaved to retinoic acid and/or retinal, which may be converted by cytochrome P450 isoenzymes to polar metabolites that are then glucuronidated (Fig. 5.1). As both astaxanthin and canthaxanthin serve as provitamin A compounds (Schiedt et al. 1985; Guillou et al. 1989; Al-Khalifa and Simpson 1988), a similar pathway as that proposed by Leo and Lieber (1999) may also be indirectly responsible for astaxanthin and canthaxanthin metabolism in salmonid fish. This hypothesis is supported by the evidence of glucuronidated metabolites of both astaxanthin and canthaxanthin in studies by Wolz et al. (1999) and Bausch et al. (1999), respectively. In addition, Boyer et al. (2000), recovered retinoic acid glucuronides in brook trout and found that metabolism through the
cytochrome P450 system was dose-dependent, increasing with increasing dietary retinoic acid inclusion.

7.6 Enzyme induction/metabolism

One possible explanation for the lack of MFO induction by carotenoids may be their structural characteristics. As stated by Gillner et al. (1985), ligands of the aromatic hydrocarbon receptor must meet strict criteria: they must be hydrophobic, planar molecules that can fit into a rectangle of 6.8 X 13.7 Å. Both astaxanthin and canthaxanthin are larger and are not planar, and thus do not meet these requirements (Weedon 1971). The observed induction by Gradelet et al. (1996) and those of Astorg et al. (1994, 1997) and Jewell and O’Brien (1999), was suggested to be due to enzymatic or oxidative cleavage products of these carotenoids which may be AH receptor ligands (Gradelet et al. 1996), although little is known about the metabolism of these compounds. The only metabolites identified conclusively to date are (rac)-3-hydroxy-4-oxo-β-ionone and (rac)-3-hydroxy-4-oxo-7,8-dihydro-β-ionone as identified in rats by Wolz et al. (1999). The metabolic pathway proposed by Wolz et al. (1999) (Fig. 6.1) may provide some insight as the cleavage of the astaxanthin molecule is not centric, and thus not likely catalyzed by 15,15’-β-carotene dioxygenase. An alternative mechanism may be a similar reduction of astaxanthin as that of eccentric cleavage of β-carotene to yield various apocarotenals (Wang et al. 1992).

It has long been known that carotenoids are substrates for lipoxidase-type enzymes (Chichester & Nakayama 1965). Lipoxygenase isolated from trout convert polyunsaturated fatty acids (PUFA) from muscle into PUFA hydroperoxides (German and Kinsella 1985), which may cause discolouration of fish flesh (Tsukuda 1972), conceivably by bleaching of carotenoids following free-radical quenching (Krinsky and Deneke 1982).
However, as yet no studies have investigated the induction of the lipoxygenase enzyme system due to dietary carotenoid inclusion.

In a study by Knight et al. (1995), the brain, gills, skin, ovary, muscle, eye, liver, spleen, heart, and gastro-intestinal tract of rainbow trout all exhibited lipoxygenase activity as evidenced by the presence of lipoxygenase-specific catabolic products. This, coupled with the observation by Kulkarni and Cook (1988) that lipoxygenase exhibits a wide range of substrate specificity, and that it can metabolize chemicals bearing diversified functions (including phenols, catechols, and hydrazine), offers an insight into its possible role in carotenoid catabolism.

According to Kulkarni and Cook (1988), the lipoxygenase enzyme system may represent an important alternative pathway to cytochrome P450 for the metabolism of endogenous chemicals and xenobiotics. Lipoxygenase activity in hepatic carotenoid metabolism has previously been proposed by Ando (1986; Fig. 7.1). Therefore, lipoxygenase may represent another important pathway for glutathione conjugate formation that could lead to detoxification of certain chemicals (Kulparni and Sajan 1997, 1999). While glutathione conjugates of carotenoids have not been identified in the literature, it may provide an alternate metabolic and excretory pathway for carotenoids. If lipoxygenases are involved in carotenoid bleaching, the interaction of lipoxygenase with various other enzyme systems (such as glutathione-s-transferase), may provide a direct excretory pathway, perhaps explaining a proportion of the observed discrepancy between the levels of absorbed versus retained carotenoids. In addition, if co-operative systems are in place, this may potentially explain why the complexity of metabolites expected in excretory tissues such as the liver and kidney have not been observed (Schiedt et al. 1985), as they are rapidly converted.
Fig. 7.1 A scheme proposed for the carotenoid metabolism in spawning-migrating Chum salmon. Circles, carotenoid-carrying lipoproteins; —, pathway proposed by Ando et al. (1985, 1986a,b); ---, pathway proposed by Hata (1978); ——, pathway proposed by German and Kinsella (1985). Reproduced from Ando (1986).
The investigation into the effects of carotenoid supplementation on liver histochemistry revealed some interesting results. Of primary interest is the significantly higher levels of recovered saturated fatty acids in the livers of carotenoid-treated trout, relative to control livers. In addition, the significantly higher levels of glycogen phosphorylase activity in carotenoid-treated fish, suggests that glycogen is being depleted in order to supply the required UDP-glucuronic acid for conjugation of what is thought to be carotenoid metabolites.

Indeed, the available evidence suggests that glucuronidation of astaxanthin and canthaxanthin metabolites, as suggested by (Bausch et al. 1999; Wolz et al. 1999) may play a significant role in their elimination. While the rates of conjugation of these two carotenoids cannot be determined from the present studies, the observed uptake profiles from the isolated liver perfusion studies (Chapter 5), appear to suggest that the earlier saturation of astaxanthin uptake, relative to that of canthaxanthin, is potentially responsible for the differences in the elimination between these carotenoids in rainbow trout as observed by Guillou et al. (1993) and Gobantes et al. (1997), where canthaxanthin is cleared at a faster rate than is astaxanthin. Further investigation is required to determine relative rates of glucuronidation between these carotenoids, and whether their degradation follows similar pathways.

The significance of the bioconversion of astaxanthin and canthaxanthin to vitamin A in rainbow trout may be underestimated in the literature. Schiedt et al. (1985) suggested that this bioconversion is quantitatively insignificant. However, as the dietary treatments used in the histochemistry studies were similar in both lipid and basal diet formulation, no differences in dietary vitamin A content would be expected. Therefore, the metabolic conversion of these xanthophylls to vitamin A may have appreciable impacts on the
induction of mucopolysaccharide synthesis, as noted by the significant elevation in mucopolysaccharides in the livers of carotenoid-fed fish. This observation requires further validation and a re-examination of the roles of carotenoids in fish health.

7.8 Antioxidant roles of carotenoids

α-Tocopherol was observed to provide selective antioxidant protection for phosphatidylserine. These results indicate that α-tocopherol may act as antioxidant protecting rod outer segment membranes from deleterious effects by a selective mechanism that diminishes the loss of docosahexaenoic acid from phosphatidylserine (Terrasa et al. 2000). It is possible that other antioxidants, such as astaxanthin and canthaxanthin, also afford selective protection from peroxidation, which may result in the preferential loss of one carotenoid over another.

In addition, some carotenoids have been shown to regulate lipid metabolism to some degree in mammals. Fatty acid Δ-5 desaturase mRNA has been shown to be regulated by dietary vitamin A and exogenous retinoic acid in rat liver (Zolfaghari et al. 2001). As both astaxanthin and canthaxanthin are provitamin A carotenoids in fish, it is possible that their metabolism into vitamin A or retinoic acid may indirectly modulate fatty acid metabolism resulting in differences in the fatty acid profiles in trout or salmon. Thus the level or rate of conversion between astaxanthin/canthaxanthin between species may account for these observed differences.

Vitamin E deficiencies in rats has been shown to increase serum and tissue concentrations of lipid peroxidation products, increase blood total and low-density lipoprotein (LDL) cholesterol levels, increase liver cholesterol concentrations and decrease blood high-density lipoprotein (HDL) concentrations (Chen et al. 1999). Similarly, carotenoids such as β-carotene have also been shown to decrease blood cholesterol and triglyceride
concentrations as well as liver cholesterol when given to spontaneously hypertensive rats (Tsai et al. 1992). The idea that lipid peroxidation could interfere with plasma lipid metabolism is supported by evidence that lipid peroxides can inhibit lipoprotein lipase activity (Wada et al. 1983). Among the mechanisms implicated from animal studies from the blood lipid-lowering effects of supplemental vitamin C, are increases in the binding and catabolism of LDL (Uchida et al. 1990). Disruption of hepatic lipoprotein metabolism at the level of cytochrome P450 could also play a role since the endoplasmic reticulum is an important organelle in lipoprotein synthesis that is highly susceptible to oxidative damage (Kubow 1998).

7.9 Vitamin E effects on carotenoid retention

Bell et al. (2000) found that a deficiency in both vitamin E and astaxanthin resulted in significant increases in the levels of recovered desaturated and elongated products of both [1-\(^{14}\)C] 18:3(n-3) and [1-\(^{14}\)C] 20:5(n-3) in isolated hepatocytes of Atlantic salmon. These observations led these authors to suggest that deficiencies in these antioxidants stimulated a conversion of fatty acids to long-chain highly-unsaturated products. These observations appear to be reciprocated in the present investigations where lower levels of saturated lipids were recovered in the liver sections from the control fish compared to rainbow trout fed carotenoid-supplemented diets (Chapter 6).

Various studies have noted interactions between astaxanthin and \(\alpha\)-tocopherol in Atlantic salmon and rainbow trout (Pozo et al. 1988; Christiansen et al. 1994, Mortensen and Skibsted 1997; Bjerkeng et al. 1999). One possible explanation for these observations may be the inhibition of CYP3A-dependent \(\alpha\)-tocopherol catabolism by astaxanthin, similar to the results observed by Parker et al. (2000) for sesamin. A hypothesis supported by the observation of elevated CYP3A (BROD) activities in liver microsomes from rats fed astaxanthin (Astorg et al. 1994). In direct contrast, Bjerkeng et al. (1999) observed that
dietary addition of α-tocopherol increased astaxanthin deposition in fillets of Atlantic salmon. Indeed, deposition was increased 8-14% in fish fed either 30 or 50 mg kg\(^{-1}\) astaxanthin by dietary addition of 800 mg kg\(^{-1}\) compared with 200 mg kg\(^{-1}\) α-tocopherol. This may indicate preferential metabolism of α-tocopherol, in effect sparing astaxanthin for tissue deposition. A likely explanation for the contradictory evidence may be mutual competitive inhibition of the enzyme system.

7.10 Future investigations

A more accurate and representative perfusion model in future investigations should employ a recirculating perfusate model as discussed above (with appropriate modifications to ensure organ viability), using a more sensitive detection mechanism such as radiolabeled carotenoid. In a recirculating perfusion system, recirculation of perfusate through the organ allows for the re-entry of substrates and metabolites into the liver for further metabolism. First, second, or multistage metabolites may emerge as a result of repetitive biotransformation processes by the liver. For this reason, the recirculating design is often employed to generate metabolites in perfusate and bile for isolation and identification purposes (Pang 1984). This design is favoured for substrates whose extractability (hepatic extraction ratio) by the liver is low; that is, only a small fraction of the substrate is removed during a single delivery through the liver (Pang 1984). The present investigations reveal a low hepatic extraction ratio (2-6%), and suggest that perfusate recirculation experiments should be developed in order to assess the rate of carotenoid degradation and subsequent identification and quantification of metabolites. Ideally this should be undertaken with radiolabeled carotenoids, and the relative radioactivity recovered from the bile and liver tissue. These experiments would offer the added advantage of determining the exact mechanism of carotenoid catabolism through the use of subcellular fractionation. While specific biliary metabolites have not been identified in rainbow trout, glucuronide metabolites from rats fed astaxanthin have been identified (Wolz et al. 1999), and similar
products may be present in the bile of rainbow trout. It should also be noted that drugs mainly excreted from the liver in the bile have molecular weights in excess of 500, while drugs with molecular weights between 300-500 are excreted both in urine and in bile (Shargel and Yu 1999). Both astaxanthin and canthaxanthin have molecular weights over 500, and are therefore both likely to be metabolized and excreted to a large extent by the liver via the biliary route.

7.11 Conclusions

While the general processes involved in carotenoid utilization are known, there is a paucity of information relating to the specific mechanisms underlying these processes. The aims of the present investigations (previously outlined) were to highlight potential differences between the two primary carotenoids used in aquaculture, namely astaxanthin and canthaxanthin, as well as potential species differences between Atlantic salmon and rainbow trout.

The present investigations have shown some differences in terms of the specific tissue accumulations between trout and Atlantic salmon. The liver and kidneys both show marked differences between species and carotenoids in terms of their relative tissue carotenoid concentrations, as well as the chromatographic profiles, which indicate the potential metabolic capacities of these tissues, with the liver playing a fundamental role in both rainbow trout and Atlantic salmon. Specifically the liver accumulated higher carotenoid levels in the Atlantic salmon compared to the rainbow trout, while the reverse was observed in the kidney tissues of these species. In addition, the rainbow trout were shown to be more efficient than Atlantic salmon in terms of carotenoid digestibility and flesh retention efficiency. Furthermore, astaxanthin was deposited at higher levels in the rainbow trout than was canthaxanthin and the reverse was observed in the Atlantic salmon.
In an attempt to determine the level of influence the liver plays in carotenoid clearance, hepatic perfusion studies were designed to investigate comparative astaxanthin and canthaxanthin uptake rates as an indicator of metabolic potential in the rainbow trout. These results appear to show that the liver potentially plays a major role in carotenoid clearance, as evidenced by the half-life estimates, which concur with the post-prandial investigations. In addition, it appears that a carotenoid-specific saturable mechanism is involved in the clearance of canthaxanthin compared to astaxanthin, which may account for the apparently better astaxanthin utilization in rainbow trout compared to canthaxanthin (Torrissen 1986, 1989; Foss et al. 1987; Storebakken et al. 1987).

While the perfusion studies were unable to determine the exact rates of carotenoid catabolism, in vivo enzyme induction studies were developed in an attempt to see whether a preferential carotenoid metabolic system plays a role in rainbow trout. Results appear to suggest that at commercially supplemented levels, the liver hepatic xenobiotic-metabolising enzyme system apparently has ample capacity for carotenoid metabolism in vivo, contrary to studies in mammalian systems. This is potentially due to the restrictions in carotenoid uptake as identified in the liver perfusion studies. Essentially, the uptake of carotenoids is regulated by the rate of lipoprotein endocytosis, as well as serum carotenoid levels. Therefore the low hepatic extraction ratio of both astaxanthin and canthaxanthin suggest that induction of enzyme systems (cytochrome P450) would only occur at supra-physiological serum carotenoid concentrations. While the cytochrome P450 enzyme system was not induced in the present studies, this metabolic pathway may still play a role in carotenoid metabolism and should be the subject of further investigation.

Further to this, the present investigations wished to determine any beneficial effects that carotenoid supplementation may have on liver metabolism as evidenced by histological and histochemical investigations. Results appear to show a slight beneficial effect, likely
due to the antioxidant capacities of these carotenoids in the liver of rainbow trout. In addition, evidence indicates that a similar metabolic pathway to that seen in rat liver is present, as evidenced by the slight elevation in glycogen phosphorylase activity. These observations seem to imply that the glucuronic acid pathway, as suggested by Sigurgisladottir et al. (1995), may be involved in the metabolism of absorbed carotenoids.

In the present investigations, astaxanthin and canthaxanthin in rainbow trout appear to show similar digestibilities (Chapter 3), and similar uptake mechanisms into the liver (Chapter 5), although astaxanthin appears to saturate faster than does canthaxanthin. The metabolic pathway for carotenoid catabolism is still unknown, however, due to the structural similarities between these carotenoids, it is unlikely that there is a carotenoid-specific enzyme system, and thus the metabolism is potentially due solely to differential saturation of the uptake mechanism. Therefore, differences in carotenoid availability for flesh deposition is regulated not by metabolism, as both carotenoids appear to be degraded to the same extent in trout (Chapter 3), but by uptake into the liver and/or kidney, in addition to potential phospholipid-catabolic specificity with which a carotenoid is associated.

In conclusion, the present investigations confirm the central role of the liver in carotenoid metabolism in salmonid fish, and suggest further research is necessary to identify the metabolic pathways that are responsible for the majority of the excretion of absorbed carotenoids. This is likely to come about through the use of radiolabeled carotenoid studies, and sub-cellular fractionation of liver tissue from carotenoid-fed fish, in addition to biochemical investigations examining enzyme systems such as lipoxygenase, cytochrome P450 monooxygenases, and glucuronidases. In terms of carotenoid pigment retention, it would appear that carotenoid uptake and subsequent metabolism via the liver does not provide the fish nutritionist with a mechanism that can be applied to enhance
pigmentation efficiency. This may not, therefore, be to the advantage of reducing pigment costs in feed manufacturing.
Appendix 1

Fig. X.1. Representative standard curve for BSA adducted fluorescamine in 96 well microplate in the extended range concentration. The fluorescence measured is best described by the quadratic polynomial \( y = 2 \times 10^{-7}x^4 + 6 \times 10^{-5}x^3 - 0.2816x^2 + 110.04x + 2407.2; r^2 = 0.99, P<0.01 \). Each value represents the mean of 6 replicates +/- SEM.
Fig. X.2. Representative standard curve for resorufin in 96 well microplate in the extended range concentration for benzoxyresorufin O-dearylase assays. The fluorescence measured is best described by a linear relationship \( y = 1967.72 + 315.54x; r^2 = 0.99, P<0.01 \). Each value represents the mean of 6 replicates +/- SEM.
REFERENCES


Braun, L., Kardon, T., Puskás, F., Csala, M., Bánhegyi, G., Mandl, J. (1997). Regulation of glucuronidation by glutathione redox state through the alteration of UDP-glucose supply


Østerlie, M., Bjerkeng, B., Liaaen-Jensen, S. (1999). Accumulation of astaxanthin all-\(E\), \(9Z\) and \(13Z\) geometrical isomers and \(3\) and \(3'\) \(RS\) optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. *Journal of Nutrition* 129: 391-398.


