GENETIC AND NON-GENETIC FACTORS AFFECTING CAROTENOID CONCENTRATION IN CATTLE TISSUES

A thesis presented for the Degree of Doctor of Philosophy
at the University of Adelaide in the Department of Animal Science

THE UNIVERSITY
OF ADELAIDE

by

Zbigniew (Zibby) Antoni Kruk
Mg.inz. (B. Ag. Sc.) Academy of Agriculture in Wroclaw, Poland
(1985)

April 2001

This thesis is dedicated to my parents Bronislawa and Mieczyslaw Kruk, my mother in-law Helena Kokot, my wife and daughters Bozena, Joanna, Natalia Kruk and to the founder of Taekwon-Do General Choi Hong Hi.

Table of Contents

List of abbreviations

ANOVA	analysis of variance
ApoE	apolipoprotein E
BC	β-carotene
BHT	butylated hydroxytoluen
BSA	bovine serum albumin
°C	temperature in Celcius
cm	centimeter
con	concentration
DNA	deoxyribonucleic acid
FCS	fat colour score
g	gram
GC	gas chromatography
h	hour
h^2	heritability
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
JJ	Jersey
KOH	potassium hydroxide
KRB	Krebs-Ringer biocarbonate buffer
l	litre
LL	Limousin
LJ	F1 cross between Jersey and Limousin
LJJJ	reciprocal cross between F1 and Jersey
LJLL	reciprocal cross between F1 and Limousin
LDL	low density lipoprotein
LUT	lutein
mg	milligram
ml	milliliter
mm	milimeter
µg	microgram
µl	microliter
µm	micrometer
min	minute
mmol	milimol

MONO	monounsaturated fatty acids
N/A	not applicable
nm	nanometer
nmol	nanomol
NaPTA	sodium phosphotungstate
N_2	nitrogen
ns	not significant
O_2	oxygen
P	probablility
PUFA	polyunsaturated fatty acids
r	correlation
Ri	ribs
rpm	rotation per minute
Ru	rump
SAT	saturated fatty acids
SD	standard deviaiton
SE	standard error
sec	second
Sh	shoulder
spec	spectrophotometry
TEM	transmission electron microscope
UV	ultraviolet
VLDL	very low density lipoprotein
v/v	volume by volume
v/w	volume by weught
yob	year of birth

Index of Figures

Index of Tables

Abstract

Yellow fat colour in cattle is caused by the accumulation of carotenoids in the adipose tissues. Since yellow fat is considered undesirable by consumers, more than $18 million is lost to Australian producers annually because carcasses are downgraded due to excessively high fat colour scores.

Genetic and non-genetic factors affecting fat colour in cattle were examined in biopsy and carcass samples of Jersey and Limousin cattle and in their F1 and backcross progeny. The animals used in the study comprised non-lactating cows managed on pastures, as well as weaners and young animals ~600 days old, raised on pastures and/or finished on a grain diet for 70-250 days. The results demonstrate that such environmental factors as geographical location (South Australia vs New Zealand), yearly changes in pasture growth, rainfall (1994-1998), diet (pasture vs grain) and season (summer vs winter) affected carotenoid content in various cattle tissues. Animals raised in New Zealand had yellower fat and more carotenoid in their adipose tissue than animals in South Australia. The concentration of carotenoids in fat was also positively associated with rainfall in both locations. However, seasonal changes did not affect the fat carotenoid concentration although there was a significant difference in the blood concentration. Feedlot animals had significantly lighter fat colour than pasture fed animals.

Other non-genetic factors had less effect on carotenoid concentration. Age did not influence fat colour in long term pasture fed cows, although age was positively correlated with carotenoid content in blood. Interestingly, animals born earlier in the year had higher carotenoid content in fat at slaughter than the animals born later in the year. Sex had no influence on fat colour.

On the other hand, genetic factors did affect carotenoid concentration in various cattle tissues. Breed had a significant impact on fat colour with Jersey and their crosses being yellower than other breed combinations. Sire influence on the variation in fat colour within the breeds was also significant. This was particularly pronounced in the Jersey cattle from New Zealand.

The study of heritability of fat colour in cattle demonstrated that this trait is lowly heritable. However, higher estimates of heritability in Jerseys demonstarate that there is more additive genetic variation in fat colour in Jerseys than in Limousins. Statistical analysis of genetic effects on fat colour recognized additive and epistatic effects as significant.

Studies of β-carotene in cattle tissues showed significant breed differences in their concentration in chylomicrons, high density lipoproteins, and adipose tissues. Moreover, Jersey cattle had a larger average adipocyte diameter and a higher number of adipocytes per gram of fat than Limousin cattle.

From these results, two models are proposed. The first hypothesizes that fat colour in cattle is controlled by a major additive gene with moderate frequency in Jerseys. The second hypothesis is that Jersey and Limousin cattle differ in the level of the enterocyte 15,15'-dioxygenase enzyme activity. Consequently, the activity of this enzyme may be the over-riding factor differentiating these breeds in fat colour and the major gene controlling β-carotene metabolic pathway.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Zbigniew A. Kruk

April 2001

Acknowledgements

It is with great pleasure that I acknowledge the assistance of all those who made it possible for me to complete this thesis.

First and foremost I would like to thank my supervisors, Professor Cynthia D.K. Bottema, Dr Wayne S. Pitchford and Dr Peter Speck for their guidance, trust, criticism and friendship during all of my years of study. In particular, I would like to thank to Cynthia for giving me a chance to work for her and then for accepting me as her student. Professor Bottema is an excellent example of a true, multi-skilled, very knowledgeable, open-minded scientist, available for scientific discussion at any time, with no hesitation of putting on gumboots and chasing cattle if it were necessary. I regard the time I spent in her laboratory as the most valuable in my scientific career. If I had not met Professor Bottema, my life definitely would taken a different path.

Many thanks to Dr Pitchford for his patience, understanding, and all of his help with statistical analyses. The large number of hours spent on discussions about the correct statistical model were very challenging and beneficial in my understanding the complicated world of statistics. These years of work with Wayne helped me to realise that a quick answer to a question is not always a good answer and the more time you put into thinking about an answer, the lower the probability of making a fool of yourself. Of course, there are some exceptions.

Although the communication with Dr Peter Speck, my external supervisor was not always easy due to his commitments, jobs and distance, he had an important input into many aspects of my project. I would like to acknowledge Peter for his influence on the experimental work, title of my thesis and his constantly repeated encouragement to complete my thesis ASAP. I can clearly see the value of his advice particularly, at this stage of my life when submitting the PhD at the age of 40.

I wish to thank my "unofficial" supervisor Dr Brian Siebert who introduced me to the very complicated and at the same time fascinating field of fatty acids. He dedicated much time and effort teaching me various techniques and correcting my papers and thesis at short notice. Brian's expertise in this area helped me to look broader on the association of carotenoids with fatty acids and gave me many ideas, which I will use in future work.

My appreciation is expressed to my friends Drs Sebely Pal and Andrew Thomson. Sebely and Andrew spent their valuable time teaching me how to separate blood lipoprotein fractions and successfully run protein gels. I would like also to thank them for their criticism regarding my ideas and work. Sometimes my discussions with Andrew were a bit emotional and upsetting, however, they were a driving force for me to prove my point or agree with his.

The completion of my thesis would not be possible without a special atmosphere created by my colleagues at work. I would like to thank Jan Cook, Helena Kojevnikoff for their friendship and being able to provide any technical help during my work in Cynthia's lab. I would like to also thank my all fellow students Dr Enoch Malau-Aduli, Toby Hughes, David Rutley, Michelle Fenton, Sara Townsend, Elke Stephens, who helped me in any aspects of my work from answering questions, preparation presentations, from making posters to going out for a drink. I really appreciate their presence. Many thanks are directed to Dr Dave Tivey and Natasha Penno for their help in video image analysis of lipoproteins and adipocyte morphology and Dr Terry Knight from New Zealand for collaboration in the project regarding the influence of vitamin A on carotenoids in cattle blood.

All work with cattle for this thesis was performed with the assistance of Mr Tony Weatherly and Mr Mick Deland. They taught me how to handle cattle, collect blood, perform biopsies and collect abattoir measurements. I appreciate their help especially, in that these procedures differed a lot from those practiced in Poland.

Special thanks must go to the J.S. Bequest Fund. Without their financial assistance, my work would not be possible.

Finally, I would like to thank all the people to whom the thesis is dedicated. Firstly, I acknowledge parents Bronislawa and Mieczyslaw, who through their hard work on their dairy farm taught me how to be an honest and hard working person. At that time, spending all school holidays working on the farm was not a pleasant exercise. However, I can see the value of this work at present. I am eternally grateful for the love and support of my wife Bozena and daughters Joanna and Natalia. During my study and the writing of this thesis, they had to sacrifice a great deal to create a comfortable atmosphere for my work and took over a number of tasks normally designated to me. I appreciate the help of my mother-in-low Helena who very often took care of our daughters when my wife worked and I was away due to my study, sampling, conferences etc. I thank her also for her financial assistance every time when required.

Last, but by no means the least, my deep appreciation goes to the man who had a very strong influence on these last 20 years of my life. General Choi Hong Hi, the founder of Taekwon-do, has been an example and a strong inspiration to me. Assisting him in a number of seminars all over the world I learned that big goals could be achievable by even a small person through full dedication, hard work and seriousness. The five fundamental tenants of the art he created (courtesy, integrity, perseverance, self-control and indomitable spirit) became my policy not only as a Taekwon-do student but also as a researcher.

Chapter 1

Literature review

Introduction

The name 'carotenoids' refers to a group of naturally occurring pigments, ranging from yellow to red in colour, which are found in all plant and animal kingdoms. They are synthesised by some bacteria, algae and higher plants. Recently, they have attracted the attention of a number of scientists because of their putative role in reducing risk of some types of cancer (Davison et al., 1993; Ziegler, 1993), having an anti-stress effect (Hasegawa, 1993), and the prevention of cardiovascular disease (Gaziano and Hennekens, 1993) and HIV infection (Coodley et al., 1993). In cattle and sheep, deposition of these pigments in adipose tissue results in yellow fat colour which is considered undesirable by consumers. Consequently, there is a significant loss of profit for Australia, because of the downgrading or rejection of such carcasses from the beef market (Browne, 1993).

Fat colour is affected by environmental factors such as diet, yearly and seasonal changes, processing methods (Walker et al., 1990), and can be improved through grain feeding. However, some animals, even after a long period of time on low carotenoid diets, still have excessively yellow fat (Strachan et al., 1993).

It has been reported that factors such as age, sex, and breed also have an impact on the fat colour of cattle and sheep (Morgan et al., 1969; Walker et al., 1990; Hayes et al., 1995). Baker et al. (1985) presented evidence for a genetic basis of yellow fat colour in sheep, hypothesising that it is inherited as a recessive trait. The between- and within-breed variation in fat colour of cattle also suggests that fat colour in cattle may have a genetic basis, although to date there is no strong evidence to prove this theory.

1.1. Overview of physical and chemical properties of carotenoids and their actions

1.1.1. Historical development of carotenoid science

The first carotene was isolated by Wackenroder from the root of carrot *(Daucus carota L.)* in 1831. Six years later, Barzelius named the yellow pigments found in autumn tree leaves "xanthophyll", and Fremy and Stokes demonstrated that these pigments also occur in the green leaves. However, the lack of precise and sensitive methods for the detection and separation of carotenoids caused stagnation in this field for almost a century. The first description of chromatographic adsorption analysis, published in 1906 by the Russian botanist Tswett, proving the inadequacy of older methods for carotenoid separation became a turning point in the research in this field.

In the early eighteen nineties, Willstatter and Mieg presented the percentage composition and molecular formula of carotene and demonstrated a connection between carotenoids and isoprene. As a consequence of this work, many analytical extraction procedures and empirical formulas were developed for lycopene, lutein, fucoxanthin and bixin between 1900-1927. The occurrence of two isomeric forms of bixin was discovered by Herzig and Faltis in 1923 and gave a new light to carotenoid research. Another important step that revolutionised the carotenoid field took place in 1930-31 when Kerrer and co-workers demonstrated the symmetrical structure of β-carotene and established its function as vitamin A precursor. This work dominated the carotenoid field until 1950, and resulted in the development of a technical process for vitamin A production on a manufacturing scale. Total synthesis of β-carotene was reported in the same year independently by Karrer and Eugster, Inhoffen and co-workers, and Milas. The Inhoffen's method is the basis for the industrial synthesis of β-carotene, which has been produced on a commercial scale since 1954. The number of known carotenoids also increased to approximately 80 during the 1950's (Kerrer and Jucker, 1950). Twenty years later, Isler (1971) reported that the number of known individual carotenoids had passed 300. At present, constant development of laboratory techniques and equipment, such as high performance liquid chromatography (HPLC), mass spectrometry (MS), resonance Raman spectroscopy and nuclear magnetic resonance spectroscopy (NMR) have allowed more than 600 carotenoids to be distinguished with approximately 60 having vitamin A potency (Olsen, 1989). Because of the abundance and diversity of carotenoids in nature as well as their association with many important medical issues related to human health and well-being, carotenoids are regarded as an important interdisciplinary field of research including chemistry, biochemistry, medicine, genetics, biology and physics.

1.1.2. Definition, nomenclature and classification of carotenoids

Carotenoiods, according to Paul Kerrer's definition (which was approved by the International Union of Chemical Science during the 14-th IUPAC Conference in London in 1947), are "...yellow to red pigments of aliphatic or alicyclic structure composed of isoprene units linked so that the two methyl groups nearest the centre of the molecule are in positions 1:6 whilst all other lateral methyl groups are in positions 1:5; the series of conjugated double bonds constitutes the chromaphoric system of the carotenoids". However, the increasing number of newly identified carotenoids which were not adequately described by this definition, and frequent usage of trivial names which were not always in accordance with the principles of nomenclature in organic chemistry, have necessitated the refinement of the existing definition. In 1965, a committee consisting of leading scientists on this field was formed by the IUPAC Commission on Nomenclature of Organic Chemistry (CNOC) and the IUPAC-

IUB Commission on Biochemical Nomenclature (CBN) which revised the carotenoid rules. In 1966, the new definition and guidelines for the nomenclature of carotenoids were presented (IUPAC Commission on Nomenclature of Organic Chemistry and the IUPAC-IUB Commission on Biochemical Nomenclature, 1974) which states that term 'carotenoids' refers to a class of hydrocarbon (carotenes) and their oxygenated derivatives (xanthophylls) which are distinguished by the following features:

(i) generally they are composed of eight 5-carbon isoprenoid units;

(ii) the units are arranged in such a way that the two methyl groups nearest the center of the molecule are in position 1:6, while other side chain methyl groups occupy the 1:5 position;

(iii) the long skeleton of conjugated C=C double bonds can be modified by hydrogenation, dehydrogenation, cyclization, oxidation, or any combination of these processes along the central chain or at the ends of the molecule;

(iv) all carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure (Figure 1.1); and

(v) some components of the class may arise from certain rearrangements of the carbon skeleton or by the removal of part of this structure

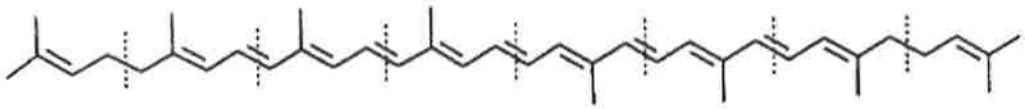


Figure 1.1. Basic acyclic $C_{40}H_{56}$ structure. All carotenoids are derived from this structure. Dotted lines separate eight isoprenoid units of the structure.

The variations in carotenoid structure and properties have created systems of sub-classification. Chemically, two classes can be distinguished: (i) carotenes made up of carbon and hydrogen; and (ii) oxycarotenoids, which also contain oxygen. Subdivisions of this second class would be (a) epoxy, (b) furanoxyl, (c) hydroxy or xanthophylls (monols, diols, polyols), (d) metoxy, (e) keto, (f) esters, etc.

A different classification system subdivides the carotenoids into (i) acyclic, (ii) monocyclic, and (iii) bicyclic. According to their function, carotenoids also can be divided into: (i) provitamins A or vitamin A precursors, (ii) vitamin A precursors, which also function as animal tissue pigmenters, (iii) animal tissue pigmenters without vitamin A activity, and (iv) compounds which do neither function (Bauernfeind, 1972).

Another classification system divides carotenoids into four main groups: (i) carotenes (C_{40}), (ii) xanthophylls, (iii) apocarotenoids and norcarotenoids with less than 40 carbon atoms in the skeleton, and (iv) carotenoids with more than 40 skeletal carbon atoms (Liaaen-Jensen, 1971). An example of numbering and structures for some carotenoids are presented in Figure 1.2.

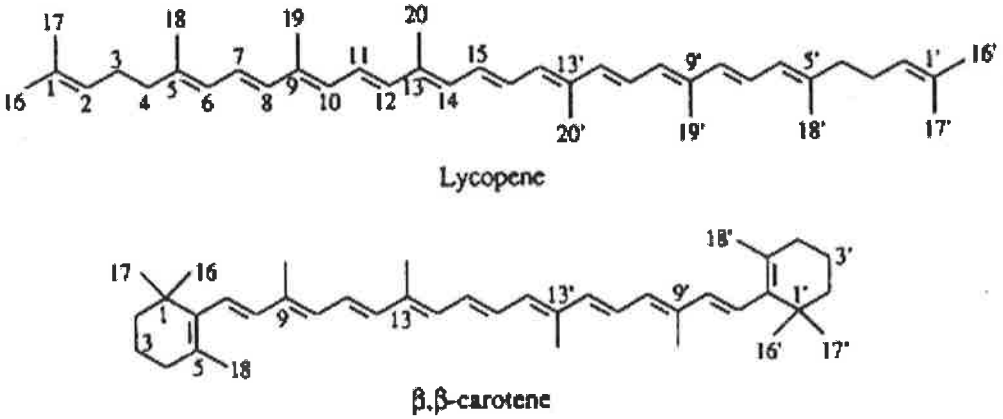


Figure 1.2. Structure and numbering of carotene stem molecule. According with the new rules, all individual names are based on the stem name 'carotene' which are specified by Greek-letter prefixes. To avoid confusion, both end group prefixes are used and cited in alphabetical order. The first prefix is separated from the second by a comma, and the second is connected to the stem name by a hyphen. Changes in hydrogenation level and the presence of oxygen-containing substituents are indicated by the standard prefixes and suffixes (hydroxy, methoxy, carboxy, oxo, aldehyde, and epoxy) used in organic chemistry. Higher carotenoids with C_{45}-C_{50} carbon atoms are named as mono- or di-substituted C_{40} carotenoids. Some carotenoids have a structure consisting of less than 40 carbon atoms, derived by the loss of a fragment(s) from the C_{40} skeleton. If these compounds have been lost from one or both ends of a carotenoid, they are called apocarotenoids. If the loss occurs within the chain, they are called norcarotenoids.

1.1.3. Synthesis, mode of occurrence and natural sources of carotenoids

More than 600 distinct carotenoids have been identified in nature (Olsen, 1989), with estimated global production at 10^8 million tonnes per year (Isler, 1971). They are synthesized by some bacteria, plants and algae. In higher plants, carotenoids are present in the chromoplasts, cell walls and in the plastids as filaments or crystals (Bauernfeind, 1972). In fruits, they are attached to proteins. In humans and other mammals, carotenoids are obtained from the diet and may be dissolved in fat.

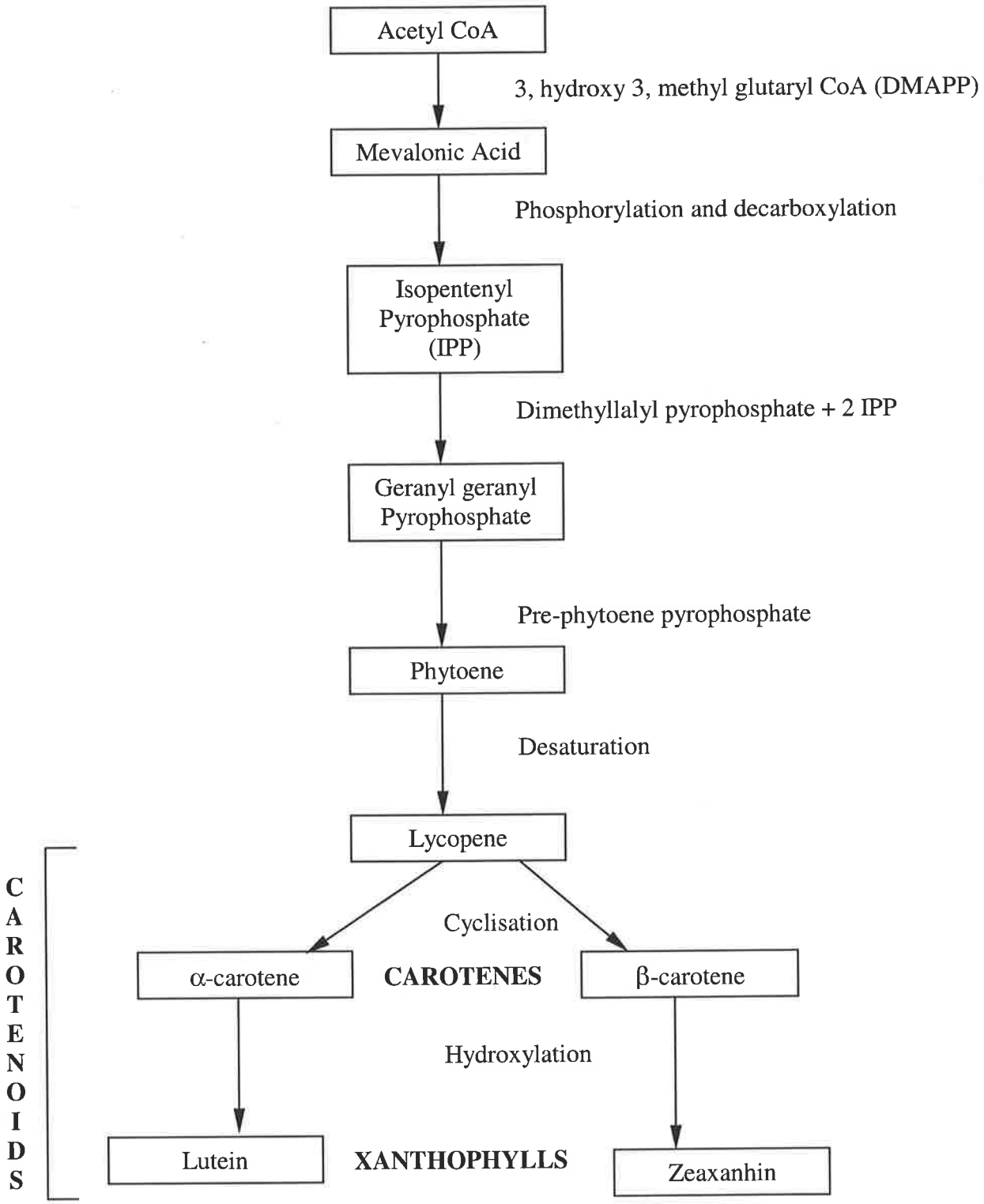


Figure 1.3. Biosynthetic scheme for some carotenoids (Bauernfeind, 1972).

Carotenoids are responsible for the colour of many fruits and vegetables (e.g. citrus, paprika, and carrot), flowers (eg. *Narcissus*), birds (eg. flamingos, ibis, and canary), insects (e.g. lady bird), and marine animals (e.g. crustaceans) (Isler, 1971, Pfander, 1992). The most abundant naturally occurring carotenoids are fucoxanthin (5,6-epoxy-3,3',5'-trihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one-3'-acetate) which is the characteristic pigment of

many marine algae, lutein (β,ε-carotene-3,3'-diol), violaxanthin (5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) and neoxanthin (5',6'-epoxy-6,7,-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol) which are major carotenoids found in green leaves, and β-carotene (β,β'-carotene) and zeaxanthin (β,β'-carotene-3,3'-diol) which are found in small concentrations in plants but are very widely distributed (Singh and Cama, 1975).

Acetate is the starting compound in carotenoid biosynthesis. Two molecules of acetyl-CoA condense to form acetoacetyl-CoA, which condenses again with acetyl-CoA, producing 3-hydroxy-3-methyl glutaryl-CoA. Through a reduction step, 3-hydroxy-3-methyl glutaryl-CoA mevalonic acid (MVA) is formed, which is further decarboxylated to form isopenyl pyrophosphate (IPP). Animals are able to condense three of the isoprene units to produce farnesyl pyrophosphate (C_{15}), which then dimerises to form squalene (C_{30}). However, animals cannot produce tetramers of the isoprene units, and consequently, are incapable of *de novo* synthesis of carotenoids (Davison et al., 1993). Plants (and some fungi) can isomerise IPP to dimethylallyl pyrophosphate (DMP) and produce a tetramer, geranylgeranyl pyrophosphate (GGP). GGP, in turn, is dimerised to form phytoene (Figure 1.3), the basic 40-carbon acyclic carotenoid structure (Bauernfeind, 1972).

1.1.4. Stereochemistry of carotenoids

Carotenoids can adopt a great number of conformations due to the isomerism of the C=C double bonds and rotation of the C-C single bonds in the polyene chain. β-Carotene, for instance, contains nine double bonds in its polyene chain, and can, theoretically form 272 isomers. α-Carotene can form 512 isomers (Bauernfeind, 1972; Olson and Krinsky, 1995) and lycopene can form 1056 (Pfander, 1992). Thus, the total possible number of carotenoids in this class reaches about 200,000 (Olson and Krinsky, 1995). Geometrical configuration around double bonds in accordance to the position of the substituent groups is called E/Z izomerisation, and is usually equivalent to *trans* and *cis* forms (Figure 1.4).

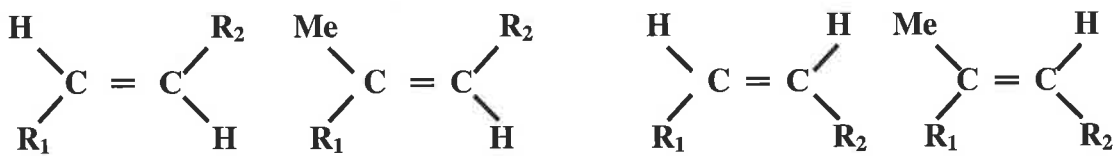


Figure 1.4. *cis* and *trans* double bonds in carotenoids.

The *cis* isomers are thermodynamically less stable than the all-*trans* forms because the *cis* double bond creates greater steric hindrance between nearby atoms of hydrogen or methyl groups. Consequently, most carotenoids in nature occur predominantly in the all-*trans* forms (Britton, 1995). This considerably reduces the number of naturally occurring isomers. For example, in the case of lycopene the number is reduced from 1056 to 72 (Pfander, 1992). All isomeric transformations of the all-*trans*-carotenoids to their *cis*-forms decreases the intensity of the pigment colour in solution, decreases the melting point, causes absorption at shorter wavelengths in the visible region of the spectrum, and causes the appearance of a new maximum (termed 'cis-peak'). Each additional double bond in the polyene chain increases the λ_{max} by 7-35 nm. (Kerrer and Jucker, 1950; Weddon, 1971; Frank, 1993).

1.1.5. Size, shape, solubility and stability of carotenoids

The size and shape of carotenoids is important in relation to their properties and functions. Generally, the all-*trans* carotenoids are extended molecules which allow them to exist in a particular preferred low-energy state. Cyclization, shortens the length of the molecule, increases the concentration of the end groups, and increases the space the molecule occupies. As a consequence of cyclization, the ability to fit into a subcellular structure may be altered (Britton, 1995). The *cis*-isomers, have less tendency to crystallise and aggregate, which can significantly influence their solubility and stability.

Generally, carotenoids are soluble in CS_2, benzene, chloroform, oils, and are very sparingly soluble in methanol and ethanol. Practically, they are insoluble in water, acids and alkalies. Ultraviolet and visible light, temperature, and atmospheric oxygen rapidly degrade carotenoid structures. To prevent degradation the following laboratory conditions have to be applied: use of dark containers with a small air-surface exchanges, low temperature, and a nitrogen atmosphere or usage of radical trapping agents, such as BHT and vitamin E (Scita, 1992).

1.1.6. Carotenoid interactions with other molecules

In vivo, carotenoids are commonly located in membranes. They are incorporated into the phospholipid bilayer and play an important role as membrane based protective antioxidants (Britton, 1995). Their localization and orientation within the membranes restricts carotenoid action and affects the properties of the membranes. For example, β-carotene and lycopene, which remain entirely within the hydrocarbon inner part of the membrane, are able to react efficiently with radicals generated in the hydrophobic part of the membrane. Zeaxanthin, whose the end groups extend into the aqueous environment, is able to effectively react with free radicals generated in the hydrophobic and aqueous phases. Generally, the presence of

any carotenoids within the membranes can affect the thickness, strength, and fluidity of the membranes, which in turn, affects the properties of the membranes as a barrier to water and permeability to other molecules (Subczynski et al., 1991). In plants, it has been proposed that β-carotene plays a physiological function in the fluidisation of chloroplast membranes during chilling stress of the photosynthetic apparatus (Strzalka and Gruszecki, 1994).

Carotenoids react with some proteins creating carotene-protein complexes. These complexes increase the stability of carotenoids, change their maximum absorption spectra, and allow them to be transported and function in an aqueous environment. Carotenoproteins have been isolated and purified from plants, cytoplasmic membranes of cyanobacteria, and some marine invertebrates. Recently, a carotenoprotein from fresh rat liver was successfully isolated and partially purified (Lakshman and Chitua, 1992).

1.1.7. Biological activities of carotenoids

Three different categories of biological activities of carotenoids can be distinguished: functions, actions, and associations (Bendich and Olson, 1989) (Figure 1.5).

Functions of carotenoids may be described as essential roles that they play under certain defined conditions. The absence of the carotenoid, therefore, leads to impaired physiological capability and possibly to death.

Actions are considered as physiological or pharmacological responses to the administration of carotenoids, which may be either beneficial or adverse but not essential for physiological well-being.

Associations are defined as correlations between carotenoids and some physiological or medical events that may or may not have a casual relationship

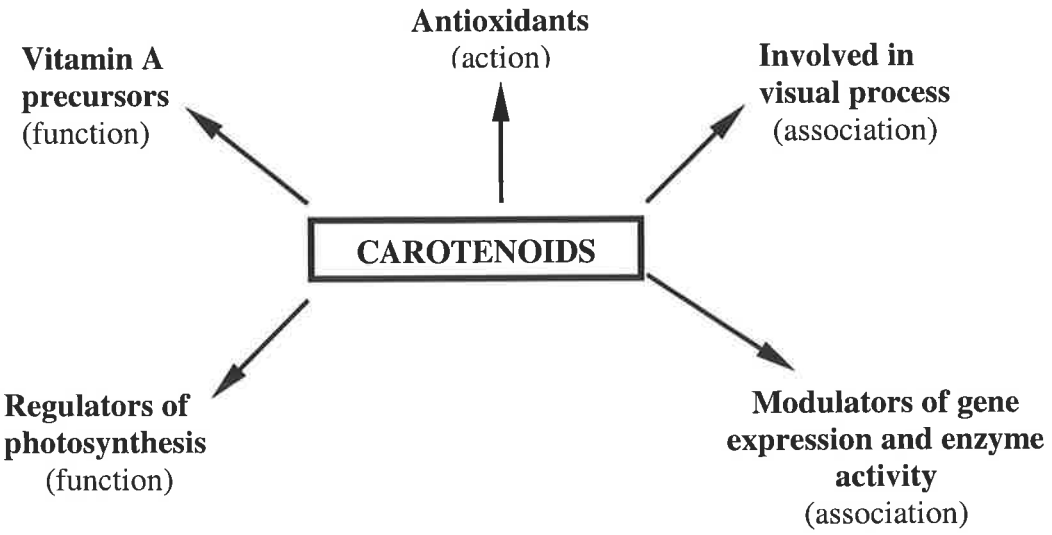


Figure 1.5. Major biological activities of carotenoids.

1.1.8. Antioxidant function of carotenoids

Pure carotenoids are susceptible to oxidation and may be broken down in the presence of even trace amounts of oxygen. The mechanism of this process is based on the ability of these pigments to quench $^1O_2$ (singlet oxygen) and is related to the number of conjugated double bonds (Palozza and Krinsky, 1992). Singlet oxygen interacts with other molecules through chemical reactions or energy transfer reactions which lead to the deactivation of singlet oxygen (Stahl and Sies, 1993). Carotenoids are the most efficient biological quenchers of $^1O_2$ and play an important role in protecting cells against DNA breakage, lipid peroxidation and enzyme inactivation. β-Carotene is reported to be a very effective scavenger of free radicals in organic solutions, especially, at comparatively low O_2 pressure (Burton and Ingold, 1984) where a single molecule may quench up to 1000 singlet oxygen molecules before becoming oxidised (Liebler, 1993). Although β-carotene effectively acts as an antioxidant *in vitro*, its function on *in vivo* systems has been recently questioned (Crabtree and Adler, 1997). The concentration of carotenoids in mammalian tissues is usually much lower compared to the model systems and may not be sufficient to provide antioxidative protection (Britton, 1995). To fully prove the antioxidant capability of carotenoids, more research is required in this area, such as establishing an appropriate animal model, developing a better understanding of the metabolic pathways for antioxidant action and analysing antioxidant catabolites not only in blood plasma but also in the urine and faeces.

1.1.9. Carotenoids as vitamin A precursors

Vitamin A (retinol) is a required nutrient for vertebrates and some invertebrate species, and can be synthesised from its precursors, the carotenoids (Figure 1.6). Of the more than 600 carotenoids identified in nature, about 50-60 have provitamin A activity. However, β-carotene is the major precursor (Underwood, 1984; Olson, 1986). The provitamin A activity of the other carotenoids varies and is less than that of β-carotene: α-carotene (50-54%), γ-carotene (42-50%), and citranoxanthin (44%). Lutein, astaxanthin, capsorubin, and bixin are inactive (Bauernfeind, 1972).

Enzymatic central cleavage of β-carotene by the enzyme carotene 15,15'-dioxygenase takes place in the intestinal mucosa and the liver, and produces two molecules of retinal (Goodman et al., 1967; Singh and Cama, 1974; Singh and Cama, 1975). This enzyme is distributed in various herbivorous and carnivorous species with the exception of the cat in which it is absent (Larkshman and Chiuta, 1992). However, the enzymatic cleavage of β-carotene is still controversial and another excentric cleavage pathway has been proposed (Wolf and Phil,

1995). This pathway is predominant in ferret intestine where retinoic acid is biosynthesised *in vivo* from β-carotene (Hebuterne et al., 1996) (Figure 1.6).

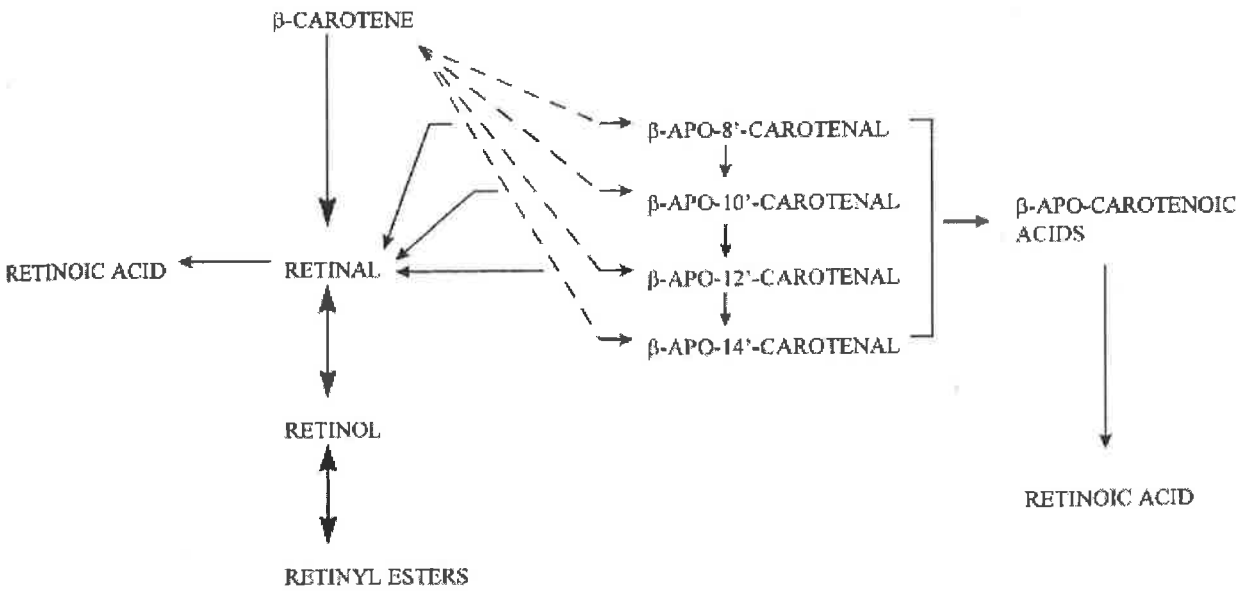


Figure 1.6. Possible cleavage mechanisms of all-*trans*-β-carotene.

1.1.10. Carotenoids and visual process

There is significant association between high intakes of carotenoid rich diets and lowered risk of cataracts. Through the quenching of singlet oxygen, lutein and zeaxanthin, present in the eye, may reduce the oxidative stress on lens proteins in humans (Bendich, 1993). Vitamin A also plays an important role in vision. The availability of vitamin A depends on the efficiency of conversion of β-carotene (1 mg of β-carotene=1667IU of vitamin A) (McDowell, 1989). Main factors affecting conversion of β-carotene to vitamin A are genetics (species, breed, strain), type of carotenoid, production level of animal, etc. A particle of vitamin A produced from the carotenoid is oxidised to its aldehyde in the eye and joins with proteins called opsons. This forms a visual pigment which consists of 11-*cis* retinoaldehyde-opsin complex. When a photon of light strikes the retina, the 11-*cis* retinoaldehyde in the opsin complexes undergoes a series of transformations that result in release of the all-*trans* form from the opsin. This breakage initiates a chain of events which result in the transmission of an impulse up to the optic nerve (Pitt, 1971; Olson, 1986) (Figure 1.7).

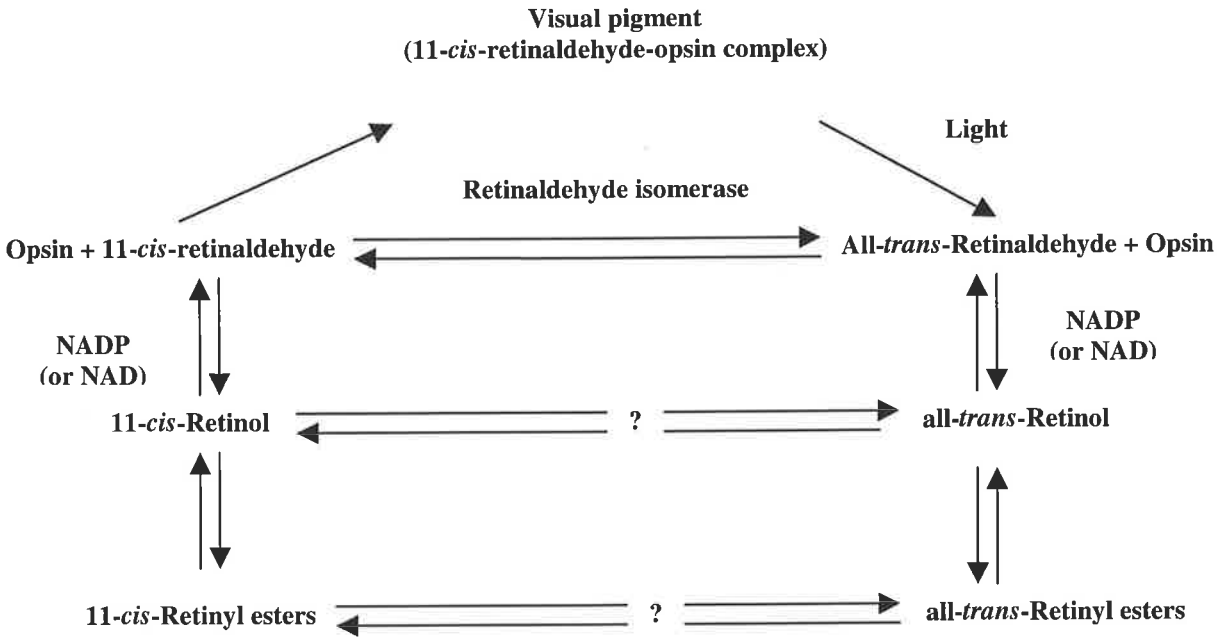


Figure 1.7. *Cis-trans* isomerisation cycle of retinoaldehyde in vertebrate eyes.

1.1.11. Carotenoids – modulation of gene expression and enzyme activity

Recently, it has been found that carotenoids may also have other biological functions including modulating gene expression and enzyme activity (Bendich, 1993). β-Carotene and cantaxanthin can inhibit genes which encode the message for production of connexin 43, a protein which is responsible for gap junction intracellular communication (Bertram, 1993). Vitamin E does not have this capacity, which suggests that the inhibition of the gene expression is independent of antioxidant activity. Retinoic acid activates the gene upon binding the retinoid receptor. However, since β-carotene does not bind the retinoid receptor, the carotenoid effect on gene expression is most likely to be independent of provitamin A activity.

Lipoxygenases are enzymes which use polyunsaturated fatty acids as substrates. They are found in plants and a wide variety of mammalian cells. β-Carotene inhibits soybean lipoxygenase in the absence of lipid substrate. This suggests that another function of carotenoids is to modulate the enzymatic activities of lipoxygenases (Parker et al., 1993).

1.2. Mammalian metabolism of carotenoids

Mammalian species do not synthetise carotenoids and are dependent upon carotenoids derived from the diet. Their bioavailability for ruminants depends on factors such as stability within the feed, forage preparation, digestibility, etc. The stability of carotenoids in feed varies and declines with storage as a result of a slow process of oxidation and polymerisation.

The rate of decline depends on temperature, amount of exposure to air and sunshine, and original content. For instance, hay stored under average conditions can lose 6-7% carotenoids per month, but when the temperature exceeds 19°C, the loss may reach 18% per month and more. Ensiling process generally preserves carotenoids, and the losses are lower than in hay (Bauernfeind et al., 1971).

In plants, carotenoids are mainly associated with membranes and lipoproteins (Olson, 1986). As a consequence, carotenoid digestibility varies and depends on many factors such as the month of forage harvest, type of forage, species of plant and the dry matter content of the forage. For dairy cattle, the digestibility of carotenoids has been estimated at 77.7% (Wing, 1969; McDowell, 1989).

1.2.1. Destruction and absorption of carotenoids in the rumen and the other parts of gastro-intestinal tract

There are contradictory reports on the destruction and absorption of carotenoids in the rumen. King et al. (1962) have reported significant loss in the recovery of carotenoids following *in vitro* incubation of β-carotene with rumen fluid. The loss of β-carotene during 9 hours of incubation exceeded 30%. The putative destruction of carotenoids in the rumen could be due to plant lipoxygenases which have the ability to bleach carotenoids. Other experiments (Larsen et al., 1993) have shown a rapid decrease in carotenoid concentrations when rumen fluid was incubated with an oxygenase *in vitro*. However, the addition of polyunsaturated fatty acids was essential. Addition of soya flour to bovine rumen had the same bleaching effect of the pigment and the extra enrichment of polyunsaturated fatty acids was not needed. It is possible that only a small part of the carotenoid content in the rumen would be exposed to the plant lipoxygenases. However, the long transit time in the rumen could possibly bleach most of the plant pigments (Larsen, 1993).

Keating et al. (1964) found no destruction of β-carotene in the rumen, unless potassium nitrate (KNO_2) was added. However, such a high level of nitrate (0.25%, 0.5% and 1.0% of rumen liquor) would never be found under usual feeding conditions.

Carotenoid content in the rumen is a consequence of the feeding history, which varies between species as a result of dietary preferences. In an experiment conducted by Yang et al. (1992), cattle, goats, and sheep were grazed on the same pasture. The total carotenoid contents in the rumen of different species varied significantly (cattle-217μg/g dry weight, goats-310μg/g, and sheep-348μg/g). Notably, the profile of the major carotenoids was

identical in each species. Lutein was the major component and β-carotene accounted for 7-9% of total carotenoids in the rumen. Generally, in the other parts of gastro-intestinal tract the concentration of total carotenoids tended to increase with passage (Yang et al., 1992). This suggests that carotenoids are poorly absorbed between the rumen and the small intestine.

1.2.2. Absorption and transformation of carotenoids in small intestine

The small intestine is believed to be the main site of absorption of carotenoids. Sheep injected with radio-labeled β-carotene in the large intestine have no sign of radioactivity in the blood, liver, and kidneys (Tucker et al., 1967). When carotenoids are injected intravenously, they are rapidly detectable in these tissues and organs.

After emulsification in the stomach, dietary carotenoids, retinal, and retinyl esters are incorporated into mixed bile salt-containing micelles. This enables the molecules to cross the water layer and interact with brush border of epithelial cells of the intestinal mucosa (Goodman and Blaner, 1984; Olson, 1986). Studies with *in vivo* rat-everted gut sacs have shown that the micellar solutions significantly enhance uptake of β-carotene and retinol over emulsions (El-Gorab et al., 1975). The *in vivo* and *in vitro* linear relationship between carotene and retinol concentrations in the perfusate with their absorption rates indicate that these components are likely to be absorbed by passive diffusion (Hollander, 1981).

Absorption of carotenoids varies and in humans has been reported to be between 5-50% (Blomhoff, 1992). Factors which influence the efficiency of absorption of carotenoids in humans include the level of dietary fat intake and carotenoid intake. A very low intake of dietary fat or an increased carotenoid intake will lower the absorption (Blomhoff, 1994). Absorption of pure β-carotene is better than β-carotene from food (Bauernfeind, 1972).

In rat studies, such factors as the level of vitamin E and water soluble vitamins, the source of protein, quantity and nature of dietary fat, emulsifiers, antioxidants, bile, age, tyroxine, and thiouracil can influence the conversion and utilisation of β-carotene (Bauernfeind, 1973). McDowell (1989) found that, in cattle, there are breed differences in absorption of carotene. The Guernsey and Jersey breeds absorbed carotene more readily, whereas Holstein was an efficient converter of β-carotene to vitamin A.

Once within the enterocytes, carotenoids are metabolised by oxidative cleavage to retinal, retinoic acid and β-apocarotenoids (Olson and Krinsky, 1995). The enzyme involved in cleavage of carotenoids is carotenoid-15,15'-dioxygenase and has been found in the liver, intestine and other organs (Olson, 1986). In some species (such as the rat, pig, goat, sheep, rabbit, and dog), almost all carotene is cleaved in the intestinal mucosa (McDowell, 1989). Tissues such as kidneys, and corpus luteum can also convert β-carotene to vitamin A, but these are less efficient than the intestine or liver (Sight and Cama, 1975). All remaining carotenoids, which have not been converted to vitamin A in the intestinal enterocytes, are packed together with cholesterol, apoproteins, and phospholipids to form chylomicrons (Shepard and Packard, 1987). After being processed in the Golgi complex, chylomicrons leave the enterocytes through exocytosis and are transported via the mesentric and thoracic ducts into the blood stream (Blomhoff et al., 1992).

1.2.3. Transport of carotenoids in the blood stream and their deposition

In the circulation, chylomicrons are catabolised rapidly (Shepherd and Packard, 1987). Some components are partly absorbed and some are exchanged between different blood fractions, thus producing chylomicron remnants (Goodman et al., 1984; Olson, 1986). The high density lipoprotein particles (HDL) are the main source of apolipoprotein C and E for the chylomicrons. Apolipoprotein C activates the enzyme lipoprotein lipase (LPL) which hydrolyses the triglyceride core of chylomicrons. This process liberates fatty acids, glycerol from the core and renders much of its surface coat. About 50% of the released components circulate within the HDL density interval. As chylomicron delipidation proceeds, the particles shrink and produce the so-called 'remnants' which are absorbed by the liver (Shepherd and Packard, 1987). What happens to carotenoids in the circulation is not fully understood.

In cattle, the main carotenoids present in the blood stream are β-carotene, α-carotene, γ-carotene, and lutein (Patterson, 1965; Yang et al., 1992). β-Carotene is approximately 60% of total carotenoids (Hidiroglou et al., 1986). The richest carotenoid blood fraction in cattle is HDL which contains 77-83 % of these pigments (Schweigert et al., 1987; Yang et al., 1992). In humans, LDL carries most of the carotenoids with β-carotene forming about 15-30% of total serum carotenoids (Davison et al., 1993). In sheep, β-carotene has not been detected in the blood. The main carotenoids present in sheep are xanthophylls (lutein, lutein-5,6-epoxide and flavoxanthin) (Hill, 1962; Payne and Twist, 1966; Crane and Clare 1975).

Carotenoids are deposited in various tissues. In humans, they are present mainly in the adipose tissue (80-85%), liver (8-12%), and muscle (2-3%) with smaller amounts in corpus luteum (60μg/g) and adrenal glands (20μg/g) (Bendich and Olson, 1989; Davison et al., 1993). The distribution of carotenoids in cattle organs is similar to humans. They can be detected in the liver (Hidiroglou, 1986), adipose tissue (Yang et al., 1992), blood plasma (Blomhoff, 1994), milk (Chanda, 1950; McGillivary, 1960; Schweigert et al., 1987), meat, kidney and corpus luteum (Gawienowski et al., 1974). After rats were injected with β-carotene, high concentrations were found in the lungs, liver, adipose tissue, pancreas, muscles and testis (Yamanushi and Igarashi, 1995).

1.3. Association of carotenoids with environmental, physiological and biochemical parameters in cattle

1.3.1. Carotenoids, diet and season

Intake and deposition of carotenoids in cattle is highly associated with diet and season. Grain products, common in ruminant nutrition, contain highly variable concentrations of carotenoids (Bauernfeind, 1972). Grains are also lower than green forages in β-carotene (Huber, 1988). In feedlot cattle, a decrease in β-carotene level of blood plasma has been reported and this level reached 17% of total carotenoids, whereas on pasture, β-carotene accounted for 63% of total carotenoids (Hidiroglou, 1986). Serum concentrations of β-carotene and retinol increase significantly in the late spring and summer months when fresh green pasture is available (Cetinkaya and Ozcan, 1991). Seasonal changes in the carotenoid concentration of milk also occur (Schweigert et al., 1987).

1.3.2. Carotenoids and HDL particles

High density lipoprotein (HDL) is the major blood fraction in cattle which transports carotenoids. Feeding polyunsaturated lipids alters the properties of the HDL and increases the concentration of β-carotene in the blood. In the experiments conducted by Ashes et al. (1982), cows were fed diets supplemented with protected sunflower oil-seed rich in linoleic acid, a polyunsaturated lipid. This resulted in a higher proportion of big HDL particles containing more β-carotene. Subsequent experiments (Ashes et al., 1984) showed that feeding cows with polyunsaturated lipids protected against rumen degradation, increasing the concentration of β-carotene in the blood but not in the milk.

1.3.3. Carotenoids and fatty acids

Zhou et al. (1993) have found a significant relationship between the concentration of carotenoids in the subcutaneous adipose tissue of cattle and some fatty acids. Total carotenoid concentration was significantly correlated with the ratio of *cis* mono-unsaturated to saturated fatty acids in subcutaneous fat ($r=0.63$; $P<0.01$). This ratio also affects fat hardness, so increased carotenoid concentration of adipose tissue is correlated with a reduction in fat hardness.

Correspondingly, a positive correlation between the total *cis* mono-unsaturated fatty acids and total carotenoid concentration also has been observed ($r=0.66$, $P<0.01$). Oleic acid (C18:1c), which is the major component of the *cis* monounsaturated fatty acids, was similarly correlated with total carotenoid concentration ($r=0.68$; $P<0.01$). The saturated fatty acids showed a negative correlation with total carotenoid concentration ($r=-0.64$; $P<0.01$).

1.3.4. Carotenoids, fertility and mastitis

The high concentrations of β-carotene (60μg/g of tissue weight) in bovine corpus luteum and the ability of the corpus luteum to synthesise retinal suggests that carotenoids may have a physiological significance in reproductive functions (Gawienowski et al., 1974). Jackson (1981) in his experiments with three groups of winter-housed Friesian cows has shown that, when plasma β-carotene levels declined, there was a direct correlation with the length of time on the supplemented diet and time of conception. The high plasma carotene concentration is not a good indicator for conception in high-yielding dairy cows (Ascarelli et al., 1985). However, under certain conditions, it can be a limiting factor. A similar conclusion has been drawn by Block and Farmer (1987). They stated that β-carotene may only improve the fertility of a dairy herd when it is the most limiting factor. In their work, β-carotene and vitamin A status of a herd were negatively correlated to calving interval and to the number of matings per conception.

Experiments conducted with supplemented β-carotene have shown no differences between the control and experimental groups of dairy heifers in the duration of standing oestrus, the length of oestrus cycle, plasma progesterone and luteinizing hormone (LH) concentrations, and the interval between LH peak and ovulation (Folman et al., 1979). Similar experiments performed on dairy cattle have shown that supplementation of β-carotene did not (i) improve reproductive efficiency, (ii) alter luteinizing hormone, progesterone, insulin, glucose, or glucagon in blood plasma, or (iii) affect the number of somatic cells in milk (Bindas et al., 1984).

Mastitis in Holstein cows has been associated with blood plasma levels of β-carotene and vitamin A. Cows with lower levels of these components had higher California Mastitis Test Scores than cows with greater β-carotene and vitamin A concentrations. The low concentrations of vitamin A and β-carotene in plasma, thus, influences the severity of mastitis in Holstein cows (Chew et al., 1982).

1.4. Carotenoids and fat colour

Subcutaneous adipose tissue is the major depot of carotenoids in mammalian species. Humans accumulate 80-85% of these pigments in this tissue (Davison et al., 1993). The major carotenoids found in cattle adipose tissue are β-carotene and lutein (Strachan et al., 1993). β-Carotene is the major carotenoid present but lutein accounts for nearly 20% of the total carotenoids (Yang et al., 1992). In sheep, lutein and lutein-5,6-epoxide have been found. However, there are differences in opinion about whether flavoxanthin or auroxanthin is a third major carotenoid in sheep fat (Hill, 1962; Payne and Twist, 1966; Crane and Clare, 1975).

The accumulation of carotenoids in the fat of cattle and sheep causes the yellow colour of adipose tissue (Payne and Twist, 1966; Crane and Clare, 1975; Kirton et al., 1975; Strachan et al., 1993; Yang et al., 1992, 1993; Zhou et al., 1993). Yellow colour is commonly evaluated in carcasses on a 4-point (Hidiroglou et al., 1986), 6-point (Walker et al., 1990), 9-point (Forest, 1981), 10-point (Strachan et al., 1993), or 11-point (Browne, 1993) scale and is a major criterion in the export and domestic markets (Walker et al., 1990; Hopkins, 1993; Hayes et al., 1995). Carcasses with excessive yellow colour are downgraded or even rejected from the market. This leads to a significant loss of profit for Australia (Walker et al., 1990; Yang et al., 1992).

The Japanese market is one of the main export markets for Australia. In 1990, 200,000 tonnes of beef and veal were exported to Japan, which accounted for 27% of the total Australian beef and veal export. However, 42% of grass-fed and 8% of grain-fed carcasses were unacceptable. Four percent of the carcasses were rejected because of undesirable fat colour (Browne, 1993). An estimation made by the Livestock and Meat Authority of Queensland showed that in 1989 about 10% of export carcasses had been downgraded due to yellow fat colour. If a reduction of 50% in the incidence of yellow fat was made, $9.2 million would be saved annually (Hayes et al., 1995).

This problem also appears in the sheep industry. Approximately 1 in 1000 New Zealand lamb carcasses are rejected from export market because of excessively yellow fat colour. As reported by Kirton et al. (1975), the 27,000 carcasses rejected from export caused a loss of $220,000 for New Zealand. The occurrence of yellow fat in Merino crossbreed sheep has been reported in Australia by Payne and Twist (1966). Kirton et al. (1975) proposed that rejection of carcasses would be overcome if those who are responsible for marketing of meat could develop a market where yellow fat was the preferred colour.

1.4.1. Correlations between methods of assessing fat colour

Generally, there are three methods of assessing the intensity of fat colour on carcasses: laboratory, subjective, and objective methods. Subjective and objective methods are most common. The accuracy of subjective measurements (using a scoring system) depends on the assessor's skills and the physical environment, whereas objective measurements using special equipment give more uniform estimates (Gaunt et al., 1994).

Walker et al. (1990) have reported a relatively low correlation between objective measurements done by chromameter and subjective assessments of fat colour using a 6-point scale ($r=0.46$). A higher correlation has been reported by Gaunt et al. (1994) where a subjective AUS-MEAT® assessment showed a correlation of $r=0.69$ ($P<0.001$) with chromameter measurements. The correlations for the P8 and rib sites were $r=0.64$ and 0.69, respectively ($P<0.001$). The same group reported a lower correlation between subjective and objective measurements in intermuscular fat ($r=0.52$, $P<0.01$). The correlation between visual score and analytical values for the amount of total carotenoids in sheep carcasses was high ($r=0.85$). However, a visual score of the subcutaneous fat on the live animal, after a small incision through the pectal skin was lowly correlated ($r=0.17$) with the visual score after slaughter (Karijord, 1978).

1.4.2. Association of fat colour, carotenoids and fatty acids

Fat colour is positively correlated with carotenoid pigments deposited in the fat tissue. Strachan et al. (1993) have shown a positive correlation between β-carotene and fat colour score in the subcutaneous and intermuscular fat in cattle ($r=0.53$ and 0.52 respectively). A significant correlation ($r=0.79$; $P<0.01$) between total carotenoids in fat tissue and objective measurements of yellow colour assessed by chromameter was also reported by Zhou et al. (1993). In sheep, Kruggel et al. (1982) have reported a significant correlation between fat colour of ram and wether lambs and lutein concentration ($r=0.52$).

Zhou et al. (1993), also found a significant relationship between yellowness of bovine adipose tissue and fat hardness. The correlation of r=0.73 indicates that yellower fat in cattle is softer. This relationship has also been reported by Kruggel et al. (1982) in sheep. A negative correlation between the firmness of fat in ram lambs and lutein concentration was found (r=-0.66).

Fatty acids are positively correlated with the objective measurement for fat (r=0.70; P<0.01) (Zhou et al., 1993). Oleic acid (C18:1), for example, was correlated with yellow fat at r=0.72. The reverse correlation existed for the saturated fatty acids and fat colour (r=-0.72) and the *trans* fatty acids (eg. C18:1t) and fat colour (r=-0.61).

1.4.3. Genetic and non-genetic factors affecting fat colour

Diet

Diet influences fat colour in cattle by causing a higher deposition of β-carotene in grass fed cattle and milk vealers than in feedlot cattle (Walker et al., 1990). Craig et al. (1959) reported that less yellow pigment was found in fat when more grain was fed in the ration. Similar results demonstrating an effect on the fat colour of Brahman cross steers by grain feeding has been shown by Strachan et al. (1993). However, the same authors (Strachan et al., 1993) reported that, even after 105-175 days of grain feeding, some Brahman cross steers still had relatively high fat colour scores. Cazes et al. (1990) have reported an effect of diet on fat colouration in sheep. There were more carcasses with yellow fat colour when animals were fed maize than when fed wheat or winter barley.

Season

Season has an impact on fat colour as well, mostly through diet. The level of β-carotene in the blood, although positively correlated with yellowness of adipose tissue in cattle (r=0.61; Strachan et al., 1993), has seasonal fluctuations (McGillivray, 1960). Hayes et al. (1995) reported more yellow fat on animals slaughtered in the dry season than in the wet season (P<0.01).

Carcass temperature

As reported by Morgan et al. (1969), fat colour intensity changes slightly from 3 to 48 h *post mortem* but increases during the first 3 hours of chiller storage. Fat colour in the freezer (-5°C) is less yellow than in the chiller (8°C). The highest objective value is at room temperature. However, the benefits of this method for improving fat colour do not balance

other negative effects caused by chilling, such as the decreased tenderness of meat (Hayes et al., 1995).

Age

Fat colour increases with age. Walker et al. (1990) have reported that the objective assessment of fat colour in cattle was similar between the dentition categories 0/4, 0/5, 2-7, but higher for cattle with eight incisors. This trend was noticed in both sexes. Earlier experiments conducted by Morgan et al. (1969) also showed that the colour intensity of fat is closely related to animal age.

Sex

Contradictory reports exist about influence of sex on fat colouration. Morgan et al. (1969) reported negligible fat colour differences between heifers and steers. On the other hand, Walker et al. (1990) demonstrated such differences between females and steers British breeds across all dentition categories. Hayes et al. (1995) reported that steers and heifers had more yellow pigment in fat than the bulls.

Sex related differences in fat colouration have been found in sheep. Subcutaneous fat tends to be yellower for rams than for wethers (Field et al., 1989). Also differences between pigment accumulation of ram lambs and wether lambs have been reported. The higher accumulation of lutein from alfalfa in ram lambs results in yellower carcasses (Kruggel et al., 1982). Yellow fat in heavy lambs can be reduced by castration when lambs are younger than 60 days of age (Kreggel et al., 1982).

Breed

Fat colour in cattle is influenced by breed. Morgan et al. (1969) confirmed that Jersey had yellower fat than Friesian and Aberdeen Angus breeds. Jersey crossed with Friesian, Charolais and Hereford breeds were intermediate in fat colour. The dairy steers, in general, had more carcasses with yellow coloured fat than the beef cattle (Barton, 1968; Walker et al. (1990).

Work in Brahmans and its crosses (Hayes et al., 1995) indicates that Brahman, Brahman x Hereford x Shorthorn, Africander x Hereford x Shorthorn and Hereford x Shorthorn had higher fat colour scores than progeny of F_1 Charolais x Brahman bulls and Africander x Hereford x Shorthorn dams.

1.4.4. Genetic basis of fat colour

There is strong evidence that the occurrence of yellow fat in sheep has a genetic basis. The hypothesis proposed by Baker et al. (1985) is that a single recessive gene causes the yellow fat colour and the yellow phenotype is due to the homozygous recessive genotype (gg). This hypothesis is based on breed differences in Norwegian sheep where the Rygja, Spealsau and Pelssau breeds have the lowest incidence of yellow fat and the Cheviot and Steiger breeds have the highest incidence. These authors also have estimated the gene frequency of lambs with yellow fat for the Cheviot breed is q=0.14.

Hill (1962) has proposed a hypothesis that modern yellow fat sheep have inherited an inability to oxidise xanthophylls, and like some primitive sheep who are deficient in the enzyme xanthophyll oxidase, may be atavistic in possessing the yellow fat. According to Karijord (1978), however, there is more than one gene coding for these enzymes which split carotenoids. Kirton et al. (1975) estimated the heritability for yellow fat colour in sheep at 0.18, based on half-sib analysis of 33 sires (Romney, Southdown, Poll Dorset, English Leicester, Lincoln and Dorset Horn).

Experiments on monozygotic twin-mates have shown that there is a marked genetic influence over the carotenoid and vitamin A metabolism within the Jersey breed (McGillivray, 1960). These findings agree with Morgan et al. (1969), who concluded that within-breed selection, cross-breeding and breed replacement can effectively reduce fat colour. The variation in the intensity of fat colour between cattle breeds also suggests that fat colour in cattle may have a genetic basis (Hayes et al., 1995), The variation in the intensity of fat colour and metabolism of β-carotene and vitamin A suggest that fat colour in cattle can be under genetic control, although the mode of inheritance is unknown.

1.5. Summary of existing gaps in knowledge

A survey conducted to identify the causes for carcass rejection prepared for the Japanese market has shown that unacceptable subcutaneous fat colour accounted for 4% of the total causes and appeared mostly in grass-fed cattle (Browne, 1993). Pastures rich in carotenoids provide high concentrations of these pigments for cattle, but the variation of their absorption has not been explained fully (Wing, 1969).

Conflicting reports exist about destruction of carotenoids in the rumen (King et al., 1962; Keating et al., 1964; Larsen et al., 1993). The lack of detailed information on the role of bile salts and polar lipids in the solubilization and absorption of carotenoids (El-Gorab et al., 1973; El-Gorab et al., 1975) does not clarify the issue about carotenoid metabolism in the digestive tract of ruminants. Relatively little is known about the efficiency of intestinal absorption of carotenoids, their conversion to vitamin A (Blomhoff, 1994), the mechanism of their action (Poor et al., 1993; Yamanushi and Igarashi, 1995) and the transport of carotenoids by the different blood lipoprotein fractions in cattle (Schweigert et al., 1987).

A low correlation between carotene concentration in plasma and fat suggests that plasma level is not a useful predictor of fat colour (Hayes et al., 1995). No correlations have been established between fat colour and the main blood subfractions carrying carotenoids in cattle.

Morgan et al. (1969) and Hayes et al. (1995) have reported a number of cattle breeds differing in fat colour, but there are no reports about Limousin, Wagyu, South Devon or Belgian Blue. The same authors also reported that the crossbreeds have intermediate fat colour. However, these reports only concerned Jersey crosses.

In light of this lack of available data, the research herein focused on four main aspects:
(i) the quantity and quality of carotenoids in fat of various cattle breeds and the suitability of commonly used extraction protocols (chapter 3);
(ii) the genetic and non-genetic factors affecting fat colour in Limousin, Jersey and various cross-bred cattle (chapter 4);
(iii) the genetics of fat colour including heritability, mode of inheritance and quantification of genetic variation (chapters 5); and
(iv) the metabolism of β-carotene and other carotenoids including their transport, deposition, absorption and association with other physiological and biochemical parameters of the animal (chapter 7).

Chapter 2

Materials and methods

2.0. Introduction

Materials and methods which were used to obtain and interpret the data for this thesis can be assigned into four major groups: animals and management (2.1), sample collection and preparation (2.2), sample analysis (2.3), and statistical analyses (2.4). Each of these groups can be sub-divided into smaller sections (Figure 2.1).

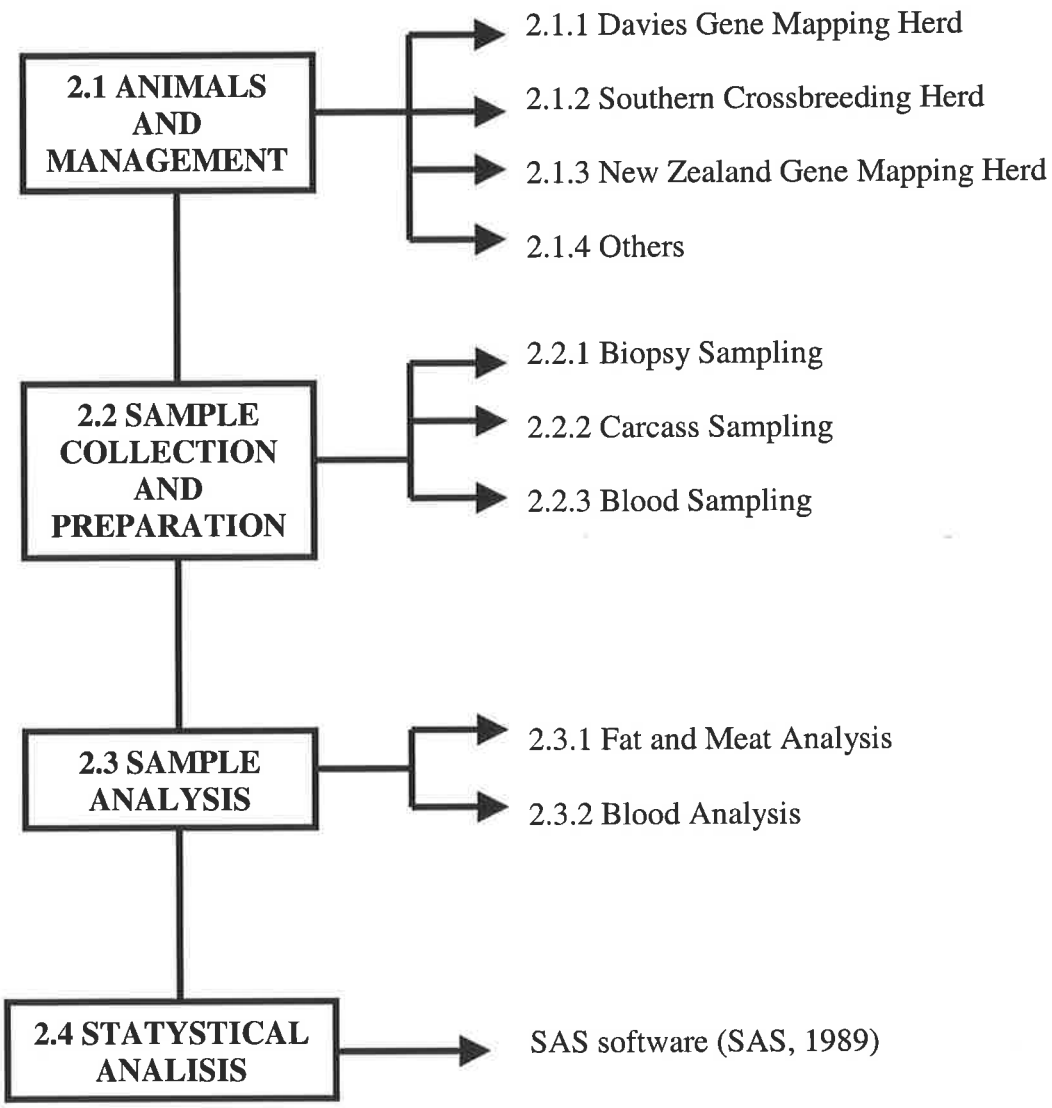


Figure 2.1. Materials and methods.

2.1. Animals and management

Three cattle herds were used to provide samples for this study: the Davies Gene Mapping Herd and the Southern Cross-Breeding Herd, both in Australia, and the Ruakura Gene Mapping Herd in New Zealand. Sporadically, some small animal groups or samples from individual animals were collected by biopsy or from abattoirs in New Zealand or South Australia (mainly SAMCOR in Adelaide or METRO MEAT in Murray Bridge).

2.1.1. The Davies Gene Mapping Herd

The Davies Gene Mapping Herd was a backcross design to study the mode of inheritance of important cattle traits and to map major genes controlling these traits. The herd was maintained at the Martindale property near Mintaro in South Australia. It comprised 143 pure Jersey (J) and 84 pure Limousin (L) cows representing a great number of genotypes from over 70 sire lines in each breed. The cows were mated to 2 Jersey and 2 Limousin bulls to produce pure Jersey (J), pure Limousin (L) and Jersey-Limousin (LJ) F1 progeny. Four Jersey-Limousin (LJ) and 2 Jersey (J) bulls were selected and mated with the Jersey (J) and Limousin (L) cows to produce ¾ Jersey (LJJJ), ¾ Limousin (LJLL) and pure Jersey (J) progeny (Figure 2.2). The animals were maintained under the same management system and grazed on pastures throughout the year with some supplement of hay from January to March.

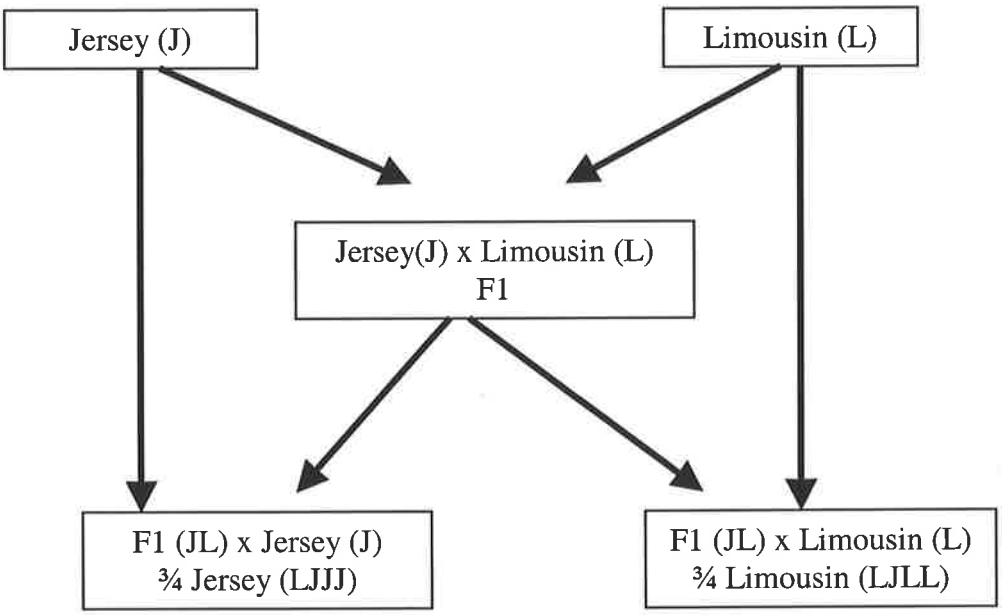


Figure 2.2. Backcross design of the Davies Gene Mapping Herd.

The progeny was weaned at 250 days of age and at the age of approximately 500 days entered a feedlot. After 60-170 days in the feedlot, the animals were slaughtered and a number of carcass traits was measured.

2.1.2. The Southern Crossbreeding Herd

The Southern Crossbreeding Herd was maintained at Struan property near Naracoorte, in south-east of South Australia. The herd was designed to quantify breed differences in a number of carcass traits and to estimate the suitability of particular breeds for different beef markets. It comprised approximately 600 mature Hereford cows which were artificially

inseminated with semen from seven sire breeds (Angus, Belgian Blue, Hereford, Jersey, Limousin, South Devon, Wagyu). Each sire breed consisted of 3-4 sires per year resulting in approximately 12 progeny per sire. The animals were maintained under the same management system and grazed pastures throughout the year with some hay supplement between January and March. Male crossbreed progeny were castrated after birth. All F1 males and females were weaned at about 250 days of age and at approximately 500 days the calves entered a feedlot. Heifers remained in the feedlot for 60-100 days whereas steers were left for 140-170 days. At the end of the lot feeding, the animals were slaughtered and a number of samples and measurements were collected.

2.1.3. The New Zealand Gene Mapping Heard

The New Zealand Gene Mapping Herd was designed as a replica of the Davies Gene Mapping Herd from South Australia. The aim was to map major genes controlling important cattle traits as well as to study the genotype by environment interactions. Semen from 3 selected crossbred (LJ) bulls from Martindale was used in Ruakura NZ to artificially inseminate pure Jersey (J) and pure Limousin (L) cows and produce ¾ Jersey (LJJJ) and ¾ Limousin (LJLL) progeny. The animals were raised on pastures, weaned at approximately 250 days of age and slaughtered at about 500 days of age, after finishing on grass. Meat and fat samples were collected and a number of measurements were made at weaning.

2.2. Sample collection and preparation

Samples for various measurements were collected from live animals using a biopsy technique (fat, meat, blood) or were obtained at slaughter from the kill floor or the chiller at the abattoir.

2.2.1. Biopsy sampling

Biopsy fat and meat samples were obtained at weaning (approximately 250 days of age). In the two consecutive years, 1995 and 1996, pure Jersey (J), pure Limousin (L), and Jersey-Limousin (LJ) F1 crossbreeds were sampled, whereas in 1997, meat and fat samples were available from pure Jersey (J), ¾ Jersey (LJJJ) and ¾ Limousin (LJLL). In 1998, there was no pure breed progeny and only backcross weaners were sampled. The detailed procedure for sampling has been described by Malau-Aduli et al., (1997) with the exception of the sampling site. The fat and meat samples were obtained from the eye muscle area between 12^{th}-13^{th} rib in 1995-1996, and fat was collected from base of the tail in 1997 and 1998. Samples were snap-frozen in liquid nitrogen, flushed with N_2 gas, then transported to the laboratory where they were stored at–20°C until required for analyses (Appendix 1).

2.2.2. Carcass sampling

Fat from carcass adipose tissue was dissected from the eye muscle area, (12^{th}-13^{th} rib), on the kill floor 5-10 min after slaughter or from the chiller within 24 hours. Samples were placed in 25 ml plastic containers, snap-frozen in liquid nitrogen, then transported to the laboratory and stored at –20°C. Slices of muscle, 1-2 cm in thickness were cut from 12^{th}-13^{th} or 10^{th}-11^{th} rib as a cross-section from the *M. Longissimus dorsi.* The samples were stored at –20°C in sealable plastic bags until analysed. Liver from two Jersey steers was acquired 10-15 min after the slaughter from the kill floor, processed, and stored at –20°C.

2.2.3. Blood sampling

Blood was drawn from the jugular vein of Jersey and Limousin non-lactating cows into 9ml vacutainer EDTA tubes (Greiner, cat. No 455036) and placed on ice. The tubes were transported to the laboratory where they were processed.

2.2.3.1. Blood fractionation

Plasma was obtained from 40-50 ml of blood as described by Yang et al., (1992). Twelve ml of plasma were used immediately for density gradient separation of chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), according to Havel et al., (1955). Separated lipoproteins were stored at –20°C under N_2 gas until required for analyses (Appendix 2).

2.3. Sample analysis

All of the major chemical analyses in this study were performed in three major tissues: adipose tissue, muscle and blood. The methods involved a number of biochemical assays for fat soluble components as well as for morphological features of the tissues under investigation.

2.3.1. Fat and meat analyses

Adipose tissue was used to measure adipocyte cell size and number and the concentration of various carotenoids. Total carotenoids were quantified by spectrophotometry, while high pressure liquid chromatography (HPLC) was used to detect and quantify individual caroteneoids and other antioxidants. In muscle, the concentration of total carotenoids and other antioxidants were measured by spectrophotometry and HPLC. Muscle sections were used to determine intramuscular fat content.

2.3.1.1. Spectrophotometric determination of β-carotene in fat and meat

Measurement of total carotenoids in adipose tissue was performed by the method described by Yang et al., (1992) with modifications as outlined in Chapter 3. Partially thawed fat was hydrolysed in 20% KOH in methanol at 65°C and carotenoids were extracted with diethyl ether from the fat and with hexane from the muscle. Extracts were washed with water, dried under the stream of nitrogen gas, resuspended in ethanol and measured in a spectrophotometer at 450 nm (Appendix 3).

2.3.1.2. Detection and quantification of carotenoids and fat soluble vitamins in fat and meat

An HPLC method was used for the detection and quantification of β-carotene, lutein, retinol and α-tocopherol in fat and meat. Samples were extracted according to the procedure described in 2.3.1.1 and, unless otherwise specified, were resuspended in hexane. A Hewlett-Packard HPLC instrument (model 1100, USA) was used. Separation was achieved using a Spherisorb ODS column (5μm, C18 250 x 4.5mm) protected by a pre-column guard cartridge. The mobile phase was a modification from Yang et al., (1987) and consisted of methanol:hexane:water (90:5:5) mixture with a flow rate of 1.2ml/min. Two ml of 5% ammonium acetate in methanol per one liter of the mobile phase was added to protect the β-carotene. Analytical standards of β-carotene, lutein, α-carotene, α-tocopherol, retinol and α-tocophetrol acetate (internal standard) were obtained from Fluka. β-Carotene, α-carotene and lutein were detected at 450nm, 446nm and 446nm, respectively, on a multiwavelength diode array detector HP 1100 series, USA. α-Tocopherol, retinol and α-tocopherol acetate were detected at 292nm, 325nm and 292nm, respectively. All stock and working solutions of the standards were stored in the dark at –20°C in a nitrogen atmosphere.

2.3.1.3. Quantification of intramuscular fat content

Prior to chemical analysis, subcutaneous and intermuscular fat were dissected from the meat and 100g of the remaining tissue was homogenised in a food processor to a fine paste. All samples were stored at -20°C. Intramuscular fat was extracted from the meat samples (10g) with chloroform:methanol (2:1) according to the method of Christie (1989) (Appendix 4).

2.3.1.4. Measurement of adipocyte size and number

Liberation of adipocytes for morphological studies was achieved by using a modification of the collagenase digestion method as described by Smith et al., (1972). Small (approximately 50-60mg) slices of adipose tissue were cut from the frozen -20°C samples using a scalpel.

The tissue slices were rinsed with 0.15M NaCl (isotonic saline) to remove the free lipid released from ruptured adipose cells during slicing. The adipose tissue (50mg) was dried on filter paper, weighed, and placed in 10 ml freshly siliconised glass tubes containing 0.5ml of Krebs-Ringer bicarbonate buffer with 4% albumin (bovine albumin, fraction V, 4%) and collagenase (5mg/ml medium; Type I, Lot26H0174, Sigma Chemical CO., St Louis, USA). The concentration of collagenase was 5mg per ml of medium. Tissue was incubated at 37°C for 3h with gentle shaking (60 cycles/min). At the end of the incubation, two aliquots of the suspension were immediately placed in a haemocytometer chamber, the slide was focused on the grid and cells in all squares were counted. Aliquots of the gently stirred adipocyte suspension after collagenase digestion were placed on a glass slide and examined under a microscope (Olympus BH-2 with CMA-D1CE Sony camera adapter, Japan). The microscope was equipped with a CCD Sony video camera and monitor attached, and the diameters of 100 cells was measured on the calibrated screen (Appendix 5).

2.3.2. Blood analyses

Fractionated blood was analysed for carotenoid, fat soluble vitamins, fatty acids, and used to determine chylomicron and HDL particle size. Results were expressed per ml of fraction or per gram of protein.

2.3.2.1. HPLC of carotenoids in blood plasma and lipoprotein fractions

A technique used for carotenoid extraction from fractionated blood was adopted from that of Yang et al., (1992) with modifications described in chapter 3. The technique principally involved precipitation of blood proteins with ethanol, followed by extraction of the carotenoids with diethyl ether. Combined extracts were washed with water and dried under the stream of pre-heated nitrogen gas. Ethanol was used to resuspend carotenoids for spectrophotometric quantification. Unless specified, the residue was resuspended in hexane for quantification and detection of carotenoids and fat soluble vitamins by HPLC.

2.3.2.2. Measurement of chylomicron and HDL particle size

The morphology of chylomicrons and HDL was studied by the droplet method as described by Forte and Nordhausen (1986). A drop of liquid from the lipoprotein fraction was placed on the Formvar-Carbon-Coated grid with a drop of 1% Sodium phosphotungstate (NaPTA, pH 7.2) for 20-30sec. The excess fluid was removed from the grid with filter paper, and the grid was inserted into a microscope chamber (TEM 100, Philips) which was connected to a digital video image camera (Gatan CCD camera, Model 673, USA). A number of images per sample (5-10) were saved with the calibration grid. Sizing of chylomicrons and HDL particles was

carried out under the magnification of 10,500x and 92,000x, respectively. Chylomicron (100) and HDL (50) particles from different micrographs of the same sample were measured manually on the monitor (Appendix 6).

2.3.2.3. Fatty acid composition of blood plasma and blood lipoprotein fractions

The fatty acid composition of blood plasma, chylomicrons and HDL was determined using gas chromatography with the method described by Christie (1989) with use of Hewlett Packard Model 5890A Series II gas chromatograph fitted with a capillary column (BPX70, SGE, Melbourne, Australia). Hydrogen was used as the carrier gas. The instrument was calibrated as described by Siebert et al., (1996). Tissue samples were extracted with chloroform/methanol (2:1 v/v), then methylated under acid conditions. Methyl esters were identified by comparing the relative retention times of the peaks from a sample with known standards. The results were expressed as percent normalised fatty acids or quantitatively as nmol/ml (Appendix 7).

2.3.2.4. Measurement of protein concentration in blood plasma and blood lipoprotein fractions

Protein concentrations of plasma and the lipoprotein fractions (chylomicrons, VLDL, LDL, HDL, LPDS) were determined colorimetrically at 595nm using the method of Bradford (1976) (Appendix 8).

2.4. Statistical analyses

Generally, data was analysed using SAS software (SAS, 1989 and SAS, 1996). The main procedures used included Proc GLM, Proc Corr, and Proc Mixed, Proc UNIVARIATE and Proc CHART.

Chapter 3

Carotenoid distribution in cattle fat

3.0. Introduction

It has been reported that β-carotene and lutein are the two predominant carotenoids present in cattle tissues (Yang et al, 1992; Kruk et al, 1998). Since many factors influence intake and metabolism of carotenoids, there is a broad range of concentrations in bovine tissues. Many methods have been developed for the extraction of β-carotene from different tissues (Khachik et al, 1992; Schmitz et al, 1991; Schweigert et al, 1987; Van Steveninck and De Goeij, 1973). However, these methods are insufficient when the concentration of β-carotene is high. Moreover, the published methods require large samples, and consequently, are not suitable for biopsy techniques where the amount of pure tissue is limited. In order to use biopsy samples and to accurately measure the extremely high concentrations of β-carotene in various tissues of Jersey cows, the existing methods were modified.

In addition to extracting the carotenoids to determine their concentration, carotenoid content of cattle adipose tissue can be also satisfactorily assessed by using subjective scoring techniques. Specially designed grading chips representing different hues of yellowness are commonly used by AUS-MEAT® assessors to describe the colour of fat (AUS-MEAT, 1990). However, their application is limited to the abattoirs where there are standard conditions for the measurement of colour (e.g. light, temperature, intermuscular fat site). A high correlation between predicted β-carotene concentration in fat with the extracted β-carotene would provide the basis for a subjective scoring system applicable in the field work.

Consequently, the aims of research in this chapter were: **(i)** to develop uniform assays for β-carotene extraction from adipose tissue, muscle, liver, blood plasma, blood lipoprotein fractions and milk containing a wide range of carotenoid concentrations (e.g. muscle, 0-0.1μg/g; blood, 0-200μg/ml in HDL), **(ii)** to optimise the assays for small sample sizes (e.g. 0.1-0.2g fat) and reduce extraction times (e.g. <24h), **(iii)** to investigate the stability of β-carotene in various tissues and ethanol solutions under different experimental and storage conditions, **(iv)** to explore the relationship between carotenoid concentrations in various adipose tissues, and **(v)** to investigate the adequacy of expressing the concentration of carotenoids in cattle fat by subjective scores.

3.1. Materials and methods

The sampling data and extraction procedures used in this chapter were derived from many different experiments and are grouped into two major studies. Study 1 involved the optimisation of carotenoid extraction methods from various cattle tissues. Study 2

investigated the distribution of carotenoids in cattle fat tissues and their relationship with subjective fat colour score. Study 2 can be divided into different experiments as follows: (a) the concentration of carotenoids in different anatomical sites of subcutaneous fat (including left and right half-carcasses as well as number of different sites of the same half-carcass), (b) the relationship between β-carotene and lutein concentration in subcutaneous and intermuscular adipose tissue, and (c) the relationship between carotenoid concentration in fat and subjective fat colour scores as assessed by AUS-MEAT® and our laboratory 5-point scale.

3.1.1. Study 1:

3.1.1.1. Sample collection and preparation

Cattle fat samples were obtained from animals slaughtered at the abattoir within 15 min after slaughter and from carcasses in the chiller 4-5 hours after slaughter. Adipose tissue representing a wide range of colour (white to yellow) was collected from the striploin (*M. longissimus dorsi*, 12^{th} - 13^{th} rib). Samples were frozen in liquid N_2, transported at -20°C, and stored under N_2 gas at -20°C until required. Meat from the *Longissimus dorsi* of a single cow (0.5kg) and the livers from 2 steers were collected within 15min of slaughter from an abattoir, frozen and stored at -20°C. Small sub-samples were taken for extractions without thawing the samples. Meat and fat biopsy samples were also obtained from Limousin and Jersey non-lactating cows, divided into 1 g sub-samples, frozen in liquid N_2 and stored under N_2 gas at -20°C.

3.1.1.2. Blood sampling and fractionation

Collection of blood and separation of blood lipoprotein fractions were performed as described in Chapter 2. β-Carotene concentrations for each lipoprotein fraction were subsequently expressed as μg/ml of blood fraction or as μg/mg protein of the lipoprotein fraction.

3.1.1.3. Apparatus and reagents

n-Hexane (95%) and diethyl ether (stabilised with ethanol 2% and water 0.5%) were purchased from Ajax Chemicals (Australia). Petroleum spirit (40-60°C b.pt.) and ethanol (99.7-100% v/v) were obtained from Merck Pty Limited (Australia). All solvents were of analytical grade and used without further distillation. High purity N_2 gas and liquid N_2 were obtained from British Oil Company (B.O.C., Australia). Absorbance of β-carotene at 450nm was determined on a Shimadzu UV-VIS Recording Spectrophotometer UV 160A (Shimadzu CO, Kyoto, Japan). Samples were dried under a stream of pre-heated N_2 (~40°C).

3.1.1.4. Extraction and quantification of carotenoids

Extractions from tissues were performed in parallel with extractions of a known amount of pure β-carotene as a control to determine the efficiency of extraction. Selected samples were also quantified by HPLC to compare the spectrophotometric results (Chapter 2). Steps for the extraction of carotenoids from all tissues were as follows (Table 3.1):

(1) An appropriate volume of a liquid sample or weight of a partially frozen sample was accurately measured.

(2) In the milk and blood samples, proteins were precipitated by the addition of ethanol and the samples vortexed for 20sec. For fat, meat and liver, samples were thawed 1-2 hours or overnight.

(3) The samples were hydrolysed by the addition of KOH in methanol (20% w/v) at 65°C.

(4/5/6) After hydrolysis, the samples were diluted in water, and the β-carotene extracted twice with organic solvents by hand shaking vigorously for 20sec.

(7) The extracts were combined, washed with water and the organic solvent fraction (upper) transferred to a fresh bottle. The extracts were then evaporated to dryness under heated nitrogen gas (~40°C).

(8) The residue was resuspended in ethanol and centrifuged at 8400 x g for 10 minutes.

(9) Using pure β-carotene as a standard, absorbance at 450nm was determined by spectrophotometry assuming that β–carotene was the major carotenoid present. β-Carotene concentrations were expressed as μg/g of tissue for muscle, liver and fat, as μg/ml of blood lipoprotein fractions or as μg/g of protein for blood and lipoprotein fractions, and as μg/ml of milk.

3.1.1.5. Statistical analysis

Statistical analysis was performed using Proc CORR (SAS 1989). Test of significance (difference from 0) at $P<0.05$ was determined by Bonferroni probabilities.

3.1.2. Study 2:

3.1.2.1. Animals and sampling

The animals used in this study were derived from 4 separate groups. Groups 1 and 2 were used to determine carotenoid concentrations at different anatomical sites (experiment a). Group 1 comprised a number of unknown animals sampled at abattoirs. Fat samples from the left and right side of carcasses were removed for carotenoid analysis. Samples were collected from the slaughter line (10-15min. after kill) or from the chiller (4-5 hours after slaughter). Also, fat from randomly selected fresh and chilled cattle carcasses (5 each) was taken from symmetrically adjacent sites (12^{th}-13^{th} rib, shoulder, rump).

Table 3.1. Steps for extraction of β-carotene from various tissues.

Procedure	Fat [a]	Meat [a]	Liver [a]	Milk	Blood fractions
Step 1 Sample measured (amount)	0.1-0.5g[b]	1.0-2.0 g	0.5 g	2.0 ml	0.05-0.2 ml[c]
Step 2 Protein ppt with ethanol (ml)	N/A[d]	N/A	N/A	6.0	0.5
Step 3 Hydrolysis with KOH	1 ml, 65°C, 40 min	2 ml, 65°C, 40 min	1 ml, 65°C, 40 min	2 ml, 42°C, 15 min	N/A
Step 4 H_2O addition (ml)	3.0	3.0	3.0	3.0	3.0
Step 5 Extraction I (ml)	diethyl ether 5.0	hexane 5.0	hexane 5.0	hexane 4.0	diethyl ether 4.0
Step 6 Extraction II (ml)	diethyl ether 5.0	hexane 3.0	hexane 5.0	hexane 4.0	diethyl ether 4.0
Step 7 H_2O wash (ml)	5.0	5.0	5.0	5.0	5.0
Step 8 Resuspension in ethanol (ml)	0.6-2.0	0.6	0.6-2.0	0.6	0.6-2.0

[a] Samples are kept partially frozen during weighing

[b] Samples visibly yellow (0.1-0.2g), samples visibly white (0.4-0.5g)

[c] 0.05 ml for HDL in cattle (125-250 mg protein/ml fraction)

[d] N/A = not applicable

Group 2 comprised 13 Jersey and 13 Limousin steers which had been raised in a feedlot for 170 days. Fat samples from the shoulder, ribs, and rump of the group 2 animals were obtained in the chiller for comparison of carotenoid content of various anatomical sites.

Group 3 included Limousin and Jersey cows (part of the Davies Gene Mapping Herd, chapter 2), Fifteen animals of each breed were used to investigate the relationship between β-carotene and lutein content in different fat tissues (experiment b). Slices of muscle and attached subcutaneous fat (1-2cm in thickness) were cut from the 10^{th}-11^{th} rib as a cross-section from the *M. longissimus dorsi*. The samples were stored at -20°C in sealable plastic bags until analysed. Prior to chemical analysis, subcutaneous and intermuscular fat were dissected from the meat, and the meat samples (100g) homogenised to a fine paste in a food processor. All samples were freeze-dried prior to analyses and stored at -20°C.

Group 4 consisted of animals from the Davies Gene Mapping Herd (1994, 1996, 1997 drops) and from the New Zealand Gene Mapping Herd (1996 drop). Analysis of adipose tissue was used to demonstrate the consistency in the relationship between carotenoid content in fat and subjective fat colour score (experiment c). Fat samples were collected at weaning (about 250 days of age – biopsy) and at slaughter (500-600 days of age).

3.1.2.2. Sample analysis

Total carotenoid concentration in experiments a, b, and c was measured spectrophotometrically as described earlier in section 3.1.1.3 under extraction and quantification of carotenoids (Study 1). In brief, adipose tissue (0.1-0.2g for yellow fat or 0.4-0.5g for visibly whiter fat) was hydrolysed with 1 ml of 20% KOH in methanol at 65°C for 40min. After hydrolysis, 3ml of water was added and carotenoids extracted twice with 5ml of diethyl ether. The combined diethyl ether extracts were washed with 5ml of water and dried under preheated nitrogen. Samples were resuspended in ethanol and absorbance at 450nm was determined on a spectrophotometer (Shimadzu UV 160A, CO, Kyoto, Japan). β-Carotene and lutein in the experiment (b) were quantified by HPLC as described in the chapter 2 and dried extracts were resuspended in hexane. Water content of the fat was calculated as the difference between fresh fat weight and the weight of the same samples after freeze drying.

3.1.2.3. Biopsy subjective fat colour score

The fat colour score of biopsied adipose tissue was assessed immediately after removal and rinsing with water. The intensity of the colour was expressed in a 5-point scale where score

of 1 was assigned to white fat and score of 5 to extremely yellow fat. Scores of 2 and 4 represented fat more intense than white and less intense than very yellow respectively, and 3 was the intermediate score.

3.1.2.4. Statistical analysis

Analysis of variance was carried out to estimate the difference between breeds, anatomical locations, and the interaction between breed and anatomical location (experiment b) using Proc GLM (SAS 1989). Least squares means and differences between means were computed. Correlations between total carotenoid concentration in different body sites were calculated (experiment a and c) using Proc CORR (SAS 1989). Test of significance (difference from 0) at $P<0.05$ was determined by Bonferroni probabilities. As the variation in carotenoid content in the carcasses was very large (by design), simple linear regression was used to test for the differences between the various anatomical sites. The hypothesis that equation slopes were not different than 1 and that the intercepts were not different than 0 was tested using a t-test.

In the experiment b, least squares analysis of variance was performed using Proc GLM (SAS 1989). The model included the fixed effects of breed, age, tissue and the interactions between breed and age, breed and tissue. The age and breed by age interactions were not significant and were removed from the model. Residual correlations between β-carotene and lutein in different fats were computed using Proc CORR (SAS 1989) after adjusting for the effects of breed and tissue.

3.2. Results and discussion

3.2.1. Study 1

ß-Carotene is a hydrocarbon which is soluble in organic solvents. Common methods for extraction of ß-carotene from various animal tissues use hexane, diethyl ether, petroleum spirit, or a mixture of these and other solvents after protein precipitation (e.g. blood plasma, lipoprotein fractions, milk) or sample homogenisation and hydrolysis (e.g. solid samples such as fat, liver, spleen, lung, etc.). After extraction with organic solvents, the extract is usually washed with water, dried, resuspended in ethanol, and the sample then measured spectrophotometrically.

In our work with cattle, many samples have to be analysed simultaneously and previously published methods were found not to be suitable. They were laborious, time consuming and the protocols differed markedly depending on the type of tissue to be extracted. Moreover,

the methods required large samples that could not always be obtained with biopsied material. Another major problem was that the concentration of ß-carotene, especially in tissues from Jersey cattle, was 10-20 fold higher than that previously reported by other authors (Yang et al, 1992; Strachan et al, 1993; Zhou et al, 1993). The high concentration contributed significantly to wide variation in the results when existing methods were used.

Our work required a standardised protocol for rapid sample analysis of minute sections of biopsied material, as well as blood and milk. The goal was to modify the existing methods for extraction of ß-carotene from various bovine tissues, making the protocols (i) reproducible regardless of ß-carotene content, (ii) uniform in terms of using similar procedures and solvents, and (iii) less time consuming so that many samples could be extracted in a short period of time.

3.2.1.1. ß-Carotene extraction of the adipose tissue

The methods currently used for the extraction of ß-carotene from adipose tissue in cattle require routinely 0.5-1.0g of fat (Yang et al, 1992; Strachan et al, 1993). This fat is hydrolysed in 20% KOH in methanol and the ß-carotene extracted 1-2 times with organic solvents. However, using 1.0g fat samples showed that two extractions with diethyl ether did not recover all the ß-carotene in some cases. A further 7-13% of the total ß-carotene was obtained by a third extraction (Figure 3.1). Reducing the amount of fat to 0.1g and reducing the solvent volume correspondingly also gave variable results. More reproducible data was obtained when 0.1-0.2g of fat was extracted using the volume of solvent recommended for 0.5g samples (Table 3.1), irrespective of ß-carotene concentration. In the case of a very white fat, where carotenoids could not be detected spectrophotometrically, 0.4-0.5g of fat was used.

Because consistent results could be obtained with 0.1g-0.2g fat, 1g biopsy samples were of sufficient size to provide the required amount of pure fat and accurately quantify carotenoids. However, loss of water during the processing of samples contributed to variation in the ß-carotene content and caused discrepancies when the results were expressed per gram of wet tissue. Freeze-drying multiple biopsy samples is time consuming. Consequently, it was found

that in order to overcome this problem, fat samples should be frozen and only partially thawed before cutting and weighing.

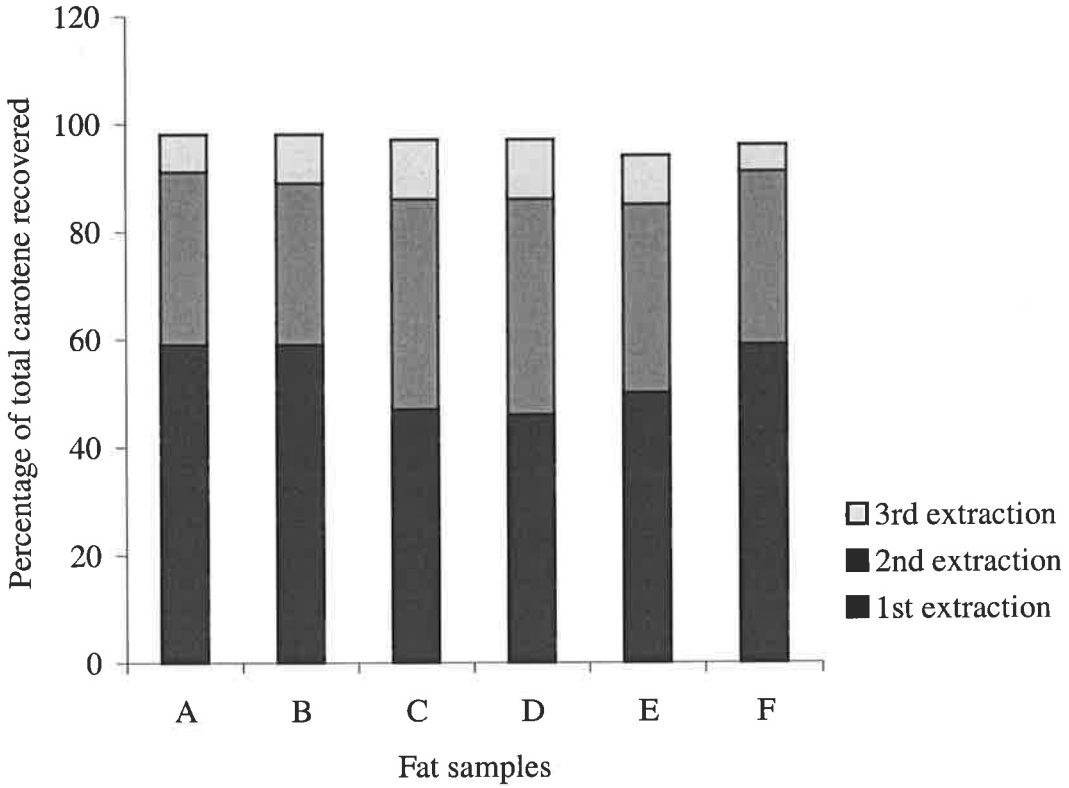


Figure 3.1. Carotene recovery from fat samples. Percentage of carotene recovery in 3 consecutive extractions with 4ml of diethyl ether from six 1.0g fat samples. (The concentration of carotene ranged from 2.9-10.8µg/g of fat, recovery varied from 94-99%).

The use of partially thawed fat samples has another advantage as the connective tissue can be visually distinguished from fat, and pure fat can be dissected more precisely. This is important because samples collected from young, lean animals usually contain a higher percentage of water and more connective tissue in comparison to fat samples from mature animals. The purity of sample was a key factor in obtaining reproducible results (Figure 3.2).

Variation can be reduced also by choosing an appropriate sampling procedure. This is because carotene appears to have a higher concentration when expressed per gram of wet fat in surface tissue compared to deeper locations. It is better to sample deeper fat in carcasses as the surface may be dry due to evaporation and solidification during the storage of carcasses in the chiller. Results indicated that when the protocol is modified as described above, consistent and accurate quantification of ß-carotene from non-freeze-dried samples (0.1-0.5g fat) is possible.

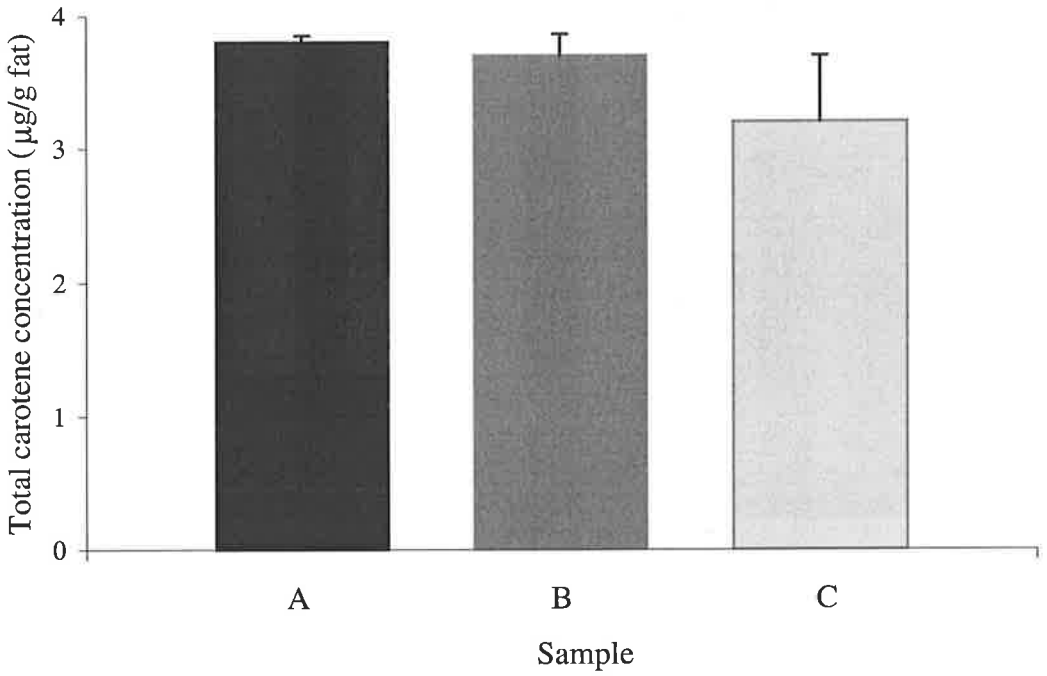


Figure 3.2. Effect of sample type on carotenoid concentration. Comparison between total carotenoid concentration in (A) pure fat, (B) contaminated fat trimmed of connective tissue and blood, (C) fat contaminated with blood and connective tissue. All samples were obtained from the same adipose tissue samples. Extractions were performed in triplicate. Bars indicate standard deviations.

Occasionally, in some samples, a solid residue remained after hydrolysis. The amount of 20% KOH in methanol, time and temperature required for hydrolysis was investigated. It was found that there was no difference between hydrolysing with 1, 2, or 3ml of 20% KOH in methanol. However, the larger volumes caused interference with spectrophotometric readings if samples were not washed several times with water. Some authors have overcome this problem by washing the samples 5 times with water (Seirer et al, 1992). Such a large number of washes is laborious, extends the extraction time and may cause loss of carotenoids. Extraction of pure ß-carotene showed that the number of washes is not significant and this step can be omitted. With fat samples, however, at least one wash with 5 ml of water was found necessary and is recommended after hydrolysis with 1ml of 20% KOH in methanol.

Changing the time (40, 60, or 90min) and temperature (65°C or 75°C) of hydrolysis did not alter ß-carotene recovery, but also did not resolve the problem of incomplete hydrolysis.

Thus, while hydrolysing samples at 65-75°C for 40-90 minutes is not detrimental, a solid residue may still remain. When this occurred, it was found that the residue could be dispersed by rapidly tapping the tube. This needed to be followed immediately by the addition of water (3ml) and extraction with diethyl ether. It was observed that there was less solid residue after hydrolysis when the measured frozen fat samples were completely thawed (1-2h or overnight at room temperature) prior to hydrolysis compared to samples which were hydrolysed immediately after removal from the freezer. Presumably if the sample was not thoroughly thawed, then the KOH could not penetrate the tissue adequately and hydrolysis was hindered.

The modified method presented herein describes important steps in sample preparation, handling, and extraction of ß-carotene from fat (Methods and Table 3.1). The small amount of tissue, higher volumes of solvents, reduced number of washes with water, and centrifugation prior to spectrophotometric readings allow reliable extraction of ß-carotene from samples with high concentrations of this pigment. This is not possible with the methods described elsewhere (Yang et al, 1992). Use of the modified methods with pure adipose tissue resulted in good reproducibility and recovery of ß-carotene ranging from 91.8 to 97.8%.

3.2.1.2. ß-Carotene extraction of liver and muscle

Analytical methods for extraction of carotenoids from spleen, liver, and lungs commonly recommend using 1-5g of tissue for homogenisation (Schmitz et al, 1991), grinding with sodium sulphate and ascorbic acid, hydrolysing with 20% KOH in methanol overnight at room temperature (Yang et al, 1992), and then extracting the pigment. When this method was used in the present study with liver samples, satisfactory recovery was achieved. However, sample preparation process was very laborious and time consuming.

Experimental work with ß-carotene showed that the temperature of 65°C did not affect the stability of this pigment for about 24h (Table 3.4). A comparison was, therefore, made between the extraction and hydrolysis of ß-carotene overnight according to method (Yang et al, 1992) and hydrolysis at 65°C for 40min. The amounts recovered were 24.6±3.5µg/g and 26.2±1.4µg/g, respectively, and were not significantly different. This accelerated the extraction and hydrolysis time and provided results within 1h.

The modified protocol for β-carotene extraction from liver was applied to muscle samples as no specific method has been previously published for β-carotene extraction from meat. It was determined that larger amounts (1.0-2.0g) of muscle tissue are required for assays compared to other tissues (Table 3.1) as the concentration of ß-carotene in bovine muscle is low (≤1.0μg/g tissue). A major impediment to using larger sample sizes is that meat samples contain relatively high concentrations of insoluble structural proteins which may interfere with the hydrolysis of these samples (Schmitz et al, 1991). Therefore, different volumes of 20% KOH in methanol, different hydrolysis times, and different temperatures were tested. All volumes of 20% KOH in methanol (2, 3, or 4ml) hydrolysed the meat after 40min at 65°C. However, 2ml was optimal as it did not require additional washes with water or interfere with spectrophotometric readings. Changes in the hydrolysis time (40min, 60min, or 90min) and temperature (65°C or 75°C) did not alter ß-carotene recovery.

Similar to adipose tissue, the repeatability of β-carotene extraction in muscle samples can be influenced by sample preparation. Muscle contains about 70% water and if the samples are frozen and thawed many times, the loss of water may significantly influence sample weight. This loss does not influence the β-carotene stability, but the concentration of ß-carotene expressed per gram of wet muscle can vary considerably. This can be overcome by freeze-drying the samples and then extracting the β-carotene. However, if the muscle is kept frozen and not completely thawed during weighing, then the time consuming step of freeze-drying can be omitted. As with adipose tissue, usage of frozen muscle for hydrolysis may not allow complete extraction of β-carotene. To overcome this problem, after weighing, muscle samples should be thawed for 1-2h or overnight at room temperature prior to hydrolysis. Weighing partially thawed or frozen samples and then thawing samples before hydrolysis gave highly repeatable results.

Various ratios of hexane to diethyl ether in the extractions of β-carotene from meat were investigated. Pure hexane gave more consistent results compared to diethyl ether (0.45±0.06 versus 0.48±0.19, respectively) with no difference between 2 or 3 extractions. Extraction with hexane has been used by other authors for these types of tissue. In this study, data showed that hexane can extract ß-carotene from both muscle and liver with good reproducibility.

However, in the liver, the concentration of β-carotene exceeds 15-fold that found in meat, so 0.5g or less of liver is recommended with 3 hexane extractions (Table 3.1). To obtain reproducible results, liver samples should also be collected from similar locations due to the variation in concentration of ß-carotene across the liver (Underwood, 1984).

During hexane extraction of β-carotene from muscle, an emulsion at the hexane-water interface may occur, especially if 4ml of hexane is used for both extractions. To prevent such an emulsion, 5ml of hexane in the first extraction and 3ml of hexane in the second extraction should be used. Also samples should be shaken for 20s by hand. If the emulsion at the interface still appeared, tapping the bottle lightly 3-4 times dispersed the emulsion.

Carotenoids, as fat soluble micronutrients, are mainly associated with fat depots in the muscle (intramuscular) and between muscle (intermuscular). To determine the carotenoid concentration solely in the intramuscular fat, it was necessary to dissect carefully all intermuscular fat before analysis. It was found, however, that there was no need to homogenise meat after the dissection of intermuscular fat (Figure 3.3). This step is laborious and time consuming, and can be omitted.

The method developed for extraction of carotenoids from liver and meat was not time consuming and allowed a large number of samples to be extracted simultaneously. The recovery of β-carotene measured in meat samples with a known amount of synthetic β-carotene was greater than 92%.

3.2.1.3. ß-Carotene extraction of blood plasma and lipoprotein fractions

A number of methods for ß-carotene extraction from blood plasma have been described (Schmitz et al, 1991; Van Steveninck and De Goeij, 1973). The techniques principally involve precipitation of proteins from blood plasma with a 1:1 (v/v) ratio of ethanol, followed by extraction of carotenoids with an organic solvent or with a mixture of organic solvents. The same protocol is also appropriate for the extraction of carotenoids from blood lipoprotein fractions. Nevertheless, when this method was applied to samples in the present study, it gave variable results. However, the concentration of ß-carotene in bovine blood plasma samples

and lipoprotein fractions were 2-10 fold higher than concentrations reported elsewhere (Yang et al, 1992; Seirer et al, 1992).

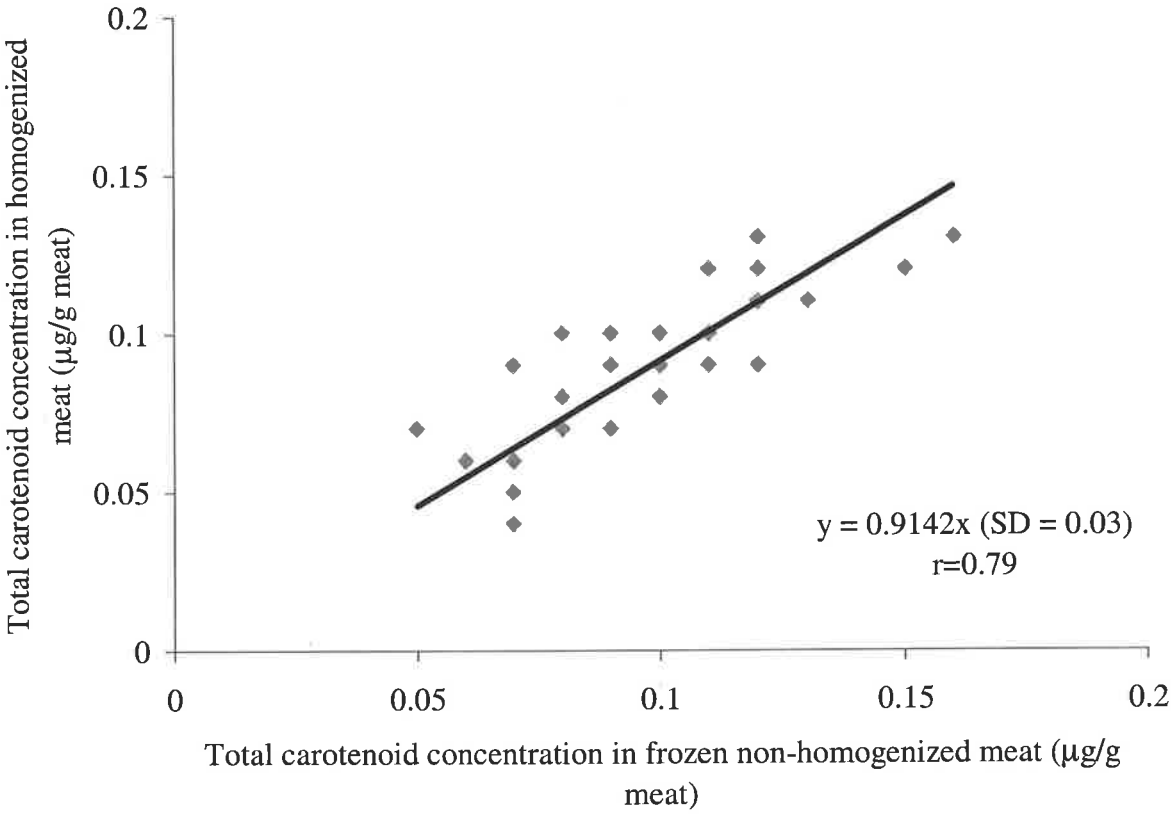


Figure 3.3. Correlation of total carotenoid concentration in homogenized and non-homogenised meat. Samples were stored in –20°C for 10 months prior homogenisation and extracted as described in Table 3.1.

Decreasing the volume of sample and precipitating the proteins with 1:1 (v/v) ratio of sample to ethanol showed an increased recovery of β-carotene, but still gave variable results. Increased volumes of diethyl ether (4ml to 6ml) to extract the ß-carotene did not improve reproducibility. However, when the ratio of sample to ethanol was increased to 1:5 (v/v), more consistent results were achieved (Figure 3.4). This suggests that the amount of ethanol used to precipitate proteins and release the carotenoids influenced the extraction. Therefore, a ratio of 1:5 or higher is recommended to obtain uniform results. Using this ratio, the variation between duplicates was found to be <1.2% compared to 9-12% using other methods (Figure 3.5).

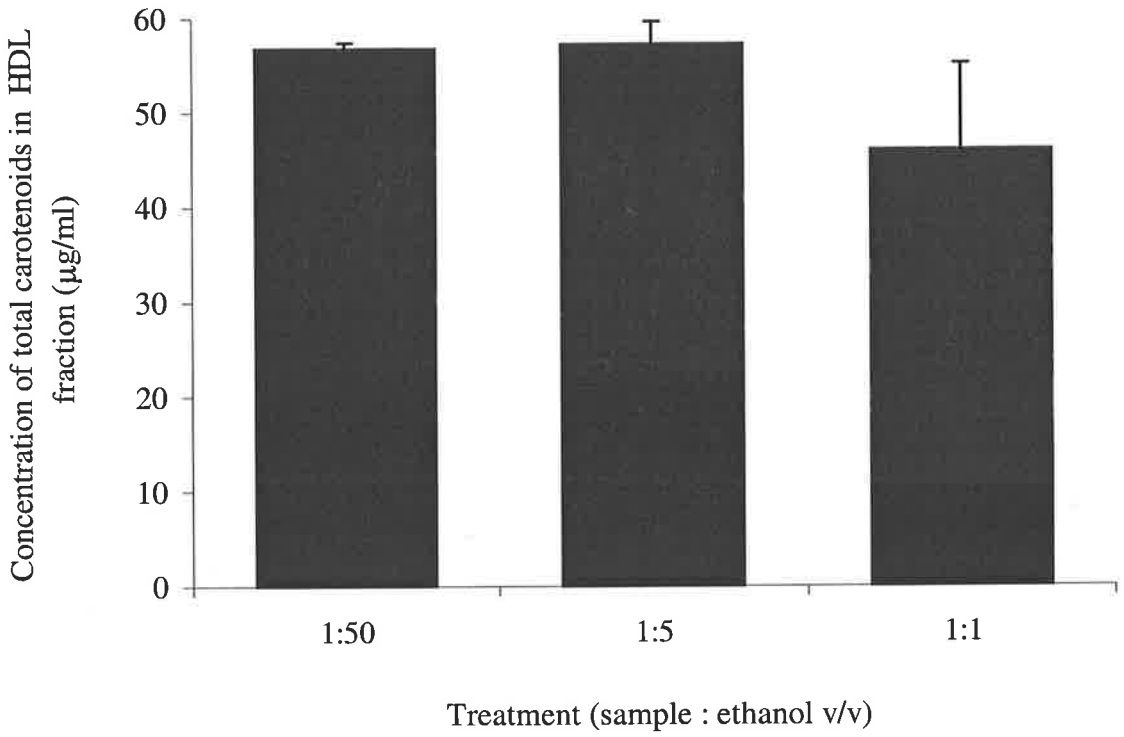


Figure 3.4. Recovery of total carotenoids from an HDL fraction with different ratios of sample to ethanol volumes. Samples extracted in triplicate. Bars indicate standard deviations.

To obtain satisfactory recovery, 100µl (700-800mg/ml protein) of blood plasma, chylomicrons (300-800mg/ml protein), VLDL (1.5-2.0mg/ml protein), and LDL (4.5-11.0mg/ml protein) were used. The concentration of ß-carotene in these fractions varied from: 2.9-30.0µg/ml (6-40µg of β-carotene/g of protein), 1.9-15.0µg/ml (5-30µg ß-carotene/g protein), <1.0 µg/ml (400-500µg ß-carotene/g protein), 1.1-23.0µg/ml (240µg - 2.1mg β-carotene/g protein), respectively. However, in the case of HDL, which is the main carotenoid transporter in cattle (Yang et al, 1992) (4-200µg ß-carotene/ml fraction, 0.2-1.3mg protein/ml fraction), a smaller volume of 50µl (125-250µg β-carotene/g protein) was found to be sufficient. In contrast, VLDL carries minute quantities of β-carotene in cattle (Yang et al, 1992), so to increase detectability, a 200µl sample was found to be necessary for extraction.

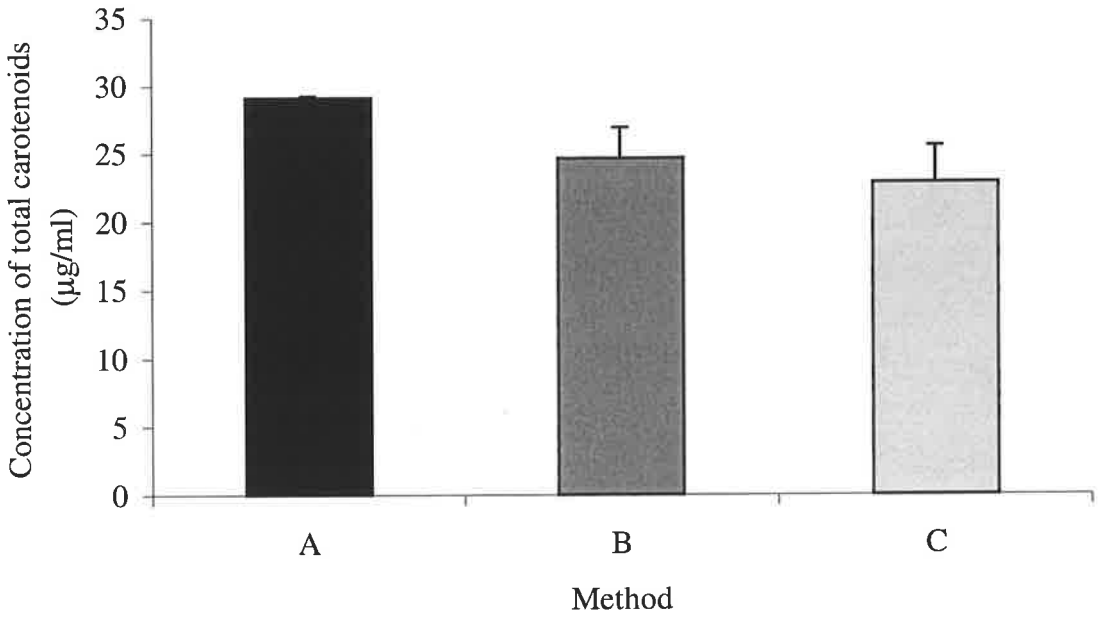


Figure 3.5. Comparison of recovery of total carotenoids from blood plasma using different methods. A) described herein; B) method of Yang et al. (1992) C) method of Van Stevenick and De Goeij (1973). Samples extracted in triplicates. Bars indicate standard deviations.

It should be noted that different volumes of lipoprotein fractions may be required for β-carotene analysis in different species. For example, in humans, LDL is the main carotenoid carrier. To obtain reproducible spectrophotometric readings, less than 100μl of this fraction from human blood is all that is required. The small volumes of blood fractions allowed for much higher recoveries (20-30% higher) than other methods (Yang et al, 1992; Van Steveninck and De Goeij, 1973).

Different solvents have been used to extract carotenoids from blood. A mixture of diethyl ether/hexane and pure diethyl ether gave very similar results. This agrees with Kachlik et al. (1986), who compared petroleum ether/acetone and diethyl ether/methanol, and did not observe significant changes in the qualitative or quantitative distribution of carotenoids. The recommended method herein involves 2 extractions with diethyl ether only. However, in some cases, 3 extractions may be needed. For instance, in HDL samples, 3 extractions were required because of the very high content of ß-carotene in this blood lipoprotein fraction in cattle, particularly if the animals had been grazing on green pastures. On the other hand, all of the ß-carotene from cattle VLDL can be obtained by only one extraction with diethyl ether.

The method developed here allows efficient extraction of ß-carotene from blood plasma and lipoprotein fractions of cattle with high concentrations of this pigment. A sample of 50-100μl (5μg-1.3mg β-carotene/g protein) used for the assay and a 1:5 (v/v) ratio of sample to ethanol resulted in better repeatability and higher recovery of this pigment (20-30% higher) compared to other methods.

3.2.1.4. ß-carotene extraction of milk

The concentration of β-carotene in milk is often estimated by procedures similar to those for blood plasma (Block and Farmer, 1987). Some authors, however, express the results per gram of milk fat. This involves the extra work in estimating milk fat content and assumes that all the β-carotene in milk fat is transported with lipid derived from plasma. Breed differences in the concentration of β-carotene in milk have been observed between Jersey, Friesian and Limousin which vary greatly in milk fat content (Townsend et al, 1997). The aim was to modify the extraction protocols which would result in a quick and precise analysis of large numbers of samples using similar procedures and equipment as for other tissues and does not rely on milk fat concentration.

The concentration of β-carotene in cow's milk is low (expressed per ml of milk) and varies in range similar to meat (0-1.0μg/ml). Milk samples (0.5ml) were extracted a number of times with petroleum ether. The results demonstrated that for all ratios of milk to ethanol, a total of 3 extractions was always more effective than 2 extractions (data not shown). However, detectability of ß-carotene from 0.5ml samples was difficult as its concentration was so low. A 2ml sample volume increased the detectability of β-carotene especially from samples with a very low carotene content.

The hydrolysis time of milk samples with 20% KOH in methanol at 42°C did not affect the recovery of β-carotene (Figure 3.6). A 1:3 ratio of milk to ethanol and 15min of hydrolysis adequately precipitated milk proteins and hydrolysed fat, releasing ß-carotene for extraction with organic solvents. Use of hexane gave comparable and less variable results than petroleum ether. The types of steps and hexane extraction make this method similar to those

modified for the other tissues and allowed the extraction of more than 100 milk samples a day at a recovery rate between 90-92%.

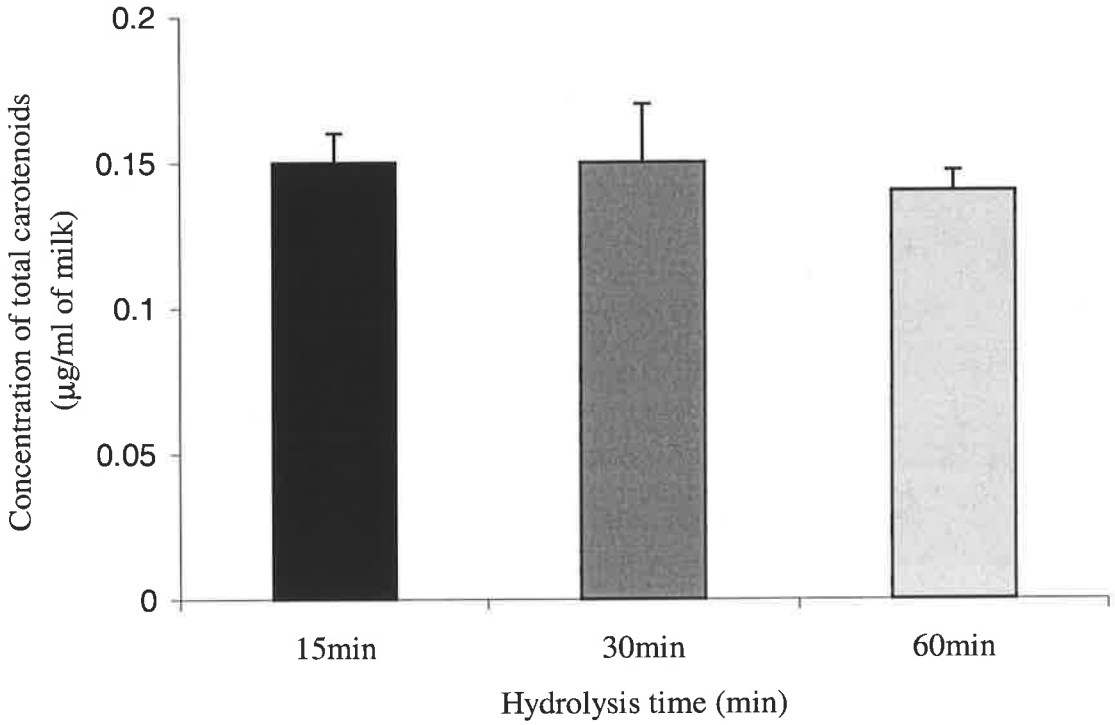


Figure 3.6. Concentration of total carotenoids recovered from milk hydrolysed for various times. Three samples were extracted in each treatment group. Bars indicate standard deviations.

3.2.1.5. Comparison of spectrophotometry vs high performance liquid chromatography

A comparison was made between the quantification of carotenoids by spectrophotometry and by high performance liquid chromatography (HPLC). The average spectrophotometric recovery of carotenoids using a known amount of β-carotene (0.5µg/ml, 1.0µg/ml, 2µg/ml) ranged from 91.0%-94.8% in blood plasma, fat, meat and milk samples (Table 3.2A). This was confirmed by HPLC recovery using α-tocopherol acetate as an internal standard. The results demonstrate that the recovery obtained by spectrophotometry is highly comparable with the HPLC data when the amount of pure β-carotene is in the range of the tissue under investigation. However, if the concentration of pure β-carotene exceeded that in the tissue by 3-5 fold (data not shown), the recovery increases to 99%. This increase is caused by the fact that the average concentration of β-carotene in a standard is almost equal to the concentration of β-carotene in samples enriched by the standard. This ratio will always be close to 1. If one wants to estimate the recovery using spectrophotometry and known amounts of pure β-carotene, this must be taken into consideration.

Table 3.2. Comparison between spectrophotometry and HPLC, β-carotene quantification. (A) Comparison of recovery based on spectrophotometry an HPLC; (B) Comparison of β-carotene and lutein percentages by HPLC as total carotenoids estimated by spectrophotometry at 450nm.

Tissue	A (Recovery)[a]		B (% of total)[a]		
	SPEC	HPLC	BC(HPLC)/TOTAL(SPEC)	LUT(HPLC)/TOTAL(SPEC)	BC+LUT(HPLC)/TOTAL(SPEC)
Blood plasma	91.0 ± 4	89.0 ± 8	77.4 ± 14.4	7.8 ± 1.2	85.2 ± 10.4
Fat	94.8 ± 3	97.1 ± 3	75.0 ± 17.3	2.5 ± 1.5	77.5 ± 19.1
Meat	92.0 ± 3	95.0 ± 3	93.3 ± 5.2	3.4 ± 1.0	96.7 ± 4.2
Milk	91.0 ± 1	86.0 ± 5	81.5± 18.9	15.8 ± 6.6	97.3 ± 9.9

Note: SPEC=spectrophotometry, HPLC=high performance liquid chromatography, BC=β-carotene, LUT=lutein, TOTAL=absorbance at 450nm, [a]=percentage of total.

Spectrophotometric readings at 450nm estimate total carotenoids and other extractable components which absorb the light at this wavelength. The ratio between β-carotene and lutein concentration quantified by HPLC and total carotenoid concentration estimated by spectrophotometry was calculated to investigate the percentage of these carotenoid components in total (Table 3.2B). Selected blood plasma, fat, meat and milk samples were extracted as described in methods, the samples resuspended in hexane and quantified by HPLC. The same samples were dried down, resuspended in a larger volume of hexane and measured by spectrophotometer. The concentration of total carotenoids was adjusted for the loss of sample due to HPLC quantification. The results demonstrate that β-carotene and lutein were major components accounting for 77.5-97.3% of total carotenoids. β-Carotene represented 75.0-93.3% of the total in all tissues. Lutein accounted for 1.0-4.4% in the meat and fat samples, and 7.8% and 15.8% in blood plasma and milk, respectively. A high positive correlation between β-carotene or β-carotene and lutein with total carotenoids was observed in all samples (Table 3.3). The lack of significant correlation between lutein and total carotenoids in blood plasma, meat and milk shows that most of the changes in total carotenoid concentration are due to the changes in β-carotene concentration. This relationship demonstrates that β-carotene content in milk, meat, fat and blood plasma samples can be estimated accurately by spectrophotometry. This is important when a large number of samples need to be quantified in a short period of time.

Table 3.3. Correlations between β-carotene, lutein and β-carotene+lutein quantified by HPLC with total carotenoids estimated by spectrophotometry.

HPLC / SPEC	Blood plasma			Fat			Meat			Milk		
	BC	LU	BC + LU	BC	LU	BC + LU	BC	LU	BC + LU	BC	LU	BC + LU
Blood	0.90 ***	0.24 ns	0.91 ***									
Fat				0.97 ***	0.79 **	0.97 ***						
Meat							0.99 ***	0.75 ns	0.99 ***			
Milk										0.95 *	0.72 ns	0.96 *

Note: HPLC=high performance liquid chromatography, BC=β-carotene, LU=lutein, ***=P<0.001, **=P<0.01, *=P<0.05, ns=not significant, SPEC=spectrophotometry.

3.2.1.6. Stability of ß-carotene under various experimental conditions

To explore factors that could influence the stability of β-carotene during sample collection, processing, assaying and storage, two concentrations of this pigment in ethanol were investigated (Table 3.4). Results showed that samples kept for 7 days at room temperature (22°C) were fairly stable with both high and low concentrations of ß-carotene. However, ß-carotene was better preserved against degradation when stored at lower temperatures (+4°C and -20°C). Protection of samples by storing under N_2 or in the dark (under aluminium foil) was not necessary at these temperatures. This agrees with Scita (1992), who reported a loss of ß-carotene stored at -20°C of only 1.55% per month during a 4-month experiment.

Table 3.4. Stability of ß-carotene in ethanol solutions under various treatments.

Treatment	Sample	Number of Days 0	1	2	5	7
Room temp	1	5.04	5.09	5.00	4.90	4.82
	2	0.5	0.5	0.50	0.48	0.48
Room temp + N_2	1	5.05	5.12	5.01	4.97	4.83
	2	0.50	0.52	0.53	0.50	0.55
Room temp + foil	1	5.03	5.02	4.98	4.92	4.88
	2	0.50	0.51	0.51	0.50	0.53
Sunlight	1	5.02	5.12	4.57	3.36	2.66
	2	-	-	-	-	-
Sunlight + N_2	1	5.09	4.56	4.37	3.24	-
	2	-	-	-	-	-
Sunlight + foil	1	5.05	5.09	4.94	4.85	4.67
	2	-	-	-	-	-
65°C	1	5.02	4.53	4.46	4.35	4.18
	2	0.52	0.52	0.53	-	-
65°C + N_2	1	5.02	5.08	4.63	4.52	4.40
	2	0.50	0.53	0.55	-	-
37°C + N_2	1	-	-	-	-	-
	2	0.49	0.48	0.50	0.53	-
37°C	1	5.08	4.94	4.85	4.67	4.96
	2	0.50	0.50	0.49	0.48	0.48
37°C + foil	1	-	-	-	-	-
	2	0.50	0.50	0.50	0.49	0.48
4°C	1	5.05	5.20	5.13	5.19	5.19
	2	0.50	0.51	0.51	0.52	0.51
4°C + N_2	1	5.04	5.16	5.23	5.10	5.04
	2	0.50	0.57	0.60	0.60	0.62
-20°C	1	5.08	5.17	5.23	5.24	5.23
	2	0.50	0.52	0.52	0.52	0.52
-20°C + N_2	1	5.04	5.17	5.15	5.01	4.97
	2	0.50	0.52	0.52	0.52	0.52

Note: sample 1 concentration of β-carotene = 5.0 μg/ml; sample 2 concentration of β-carotene=0.5μg/ml; N_2=samples stored under nitrogen; foil=samples protected with aluminium foil; sunlight=samples at 22°C exposed to sunlight 10-14h/day. Each treatment group consisted of 6 samples.

Incubating β-carotene at 65°C lowered the spectrophotometric readings of a high concentration solution within 24h. Storing the samples under N_2 extended the stability of β-carotene to ~30h. However, a noted decrease of absorption at 450nm began after 90min of incubation at 65°C (data not shown). These results suggest that hydrolysis of samples at 65°C

for 40min should not affect the stability of ß-carotene during this treatment, but that hydrolysis time should not exceed 90min.

Carotenoids are easily oxidised when exposed to intensive light and high temperature (Scita, 1992). In the study herein, the concentration of ß-carotene in samples exposed to sunlight declined constantly after 24h, and N_2 had no protective effect. Samples wrapped with aluminium foil extended the stability to approximately 72h. These results suggest that ß-carotene in ethanol solution is stable for about 7 days. However, under certain conditions (intensive light and high temperature), the stability declines and samples have to be protected with N_2 or aluminum foil.

The stability of ß-carotene in various tissues was also measured. The recovery of ß-carotene from fat samples with a range of concentrations varying from 1.0-12.5μg/g (Figure 3.7) did not differ after 12 months of storage in -20°C. During this time, samples were frequently thawed and re-frozen. Meat, blood plasma, and blood lipoprotein fraction samples stored at -20°C without N_2 also had the same spectrophotometric readings after 4 and 12 months of storage (data not shown). These results show that -20°C is an appropriate temperature to store blood plasma, fat and meat samples for long periods of time. However, samples can remain at room temperature (22°C) or be heated to 65°C for a short period of time without altering the ß-carotene recovery.

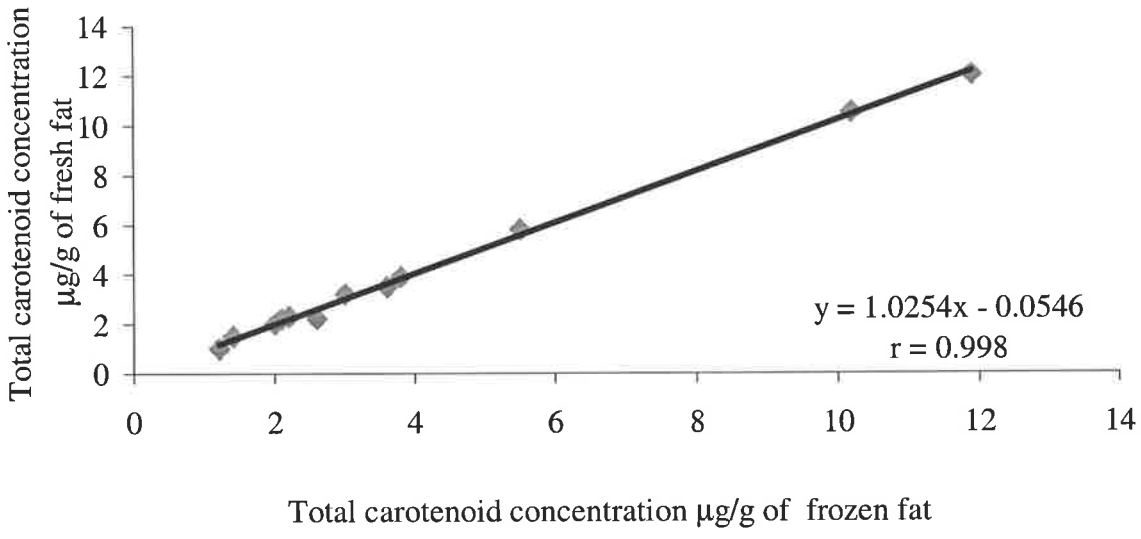


Figure 3.7. Total carotenoid recovery from fresh vs frozen fat samples. Fat samples (1-12) with a range of total carotenoid concentrations were analysed as fresh samples and as frozen samples in duplicate. Frozen samples were stored at -20°C for 12 months.

3.2.1.7. Ethanol solubility of ß-carotene

Standard solutions were prepared by an appropriate dilution of a stock of ß-carotene in chloroform into ethanol. The absorbance at various concentrations, ranging from 1μg/ml to 15μg/ml, was measured and standard curves plotted. The concentration of ß-carotene in ethanol was linear from 0μg/ml to about 5μg/ml (Figure 3.8). The ethanol was saturated with ß-carotene at higher concentrations as ethanol solubilises ß-carotene only moderately. This has to be taken into consideration when resuspending extracted samples in ethanol. There are other solvents such as benzene and hexane, which more readily solubilise carotenoids. However, these solvents are highly volatile and the concentration increases with time. Consequently, they are not suitable for work with large number of samples extracted simultaneously.

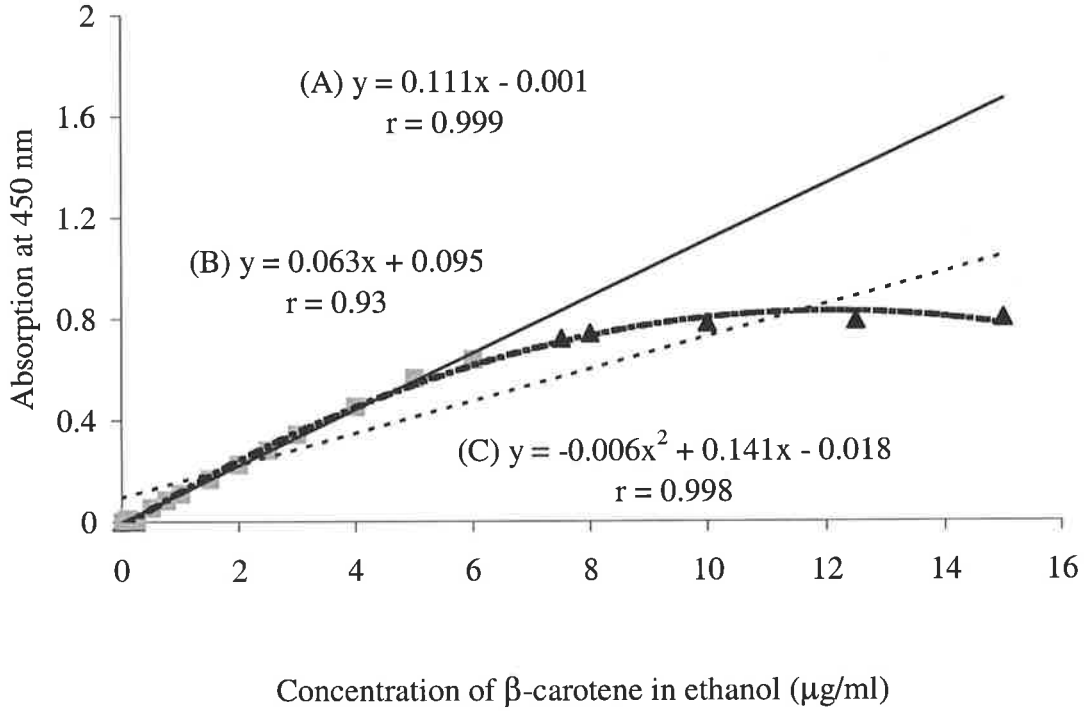


Figure 3.8. Saturation of β-carotene in ethanol. The concentration of β-carotene ranged from 0-15µg/ml; (A) represents a linear relationship between 0-5µg/ml (solid line), (B) represents linear relationship between 0-15µg/ml (doted line), (C) represents quadratic relationship between 0-15µg/ml (thick doted line).

3.2.2. Study 2

3.2.2.1. Carotenoid content in different anatomical sites

The difference in fresh or chilled fat colour between the left and right sides of the carcasses was determined for range of carotenoid concentrations from 0.9-4.5µg/g in fresh fat and 0.8-3.0µg/g in chilled fat (Figure 3.9). The test for differences between the equations showed that

the slopes were not different from 1 and intercepts were not different from 0. The correlations between the left and right sides of the carcasses were high and significant in both fresh and chilled fat (r=0.99 and r=0.97, respectively).

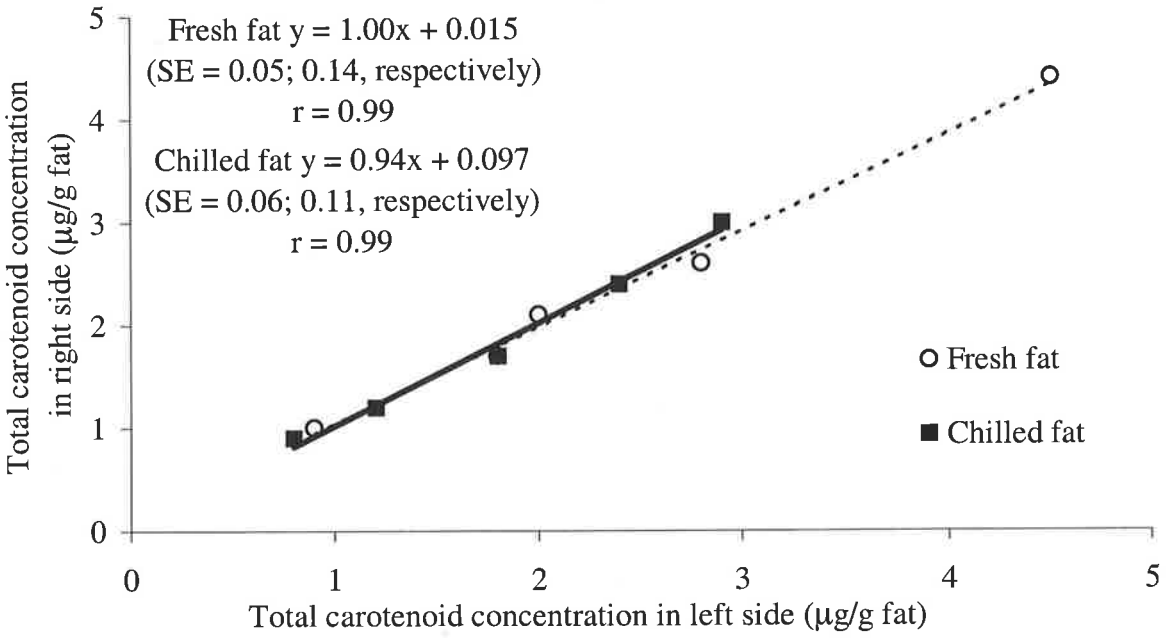


Figure 3.9. The relationship of total carotenoid concentration between left and right half-carcasses in fresh and chilled adipose tissue of cattle.

Total carotenoid concentration measured in left and right half-carcasses showed no difference between fresh and chilled fat. These findings agree with the other authors (Seirer et al, 1992) who did not find differences when measuring fat colour using a chromameter. However, they reported a significant difference between fresh and chilled carcasses. Such differences in carcass fat colour is probably due to the changes in the chiller (eg. fat drying and solidification) which lower the chromameter readings. Extraction and measurement of total carotenoids does not have these limitations.

The concentration of total carotenoids in rib, rump and shoulder wet fat did not differ significantly between the sites (Figure 3.10). No difference in these sites was also observed when the carotenoid content was expressed per gram of dry fat (data not presented). The concentration of total carotenoids in dry fat samples was higher. Breed had an effect on carotenoid concentration in fat. Jersey steers were higher in concentration compared to Limousin steers in both wet and dry fat (0.96 ± 0.03µg/g vs 0.33 ± 0.03µg/g and 1.62 ± 0.06µg/g vs 0.68 ± 0.06µg/g, respectively). Breed by site interaction was not significant for wet and dry fat samples.

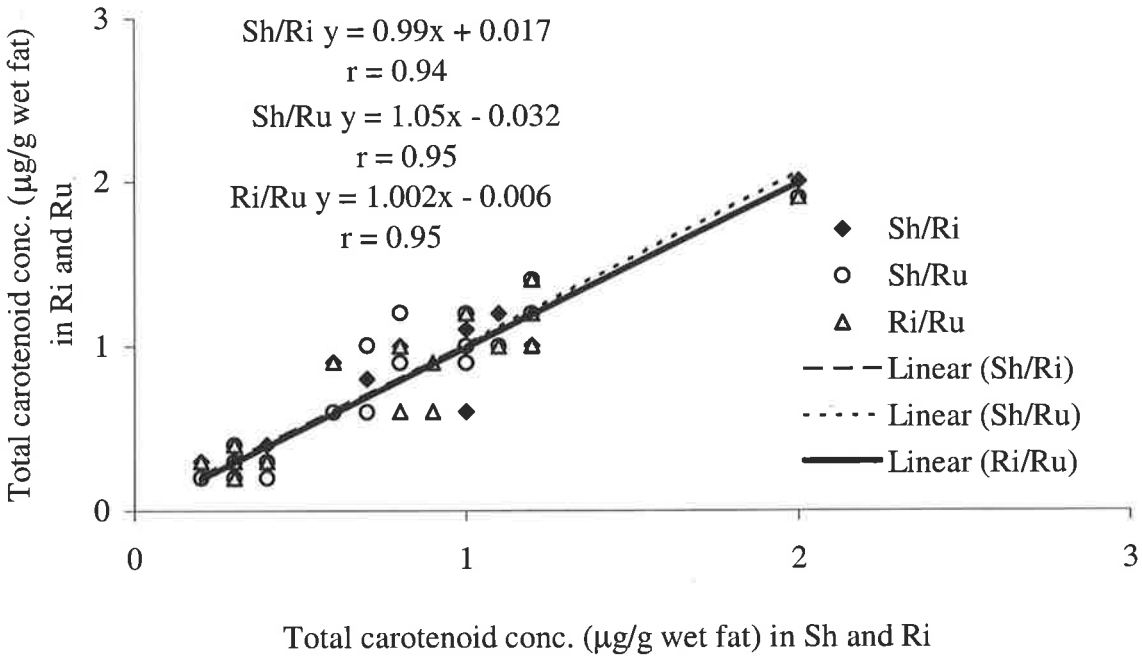


Figure 3. 10. Relationship of total carotenoid concentration between shoulder (Sh), ribs (Ri), and rump (Ru) in wet cattle fat.

Water content varied between the 3 sites tested with the lowest values for rump samples (8.9 ± 0.72%). The difference was significant ($P<0.01$) when water content was compared between the rump and ribs and shoulder (11.8% and 12.1% ± 0.72, respectively). Breed also had an effect on water content in fat with Jersey having significantly higher percentage of water than Limousin steers (12.3% and 9.5% ± 0.59, respectively). Breed by site interaction was not significant.

The shoulder, 10^{th}-13^{th} ribs, and the base of the tail are commonly sampled sites for fat in cattle. Total carotenoid concentration measured in these sites did not vary between the sites when the results were expressed per unit of dry or wet fat. Even the difference in water content between rump and the other sites observed in the studies reported here did not influence carotenoid measurement. The difference in water content was presumably caused by the conditions in the chiller. The rumps were closely situated to the air inlet and the direct flow of a cold air dried the fat faster than in other locations.

The lack of difference in carotenoid concentration between anatomical sites was observed regardless of the breed and range of carotenoid concentrations. Even such diverse breeds as Jersey and Limousin showed no difference in carotenoid concentration between the various anatomical sites. Thus, carotenoid concentration does not differ between various anatomical sites of subcutaneous fat in cattle and results can be compared even when the samples derive from different subcutaneous fat sites.

3.2.2.2. Relationship of carotenoid concentration between different adipose tissues

Two carotenoids, β-carotene and lutein, were measured separately in subcutaneous and intermuscular adipose tissues over a wide range of concentrations (1.7-47.6μg/g for β-carotene and 0.2-3.2μg/g for lutein). The concentration of β-carotene in the subcutaneous fat did not differ from that in the intermuscular fat (11.6 ± 2.1 vs 13.4 ± 2.2μg/g of fat, respectively). The correlation was positive and high (r=0.87, P<0.001). A similar trend was observed in the lutein content. The increased concentration of lutein in the subcutaneous adipose tissue was accompanied by similar increase in the intermuscular fat (r=0.93) and the relationship was statistically significant. These results demonstrated that there was no difference in the concentration of β-carotene and lutein in subcutaneous and intermuscular adipose tissues. Yellowness of intermuscular fat was used to subjectively assess fat colour score by AUS-MEAT® assessors. Lack of a difference between these two adipose tissues would allow the use of both tissues for this assessment, especially if the layer of intermuscular fat is thin.

3.2.2.3. Carotenoids and subjective fat colour scores

Subjective fat colour score estimated according to AUS-MEAT® specifications was moderately or highly correlated with total carotenoid content in carcass fat (Table 3.5) and the correlation was similar to that observed by other authors (Zhou et al, 1993). Subjective fat colour score assessed in our 5-point scale was moderately to highly correlated with total carotenoid concentration at biopsy (Table 3.5).

Table 3.5. Correlations between total carotenoid concentration and subjective fat colour scores in weaner biopsy and carcass fat.

	Fat colour score (FCS)	
	Biopsy (5 point scale)	Carcass AUS-MEAT®
Biopsy: Total carotenoid concentration (μg/g fat)	0.57*** (1996) 0.82*** (1997) 0.63*** (1998) 0.65*** (1997, NZ)	
Carcass: Total carotenoid concentration (μg/g fat)		0.63*** (1996)

Note: total carotenoids expressed as μg/g of wet fat, years represent year of biopsy sampling
NZ = New Zealand.

Subjective estimates depend on assessors skills and may not be highly accurate (Gaunt et al, 1994). However, the high and highly significant correlations from biopsy samples demonstrate that the relationship is very constant regardless of the year of sampling, breed of cattle, and geographical location. Higher correlations between biopsy fat colour score and biopsy total carotenoid concentration show that even if the 5-point scale is not as precise as the AUS-MEAT® 10 point scale, it does assign animals to different fat colour groups with accuracy.

3.3. Summary

(i) Carotenoid extraction assays were developed to give consistent and highly repeatable results with recovery of ß-carotene ranging from 91.0-94.8%. The assays were very similar regardless of tissue extracted. The small amounts of sample required for these assays made them suitable for biopsy techniques. Multiple repetitions with large numbers of extractions were able to be conducted simultaneously. Investigation of the extraction steps avoided a number of methodological problems. Another important advantage of these refined methods is that they demonstrate good recovery of ß-carotene from samples with high concentrations.

(ii) Storage of samples for carotenoid measurement in the dark at -20°C in N_2 atmosphere demonstrated that such conditions protect these oxidisable substances for at least 12 months.

(iii) The high percentage of β-carotene in total carotenoids and the strong correlation between spectrophotometry and HPLC estimates demonstrate that β-carotene is the major component absorbed at 450nm in the tissues of Jersey and Limousin cattle and can be satisfactorily measured by spectrophotometry.

(iv) Carotenoid concentration does not differ between various anatomical sites of subcutaneous fat in Jersey and Limousin cattle. Therefore, the results can be compared directly between different studies where carotene concentrations were estimated in various anatomical locations.

(v) Different adipose tissues (subcutaneous and intermuscular) do not differ in β-carotene and lutein content within the investigated range of concentrations. The lack of a difference makes it possible to use the subcutaneous fat for estimating subjective fat colour scores in field work or at slaughter if the layer of intermuscular fat is thin.

Chapter 4

Genetic and non-genetic factors affecting carotenoid concentration in cattle fat

4.0. Introduction

The colour of cattle fat is influenced by various environmental and genetic factors (Hayes et al. 1995). A number of studies, which demonstrate the effect of breed, age, and diet on fat colour have been conducted using different breeds (Morgan et al., 1969; Forrest, 1981; Walker et al., 1990). Jersey and Limousin are breeds of cattle divergent in many metabolic and market traits. The Jersey are dairy cattle which mature early. They deposit large amounts of intramuscular fat (marbling), particularly noticeable in the eye muscle (*M. longissimus dorsi*). The fat is frequently yellow in colour. In contrast, the Limousin are late maturing beef cattle with low marbling and pale or white fat (Cundiff et al., 1988). Factors affecting fat colour in various environments in these diverse breeds were studied in three experiments. Experiment 1 investigated the influence of age, breed, year and season using mature cows, experiment 2 examined the effects of sex, year, breed, sire and geographical location in young weaners, and experiment 3 explored the effect of breed and sire using a range of sire breeds.

4.1. Materials and methods

4.1.1. Animals and management

Pure Jersey and Limousin cows aged between 20-124 months and 25-141 months, respectively, were used in experiment 1. The cows were a part of the J.S. Davies Cattle Gene Mapping Herd maintained at the Martindale property, Mintaro, South Australia (Chapter 2).

In experiment 2, two cattle herds were utilised: 286 Jersey backcross and Limousin backcross weaners from Martindale which were born in April/May 1996 and 1997, and 404 backcross weaners from New Zealand which were born in August 1996 and 1997. The animals grazed pastures supplemented with hay when necessary.

The animals for experiment 3 were derived from the Southern Crossbreeding Project and represented 7 sire breeds with 6-17 (average 12) progeny per sire (Chapter 2). The group comprised of 654 male and female calves born in 1994 and 1995. The calves were situated in two locations: Struan, 14km south of Naracoorte SA with average rainfall of 540mm and heavy textured soils, and Wandillo, near Mt Gambier, with 600mm average rainfall and lighter sandy soils. Progeny of the same breeds and sires were held at each location. After weaning at about 250 days of age, calves from Wandillo were transported to Struan. The Struan herd was divided into three groups and run in different paddocks, which varied in soil type, ground water level, etc. At approximately 450 days of age, the animals were placed in a

feedlot where they were grain fed for 70 days (heifers born in 1994 and 1995) and 170 days (steers born in 1994) before being slaughtered.

4.1.2. Sample collection and preparation

For experiment 1, fat was collected by biopsy sampling [198 cows in January-April 1994 (56 animals), April 1995 (30 animals), April 1996 (30 animals), and January 1998 (163 animals)]. To investigate seasonal differences, the same 30 cows sampled in April 1995 (with exception of 2) were re-sampled again in November 1995.

Biopsy fat samples from the animals used in experiment 2 were obtained at weaning when the animals were about 250 days of age. Weaner animals at Martindale were sampled in January/February whereas New Zealand cattle were sampled in July but calves were similar age. Details of the biopsy sampling procedure are described in Materials and Methods (Chapter 2).

Carcass fat samples from animals used in the experiment 3 were collected from carcasses stored in the chiller on the day after slaughter. Fat was placed in plastic vials and stored at -20°C.

4.1.3. Sample analysis

Total carotenoid concentration in fat was estimated spectrophotometrically as described in chapter 2. Fat colour score at biopsy was estimated on a 5-point scale ranging from 1 to 5. Fat colour at slaughter was assessed by AUS-MEAT® assessors on a 10-point scale.

4.1.4. Statistical analyses

The data was analysed using the GLM, MIXED, and CORR procedures in SAS, 1989. In experiment 1, Proc GLM was run using a model which included fixed effects of year (1994, 1995, 1996, 1998), age (20-141 months, Table 4.1), breed (Limousin or Jersey). Interactions between breed and year, breed and age, breed and year and age were also included (Table 4.2). The breed by age, year by age and breed by age by year interactions were not significant and were removed from the model. Season was treated as a separate year and run in one analysis (e.g. 1995 summer and 1995 winter).

The model used in experiment 2 included year, geographical location, sex, the interaction between year and location, the interactions between year and sex, location and sex, year by location by sex, breed, the interactions between year and breed, location and breed, sex and

breed, year and location and breed, with sire nested within location and nested within breed by location. Sex, breed by sex, and year by breed interactions were not significant and were removed from the model (Table 4.2).

Table 4.1. Average age of Jersey and Limousin cows.

Year	Breed			
	n	Jersey	n	Limousin
1994	34	42.3 ± 20.0	22	46.2 ± 19.0
1995	15	66.0 ± 20.0	15	54.5 ± 14.1
1996	15	66.5 ± 25.0	15	75.8 ± 21.8
1998	68	66.6 ± 29.2	58	84.3 ± 20.1

Note: n=number of cows investigated in each year, values=average age ± standard deviation, where age expressed in months.

Table 4.2. ANOVA for total carotenoids and fat colour score (experiments 1, 2 and 3).

Effect	Experiment 1		Experiment 2		Experiment 3	
	Carotene	FCS	Carotene	FCS	Carotene	FCS
Year	***	***	***	ns	-	-
Location (geographical)	-	-	***	***	***	***
Year x location	-	-	***	*	-	-
Year x sex	-	-	**	*	-	-
Sex x location	-	-	**	ns	-	-
Year x sex x location	-	-	*	ns	-	-
Breed	***	***	***	***	***	***
Location x breed	-	-	***	*	ns	ns
Year x location x breed	-	-	**	***	-	-
Sire(location)	-	-	***	***	-	-
Sire(location x breed)	-	-	*	ns	-	-
Breed x year	ns	***	-	-	-	-
Year x age	ns	**	-	-	-	-
Cohort	-	-	-	-	***	***
Location x cohort	-	-	-	-	**	*
Breed x cohort	-	-	-	-	*	*

Note: ns = not significant, ***=P<0.001, **=P<0.01, *=P<0.05, Cohort=year x sex, FCS=fat colour score.

In experiment 3, year and sex were partially confounded. To enable the computation of means, three cohorts were created: cohort 95M which comprised only males born in 1995 which were grain fed for 170 days, cohort 95F comprising females born in 1995 and grain fed for 70 days, and cohort 96F comprising females born in 1996 and fed for 70 days on feedlot. Means were computed using Proc GLM and the final model (after removing all non-significant interactions) included: location, post-weaning treatment, cohort, the interaction between location and cohort, breed, and the interaction between breed and cohort (Table 4.2). To estimate the sire variance component, Proc MIXED was run where sire was nested within breed and was fitted as a random effect. The raw correlations between total carotenoids and subjective fat colour score were computed using Proc CORR.

4.2. Results and discussion

4.2.1. Carotenoids, fat colour and age

In order to examine the effects of age on fat colour, cows of different ages maintained under the same feeding regime were biopsied for subcutaneous fat samples (experiment 1). It was observed that carotenoid content in fat and fat colour score increased with age. However, the differences between young and old cows were not statistically significant (Table 4.2). The same trend appeared in both cattle breeds. The Jersey and the Limousin cows did not differ in the range of ages in each year of investigation (Table 4.1). The increase in carotenoid concentration affected both breeds in the same way as the interaction between breed and age was not significant and the equation slopes (describing the concentration of carotenoids and fat colour score with age course) were not different from each other.

The lack of difference in fat colour between young and old cows confirms the study of Morgan et al., (1969), in studying Jersey, Friesian, Aberdeen Angus, and various crossbreeds, reported that fat colour intensity appeared unrelated to animal age. The concentration of carotenoids in the present study was higher than those of Morgan *et al.,* and ranged from 0.8-23.7μg/g fat vs 1.4-8.0μg/g fat, respectively. This demonstrated that age did not affect fat colour even if the concentration of carotenoids was very high.

In contrast, other studies have demonstrated a higher fat colour score in older animals. For example, in the study conducted by Shemeis et al., (1994) using Danish cull cows of three age groups, there was a significant difference in fat colour between the age groups as measured on a 5-point scale on carcass. The difference in findings could be due to the fact that in present study, the data set was 2.8 fold larger, cows were not divided into the age groups, fat colour and carotenoid concentration were assessed on biopsy samples and the

proportion of mature cows was higher than in the Danish study (61.8% vs 22.4%, respectively). Hayes et al., (1995) also reported an influence of age on female fat colour in various breeds and cross breeds including Brahman cattle. However, they had lower fat colours compared to the present study (2.87 average on a 10 point scale).

These opposing results of the different studies could be explained by different experimental conditions, and breeds of animal used. However, it should be noted that age influenced the yellowness of fat when the concentration of carotenoids in fat or chromameter values were low. In this experiment and the study conducted by Morgan et al., (1969), the concentration of carotenoids was extremely high and Jersey cattle were present in the both studies.

4.2.2. Carotenoids, fat colour and sex

It was found that sex did not have an impact on total carotenoid concentration and fat colour in JJJL and LLLJ weaners (experiment 2). Males had 1.30 ± 0.04μg of carotenoids per gram of fat and an average fat colour score of 1.74 ± 0.04, whereas females had 1.27 ± 0.04μg of carotenoids per gram of fat and an average fat colour score of 1.72 ± 0.04. However, a difference between the sexes appeared in different geographical locations as the sex by location interaction was highly significant (Table 4.2). Steers in Australia (Davies Gene Mapping project) did not differ from heifers in total carotenoid concentration (0.88 ± 0.06μg/g and 0.81μg/g ± 0.07, respectively) and in the subjective fat colour score (1.88 ± 0.05 and 1.88 ± 0.06, respectively). In 1997, there was no difference in carotene concentration in fat between different genders in New Zealand. However, in 1998, sex differences were observed where males had higher total carotenoid concentration and fat colour scores than females (2.19 ± 0.08μg/g, 1.57 ± 0.07 and 1.79 ± 0.08μg/g, 1.39 ± 0.07, respectively). However, the growth of the weaner steers in 1998 was greatly affected by an outbreak of facial eczema. There are no reports available about the influence of this disease on carotene metabolism or fat colour in cattle but this factor cannot be excluded and may have caused the sex differences which were observed only in 1998 in New Zealand.

Variable reports exist about sex influence on fat colour in cattle. Walker et al., (1990) reported yellower fat in females of British breed cattle than in castrates across all age categories. Higher fat colour scores in heifers were also reported by Hayes et al., (1995) in Brahman and Brahman cross cattle. In contrast, Morgan et al., (1969) did not find such differences between heifers and steers, although they reported inconsistent differences in the 9 and 21 months slaughter groups. The results from this study cannot be compared directly with the other studies due to differences in breeds, techniques in the estimation of fat colour,

and environment. However, use of Jersey cattle, high carotenoid content in fat, and lack of sex differences in general show similarity with Morgan's study. The sex difference demonstrated between cattle in New Zealand was also associated with a 2.1 fold higher concentration of carotenoids in fat than in Australia.

4.2.3. Carotenoids, fat colour and year

Variation in total carotenoid concentration was observed in different years in experiments 1 and 2. In experiment 1, the highest concentration of carotenoids in the cows appeared in 1994 and 1998 (Figure 4.1). There was no significant difference between these years. In years 1995 and 1996, the concentration decreased reaching a level which was significantly different from 1994 and 1998. However, the carotenoid content in fat did not vary significantly between 1995 and 1996. Both cattle breeds were affected in the same way as the interaction between breed and year was not significant.

Fat colour score in experiment 1 followed a similar pattern to total carotenoid concentration in cows (Figure 4.2). The years 1994 and 1998 had the highest values and they differed from each other. Years 1995 and 1996 had lower fat colour. Fat colour scores did not differ significantly between 1994, 1995 and 1996. Breed by year interaction was significant in subjective fat colour score. In all years, Jersey cows were higher in fat colour score except 1998 where the difference between breeds was not statistically significant.

In experiment 2, a higher concentration of carotenoids in fat of weaners was observed in New Zealand weaners in 1998 than in 1997 (1.99 ± 0.06 and 1.48 ± 0.04, respectively). On the other hand, Australian weaners did not differ in years (0.84 ± 0.06 and 0.84 ± 0.07, respectively).

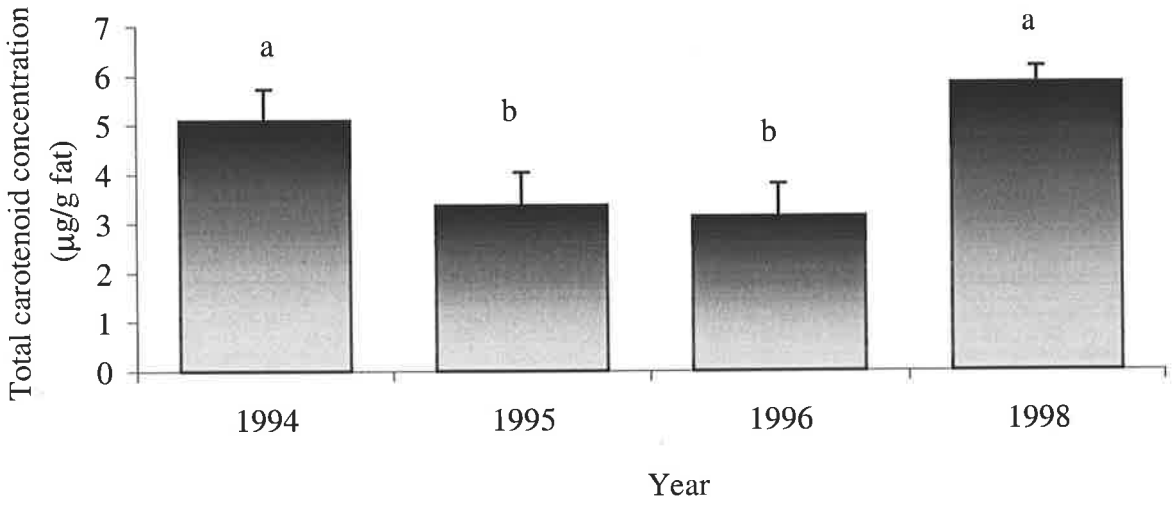


Figure 4.1. Year effect on carotene concentration in cattle.

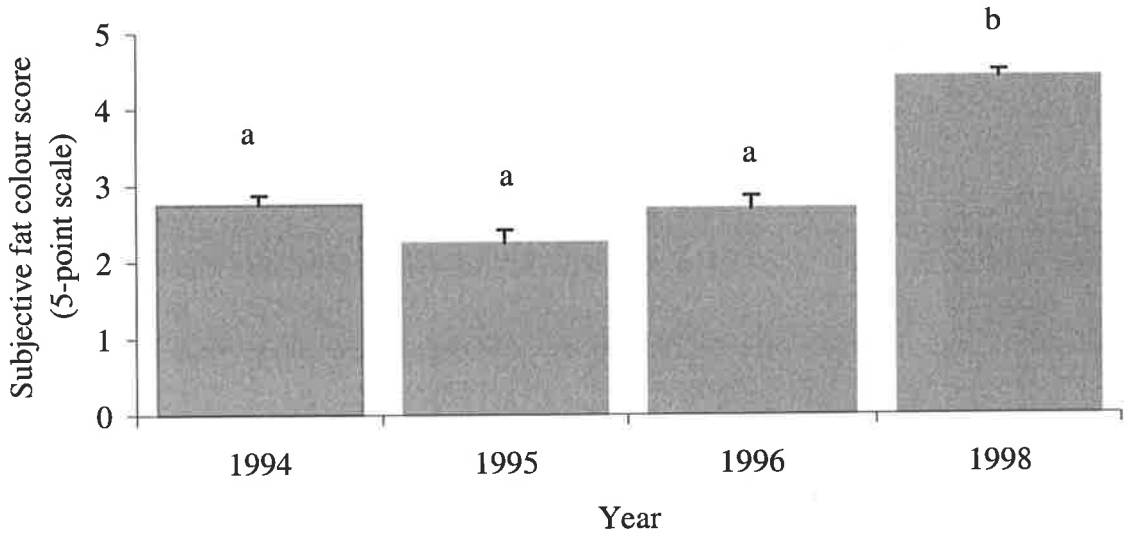


Figure 4.2. Year difference in subjective fat colour score.

Nakama et al., (1986) concluded that the concentration of carotene in cattle is dependent upon the amount of green grass grazed by cattle. Variation in carotene concentration in various grasses exists and is influenced by a number of environmental and climatic conditions (Visser and Blair, 1991). A significant difference in rainfall between the 2 years was observed in New Zealand during the period of birth to sampling. In 1997, the rainfall was lower than in 1998 (1038mm vs 1238mm, respectively). The lack of a difference in carotene content in the Australian weaners was accompanied by a lack of difference in rainfall between 1997 and 1998 (545.4mm and 490.5mm, respectively). There was a high correlation between the concentration of total carotene and rainfall between the month of birth and sampling (r=0.99, Figure 4.3). Rainfall is only one of many factors influencing the growth of green pasture, but in this case could be the major factor.

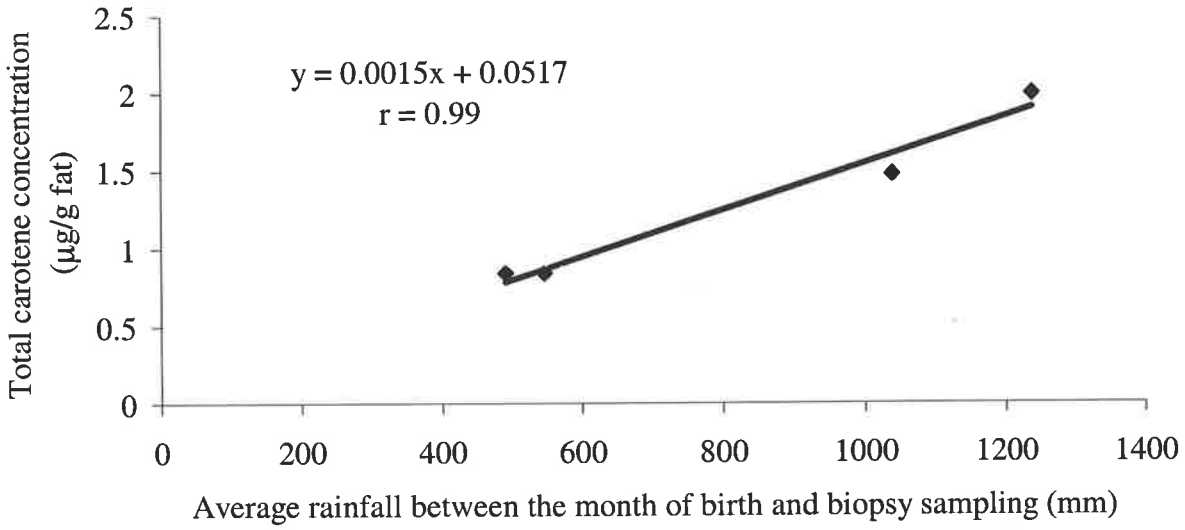


Figure 4.3. Correlation between rainfall and carotene concentration in subcutaneous fat. (Australian and New Zealand samples in 1997-1998).

The difference in concentration of carotenoid in fat between different years in the cows of experiment 1 cannot be explained only by the amount of rainfall in these years. The highest concentration of carotene in 1994 and 1998 were accompanied by the lowest total rainfall (366mm and 523mm, respectively). In 1995 and 1996, when carotene content was significantly lower, the rainfall was higher (607mm and 627mm, respectively). Due to the fact that cows spent a longer period of time at pastures compared to the weaners, they may have been affected by other factors (McGillivray, 1960).

4.2.4. Carotenoids, fat colour and geographical location

The mean concentration of carotenoids in fat in New Zealand weaners was 2.1 times higher than that in Australian weaners (1.73 ± 0.04μg/g and 0.84 ± 0.05μg/g, respectively). The New Zealand location values were greater regardless of year of sampling, breed of animal, sire or sex. In New Zealand, there was also 1.9 times higher rainfall than in Australia (1045.5mm vs 566.6mm, respectively) which accounted for greater growth of green pastures and greater abundance of carotene. Moreover, other locational factors such as average length of growing season, available water capacity in soils, soil type, soil characteristics of drainage and depth, relief and vegetative cover differed between these geographical locations (data not presented here). On the other hand, the results from New Zealand have to be carefully interpreted when compared with South Australia as the New Zealand weaners spent 11 months on the grass while South Australian weaners only 8. This three months period of time could be crucial in accumulation of carotenoids in fat, especially in the animals with a fully developed rumen.

Pre-weaning location also had an influence on fat colour of grain fed cattle (experiment 3). Weaners from Wandillo, which was characterised by higher annual rainfall and light textured soils, had higher carotene content in the fat than weaners from Struan characterised by lower annual rainfall and heavy textured soils (1.48 ± 0.04μg/g fat vs 1.30 ± 0.03μg/g fat, $P<0.01$, respectively). The progeny of 7 sire breeds which were fed on different type of pastures (Experiment 3) varied significantly in total carotenoid content in 1996 (1.52 ± 0.06μg/g fat vs 1.15 ± 0.05μg/g fat, $P<0.001$, respectively). Such differences in 1996 were also observed in a 10-point scale subjective fat colour score (0.28 ± 0.06 vs 0.02 ± 0.05, $P<0.001$, respectively). The crossbreeds did not differ significantly between the 1995 female or male cohorts.

4.2.5. Carotenoids, fat colour and season

Total carotenoid content in the fat of Jersey and Limousin cows did not vary significantly between summer and winter (3.36 ± 0.67μg/g and 3.18 ± 0.66μg/g, respectively). This was confirmed by subjective fat colour score which also did not differ between these two seasons (2.24 ± 0.17 in summer and 2.41 ± 0.17 in winter). Given the alteration of feed between seasons, it is most likely that the turnover of carotenoids in the adipose tissue is not a rapid process, unlike in blood. Blood plasma levels reflect dietary carotenoid intake over the recent few weeks, whereas tissue levels indicate longer-term intake (Davison et al., 1993). Yang et al., (1993) reported that after 8 weeks on low carotenoid diets, carotenoid concentration in fat and fat colour were not affected. This was confirmed by McCaughey and Cliplef (1996), who observed no changes in fat colour when steers were grazing on alfalfa/grass pastures and finished for 75 days on grain. The slow turnover can account then for the lack of effect of the summer feed on the reduction of total carotenoids in adipose tissue of the Martindale cows. Thus, while geographical location and environmental factors such as rainfall can affect fat colour, small seasonal changes in diet will not result in large differences in adipose carotenoid concentration.

4.2.6. Carotenoids, fat colour and breed

Breed differences in carotenoid concentrations were observed in all 3 experiments. In experiment 1, Jersey cows had 79% higher concentration of carotenoids than Limousin cows (5.29 ± 0.37μg/g fat and 2.95 ± 0.39μg/g fat, respectively). Fat colour score (FCS) followed the same pattern and was 46% higher in the Jersey cows (3.44 ± 0.09) than in the Limousin cows (2.35 ± 0.09).

Higher values for carotenoid and FCS were also recorded in Jersey reciprocal crosses when Jersey cows were crossed with Jersey by Limousin F1 bulls (experiment 2). The Jersey backcross weaners from New Zealand had 56% higher carotenoid content in their fat than Limousin backcross weaners (2.12± 0.04 and 1.36 ± 0.06, respectively). This large difference was not noted in the backcrosses from Australia, where LJJ weaners were only slightly higher (15%) from LJLL weaners (0.9 ± 0.07 and 0.78 ± 0.07, respectively). Correspondingly, the difference between the backcrosses in fat colour scores just failed significance ($P<0.06$) in the Australian herd (1.97 ± 0.06 in LJJ and 1.79 ± 0.06 in LJLL) but was significant in the New Zealand herd (1.81 ± 0.03 for LJJ and 1.36 ± 0.06 for LJLL).

In the Southern Crossbreeding Project, there were significant differences between the breeds in both total carotenoid concentration and fat colour score (experiment 3, Table 4.3). Jersey

crosses had the highest carotene content and subjective fat colour score followed by Belgian Blue crosses. The difference between these crossbreeds was significant. Wagyu crossbreed ranked third for total carotenoid content followed by South Devon. Wagyu x Hereford differed significantly from Jersey x Hereford and Belgian Blue x Hereford, but did not differ significantly from South Devon x Hereford. These two crossbreeds did not differ significantly from Limousin x Hereford and Angus x Hereford which ranked fifth and sixth, respectively. Wagyu x Hereford cattle were significantly higher than pure Hereford which ranked the lowest. South Devon x Hereford did not differ from pure Hereford cattle. The lowest two crossbreeds, Limousin x Hereford, Angus x Hereford, and pure Hereford did not differ significantly.

The ranking of different breeds for fat colour score was similar to the carotene content ranking list. Wagyu x Hereford cross ranked third. There were no significant differences between Hereford, Angus x Hereford, South Devon x Hereford, and Limousin x Hereford which ranked fourth to seventh, respectively.

Table 4.3. Breed differences in total carotenoid concentration (A) and subjective fat colour score (B) in Struan Hereford crossbreeds.

A		B	
Total carotenoid concentration (µg/g fat)		Subjective fat colour score (AUS-MEAT® 10-point scale)	
Sire breed	Mean ± SE	Sire breed	Mean ± SE
Jersey	1.94 ± 0.05^{a}	Jersey	0.52 ± 0.05^{a}
Belgian Blue	1.53 ± 0.05^{b}	Belgian Blue	0.26 ± 0.05^{b}
Wagyu	1.35 ± 0.05^{cf}	Wagyu	0.16 ± 0.05^{bc}
South Devon	1.31 ± 0.06^{cdf}	Hereford	0.14 ± 0.06^{bcd}
Limousin	1.23 ± 0.05^{cdeg}	Angus	0.11 ± 0.05^{bcde}
Angus	1.22 ± 0.06^{cdef}	South Devon	0.07 ± 0.05^{cdef}
Hereford	1.15 ± 0.06^{ef}	Limousin	0.05 ± 0.05^{cdef}

Note: SE = standard error, $^{a-g}$ = within column means followed by the same letter are not significantly different (P>0.05).

Fat colour rankings were the same regardless of year of birth, days in feedlot or sex. In contrast, carotenoid concentration differed as the animals were assigned to different cohorts

(Table 4.4). Jersey x Hereford and Belgian Blue x Hereford heifers which were lot fed for 70 days, had the highest carotenoid concentration compared with the other cross-breeds. They did not differ from each other but significantly differed from the other breeds. The remaining breeds in this cohort did not differ.

Table 4.4. Mean total carotenoid concentration (μg/g fat) in subcutaneous adipose tissue of seven sire breeds assigned to different cohorts.

Cohort		
95F	95M	96F
JxH: 2.03 ± 0.08^{a}	JxH: 1.79 ± 0.09^{a}	JxH: 2.00 ± 0.09^{a}
BxH: 1.83 ± 0.08^{ab}	BxH: 1.31 ± 0.08^{b}	WxH: 1.44 ± 0.10^{b}
LxH: 1.49 ± 0.09^{c}	AxH: 1.23 ± 0.09^{bc}	BxH: 1.43 ± 0.10^{bc}
SxH: 1.45 ± 0.11^{cd}	WxH: 1.21 ± 0.08^{bcd}	SxH: 1.38 ± 0.08^{bcd}
HxH: 1.44 ± 0.12^{cde}	LxH: 1.11 ± 0.08^{bcde}	LxH: 1.08 ± 0.08^{e}
AxH: 1.40 ± 0.10^{cdef}	SxH: 1.08 ± 0.09^{cdef}	AxH: 1.01 ± 0.09^{ef}
WxH: 1.39 ± 0.08^{cdef}	HxH: 1.02 ± 0.09^{cdef}	HxH: 0.97 ± 0.10^{ef}

Note: 95F=female born in 1995 and grain fed for 70 days, 95M=male born in 1995 and grain fed for 170 days, 96F=female born in 1996 and grain fed for 70 days, AxH=Angus x Hereford, BxH=Belgian Blue x Hereford, HxH=pure Hereford, JxH=Jersey x Hereford, LxH=Limousin x Hereford, SxH=South Devon x Hereford, WxH=Wagyu x Hereford, $^{a-g}$ =within column means followed by the same letter are not significantly different (P>0.05).

Cohort 95M included only male cattle fed for 170 days on a high grain diet. Jersey x Hereford steers had significantly higher carotenoid concentration than the other breeds. Belgian Blue x Hereford ranked second but were not significantly different from Angus x Hereford, Wagyu x Hereford, and Limousin x Hereford which ranked as third, fourth and fifth, respectively. South Devon x Hereford and pure Hereford ranked last and did not differ significantly.

Among females born in 1996, Jersey x Hereford crosses had higher carotenoid concentration in adipose tissue followed by Wagyu x Hereford, Belgian Blue x Hereford, and South Devon x Hereford. The difference between these three crossbreeds was not significant. The remaining three crossbreed heifers ranked last and did not differ significantly between each other.

It has been reported by Morgan et al., (1969) and Barton et al., (1994) that Jersey cattle have more yellow fat than Friesian, Aberdeen Angus, and Jersey crosses with Friesian, Charolais, and Hereford. The present study demonstrates a difference between Jersey and Limousin cattle. The higher results of Jersey cattle over the other beef breeds in fat colour extended to their reciprocal crosses with Limousin cattle as well as F1 crosses with Hereford cows. This confirms the findings of Walker et al., (1990) that dairy cattle breeds have yellower fat than British and European beef breeds. The lack of difference between LJJJ and LJLL in Australia and significant difference between these cross-breeds in New Zealand can be explained by different environmental conditions in these two locations.

Grain feeding reduces fat colour in cattle (Forrest, 1981; Strachan et al., 1993). The progeny from six crossbreeds with Hereford cows in the experiment 3 ranked similarly regardless of the length of time in feedlot. Jersey crosses had the highest carotenoid concentration followed by Belgian Blue cross. The difference between the remaining crossbreeds was not significant in the majority of crosses. The Limousin x Hereford cattle ranked between the 3-5 positions and did not differ significantly from the other breeds.

4.2.7. Carotenoids, fat colour and sire

Sire effect on carotenoid concentration and subcutaneous fat colour was investigated in the experiments 2 and 3. In experiment 2, four LLJJ sires were used to produce Jersey backcross and Limousin backcross progeny in two consecutive years in Australia. The sire differences in carotenoid concentration were not significant except sire 398, which had the highest total carotene content among Australian sires and just missed the significant level ($P<0.06$, Figure 4.4). There was no difference in subjective fat colour score between the sires (Figure 4.5). In contrast, the three F1 sires, which were used to obtain the backcross progeny in the New Zealand herd, did differ significantly in both total carotenoid content and fat colour (Figures 4.4 and 4.5).

For total carotenoid content of the New Zealand Jersey cross weaners, sire 402 ranked highest followed by sires 394 and 417. The difference between all sires was significant. In the New Zealand Limousin crosses, the sires were ranked in the same order as for Jersey backcross calves and were significantly different except sires 394 and 417 (Figure 4.6).

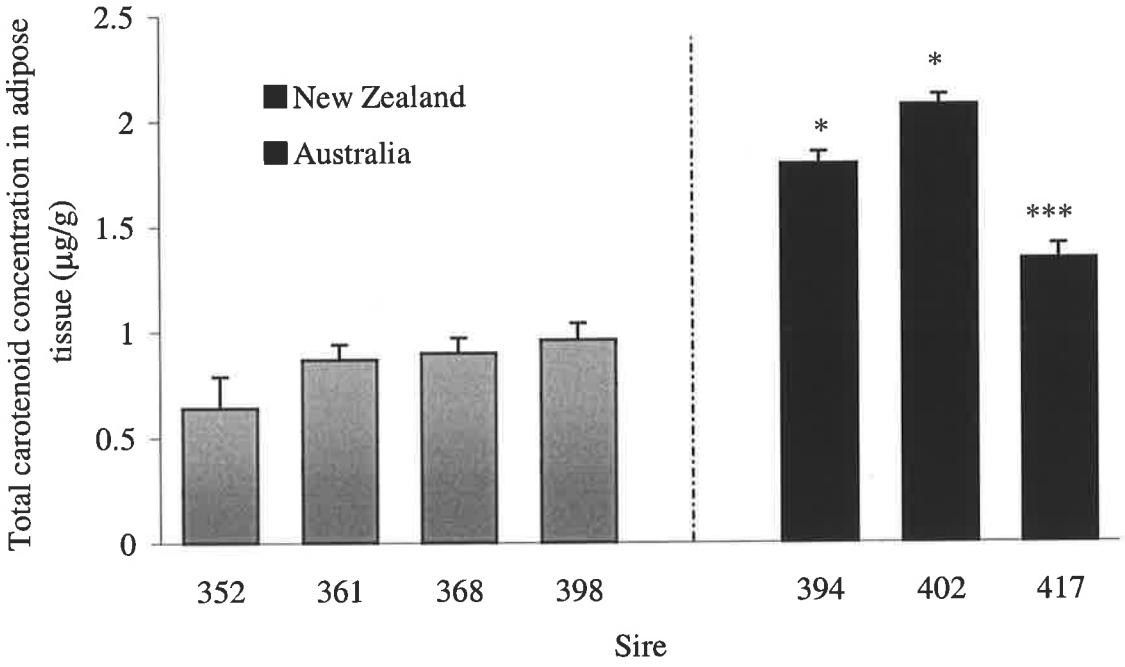


Figure 4.4. Sire differences in adipose carotenoid concentration of cattle in Australia and New Zealand. * = P<0.05, *** = P<0.001 within the location.

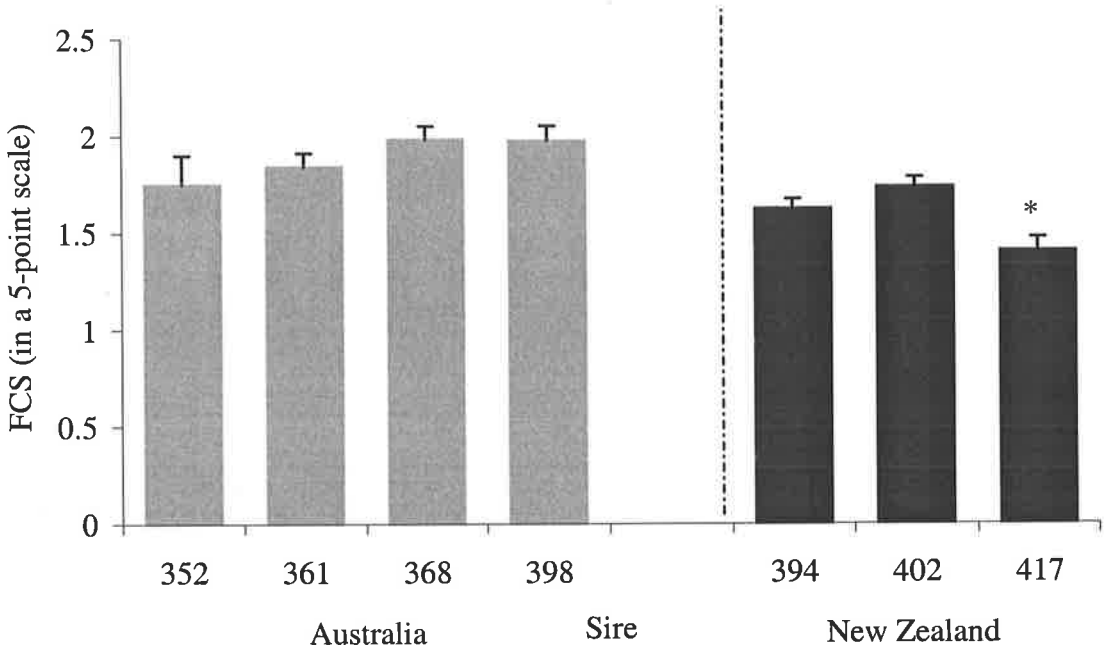


Figure 4.5. Sire differences in fat colour score (FCS) in Australia and New Zealand. * = P<0.05, within the location.

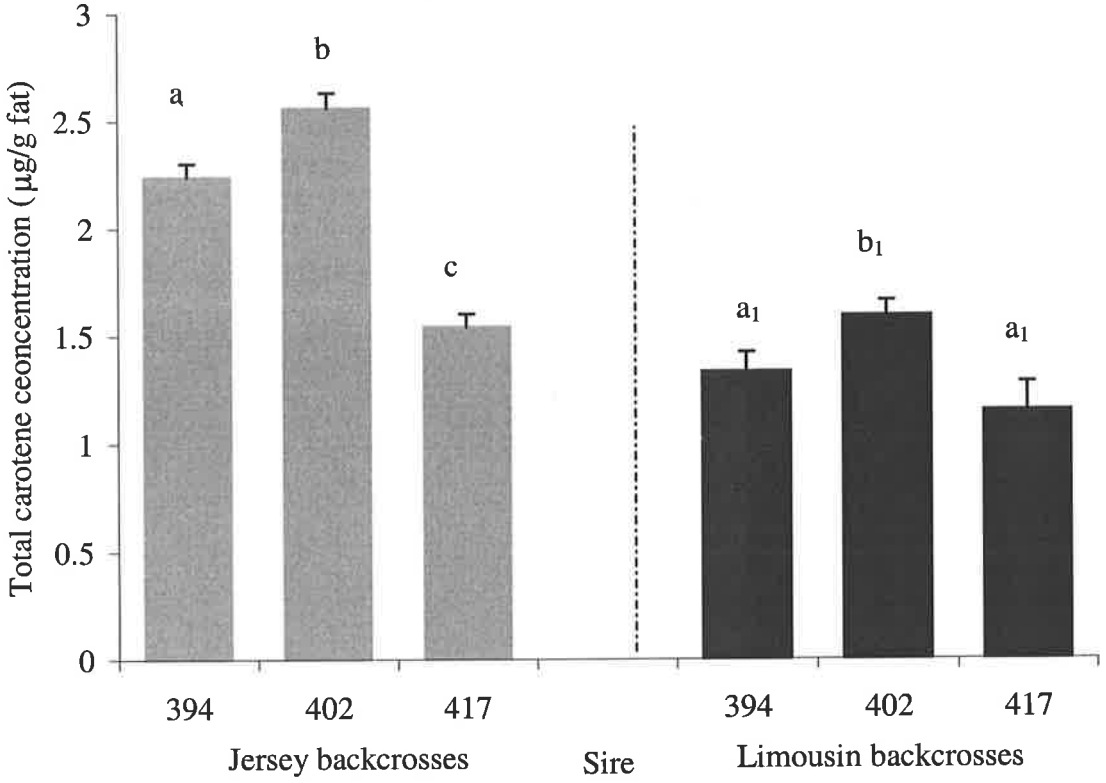


Figure 4.6. Sire differences in adipose carotene concentration in New Zealand backcrosses. (a, b, c = significantly different within the location).

In the Australian herd, no significant difference in carotene concentration was noted between sires except sire 398 in Jersey backcrosses, which just missed statistical significance (P<0.08, Figure 4.7). The difference among the other sires within both crossbreeds was not significant.

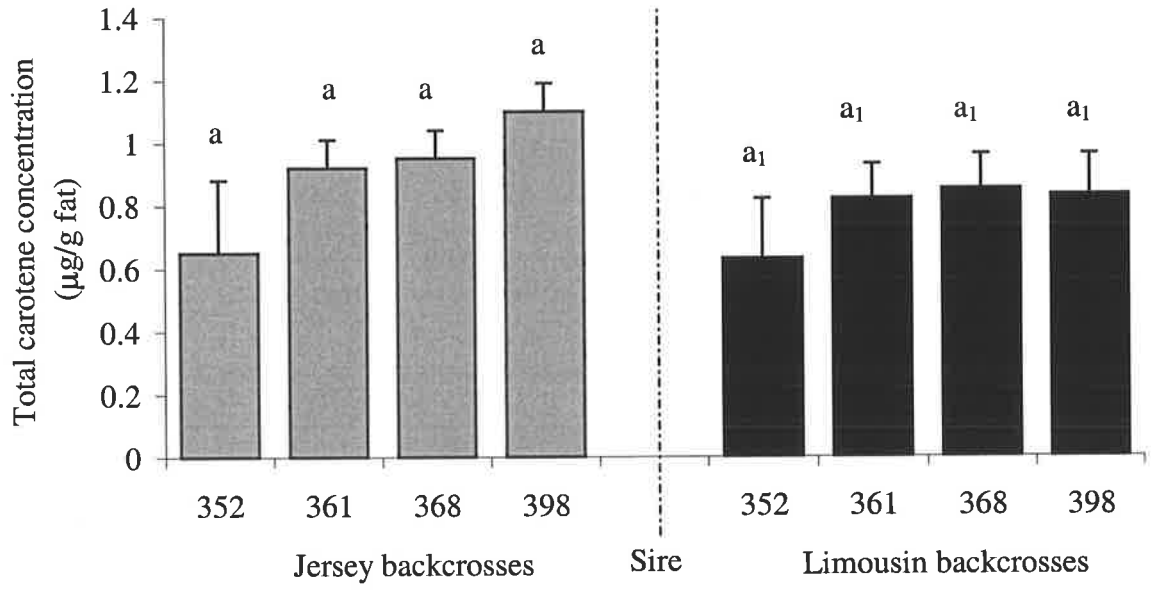


Figure 4.7. Sire differences in adipose carotene concentration in Australian backcrosses.

In experiment 3 which involved 52 sires from seven sire breeds, no significant sire influence within breed was detected in fat colour or total carotenoid concentration in adipose tissue. The sires within each breed did not differ significantly.

Within breed variation in fat colour was observed in the study of Barton (1968) and Morgan et al, (1969). This was also observed herein (experiment 2). In New Zealand, the variation in total carotenoid concentration was broader than in Australia and was due to the differences between sires. This finding clearly demonstrates that differences between the sires can account for within breed variation in fat colour. Moreover, all three sires from New Zealand were ranked in the same order regardless of the backcross. It is interesting to note that the difference between sires appeared only when the carotenoid concentration was high.

4.3. Summary

(i) An increase in total carotene in fat and subjective fat colour score with increased age was observed, however, this trend was not statistically significant. The same tendency appeared in both breeds regardless of the year of sampling or carotene content.

(ii) Sex did not influence fat colour among weaners in different environments with the exception of the New Zealand 1998 weaners. The sex difference which appeared in New Zealand in 1998 could be due to the other factors such as pasture related diseases which affected performance of weaners in that year.

(iii) Year changes in climatic conditions which affect plant growth, influenced the availability of carotenoids in grass fed cattle. This was most pronounced in the young weaners which were dependent on pasture carotene supplementation from the moment of birth until sampling. Rainfall appeared to be one of the climatic factors which contributed to the incidence of fat colour and was highly correlated with carotene concentration in adipose tissue.

(iv) The differences in climatic conditions can be extended to explain the divergence between geographical locations. Jersey and Limousin cross weaners in New Zealand had markedly higher carotenoid concentration than weaners of the same crosses raised in South Australia. Pre-weaning location affected fat colour in young animals even if they were finished on low carotene diet for up to 170 days in a feedlot.

(v) On the other hand, seasonal differences in feed quality and quantity did not affect fat colour in the Australian cows. This can be explained by the slow turnover of this nutrient in the adipose tissue.

(vi) In addition to the environmental effects, breed also had an influence on total carotene concentration in fat and subjective fat colour score with Jersey cattle having the highest concentration. The higher content of carotene in Jersey cattle was extended to their crosses with Hereford cattle as the Jersey crossbreeds had the highest carotene content in adipose tissue and were significantly different from the other six breeds under investigation. On the other hand, Limousin cattle had the one of the lowest carotene content and did not differ from the other beef breeds when crossed with Hereford cows. Sire effects on fat colour were significant and particularly pronounced in the Jersey cattle in New Zealand.

In conclusion, the availability of carotenoids can be responsible for the variation in results obtained from different experiments. The response in fat colour of animals raised on rich carotene diets differed to animals raised on low carotenoid diets. However, that response is moderated by genetic effects.

Chapter 5

Heritability and mode of inheritance of fat colour in cattle

5.0. Introduction

The variation in fat colour or carotenoid concentration in fat between different cattle breeds, as well as within breeds in the same environment (chapter 4) suggests that both genetic and environmental factors influence this trait. Additional evidence which supports the hypothesis that fat colour has a genetic component comes from feedlot experiments. In a study conducted by Strachan et al. (1993), some animals even after a long period of time in the feedlot still had excessively yellow fat (fat colour scores of 4 and 6 on the 10 AUS-MEAT® scale).

Despite the strong evidence for a genetic effects influencing cattle fat colour, a model for the mode of inheritance has not been proposed. Three major experiments were designed to develop such a model: Experiment 1 which estimated the heritability and genetic effects of yellow fat colour in cattle, Experiment 2 which implemented several approaches to detect potential carriers of the yellow fat allele, and Experiment 3 which investigated these potential carriers of the yellow fat allele(s).

5.1. Materials and methods

5.1.1. Experiment 1:

Animals and management

To estimate heritability for fat colour and total carotenoid concentration in fat, 2 groups of animals were used. Group 1 consisted of 1124 crossbreed animals (chapter 2) from the 4 year "Southern Crossbreeding Project". The animals were raised on pastures, weaned at ~250 days, and slaughtered at 500-600 days of age after a high concentrate diet for 90-170 days. The second group comprised Jersey backcross (LJJJ) and Limousin backcross (LJLL) weaners 250 days of age from Martindale in South Australia, pure Jersey and Limousin cows (dams of the weaners) and F1 (JJLL, LLJJ) bulls (sires of the weaners). The animals were raised on pastures with supplement of oaten hay during the summer. Some cows, which were not sampled by biopsy in January, were culled in June and sampled after the slaughter.

Sample collection, preparation and analyses

In group 1, fat colour score was assessed in carcasses on a 10-point scale by AUS-MEAT® assessors. In group 2, fat was collected using a biopsy technique during 3 days of sampling and handled as described in the chapter 2.

Statistical analysis.

Data from the group 1 was analysed using the MIXED Procedure (SAS, 1996). Animals were assigned into four birth groups, depending on when they were born within the 7 week calving season (early, mid 1, mid 2 and late). Day of birth was also fitted as a linear covariate within these four groups. Cohort groups were formed based on birth year (1994-1997), sex (heifers or steers), birth location (Struan or Wandilo) and post weaning group. In the data set, 34 cohort combinations were created. Sire was fitted as a random effect nested within breed and breed differences were tested against the sire mean square. Significance was defined as $P<0.05$. Heritability was estimated as four times the sire variance divided by the total variance.

Heritability in group 2 was estimated by plotting concentration of carotenoids in the cows, in the mid-parents or in the bulls against the concentration of carotenoids in the weaners, separately for Jersey and Limousin cattle. Slopes of the equations generated from each data set represented heritability (h^2) when carotenoids in the weaners were plotted against mid-parents or $½ h^2$ when weaner data was plotted against the separate dam or sire data.

5.1.2. Experiment 2:

Animals and management

The animals used in this experiment were a part of the J.S. Davies Cattle Gene Mapping Herd maintained at Martindale in South Australia. They were born in April/May in 1995 and 1996, and represented 5 breed combinations: pure Jersey (J), pure Limousin (L), F1 (JJLL, LLJJ) and backcrosses to the Jersey (LJJJ) and the Limousin (LJLL). The group born in 1995 consisted of 2 cohorts: females, which were finished in the feedlot for 70 days and slaughtered at 16 months of age, and males, which were finished in the feedlot for 170 days and slaughtered at 25 months of age. The 1995 animals comprised pure Jersey, pure Limousin and F1 cattle. The animals born in 1996 (males and females) were divided into 2 cohorts. Cohort one, consisting of pure Jersey, LJJJ and LJLL cattle which were slaughtered after pasture feeding at the age of 20 months and the second cohort, consisting of LJJJ and LJLL cattle, slaughtered after 240 days on a high grain diet (33 months of age). There were no sires common across the two cohorts.

Sample collection, preparation and analyses

Adipose tissue biopsy samples from 250 days old weaners (341 animals) were collected in 1996 and 1997 from the base of the tail and processed as described by Malau-Aduli et al., (1995) except that tissue was removed with use of a scalpel. Fat from slaughtered animals

(500-600 days old) was collected from the 12^{th}–13^{th} rib area on the day of slaughter from the chiller (179 animals). Both steers and heifers were fed to the same average fat depth. Total carotenoid content in the fat samples was analysed as described in Chapter 2. Fat colour score (FCS) of adipose biopsy samples was assessed immediately after removing the fat from the biopsy site and rinsing with water. Fat colour score from carcasses was assessed according to AUS-MEAT® specifications on a 10-point scale. All biopsy and carcass fat samples were placed in the plastic vials, and stored at -20°C under N_2 gas until analysed.

Statistical analysis.

Least squares analysis of variance was carried out separately for classes of fat colour score at weaning, fat colour score at slaughter and days in feedlot, using Proc GLM (SAS 1989). Least squares means and differences between means were computed. Correlations between total carotenoid at weaning and at slaughter as well as fat colour scores were calculated using Proc CORR (SAS 1989). Simple linear regression was used to test for the difference between FCS and carotenoid concentrations at weaning and slaughter. The hypothesis that the equation slopes were not different from each other was tested using a t-test. The concentration of total carotenoids in biopsy fat was also tested against total carotenoid concentration in carcass fat and cohort, and the solutions for this model were obtained.

5.1.3. Experiment 3:

Animals and management

The animals from the J.S. Davies Cattle Gene Mapping Project (453) were used in this experiment. They were born between 1994 and 1998 and represented 5 crossbreed groups: pure Jersey, pure Limousin, F1 crosses between the Jersey and Limousin and reciprocal back-crosses between F1 bulls and Jersey or Limousin cows. The animals were raised on pastures, weaned at the age of ~250 days, and after ~90 days in the feedlot were slaughtered.

Sample collection, preparation and analyses

Fat samples were collected at weaning using a biopsy technique and at slaughter as described under Experiment 1 of this Chapter. Total carotenoid concentration was assessed spectrophotometrically as described in the chapter 3.

Statistical analysis

Data from this experiment was analysed using least square means with Proc GLM (SAS 1989). As breed, sampling and year of birth (yob) were partially confounded, the final model included fixed effects of sample (biopsy and carcass fat), yob (1994-1997) nested within

sample, sex (male and female), breed (JJJJ, LJLL, LJJJ, LLJJ, LLLL), day of birth as a covariate, and the interactions between sex and sample, yob and sex nested within sample, dob and yob nested within sample, breed and sample, breed and yob nested within sample, breed yob and sex nested within sample. Least square means and differences between means were computed. Estimation of genetic effects (Dickerson, 1969) such as additive (JJ-LL-XJ+XL), maternal [(LL-JJ)/2+XJ-XL], heterotic (LJ-LL-XJ+XL) and epistatic (2(XJ)-LJ-LL) was carried out using Proc GLM (SAS, 1989) with a similar model as presented above excluding the interactions between breed and sample, breed and yob nested within sample, breed, sex and yob nested within sample. To test the distribution of carotenoids in each breed and calculate the "outliers" from normal distributions, Proc UNIVARIATE and Proc CHART (SAS, 1989) were applied. Calculation of breed and sire influence was performed with Proc GLM where the model included the fixed effect of breed, sire and the interaction between breed and sire.

5.2. Results

5.2.1. Heritability of fat colour (experiment 1)

Breed effects (chapter 4) on carotene adipose tissue concentration suggest that fat colour is influenced by genetic factors. Heritability is a parameter which estimates the proportion of the total variance of a character attributable to additive genetic as opposed to environmental and non-additive genetic factors. There was a high correlation between subjective fat colour score and total carotenoid concentration, and these variables were used herein to estimate the heritability of this trait.

5.2.1.1. Heritability of fat colour based on AUS-MEAT® subjective fat colour score

Heritability, based on 1124 fat colour scores derived from the 1994-1997 Southern Cross Breeding Project slaughtered animals, was low (h^2 = 10%), indicating that fat colour is under a large environmental influence. This confirms the prediction made when comparing the differences in fat colour between New Zealand and South Australia. The weaners in New Zealand, which were raised on a diet rich in carotenoids, had significantly higher fat colour scores or total carotenoids than their counterparts in South Australia.

A moderate heritability (32±11%) based on an 11 point scale subjective score at slaughter was reported by Hayes et al. (1995) in Brahman, Hereford, Shorthorn and Africander crosses. This confirmed our findings that improvement of fat colour through selection is possible, however, there is also a substantial environmental variation.

It was interesting to note that day of birth had an influence on fat colour at slaughter. From 4 birth groups [group 1: 0-74 days of year (doy), 2: 74-96 doy, 3: 96-118 doy, and group 4: more than 118 doy], a significant difference was observed between group 4 animals and the other groups ($P<0.05$). Animals from group 4 (the youngest group) had approximately 26% lower subjective fat colour score than animals from the other groups possibly because they have consumed less carotenoids in total.

5.2.1.2. Heritability of fat colour based on total adipose carotenoid concentration

The concentration of total carotenoids in adipose tissue of animals from the J.S. Davies Gene Mapping Herd in Martindale in 1998 was also used to estimate the heritability of fat colour. The available data included information from dams, sires and offspring. This allowed heritability to be estimated by offspring-parent regression. The slope of the linear regression of offspring on mid-parent gave a heritability estimate of 6%. (Figure 5.1). The low h^2 demonstrated that, similar to the animals from the Southern Cross-Breeding Project at slaughter, this trait at weaning is barely affected by additive gene(s).

It has been reported that dairy breeds have yellower fat than beef breeds (Walker et al., 1990). Therefore, it was of interest to estimate the heritability separately for the Jersey and Limousin crosses. Heritability in these crossbreeds was estimated by plotting offspring measurements against sires, dams and mid-parent data. For Jersey cattle, these estimates were higher than those for Jersey and Limousin combined (Table 5.1, Figures 5.2, 5.3, 5.4). The opposite was observed when evaluating the heritability in the Limousin population (Figures 5.5., 5.6., 5.7.) indicating more genetic variation for fat colour in Jersey than Limousin. The values for mid-parent-offspring and dam-offspring were lower than for Jersey cattle and the Jersey and Limousin cattle together. The value estimated based on sire-offspring was the highest in Limousin. However, these values have very low accuracy because only 3 F1 sires were used. The breed differences in heritability showed that Jersey cattle contributed more to the variation in fat colour than Limousin cattle. The heritability of 2% in Limousin dam-offspring estimate showed that there was only a small additive effect in the Limousin population. The higher heritability for sire-offspring observed in the Limousin cattle suggested that the biggest influence on gene frequency in this population derived from F1 Jersey x Limousin sires which introduced the Jersey yellow fat gene(s) to the Limousin backcross progeny.

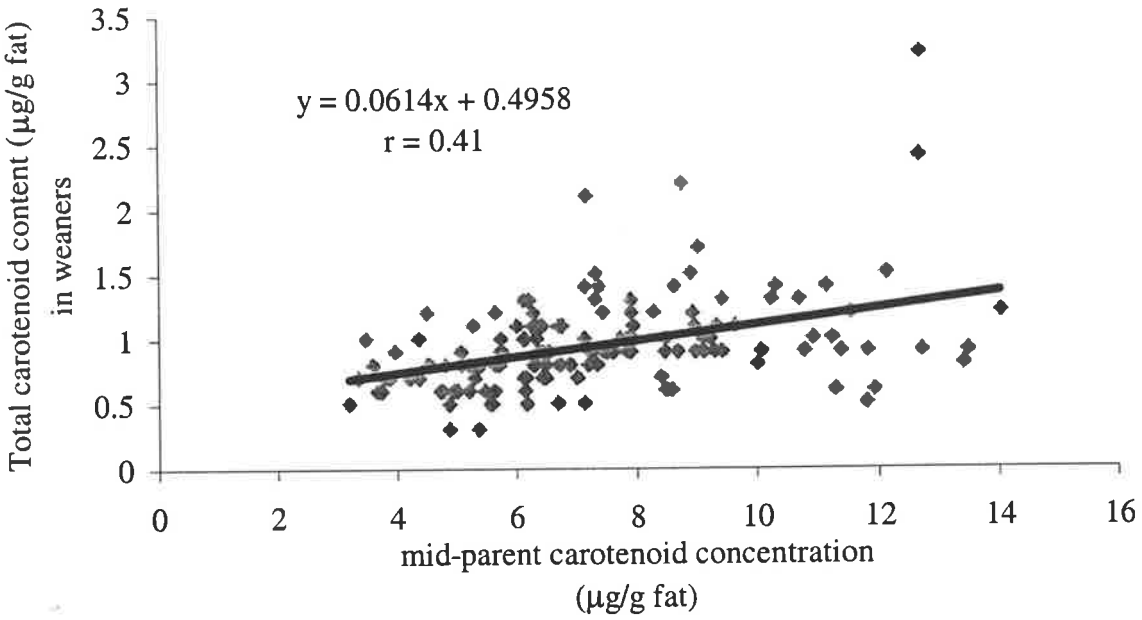


Figure 5.1. Regression analysis of total carotenoid concentration (µg/g fat) in mid-parent and offspring.

Table 5.1. Heritability estimates in Jersey and Limousin backcrosses based on regression for mid-parent-offspring, sire-offspring and dam-offspring.

Method	Jersey	Limousin	Jersey + Limousin
Dam-offspring	8%	2%	6%
Sire-offspring	21%	33%	23%
Mid-parent-offspring	9%	4%	6%

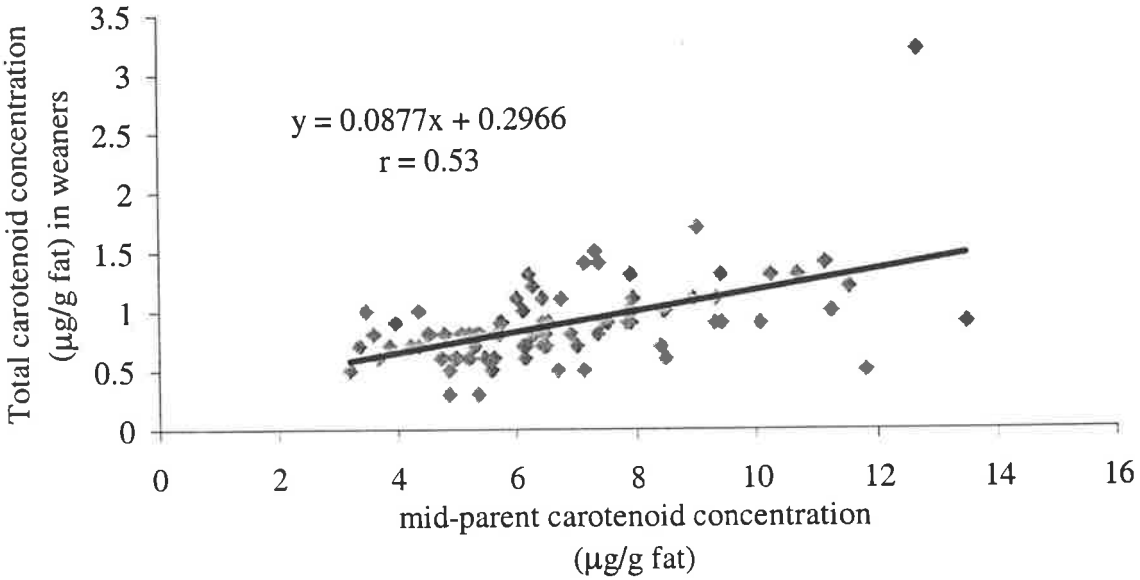


Figure 5.2. Regression analysis of total carotenoid concentration (µg/g fat) in mid-parent Jersey cows and F1 (Jersey x Limousin) bulls and offspring (LJJJ).

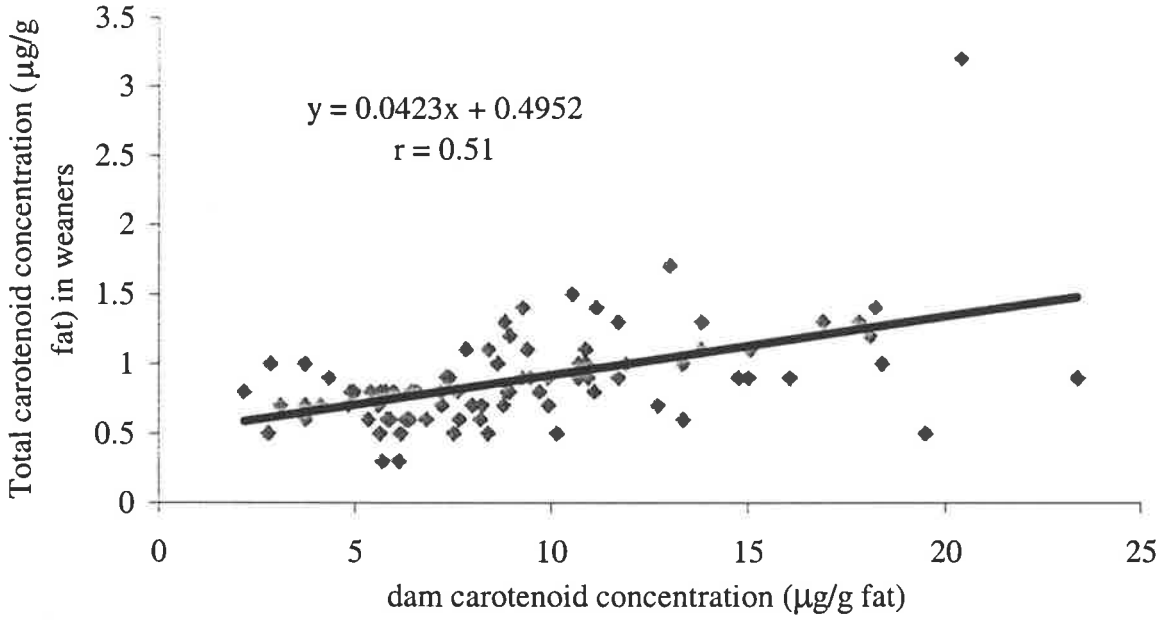


Figure 5.3. Regression analysis for total carotenoid concentration (µg/g fat) in Jersey dams and offspring (LJJJ).

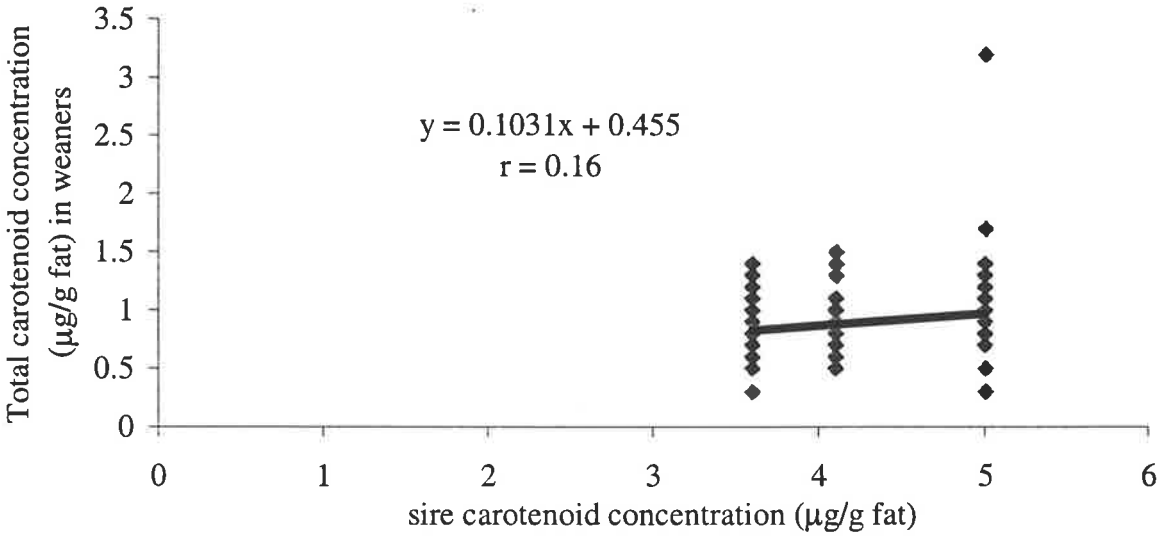


Figure 5.4. Regression analysis for total carotenoid concentration (µg/g fat) in F1 (Jersey x Limousin) sires and offspring (LJJJ).

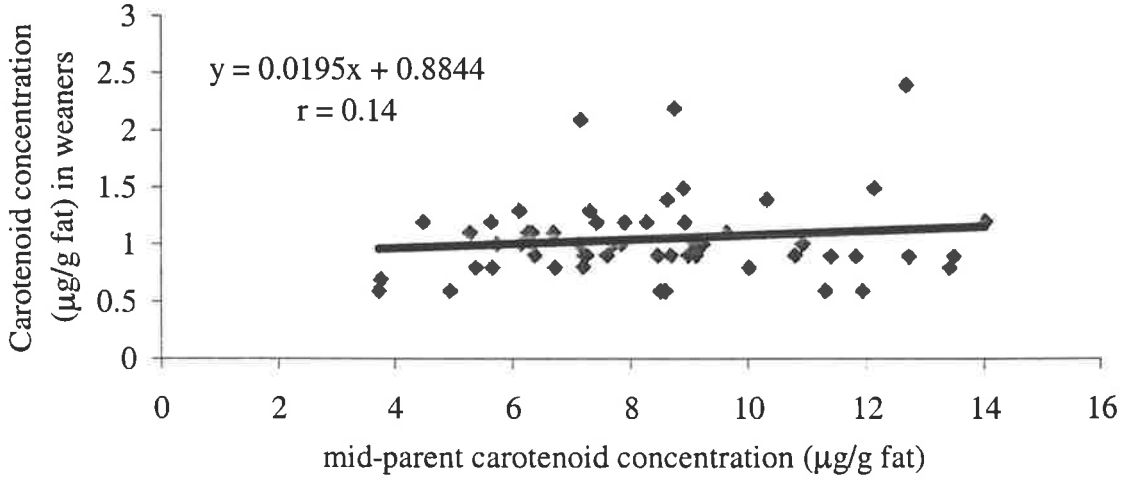


Figure 5.5. Regression analysis of total carotenoid concentration (µg/g fat) in mid-parent Limousin cows and F1 (Jersey x Limousin) bulls and offspring (LJLL).

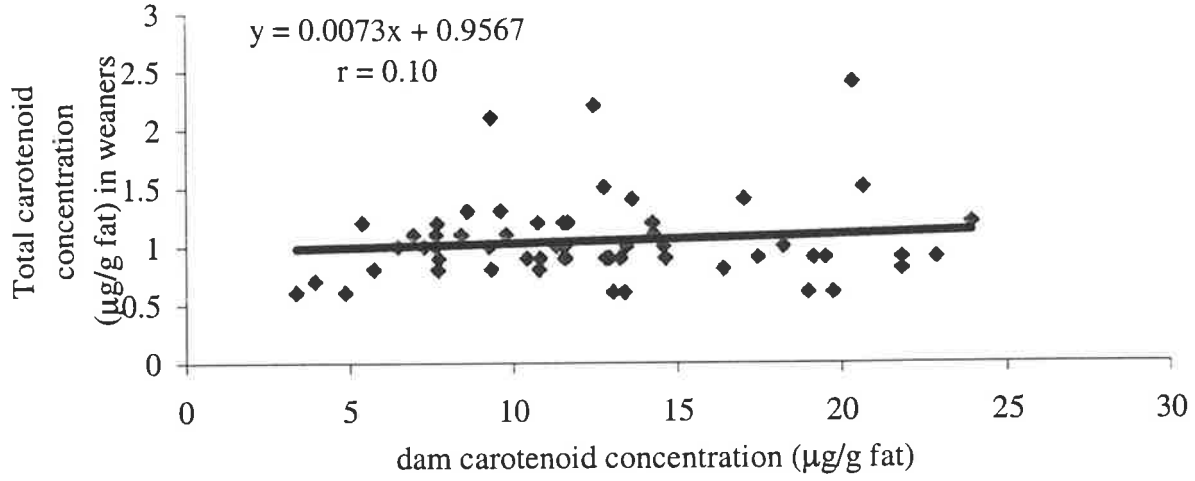


Figure 5.6. Regression analysis for total carotenoid concentration (µg/g fat) in Limousin dams and offspring (LJLL).

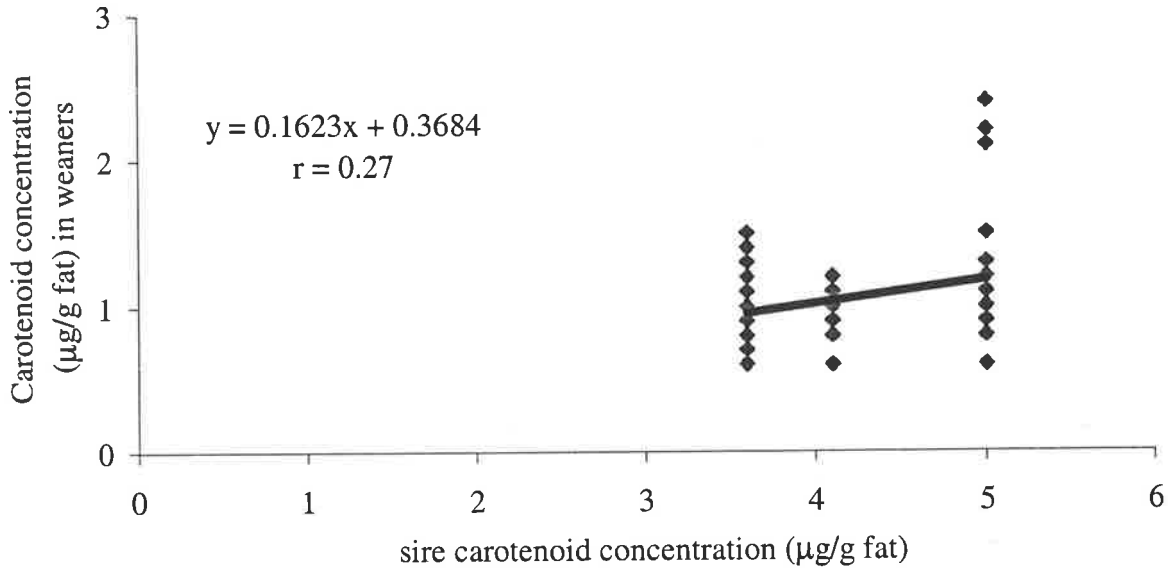


Figure 5.7. Regression analysis for total carotenoid concentration (µg/g fat) in F1 (Jersey x Limousin) sires and offspring (LJLL).

5.2.1.3. Genetic effects influencing fat colour

Based on the available data from the Davies Gene Mapping Herd, four major genetic effects involved in crossbreeding were examined: (i) direct (individual) genetic (g^I), maternal genetic (g^M), individual heterotic (h^I) and epistatic (e^I). The breed combinations used in this experiment contained specific percentages of Jersey genes as follows: JJxJJ=100%, JJxJL=75%, JJxLL=50%, JLxLL=25% and LLxLL=0%. Assuming that there were only additive (direct) gene effects on fat colour, then the performance of each cross would be expected to be a function of the percentage of Jersey or Limousin genes only. However, other effects may play a significant role and they were estimated as deviations from the direct effects as a function of Jersey or Limousin percentage.

The genetic effects for individual breed crosses were calculated by weighting for the breed means (Table 5.2). Analysis of the statistical model showed that maternal and heterotic effects were not significant (Table 5.3). Only additive and epistatic effects affected fat colour. The residual means from the statistical model demonstrated that crossbreeding reduced the carotene concentration in F1 crosses (Figure 5.8, Figure 5.9). The F1 crosses between Jersey and Limousin cattle were intermediate in fat colour and the backcrosses were closer to their pure breed ancestors. Pure Jersey cattle differed significantly from pure Limousin and Jersey-Limousin crosses. Pure Limousin differed from other breeds and crosses except LJLL. All crossbreeds between Jersey and Limousin cattle differed from each other with the exception of LJLL and LL JJ.

Table 5.2. Breed crosses and genetic effects.

	g^I_J	g^M_J	h^I_{LJ}	e^I_{LJ}	g^I_L	g^M_L
JJJJ	1	1	0	0	0	0
LLJJ	½	1	1	0	½	0
LLLL	0	0	0	0	1	1
LJJJ	¾	1	½	½	¼	0
LJLL	¼	0	½	½	¾	1

Note: $g^I_L = -g^I_J$, $g^M_L = -g^M_J$

Table 5.3. Genetic effects on fat colour.

Genetic effect	mean	SE	significance
Additive	0.47	0.084	P<0.0001
Maternal	-0.08	0.062	ns
Heterotic	-0.04	0.080	ns
Epistatic	-0.40	0.193	P<0.05

*Note:*SE = standard deviation, ns = not significant

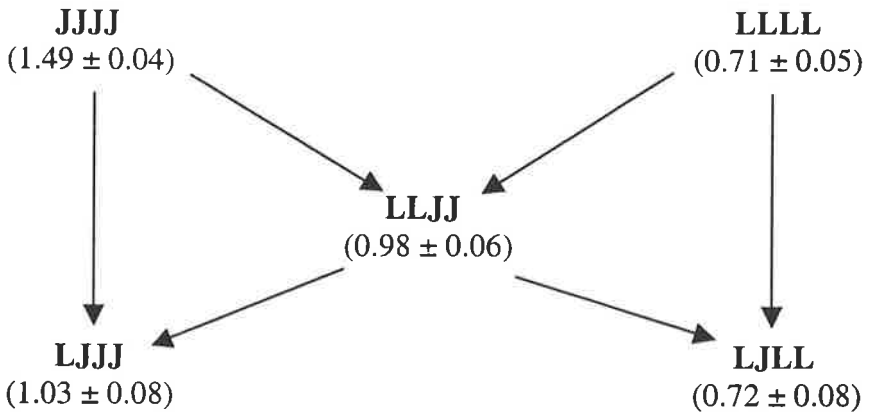


Figure 5.8. Diagrammatic representation of crossbreeding effect on total carotenoid concentration in cattle adipose tissue (μg/g fat).

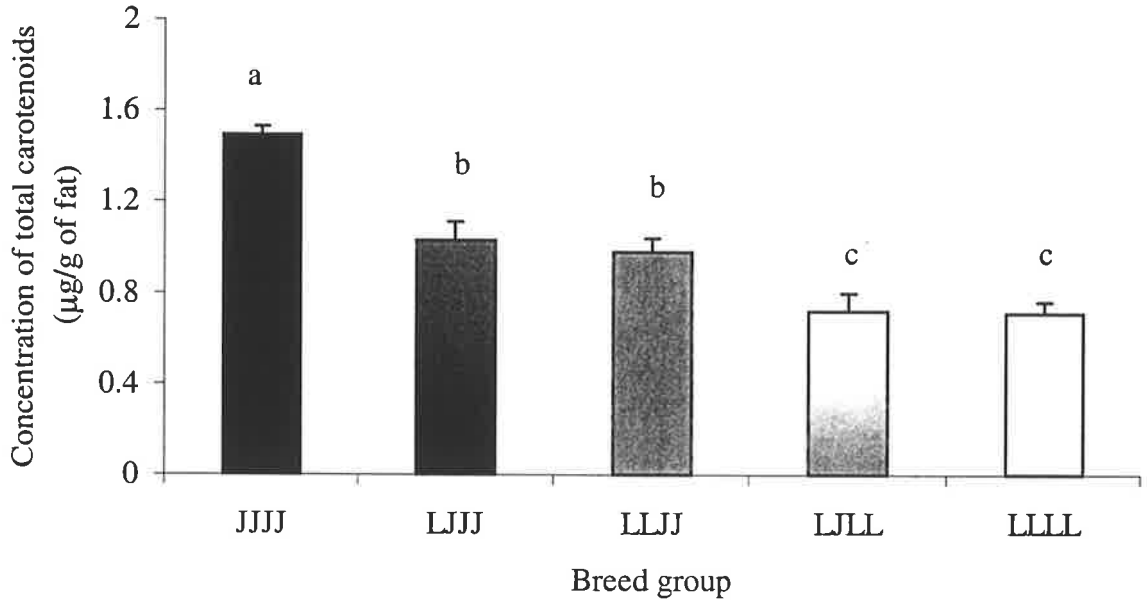


Figure 5.9. Difference in total carotenoid concentration (μg/g fat) between Jersey and Limousin cattle and their crosses. Letters a-c = the same letter denote the data which is not significantly different (P>0.05).

5.2.2. Methods of detecting carriers of the "yellow fat" gene (experiment 2)

The data analysis from experiment 1 suggests that Jersey yellow fat gene(s) were introgressed into the F1 progeny and the Limousin backcrosses (LJLL). Three methods were applied to distinguish the animals which could be potential carriers of yellow fat gene(s): (1) based on residual values which were derived from equating the normal distribution of all crossbred groups after statistical analysis using Proc GLM followed by Proc UNIVARIATE to estimate distances, (2) based on visual assessment of fat colour and carotenoid concentration in adipose tissue of biopsy and carcass samples and the ability to reduce fat colour if fed a low

carotenoid diet, and (3) based on "outliers" of normal distributions as plotted for each breed and age group (weaners, cows, bulls) separately.

5.2.2.1. Detecting carriers based on residual values

The distribution of residual values for total carotenoid concentration was adjusted for the effects of sample, year of birth, sex, day of birth and breed (Figure 5.10). The test statistic (W) with a small value (less than 1) indicated that the data was not normally distributed (Table 5.4). The probability of Pr=0.0001, which was very close to 0, confirmed the hypothesis of a non-normal distribution. The skewness and kurtosis were different from 0 (1.89 and 6.25, respectively) which provided additional evidence that the distribution of total carotenoid concentration values in fat of Jersey, Limousin and various crossbreeds between these pure breeds was not normal. The distribution was skewed right and heavily tailed (Figure 5.11).

Univariate procedure (Histogram)

Variable = residual total carotenoid concentration (µg/g fat)

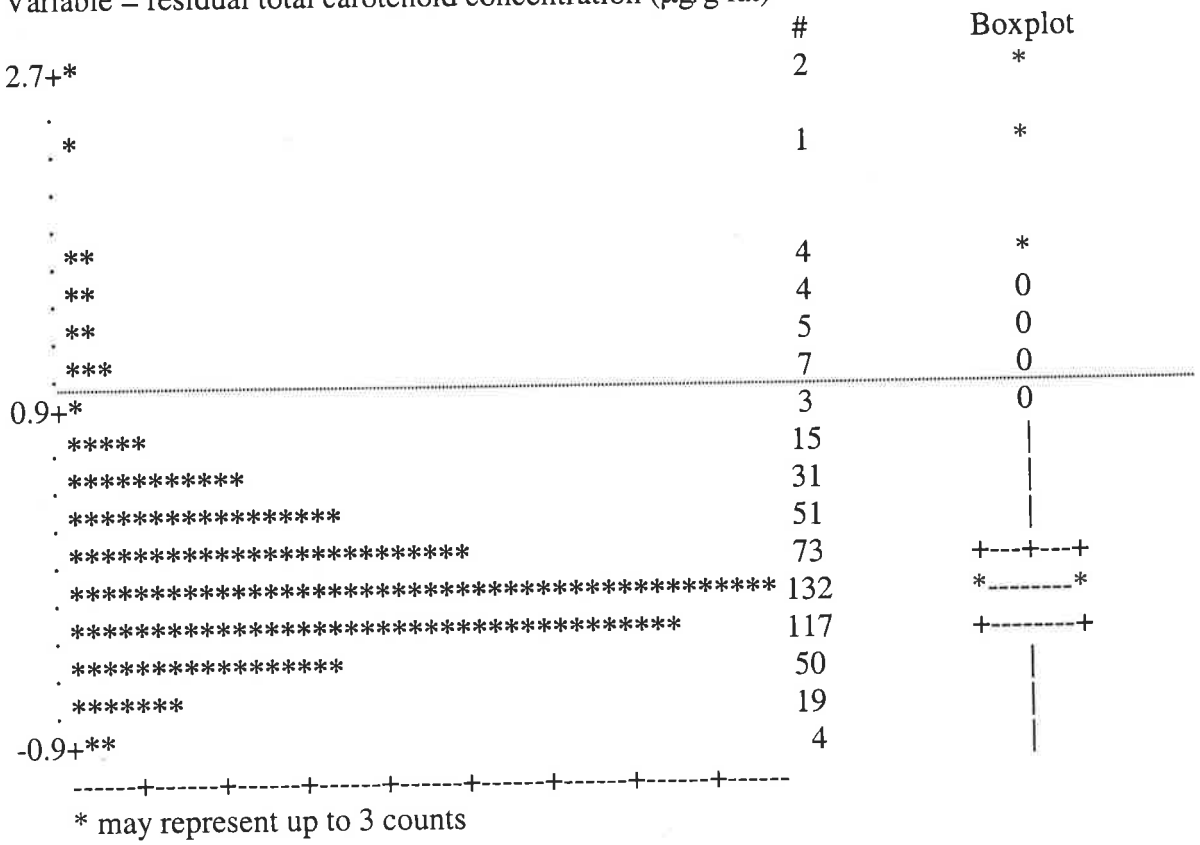


Figure 5.10. Histogram and boxplot of residual total carotenoid concentration in fat of Jersey (JJJJ), Limousin (LLLL), Jersey by Limousin F1 cross (LLJJ) and reciprocal crosses between F1 x Jersey (LJJJ) and F1 by Limousin (LJLL). The doted line at 0.9 separates "outliers" from the normal distribution.

A total number of 23 "outliers" was selected from the tail of the distribution using this method (Table 5.5). It is interesting to note that breeding of Limousin cows with pure

Limousin bulls did not result in appearance of any "outliers". However, the Limousin bulls crossed with Jersey cows did produce some yellow fat "outliers" although there were very few progeny in this breed group. Crossing pure Jersey bulls with Limousin cows also did not produce "outliers". However, there were 15 "outliers" from mating Jersey bulls with Jersey cows. No "outliers" were present when F1 crossbred bulls were mated with either Jersey or Limousin cows except bull 398 which produced 5 "outliers" with Jersey cows.

Table 5.4. Parameters for analysis of carotenoid distribution in Jersey and Limousin cattle and their crosses.

Breed / Test	JJJJ	LJJJ	LLJJ	LJLL	LLLL	All breeds
Skewness	1.26	2.86	1.91	0.33	0.53	1.89
W:Normal	0.92	0.78	0.84	0.98	0.97	0.87
Kurtosis	2.72	14.18	5.05	2.04	1.04	6.25
Pr<W	0.0001	0.0001	0.0001	0.6784	0.2551	0.0001

Note: JJJJ=pure Jersey cattle, LLLL=pure Limousin cattle, LLJJ=F1 crossbreed between pure Jersey and pure Limousin cattle, JJJL=reciprocal crosses between F1 bulls and pure Jersey cows, LLLJ=reciprocal crosses between F1 bulls and pure Limousin cows, W=test statistic ranging between 0 and 1 (0<W≤1), Pr=probability value.

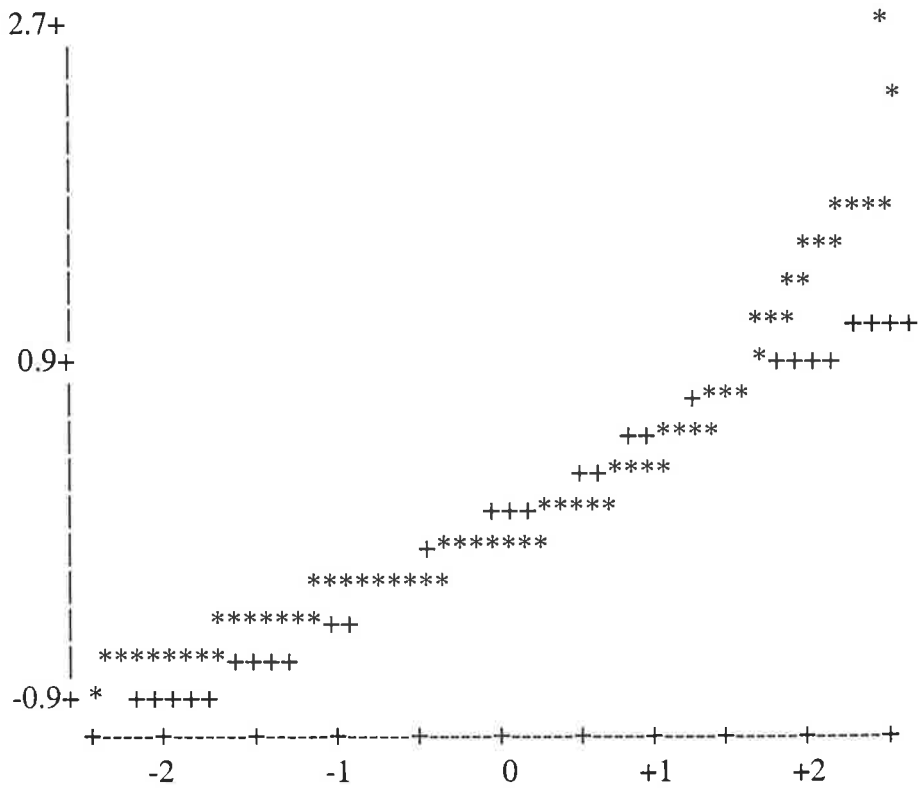


Figure 5.11. Normal probability plot for total carotenoid concentration in Jersey, Limousin, and various crossbreeds between the Jersey and the Limousin pure breeds. (The + signs form straight line and the asterisks represent the sample. A large number of + signs indicates a non-normal distribution).

Table 5.5. Pedigrees of "outliers".

Sire #	Breed of Sire	Breed of Dam	# of outliers	Percentage*
74	LL	LL	0	0
75	LL	LL	0	0
74	**LL**	**JJ**	**1**	3.0
75	**LL**	**JJ**	**2**	11.1
77	JJ	LL	0	0
78	JJ	LL	0	0
77	**JJ**	**JJ**	**6**	12.5
78	**JJ**	**JJ**	**9**	28.1
352	LJ	LL	0	0
352	LJ	JJ	0	0
361	LJ	LL	0	0
361	LJ	JJ	0	0
368	LJ	LL	0	0
368	LJ	JJ	0	0
398	LJ	LL	0	0
398	**LJ**	**JJ**	**5**	13.2

Note: Boldface=sires producing "outliers" progeny, *-percentage represents number of outliers within each breed group.

5.2.2.2. Detecting carriers based on visual assessment

The high correlation between total carotenoid content in the adipose tissue and subjective fat colour scores in our 5-point and AUS-MEAT®'s 10-point scale (Chapter 3) suggested that visual assessment could be a precise predictor for fat colour. Moreover, this method would be of great importance as the excessively coloured carcasses are downgraded based on the visual scoring system in the abattoir. To be able to define and select "outliers" using this method, the relation between fat colour score and carotenoid concentration needed further investigation.

Higher concentrations of total carotenoids in biopsy and carcass adipose tissue were accompanied by higher subjective fat colour scores (Table 5.6). Correlations between the measurements were high and there was no significant difference between the equation slopes describing these correlations (Figure 5.12). There was a significant difference between the range of carotenoid concentration in biopsy fat as assigned to different subjective fat colour score classes (Table 5.6). A similar trend to biopsy samples was observed at slaughter where the range of carotenoid concentration within the various subjective fat colour scores differed significantly with the exception of a few adjacent scores. There were also positive correlations between carotenoid content in biopsy fat and in carcass fat as well as between fat colour score at weaning and at slaughter ($r=0.58$, and $r=0.70$ respectively, $P<0.001$). The data set used to estimate the AUS-MEAT® 10-point scale fat colour score comprised the entire range of scores except the scores of 6 and 9.

Table 5.6. Means for total carotenoid concentration in adipose tissue (μg/g of fat) as assigned to different subjective fat colour scores at biopsy and at slaughter.

FCS	(A) Biopsy Samples		(B) Carcasses Sampling	
	Total carotenoids	n	Total carotenoids	n
0	-	-	1.18 ± 0.09^{a}	76
1	0.73 ± 0.04^{a}	63	1.26 ± 0.18^{a}	52
2	0.93 ± 0.02^{b}	229	$1.90 \pm 0.18^{b\,c}$	20
3	1.32 ± 0.05^{c}	34	$2.14 \pm 0.19^{c\,d}$	11
4	2.08 ± 0.14^{d}	5	2.49 ± 0.22^{d}	8
5	2.65 ± 0.10^{e}	10	3.20 ± 0.28^{e}	6
7	-	-	4.52 ± 0.28^{f}	5
8	-	-	6.20 ± 0.63^{g}	1

Note: n=number of animals, $^{a\text{-}h}$ within column means followed by the same letter are not significantly different ($P>0.05$).

A significant difference was observed when comparing fat colour score in carcasses of grass fed and grain fed cattle. The grass fed animals had higher fat colour score than animals fed on grain for 70 or 170 days. The trend for fat colour score observed in different feeding groups was similar for total carotenoid content based on the raw data (Figure 5.13), where more carotene was present in the fat of grass fed animals. This was confirmed by the lack of a difference between the slopes of the equations describing the relationship between fat colour score at biopsy and slaughter (Figure 5.14). The equations also demonstrated that increased carotenoid content at biopsy was accompanied by the same increase at slaughter, regardless of sex, year of birth or feeding system. Correlations between concentration of carotenoids in biopsy and carcass fat were high and similar in different feeding groups. Higher fat colour

scores were found in Jersey and Jersey crosses, whereas pure Limousin and ¾ L had fat colour scores of 0 and 0-2, respectively.

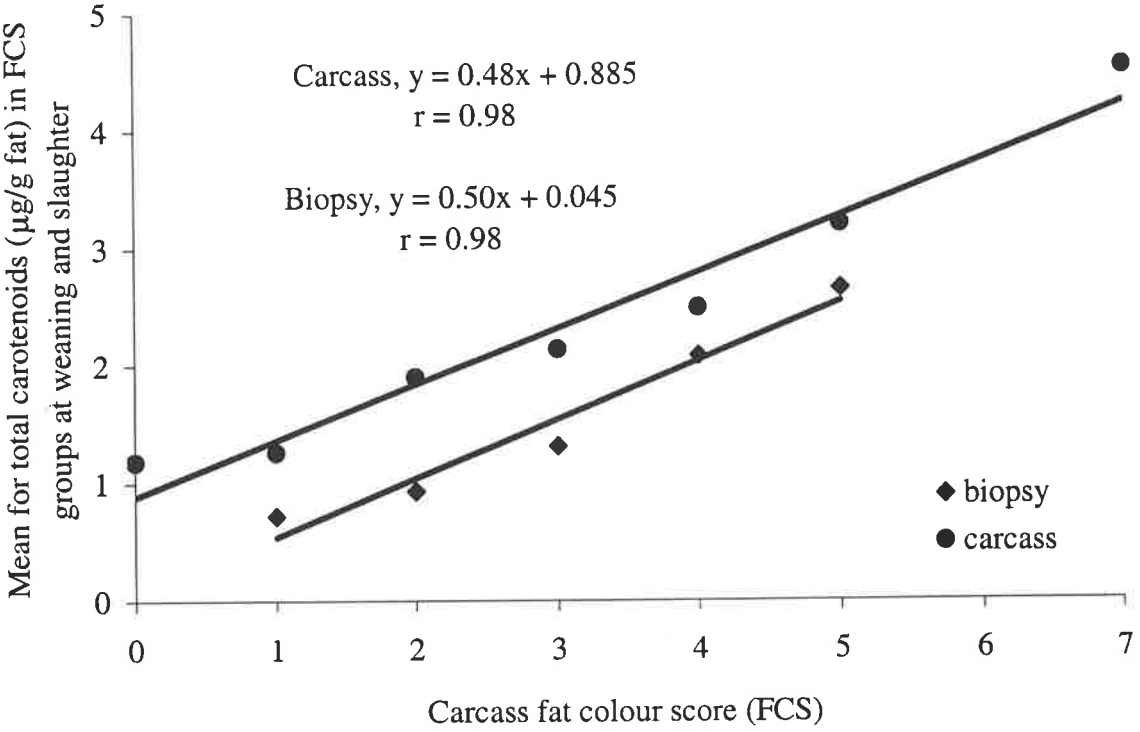


Figure 5.12. Correlation between total carotenoid concentration in fat and subjective fat colour score.

Walker et al. (1990) reported that high value meat markets accept fat colour scores of 1 and 2 (in a 6-point scale) without penalizing carcasses for excessively yellow fat. Carcass fat colour scores of 3 (total carotenoid concentration ~ 2.1µg/g fat in herein study) and higher in a 10-point scale were judged as yellow. At weaning, fat colour scores of 4-5 were considered as very yellow or orange (equivalent to a total carotenoid concentration of ~ 1.9µg/g fat). Consequently, "outliers" were those animals which had a fat colour score of 4-5 at weaning and carotene content ≥1.9µg/g fat or a fat colour score of >3 and carotene content ≥2.1µg/g fat at slaughter after 70-170 days of feedlot. Selection of "outliers" which were slaughtered after finishing on pastures (48 animals) was based on the weaning data and on the distribution of fat colour and carotene concentration at slaughter (Figure 5.15).

Applying these criteria, 21 from carcass samples and 18 from biopsy samples were selected. These data were in agreement with the number of yellow fat "outliers" obtained from the statistical analysis. It is important to note that almost all animals which were considered as "outliers" at weaning were also the "outliers" at slaughter except 3 weaners which had lower

fat colour scores or carotene content at slaughter than the minimum. Some animals were not considered as "outliers" at biopsy but were definite "outliers" at slaughter. This could be due to the fact that day of birth and year of birth had an impact on fat colour at weaning and slaughter ($P<0.001$). The animals which were born in a dry year with low carotenoid content in grass or born later during the year may not have accumulated large amounts of carotenoids even if they had a predisposition.

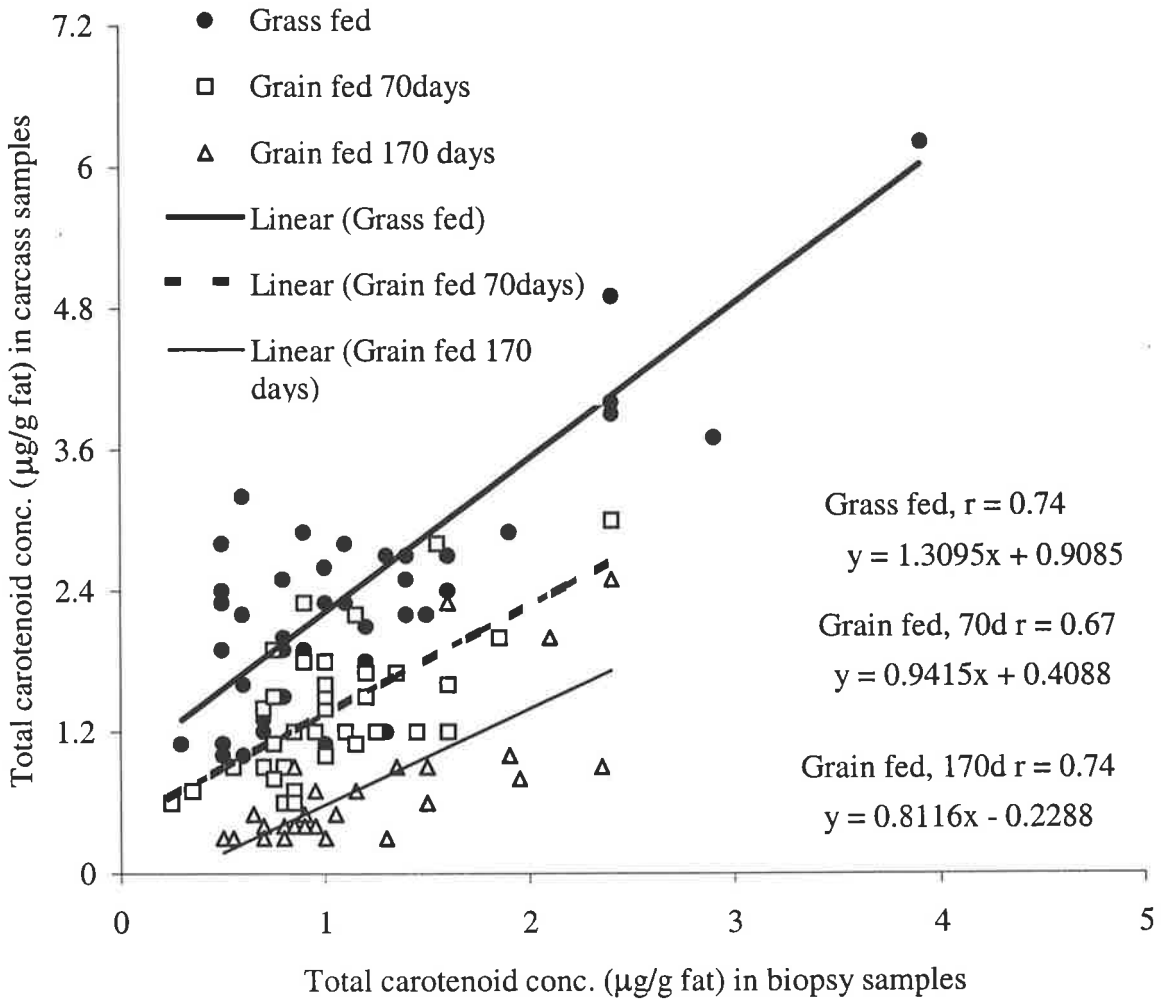


Figure 5.13. Relationship between carotene concentration at weaning and at slaughter.

There were no "outliers" from Limousin cows (Figure 5.15). Two Limousin bulls, 74 and 75, produced 1 (1.3%) and 3 (6.1%) "outliers" respectively, when mated to Jersey cows. Two Jersey bulls, 77 and 78, produced 6 and 9 "outliers", respectively, with Jersey cows. No "outliers" were present when F1 bulls were mated to Limousin and Jersey cows, except bull 398 which had 5 yellow fat progeny with Jersey cows (Table 5.5).

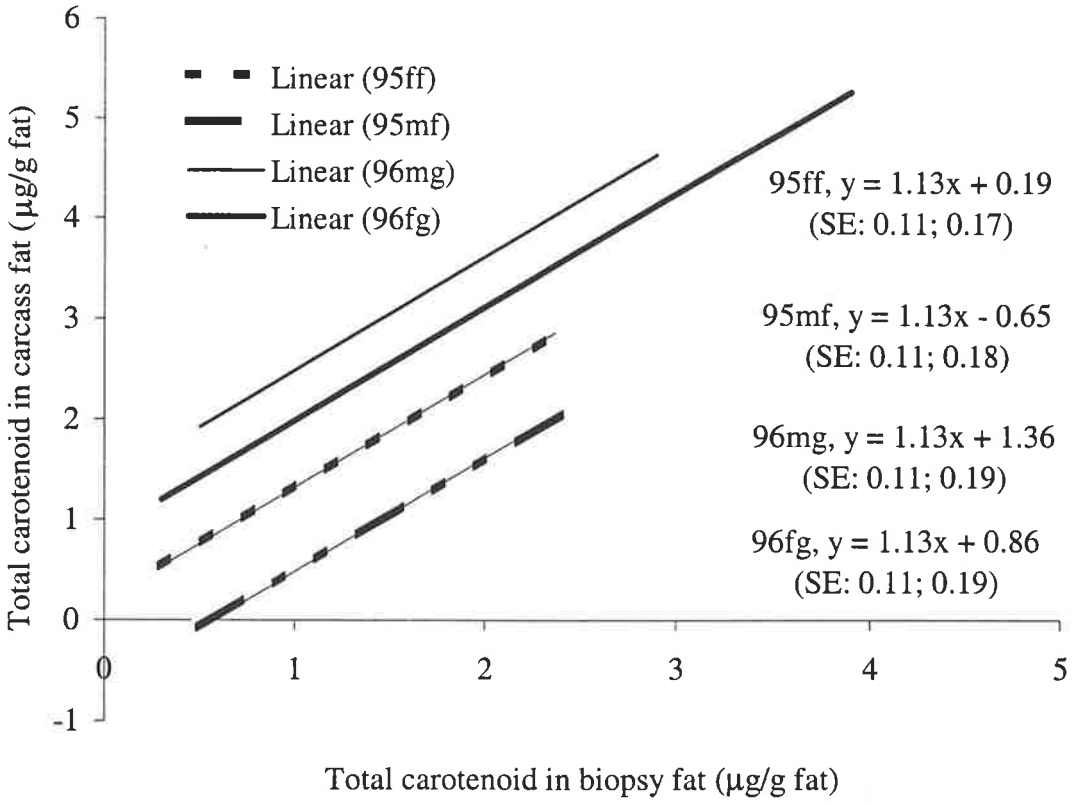


Figure 5.14. Relationship between carotenoid concentration in biopsy and carcass fat in different cohorts. 95ff=lotfed females born in 1995, 95mf=lotfed males born in 1995, 96fg=grassfed females born in 1996, 96mg=grassfed males born in 1996.

5.2.2.3. Detecting carriers based on distributions

Cows and bulls after several years on pasture accumulated 5-20 fold more carotenoids in their fat than weaners. Therefore, selection based on the level of carotenoid concentration at weaning was not applicable for older animals. Consequently, the cow "outliers" were selected from the tails of the distributions for each breed and sex separately. The distributions for the Limousin and the normal (hypothetical) for the Jersey cows were similar in shape, slightly skewed right (Figure 5.16). The Limousin distribution overlapped the normal distribution for Jersey cows. However, the full distribution for the Jerseys was widely spread and contained an irregular peak at the high end. Since pure Limousin weaners were not "outliers" and the Limousin cows with the highest carotenoid concentrations were still within the normal distribution of the Jerseys, those Jersey cows in this irregular peak were regarded as "outliers" in cow population. This resulted in identifying 11 Jersey cows as potential carriers of yellow fat gene(s).

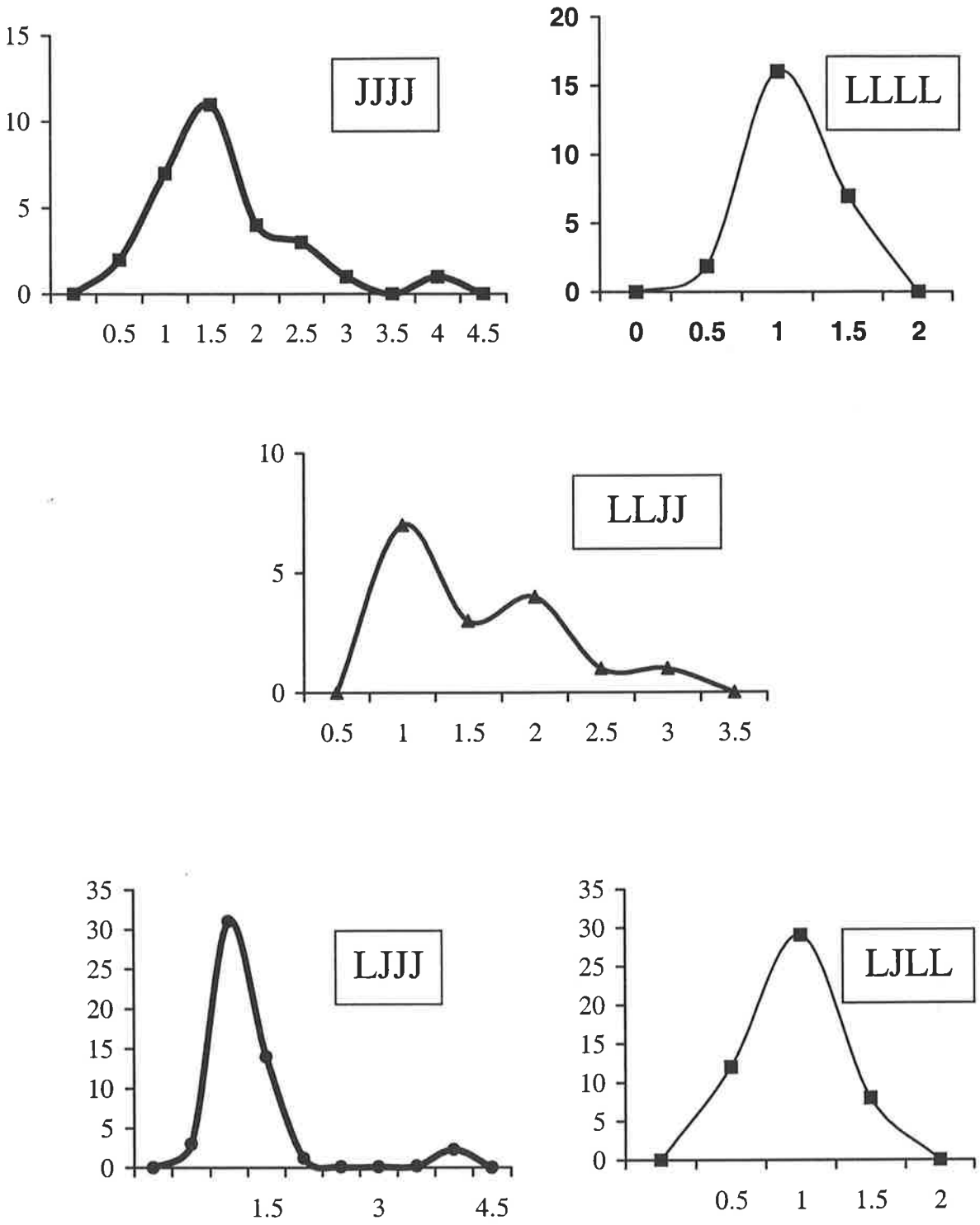


Figure 5.15. Distributions for carotenoid concentration in various progeny breeds. JJJJ = pure Jersey, LLLL = pure Limousin, LLJJ = F1 cross between Jersey and Limousin, LJJJ = Jersey reciprocal cross, LJLL = Limousin reciprocal cross. (X-axis represents concentration of carotenoids in μg/g of fat and Y-axis represents number of animals).

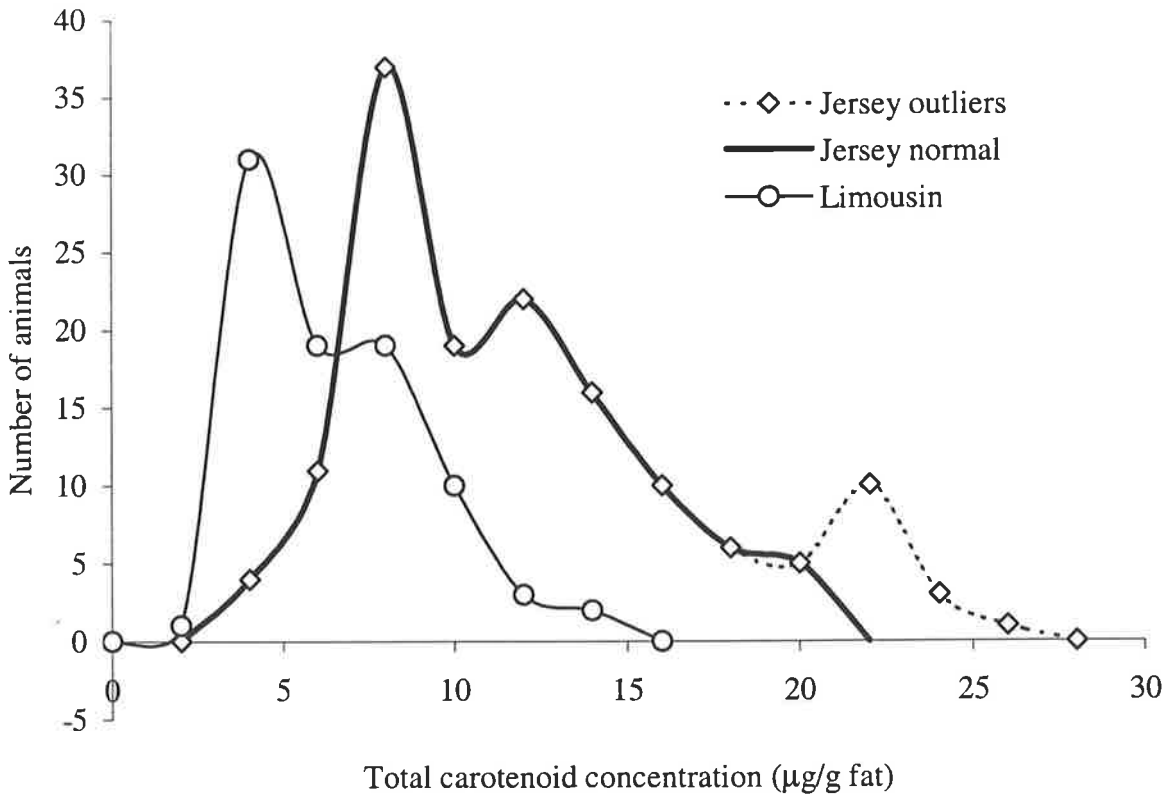


Figure 5.16. Distribution of total carotenoid concentration in the fat of Limousin and Jersey cows. Cows ranged between 22-141 months of age.

A similar approach was applied to the sire population which consisted of four F1 Jersey x Limousin crossed bulls sampled in the same year. As there were only four sires, however, the "outliers" were chosen based on their progeny. The number of "outliers" present in weaners as a crossbreeding effect of individual sire was examined. Among these four sires, only the progeny of the sire 398 were "outliers" in 1997 and 1998 (Appendix 9). Moreover, the concentration of carotenoids in the fat of this sire was the highest among all sires investigated (sire 352=2.4µg/g fat, sire 368=3.6µg/g fat, sire 361=4.1µg/g fat and sire 398=5.0µg/g fat). Therefore, sire 398 was selected as a potential carrier of yellow fat gene(s).

5.2.3. Analysis of "outliers" (experiment 3)

Additional information about the inheritance of fat colour in cattle was obtained by analysing the carotene distributions in Jersey, Limousin cattle and their crosses and studying the pedigrees of the "outliers". Using the heritability estimates and genetics effects on fat colour, a hypothetical model of the inheritance of fat colour in cattle was developed.

5.2.3.1. *Analysis of carotenoid distribution in Jersey, Limousin breeds and their crosses*

Generally, two major trends, which distinguished pure Limousin and LJLL from pure Jersey, JJLL and LJJJ crosses, were observed. The test statistic which compares the shape of the sample distribution with the shape of a normal distribution differed for Jersey and Jersey crosses from that for the Limousin and LJLL. The values were lower for the Jersey and Jersey crosses with the probability close to 0 (Table 5.4). This provided evidence that in pure Jersey, JJLL and LJJJ crosses, carotene distribution was not normal, whereas in Limousin and LJLL the distribution was normal. Additional evidence for a non-normal distribution in Jersey crosses derived from the analysis of skewness and kurtosis (Table 5.4). These parameters were higher for all Jersey crosses (50-100%). The analysis of normal probability plots and Stem-and-Leaf plots (data not presented here) for each breed group showed that in pure Jersey and LJJJ the distributions were skewed right and heavily tailed. A similar trend was observed in the JJLL cross where the distribution was also skewed right but had a smaller tail. In pure Limousin and LJLL, the distributions were normal with a tendency to right skewness in the LJLL backcross.

5.2.3.2. "Outlier" pedigrees

Four sires at Martindale and three sires in New Zealand were used to generate offspring from Jersey and Limousin cows (Table 5.5). Only five resulted in "outliers" when mated with Jersey cows. Among the F1 sires, one sire number 398, produced 5 "outlier" progeny at Martindale. This sire was the offspring of bull 78 which produced "outliers" in matings with Jersey cows. The dam of the sire 398 was not an "outlier" in the cow population and had little carotene in the fat. The remaining F1 sires from Martindale did not produce "outliers". Two of three sires used in New Zealand were considered as "outliers" in fat colour and all the "outlier" progeny (data not presented) derived from these two sires. The three sires which produced "outliers" in Australia and New Zealand were the progeny of the two Jersey sires, numbers 77 and 78, which produced all the "outliers" in the pure breed population.

The pedigrees of all progeny "outliers" were examined (Appendix 9). There were 26 dams of "outliers". Four cows from the dams of the "outliers" (19.2%) were considered as "outliers" in the cow population (section 5.2.2.3). All "outlier" progeny derived from at least one parent which was an "outlier" within parental population with one exception (Jersey cow 107 mated to Limousin bull 75 produced "outlier" offspring 461). [This progeny had a low concentration of carotenoid at weaning but a high concentration at slaughter. However, the high concentration of total carotenoid at slaughter was not in agreement with the AUS-MEAT® colour score of zero].

When comparing "outliers" with their half-sib "non-outliers", the majority of "non-outliers" (67.6%) were a result of crossbreeding between "non-outlier" parents. The remaining 32.4% had one parent as an "outlier" within the parental population.

5.4. Discussion

A proposed model for the inheritance of fat colour in cattle is based on the following:

- Limousin weaners did not have excessively yellow fat colour as confirmed by the lack of "outliers" present within their population. Limousin cows had whiter fat than Jersey cows and their distribution of fat colour overlapped the distribution of the Jersey population.
- The distribution for pure Limousin cattle was normal indicating a uniform population.
- The Jersey population had animals with extremely yellow fat which could not be reduced even after 240 days on a low carotenoid diet.
- The distribution of total carotenoid concentration in fat was not normal in Jersey purbreeds, but was skewed right and heavily tailed.
- Two sub-populations could be distinguished within Jerseys, one which had low carotene content and was normally distributed, and another which consisted of animals with extremely yellow fat.

These results suggest that Limousin cattle do not possess a yellow fat allele, while the Jersey cattle population has an allele resulting in yellow fat "outliers". Therefore, a possible model describing fat colour in cattle would be that Limousin cattle are homozygotes for white fat (WW) and Jersey cattle represent all genotypes: homozygotes for white fat (WW), heterozygous for white/yellow fat (WY) and homozygotes for yellow fat (YY). However, on average the crossbred progeny between Jersey and Limousin cattle were intermediate in fat colour. This observation and the distribution analysis provide evidence that fat colour in cattle is not caused by a simple recessive gene as reported in sheep. The different level of fat colour in the heterozygote (F1) population suggests an influence by other gene(s) or additive effects. In addition, the lack of a sex difference in fat colour between males and females and the presence of yellow fat "outliers" in both sexes removes the possibility of a sex influenced trait.

Nevertheless, if fat colour was a trait affected by a number of genes with large effects, then more variation would be expected within the Limousin breed with some "outliers". Based on the data from five years, there was little variation in the Limousin breed. Consequently, an assumption can be made that yellow fat colour is affected by a single major gene. Since

mating Jersey bulls with Jersey cows or F1 bulls with Jersey cows resulted in the presence of fat colour "outliers", the gene should be present in Jersey population in both bulls and cows.

The heritability of fat colour was low in general, but higher for Jersey cattle. The heritability estimates based on Limousin dams was close to zero. This and the two significant gene effects from statistical model (additive and epistatic) indicate a high level of homozygosity in Limousin population. The higher heritability estimates from F1 sires showed that the sire influence on the genetic pool was substantial. The distribution and means of the ¾ Limousin cattle suggest a large impact of Jersey yellow fat gene when introduced into the Limousin population via the F1 sires, providing further evidence of a major gene in the Jersey population.

The appearance of "outliers" among progeny resulted from mating animals where at least one of the parents was an "outlier" for fat colour. No "outlier" progeny were derived from "non-outlier" parents. This indicates that the "outliers" are likely to be homozygotes for the yellow fat allele.

If the "outliers" are assumed to be homozygous (YY), it is possible to estimate the frequency of the yellow fat allele in Jerseys. Three estimates can be made using the carotenoid concentration data from:

1/ Jersey cows

2/ Sire 77 & 78 pure Jersey progeny, and

3/ Sire 398 Jersey backcross progeny.

For the cow estimation, if it is assumed that the Jersey population is in Hardy-Weinberg equilibrium and that yellow fat "outlier" animals are homozygotes (YY), the frequencies of genotypes YY, WY, WW, respectively, in the Jersey population can be estimated with the following formula:

$$p^2 + 2pq + q^2 = 1$$

Therefore, p^2 = (# Jersey cows "outliers")/(total # Jersey cows) = 11/144 = 0.08, so p = **0.28**.

For the sire progeny estimations, it is assumed that Jersey sire 78 is homozygous because of the high frequency of progeny "outliers" from this sire. Therefore, all progeny produced with Jersey cows would be only homozygous or heterozygous in yellow fat allele (WW=0, WY=q,

YY=p. The number of "outliers" resulting from mating the sire 78 and Jersey cows was p = (# pure Jersey 78 progeny "outliers")/(total # pure Jersey 78 progeny) = 9/32 = **0.28**.

The other Jersey sire, 77, is likely to be heterozygous, as he had fewer "outlier" progeny. Therefore, his progeny would possess some white fat alleles [WW=0.5q, WY=0.5(p+q), YY=0.5p]. From the progeny data of sire 77, 1/2p = (# pure Jersey 77 progeny "outliers")/(total # pure Jersey 77 progeny) = 6/48 = 0.13, so p = **0.26**.

Sire 398 would be heterozygous (WY) by definition as his sire was Jersey 78 (YY) and his dam was a Limousin cow (WW). Analogically, from his progeny data, 1/2p = (# Jersey backcross 398 progeny "outliers")/(total # Jersey backcross 398 progeny) = 5/38 = 0.13, so p = **0.26**. From all of these calculations, the yellow fat allele frequency in the Jersey population is approximately **0.27**.

In conclusion, fat colour in cattle appears to be controlled by a major additive gene which is moderate in frequency in the Jersey breed (p~0.27). There was no evidence for additional genetic variation, and the remaining variation must be primarily environmental.

5.5. Summary

(i) Heritability of fat colour was low in Martindale cattle when estimated on total carotenoid concentration in fat, and in Struan cattle when estimated heritability based on subjective fat colour score at slaughter. Higher heritability estimates for Jersey cattle indicated a large influence of this breed on fat colour or a major gene existing in this breed only.

(ii) Statistical analysis of genetic effects on fat colour recognised additive and epistatic effects as significant but not maternal or heterotic effects.

(iii) Three major criteria were used to distinguish fat colour "outliers". The number of animals selected based on these criteria were in agreement, regardless of the criterion applied.

(iv) There are breed differences in distribution of carotene in cattle fat. Limousin crosses had a normal distribution, whereas the distribution for Jersey crosses was not normal, skewed right and tailed.

(v) Pedigree studies showed that the animals with excessively yellow fat derived from parents where at least one of them was regarded as an "outlier" in fat colour in the parental population.

The proposed model for inheritance of fat colour in cattle suggests that this trait is controlled by a major additive gene with a moderate frequency in Jersey population. However, the variation observed in the concentration of carotene in cattle adipose tissue is mainly influenced by dietary intake of carotenoids.

Chapter 6

Carotenoids in cattle muscle

6.0. Introduction

Meat consumption, especially in well developed countries, has a significant influence on supplementation of basic nutrients to the human body. However, there is little data on the carotenoid content of meat. In cattle, carotenoids are mostly deposited in subcutaneous fat and liver (Hidiroglou, 1986; Yang et al., 1992) although they have been detected in other organs and tissues (Gawienowski et al., 1974). No information is available on the influence of season and other genetic and non-genetic factors on carotenoid concentration in cattle muscle. If the concentration of carotenes in the muscles of cattle is as high as that in fat and liver, then meat could be an important source of carotenoids in the human diet. Therefore, the aim was to investigate carotene content in cattle muscle and factors which influence the concentration.

6.1. Materials and methods

Animals and management.

Three groups of cattle were used in the study: 469 steers and heifers from Southern Cross Breeding Project born in 1994 and 1995, 30 non-lactating cows from the Gene Mapping Project sampled by biopsy in autumn and spring in 1995 and 30 culled cows from Gene Mapping Project sampled in 1998 at slaughter. The steers and heifers were raised on pastures (see chapter 2), weaned at 250 days and finished in the feedlot for 70 days (heifers) and 140 days (steers). The cows were from the J.S. Davies Gene Mapping Herd and represented two breeds, Jersey and Limousin (see Chapter 2). The cows were kept on pastures and supplemented with hay during the summer.

Sample collection, preparation and analyses.

Fat and meat samples from 30 cows were collected from the *M. longissimus dorsi* by biopsy in 1995 (Chapter 2). Meat and fat samples from the Southern Crossbreeding Project animals and 30 Martindale cows (1998) were collected from the striploin of carcass on the day after slaughter (Chapter 2).

Biopsy fat and meat samples were snap-frozen in liquid nitrogen, flushed with N_2 gas, transported to the laboratory and stored at–20°C until required for analysis. Slices of muscle, 1-2 cm in thickness with attached subcutaneous fat were cut from the 12^{th}-13^{th} or 10^{th}-11^{th} rib region as a cross-section from the *M. longissimus dorsi.* The samples were stored at –20°C in sealable plastic bags until analysis.

Total carotenoid concentration in fat and fat-trimmed muscle from the 1995 Struan and Martindale samples was estimated spectrophotometrically (Chapter 2). Concentration of β-carotene and lutein in fat and fat-trimmed muscle muscle from cows sampled in 1998 was assessed by HPLC (Chapter 2). In brief, separation was achieved using a Spherisorb ODS column (5μm, C18 250 X 4.6mm) protected by a pre-column guard cartridge. The mobile phase was a methanol:hexane:water (90:5:5) mixture with a flow rate of 1.2ml/min. Analytical standards of β-carotene, lutein and alpha-tocopherol acetate (internal standard) were obtained from Fluka (USA). Prior to chemical analysis of carcass samples, subcutaneous and intermuscular fat were dissected from the meat, and the meat samples (100g) homogenised in a food processor to a fine paste. All samples were freeze-dried prior to analyses and stored at -20°C.

Statistical analysis

Southern Cross data was analysed using Proc GLM Proc MIXED and Proc CORR (SAS, 1989) (Chapter 4, experiment 2). The final model fitted with Proc GLM included year, sex, breed and sire nested within breed as a random effect. Data derived from 30 Martindale cows sampled in two seasons was analysed using Proc GLM and the final model included season, age, breed. A number of two way interactions were tested (breed and season, breed and age and season by age by breed) but removed from the model because they were not significant. Age, breed and age by breed interaction was the model used for analysis of differences in 30 Martindale culled cows sampled in 1998 at slaughter. Age and the interaction between age and breed were not significant. Consequently, the final model included only breed as a main effect. Least squares means and differences between the means were computed. Residual correlations between total carotenoids in meat and fat as well as between β-carotene and lutein in fat and meat were estimated by adjusting for the appropriate effects in each model.

6.2. Results and discussion

6.2.1. Breed, sex, age and seasonal differences in carotenoid concentration in muscle

Breed differences were observed in the total carotenoid concentration of cattle muscle. Jersey and Limousin cows from Martindale as well as the seven sire breeds crossed with Hereford cows from Struan differed significantly in the carotenoid content of *M. Longissimus dorsi.* Jersey cows had higher concentration of total carotenoid than Limousin cows (0.46μg/g of muscle and 0.27μg/g of muscle, respectively). Higher values for Jersey cattle were also observed in their crosses with Hereford cows. Jersey x Hereford crossbred cattle had a significantly higher concentration of total carotenoids compared to six other crossbreeds (Figure 6.1). Pure Hereford, South Devon x Hereford, Angus x Hereford and Belgian Blue x

Hereford were the next crossbreeds in ranking, although they did not differ significantly between each other. Limousin x Hereford and Wagyu x Hereford, which were allocated in the last place of the breed ranking, did not differ from each other but differed significantly from the remaining breeds.

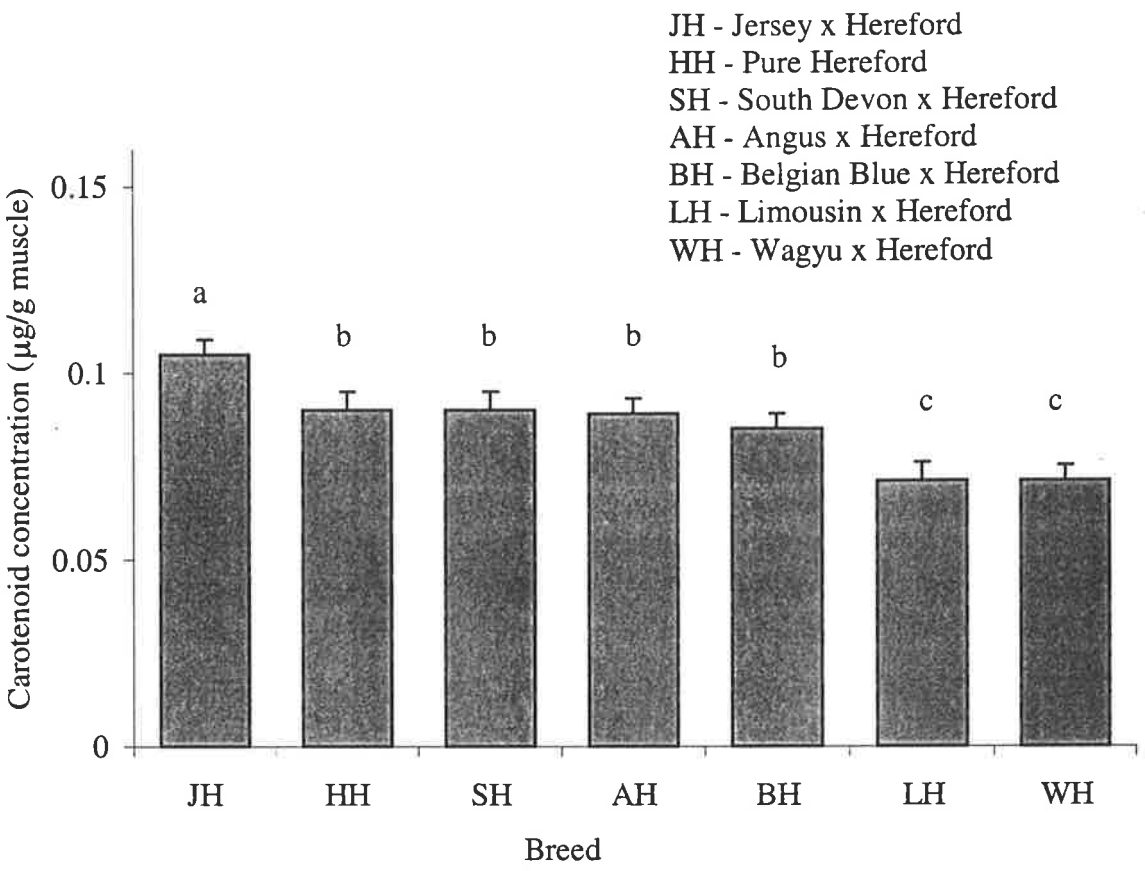


Figure 6.1. Breed differences in total carotene content in *M. longissimus dorsi*. The same letters (a-c) represent not significantly different values (P>0.05).

Length of time in the feedlot influenced the carotenoid content in fat (Chapter 5). Steers feedlotted for a long period of time (140 days) also had significantly lower total carotenoid concentration in their muscle compared to heifers which spent only 70 days in the feedlot (Figure 6.2). Jersey x Hereford heifers had the highest concentration of total carotenoids, which was significantly different from that of the other breed types. Jersey x Hereford steers also had the highest concentration, but they only differed significantly from the Wagyu x Hereford crossbreeds. No difference between other crossbred steers was observed. In contrast, Limousin x Hereford and Wagyu x Hereford heifers had the lowest carotene content and differed significantly from the other crossbred heifers.

Seasonal fluctuations in total muscle carotene were observed in the cows. In the dry season (summer/autumn), the concentration was significantly lower than in the wet season (winter/spring) (0.27µg/g of muscle and 0.46µg/g of muscle, respectively). Interestingly,

seasonal fluctuations in total carotene was not observed in subcutaneous fat from these same animals (Chapter 4). The observed difference was similar in both breeds as the breed by season interaction was not significant.

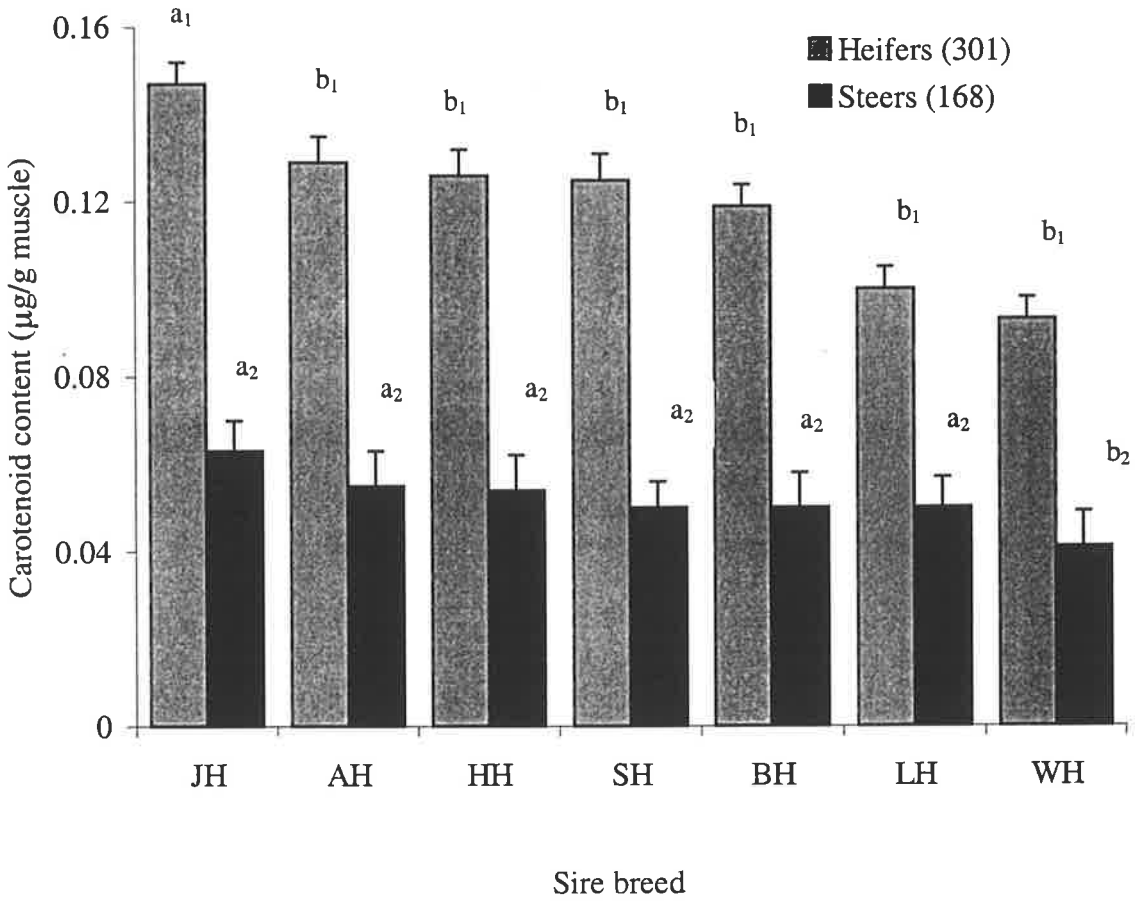


Figure 6.2. Sex differences and breed ranking in total carotene content in muscle. (Breed description as in Figure 6.1; The same letters: a_1-b_1 and a_2-b_2, represent not significantly different values). Legend values in brackets indicate the number of animals.

Age affected carotene concentration in muscle of non-lactating Jersey and Limousin cows. Older cows had more carotene in their muscle than young cows (Figure 6.3). No seasonal or breed influence on age was observed as age by season, age by breed and age by breed by season interactions were not significant. The Jersey and Limousin cows did not differ significantly in age (5.4 ± 1.7 and 4.5 ± 1.2 years, respectively). However, Jersey cattle in contrast to Limousin cattle are an early maturing breed and may metabolise carotenoids differently. On the other hand, the age of cows of both breeds demonstrated that the cows of both breeds had reached maturity and yet the difference between these breeds was still observed. The difference between Jersey and Limousin cattle was also pronounced in the Southern Cross Breeding Project where the animals were the same age. The progeny of Jersey sires crossed with Hereford cows were significantly different from the progeny of

Limousin sires crossed with Hereford cows. This was true in both sexes. It is important to point out that there was also a 3-7 fold difference in carotene concentration between the studies demonstrating that both Jersey and Limousin cattle can have diverse carotene concentrations in muscle.

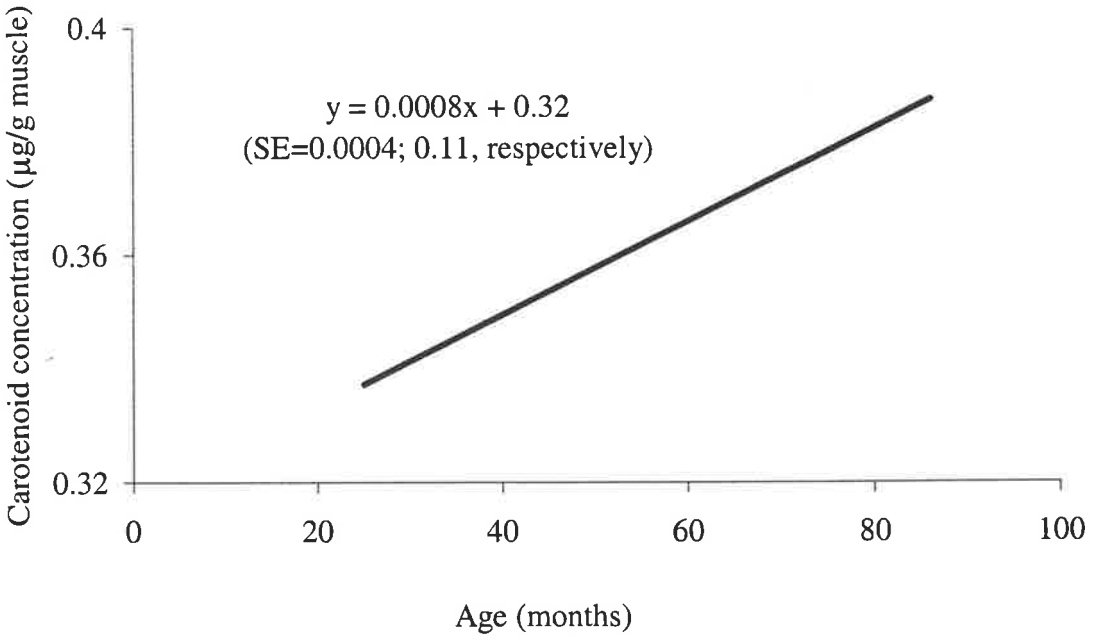


Figure 6.3. Relationship between total carotene concentration in muscle and age of cows.

6.2.2. Correlations between carotenoids in meat and fat

A moderate but highly significant correlation was observed between total carotenoid concentration in muscle and total carotene content in subcutaneous adipose tissue of Struan steers and heifers ($r=0.23$, $P<0.001$). Also fat colour score of these animals was positively correlated with total carotene in muscle ($r=0.32$, $P<0.001$). A moderate correlation between carotene in muscle and subcutaneous fat of Martindale cows sampled in 1995 was also noted, however, it was not significant ($r=0.19$, $P<0.09$). Low and non-significant correlations were found between intramuscular fat content in muscle, marbling and total carotene in muscle ($r=0.05$ and 0.06, respectively). The average AUS-MEAT® marbling scores differed between the sexes and were 1.37 for heifers lotfed for 70 days and 1.23 for steers lotfed for 140 days. The difference was statistically significant ($P < 0.01$).

The positive correlation between total carotene in muscle and subcutaneous adipose tissue led to a more detailed investigation of a relationship between β-carotene and lutein in muscle with these components in the adipose tissues. A number of high and positive correlations

were observed with various fat tissues (Table 6.1). Within the muscle, there was a positive correlation between β-carotene and lutein. Muscle β-carotene was also positively correlated with β-carotene in subcutaneous and intermuscular fat, and the correlations were high. Furthermore, β-carotene in subcutaneous, intermuscular and intramuscular fats was positively correlated with lutein content in the muscle. There was no correlation between lutein in muscle and lutein in the three adipose tissues. This was due to the fact that lutein accounted for only a small percentage of total carotene in these tissues with relatively high variation within each tissue (Chapter 3).

Table 6.1. Correlation between β-carotene and lutein in muscle and different adipose tissues.

Tissue		Meat		Subcutaneous		Intermuscular		Intramuscular	
		BC	LU	BC	LU	BC	LU	BC	LU
Meat	BC	X							
	LU	0.64	X						
Subcutaneous fat	BC	0.84	0.46	X					
	LU	ns	ns	ns	X				
Intermuscular fat	BC	0.85	0.49	0.87	ns	X			
	LU	ns	ns	ns	0.93	ns	X		
Intramuscular fat	BC	ns	0.64	0.66	ns	0.90	ns	X	
	LU	ns	ns	ns	0.73	ns	0.80	ns	X

Note: BC=β-carotene, LU=lutein, ns=not significantly correlated (P<95%)

6.2.3. Prediction of intramuscular fat content based on carotenoid concentration

The intramuscular fat concentration in the *Longissimus dorsi* was low and just missed statistical significance between these two breeds (2.25±0.38% in Jerseys and 1.33±0.45% in Limousin, respectively). The high positive correlations of β-carotene and lutein between subcutaneous, intermuscular and intramuscular fat were found and indicated that their deposition is similar in all types of adipose tissues (Table 6.1). Since β-carotene and lutein are fat soluble, poorly soluble in water and are highly correlated in their adipose depots, the vast majority of the carotenoids are likely to be found in fat cells rather than muscle cells. Hence, the carotenoid content of meat should be directly related to the fat content of the meat. If true, it should be possible to predict intramuscular fat content based on these fat soluble carotenoids.

To obtain the predicted fat content, β-carotene or lutein concentration in the meat (expressed as μg/g meat) was divided by its concentration in the subcutaneous fat (expressed as μg/g of subcutaneous fat) according to the equation:

$$\text{Predicted fat content} = \frac{\text{concentration of } \beta\text{-carotene (or lutein) in the meat } (\mu g/g)}{\text{concentration of } \beta\text{-carotene (or lutein) in the subcutaneous fat } (\mu g/g)}$$

The correlations between measured fat content and the fat content predicted for β-carotene and lutein were 0.9997 and 0.9978, respectively. Thus, the predicted fat content estimates were very highly correlated with measured fat content. This finding supports the hypothesis that β-carotene and lutein concentration in *M. Longissimus dorsi* must be highly associated with intramuscular fat (marbling).

The study was repeated on animals of the same age and sex which had very low carotene in the adipose tissue (data not presented here). In most cases, β-carotene was not detectable in the meat. However, the correlation between extracted total lipids and fat content predicted on lutein was significant ($r=0.48$, $P<0.01$), but not as high as in cows. The concentration of carotenoids in meat was five-fold lower.

A high correlation between total lipids extracted from meat and the predicted fat content in meat based on β-carotene and lutein show clearly that the HPLC quantitation of carotenoids in meat and adipose tissue can be an accurate measurement of fat content in cattle with high concentrations of these pigments. This is invaluable because there is no other accurate method of estimating fat content (marbling) in live animals. Since total lipid extraction is tedious, time consuming and requires a large quantity of meat, the prediction of fat content based on β-carotene and lutein can also be a competitive substitute for fat quantitation in slaughtered animals.

6.3. Summary

(i) Breed differences in total carotenoid concentration, as well as β-carotene and lutein concentrations, are evident in cattle muscle. The concentrations of these nutrients are also influenced by season and age of the animal.

(ii) Jersey and Limousin cattle differ not only in carotene content in their fat (Chapter 4), but also in muscle.

(iii) Breed differences in carotene concentration in muscle were observed regardless of carotenoid level.

(iv) Differences observed in carotene content between steers and heifers were probably due to the differences in the intramuscular fat content of the muscle.

(v) Carotenoid concentration in the eye muscle of cows with extremely high concentrations of these pigments was largely correlated with the intramuscular fat content (marbling). In cattle with extremely low carotenoid content in their tissues, only a certain portion of carotenoids was associated with intramuscular fat, whereas the rest would be associated with cell membranes.

Chapter 7

Absorption, transport and deposition of carotenoids in cattle tissues

7.0. Introduction

Prior to appearance of carotenoids in the blood stream, they are absorbed in the small intestine and undergo various changes in the enterocytes. Provitamin A carotenoids can be converted to retinal in a process catalysed by the enzyme 15,15'-β-carotene dioxygenase (Singh and Cama, 1974). Two metabolic pathways are proposed for this oxidative reaction:

- central cleavage which yields two molecules of retinal per one molecule of β-carotene consumed, and
- eccentric cleavage resulting in one molecule of retinal and various β-apo-carotenals (Hebuterne et al., 1996; Nagao et al., 1996), (Figure 1.6, Chapter 1).

Retinal can be then converted to retinoic acid and/or retinol (Hebuterne et al., 1996) and together with other dietary micronutrients (carotenoids, triacylglycerols, unesterified and esterified cholesterol, phospholipids, apoliporoteins, unesterified and esterified retinol, other fat soluble vitamins) are incorporated into the large particles (35-250nm in diameter) called chylomicrons (Blomhoff et al., 1992). Chylomicrons enter the blood stream *via* thoracic ducts and undergo rapid structural changes (Shepherd and Packard, 1987). Carotenoids as lipophilic nutrients (Parker, 1996) are exclusively associated with plasma lipoproteins, macromolecular aggregates, that transport water insoluble nutrients through the blood stream from their site of absorption or synthesis to peripheral tissues (Miller and Small, 1987). In cattle, the distribution of carotenoids in various lipoprotein fractions has been reported in Simmental, Holstein-Friesian (Schwaigert et al., 1987) and Hereford breeds (Yang et al., 1992). No such reports are available from Jersey and Limousin, the breeds of interest in this study.

Seasonal variation of β-carotene in blood has been reported by Cetinkaya and Ozcan (1991). They observed that serum β-carotene and retinol concentrations were higher in June and July (northern hemisphere) than in other months when the cows were in pasture. However, the data did not include the influence of season on the proportional distribution of carotene in different lipoprotein fractions.

A number of morphological parameters such as lipoprotein particle size, adipocyte size and adipocyte cell number may play a crucial role in carotenoid absorption or deposition. Reports on size variation in lipoprotein particles carrying different amounts of carotene (Ashes et al., 1984) as well as breed differences in adipocyte cell size in cattle (Miller et al., 1991) have been published. However, it was interesting and important to examine these parameters in Jersey and Limousin cattle acting in different environments as these breeds differed markedly in carotene content. Therefore, this chapter focuses on the following issues:

(i) factors affecting carotenoid concentration in cattle blood plasma,

(ii) influence of vitamin A on carotene status in cattle blood,

(iii) association of fatty acids and fat soluble vitamins with carotenoids in different lipoprotein fractions,

(iv) breed differences in the distribution of carotenoids in various lipoprotein fractions,

(v) breed differences in chylomicron and HDL morphology, and

(vi) breed differences in adipocyte cell size and number.

Examining these parameters may help identify which part of the carotenoid metabolic pathway leads to breed differences in adipose carotenoid concentration.

7.1. Materials and methods

Animals and management.

Cattle from Australia and New Zealand were used to provide the data for the research described in this chapter. Thirty non-lactating Jersey and Limousin cows from the Martindale property (South Australia) sampled in two different seasons in 1995 (Chapter 2 and 4) were analysed for carotenoids, fatty acids and fat soluble vitamins in blood, blood lipoprotein fractions as well as the morphology of chylomicron and high density lipoprotein particles (Experiment 1). Additionally, another group of thirty Jersey and Limousin non-lactating cows from Martindale was sampled in April 1996 to confirm the data from April 1995, since the recovery of carotenoids from the April 1995 samples was lower than from November sampling. Some of the cows sampled in 1996 were also sampled in 1995 but the majority were sampled for the first time. The cows did not differ significantly in age and grazed pastures supplemented with oaten hay during summer.

The influence of vitamin A on carotene metabolism in cattle was investigated using eight Angus and eight Hereford-Friesian ten month old steers raised on pastures in Palmerston North, New Zealand (Experiment 2). The steers were derived from one sire for each breed and were randomly allocated into two treatment groups, a control and a vitamin A group. The allocation of the steers to different treatment groups was based on the total carotene concentration in blood plasma before the beginning of the experiment. The vitamin A group was supplemented with 1 million IU of vitamin A in the form of pellets. The pellets were made in a commercial feed mill. Retinyl acetate was used as active form of vitamin A and supplied in Lutavit 500+ provided by BASF®. Steers were fed 1kg of pellets/head/day.

For the analysis of adipocyte morphology, 26 Jersey and Limousin steers from Martindale (13 per breed) born in 1995 were used (Experiment 3). They were raised on pastures, weaned at ~250 days of age and fed a low carotene diet in the feedlot for 170 days.

Sample collection, preparation and analyses.

Fat samples from the Martindale cows were collected using a biopsy technique (Chapter 2). Blood was collected from the jugular vein. Total carotene concentration in these samples was estimated spectrophotometrically (Chapter 3). Individual carotenoids and fat soluble vitamins [vitamin A (retinol) and E (α-tocopherol)] were quantified by HPLC (Chapter 3). Separation of blood lipoprotein fractions was achieved by repetitive preparative ultracentrifugation using a Beckman L8 ultracentrifuge fitted with a 70.1 Ti rotor (Havel et al., 1955, Chapter 2). The lipoprotein fractions were removed by using a tube slicer and stored under nitrogen gas at –20°C if used within 3-4 months or at –70°C for a longer period. Protein concentration in plasma and lipoprotein fractions was quantified by the method of Bradford (1976).

The measurement of lipoprotein particles was conducted by the use of a electron microscopy technique described by Forte and Nordhausen (1986) (Figure 7.1). The technique involved negative staining of the background of the sample with sodium phosphotungstate (NaPTA) on standard formvar-carbon-coated grids. The grid was inserted into the microscope specimen chamber (TEM 100, Philips) and examined under 10,500x magnification for chylomicrons and 92,000x for HDL (acceleration voltage of 80kV, and small objective aperture 50μm, Chapter 2). The images of the lipoproteins were visualised and saved utilising the Digital Micrograph 2.1 Macintosh computer program. The measurement of 100 chylomicron and 50 HDL particles was performed manually by the VP32 computer program after calibration of the scale using the calibration bar from the electron microscope. Results were expressed in nm.

Fatty acid composition was determined as described by Christie (1989). Lipids were extracted with chloroform:methanol (2:1/v:v) (Chapter 2). Fatty acids were esterified with acidic methanol (1% H_2SO_4 in methanol), the extracted esters were dried under N_2 and dissolved in 100μl of iso-octane (trimethyl pentane). Samples were stored at 4°C (short term) or –70°C (long term) prior to gas chromatography analysis. Results were expressed as a proportion of fatty acids in total or nmol/g of protein.

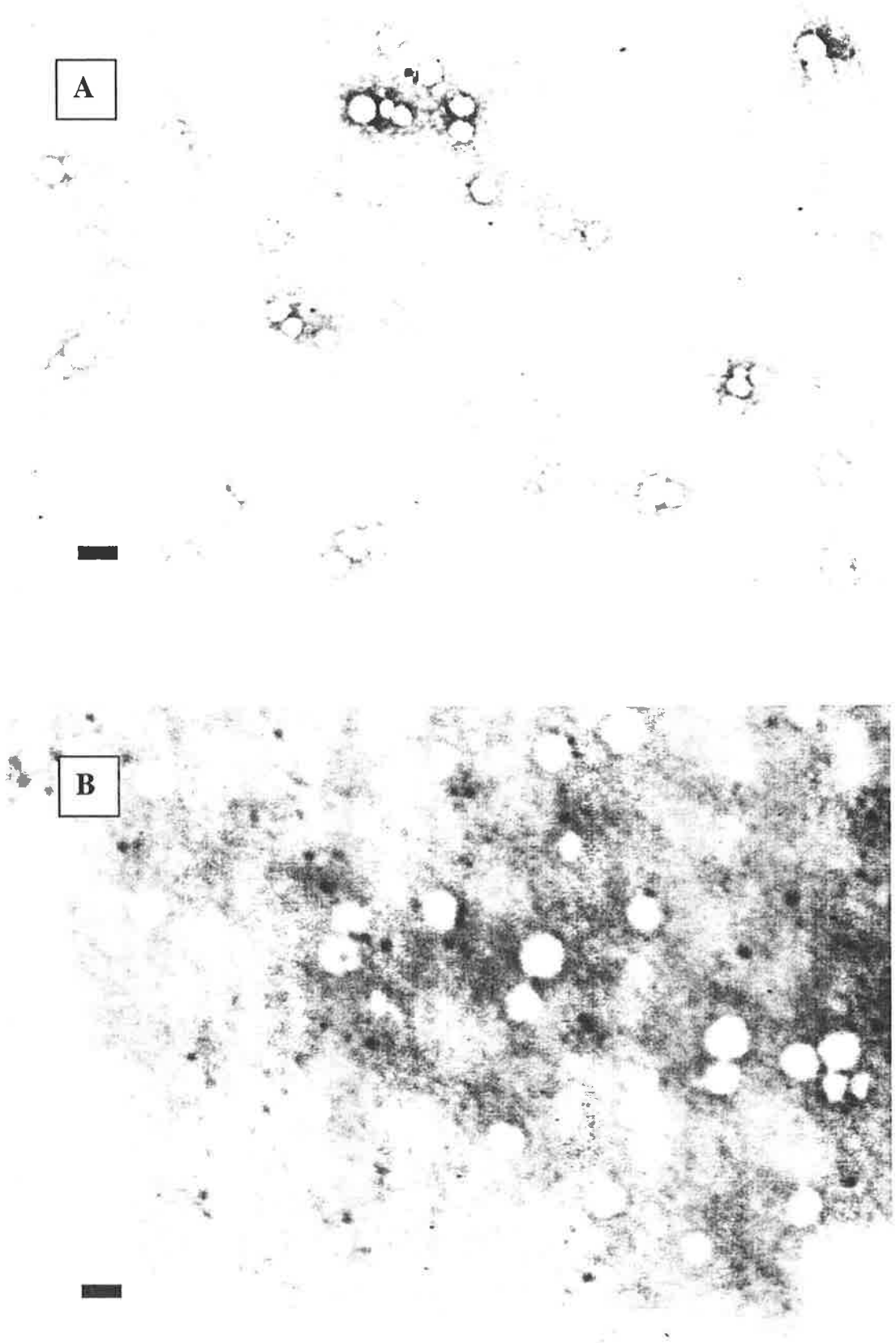


Figure 7.1. Electron micrographs of chylomicrons and HDL particles. *Note:* A-electron micrograph of chylomicrons, magnification 10,500x, scale bar represents 100nm, B-electron micrograph of HDL, magnification 92,000x, scale bar represents 10nm.

Adipose tissue of 26 Martindale steers was obtained from shoulder, ribs and rump from the chiller after slaughter (Chapter 2). Total carotenoid concentration in fat and blood plasma was measured spectrophotometrically.

Adipocyte liberation was conducted as described by Smith et al., (1972) with modifications described in Chapter 2. Fat specimens (~50mg) were incubated in freshly siliconised tubes containing 0.5ml of Krebs-Ringer bicarbonate buffer with 4% albumin (bovine albumin, fraction V) and collagenase (Type I, Lot 26 H0174, Sigma Chemical CO., St Louis, USA) added at a concentration of 5mg/ml of medium. The tubes were incubated at 37°C for 2.5 hours in the incubator while shaking samples gently (60 cycles/min). When the incubation was completed and adipocytes almost completely freed, the specimens were gently stirred and 50µl aliquots of the cell suspension were immediately placed in a heamocytometer. All adipocytes in each of 25 squares in chamber were counted (magnification 200x). Results were expressed as number of cells/g fat. An aliquot of cell suspension (100µl) was gently stirred and placed on a siliconized glass slide. One hundred cells were examined under the microscope (Olympus BH-2) with CMA-D1CE camera adapter (Sony, Japan), equipped with a CCD Sony camera and monitored under a final magnification of 670x. The cells were assigned into 10µ diameter interval classes based on the calibration mark on the monitor (Calibration for cell sizing was performed on the screen of the monitor using a calibration slide. After focusing, the slide scale with 10µ intervals was marked on the monitor). The results were expressed as % of adipocytes in each size class.

Blood from New Zealand steers was collected from the jugular vein before the trial (to assign steers into different treatment groups) and at –14, 0, +14, +28 days of the experiment. Plasma and chylomicron fractions were obtained by the method of Havel et al., (1955) as described above. Lutein, β-carotene, α-carotene, retinol and tocopherol standards were used for HPLC to detect these nutrients in blood plasma and chylomicrons (Chapter 2).

Statistical analysis

Percentage distribution of carotenoids in blood lipoprotein fractions was calculated by multiplying carotene concentration (expressed per ml of a fraction) by the fraction volume and dividing by the carotene concentration in plasma (µg/ml) and then multiplying by the volume of plasma used. The results were multiplied by 100 to express the data in percentage terms. Carotene recovery was the ratio of the sum of carotene concentration in all blood lipoprotein fractions multiplied by the fraction volumes for total carotene concentration in blood plasma.

To test differences in carotene concentration, fatty acid profiles and fat soluble vitamin content as well as chylomicron and HDL particle size in the 30 Martindale cows, least squares analysis of variance was carried out using Proc GLM of SAS (SAS, 1989). The model included the fixed effects of age, breed, season and the interactions of age by breed, breed by season, age by season and age by breed by season. For the analysis of adipocyte morphology, the model included breed, anatomical site and breed by site interaction. However, the interaction between breed and anatomical site was not significant and was removed from the model. The model for the analysis of the influence of vitamin A on carotene in cattle included treatment, breed and the interaction between breed and treatment. Residual correlations between the variables in all of the analyses were estimated using Proc CORR (SAS, 1989).

7.2. Results and discussion

7.2.1. Carotenoids in cattle blood (Experiment 1)

7.2.1.1. Factors affecting carotenoid concentration in blood plasma

When the concentration of total carotenoids in plasma was expressed in μg/ml of plasma, only the season and breed had a significant influence but not age. The Jersey cows had significantly more carotene in blood plasma than the Limousin cows (13.72μg/ml ± 0.59 and 10.62μg/ml ± 0.59, respectively) and this occurred in both summer and winter (Figure 7.2) The concentration of carotenoids in both breeds was higher in winter when green pastures were available to the cows (18.0μg/ml ± 0.57 and 6.34μg/ml ± 0.58, respectively). The interactions between the main effects were not significant which demonstrated that the breeds were affected in a similar way in both seasons.

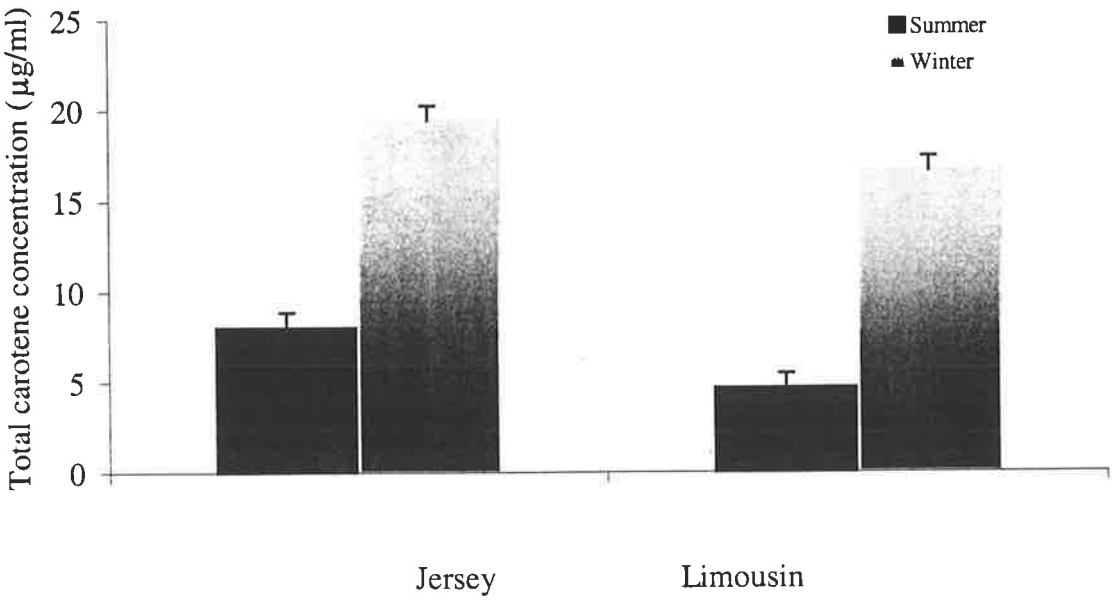


Figure 7.2. Breed by season interaction in carotene concentration in blood plasma

The results confirmed work of Cetinkaya and Ozcan (1991) who demonstrated a significant increase in serum β-carotene concentration when green and fresh pasture was available to Holstein and Brown Swiss crossed cows. Breed differences in plasma carotene concentration were also reported by McGillivray (1961) who showed that higher levels of carotenoids were found in lactating Guernsey cows followed by Jersey (60% of Guernsey level), Friesian, Ayrshire and Shorthorn. The difference between Jersey and Limousin non-lactating cows in this study demonstrated that the Jersey cows were still higher in plasma carotenoid concentration than the Limousin, even when carotenoids are not secreted by mammary glands during milk production.

In order to compare results from the plasma and different lipoprotein fractions, the carotene concentration per gram of protein was also calculated. The lipoprotein separation method, which required overlaying of the plasma with variable volumes of different density gradients, necessitated the expression of the results based on a constant component of blood. Thus, if expressed in relation to protein, the results could be compared between different studies and between blood fractions. Recovery of protein was estimated as the difference between protein concentration in blood plasma multiplied by the plasma volume used for lipoprotein separations, and the sum of volumes of particular lipoprotein fractions multiplied by their protein concentrations. The recovery of protein was between 81.9–90.1%. The concentration of proteins in blood plasma did not differ between Jersey and Limousin cows (575.2 ± 15.1mg/ml and 584.3 ± 15.6mg/ml, respectively) and was not affected by age, breed, season or all of the interactions between these main effects.

When the plasma carotene concentration (μg) is expressed per gram of protein, three major factors (age, breed and season) had an impact on plasma total carotenoid concentration in the Martindale cows. Within the age range between 25 and 86 months, older cows had lower concentrations of carotenoids compared to young cows (Figure 7.3). Age influence on total carotene concentration, when the results are expressed as carotenoid μg/ml of plasma, just missed significance ($P<0.06$). The age influenced both breeds in the same manner, as the breed by age interaction was not statistically significant.

These findings agree with the report of McGillivray (1960) who concluded that blood carotenoid levels increases up to about three years of age, and thereafter, remains relatively constant throughout the adult life of the animal. The lack of significant interaction between breed and age demonstrated that Jersey and Limousin cows, though different in carotene plasma content, were both affected in the same way by age. The cows from Martindale were

older than 3 years, except for three Limousin cows, which were 25, 30 and 30 months of age. Carotene levels in McGillivray's experiment were slightly higher as Jersey cows were predominantly used in that study.

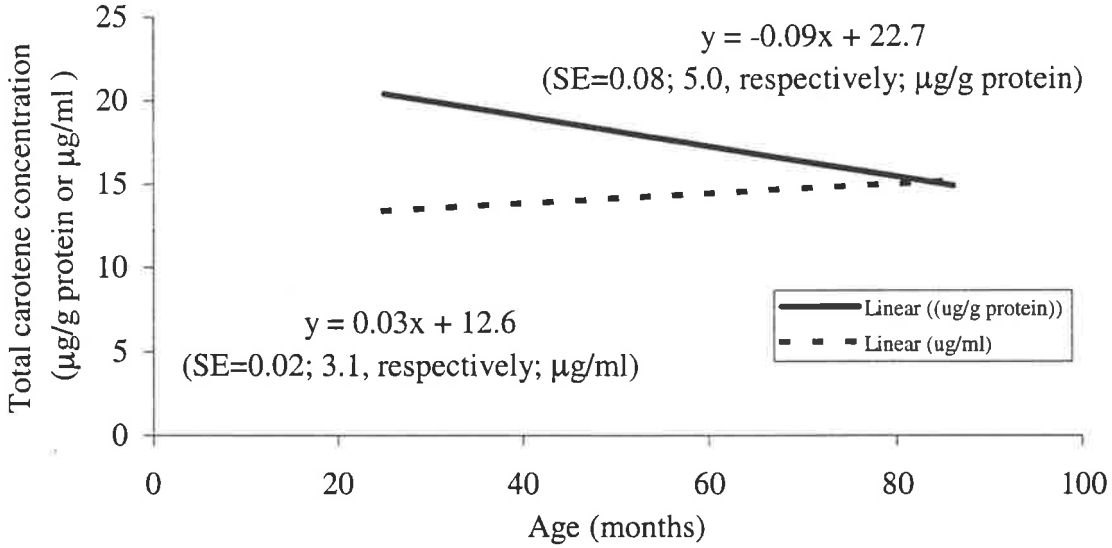


Figure 7.3. Age effect on plasma carotene concentration in Jersey and Limousin cows. Results expressed as µg/ml of plasma and µg/g of plasma proteins.

7.2.1.2. Carotenoid distribution in blood lipoprotein fractions.

The distribution of total carotene in the blood lipoprotein fractions in Jersey and Limousin non-lactating cows was determined (Table 7.1). The most abundant in carotenoid fraction was the high density lipoprotein (HDL), followed by low density lipoprotein (LDL), chylomicrons and very low density lipoprotein (VLDL) fractions. The distribution between fractions was consistent in both breeds, although there was a significant difference in carotenoid concentration in all major fractions between the breeds.

Table 7.1. Distribution of carotene in blood lipoprotein fractions (percentage).

Blood fraction	Summer 1995		Winter 1995		Summer 1996	
	Jersey	Limousin	Jersey	Limousin	Jersey	Limousin
Plasma[a]	8.0±0.8	4.7±0.8	19.4±0.8	16.6±0.8	7.2±0.8	5.2±0.8
Chylomicrons	7.5	6.6	4.1	3.6	6.2	5.7
VLDL	nd	nd	0.3	0.3	nd	nd
LDL	6.0	9.9	7.1	10.9	5.6	7.4
HDL	86.5	83.5	88.5	85.2	88.3	87.9

Note: VLDL=very low density lipoproteins, LDL=low density lipoproteins, HDL=high density lipoproteins, nd=not detected (delectability level <0.3µg/ml), [a]=plasma carotene concentration expressed in µg/ml while other values expressed as percent in total.

A similar distribution was observed in different seasons with most of the carotene present in HDL fraction, followed by LDL and chylomicrons. In the majority of samples, carotenoids were not detectable in the VLDL fraction in the summer, but were detectable in winter. Since the carotenoids in the VLDL fraction accounted for only ~1% of total carotenoids in blood, their concentration in summer was less than the detectable level (<0.3μg/ml). There also was no difference in distribution of carotenoids in blood fractions between summer of 1995 and 1996 (Table 7.1). This allowed the results from 1995 summer sampling to be used for further analysis even though the recovery of carotenoids was 16.9% less than in summer 1996 and 18.6% less than in November 1995. The advantage of using results from 1995 was that the cows available for sampling in both seasons were the same except for two animals. In summer 1996, the majority of the cows were sampled for the first time and had not been sampled in winter of the previous year. To avoid significant genotypic differences between the cows, the data from the same animals sampled in both seasons were adjusted for differences in recovery.

Trace amounts of carotenoids were detected in the lipoprotein depleted serum (LPDS) when 3 ml of this fraction was extracted. It accounted for ~0.6% of total carotenoids present in the blood. However, the concentration of carotene in this fraction was probably due to contamination during the removal of the HDL. This was confirmed by snap freezing LPDS immediately after the removal of HDL and extracting carotenoids from various LPDS layers. The only layer containing carotenoids was the one adjacent to the HDL fraction (approximately 3ml of LPDS). This confirms the finding that carotenoids in blood are transported exclusively by the lipoproteins (Erdman et al., 1993).

The distribution of carotenoids in blood ,lipoprotein fractions reported for Jersey and Limousin cows is in agreement with the other studies. The predominant fraction (83.5-88.7%) carrying carotenoids in cattle is HDL (77-83% β-carotene; Schwaigert et al., 1987 and 81.4% of carotenoids; Yang et al., 1992). In contrast, the VLDL fraction contained the lowest amounts of carotene. Schwaigert et al., (1987) and Yang et al. (1992) observed that there was no chylomicron fraction in lactating cows or steers. The presence of ~6.0% of carotenoids in chylomicron size particles (67.2 ± 4.2nm in diameter) herein demonstrated the presence of chylomicrons in non-lactating cows. Notably, in Yang's experiment, there were only three steers used for the acquiring data and the concentration of carotenoids was ~6 fold lower. In Schwaigert's study, chylomicrons were not detectable and the cows were producing milk during sampling. The difference between the studies in plasma lipoprotein distribution could be due to the physiological state of the animal as observed by Raphael et al., (1973).

Moreover, using different techniques for detection and separation of lipoprotein fractions may affect the presence of chylomicrons as demonstrated by Ferreri and Elbein (1982).

7.2.2. Absorption of carotenoids in cattle

Various dietary and physiological factors influence carotenoid concentration in blood plasma with vitamin A decreasing the concentration (Knight and Death, 1996). However, the reduction of carotenoids in blood plasma and liver did not affect fat colour in cattle even if the animals were supplemented with different doses of vitamin A. This effect of vitamin A on carotenoid concentration in milk fat and blood plasma was recognised as early as in the beginning of 1940's (Deuel et al., 1941, 1942). However, the mechanism of its action is still not known.

7.2.2.1. Influence of vitamin A on carotene in blood

Supplementation with vitamin A decreased β-carotene and lutein concentration in blood plasma (Experiment 2). The differences between treatment and control groups in both carotenoids appeared within the first fortnight of the trial and were statistically significant (Figure 7.4, Figure 7.5). The concentration of vitamin A in the blood plasma was constant through the whole experiment and did not vary between the treatments (Figure 7.6).

A similar trend appeared in the chylomicrons, where there was no difference in vitamin A concentration between the treatment groups (Figure 7.7). The concentration of β-carotene in the chylomicrons decreased in the vitamin A treated animals reaching a significantly different level within the first two weeks of the treatment (Figure 7.8). The concentration of lutein followed the same trend. However, a significantly different level of lutein was only observed ($P<0.0001$) at the end of the experiment (Figure 7.9).

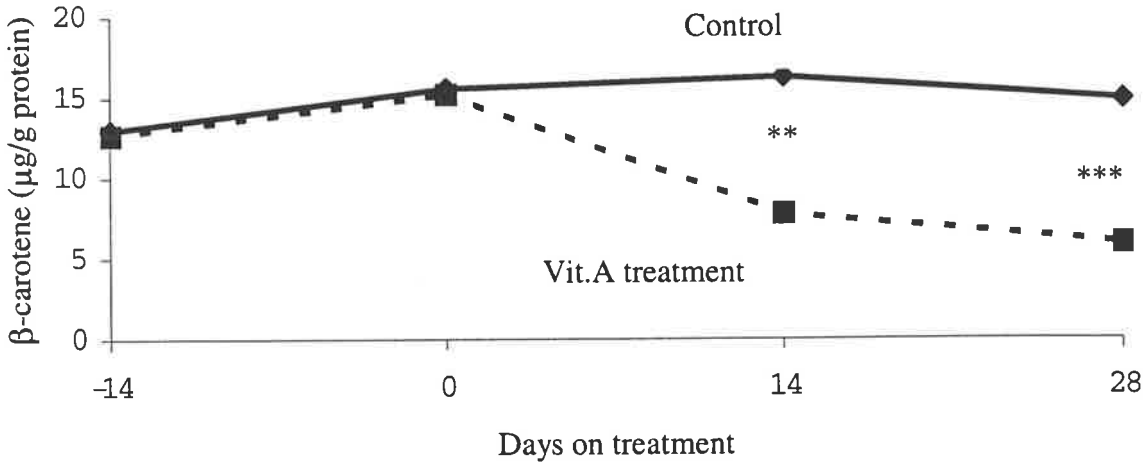


Figure 7.4. Influence of vitamin A supplementation on β-carotene concentration in blood plasma. ** = P<0.01, *** = P<0.001.

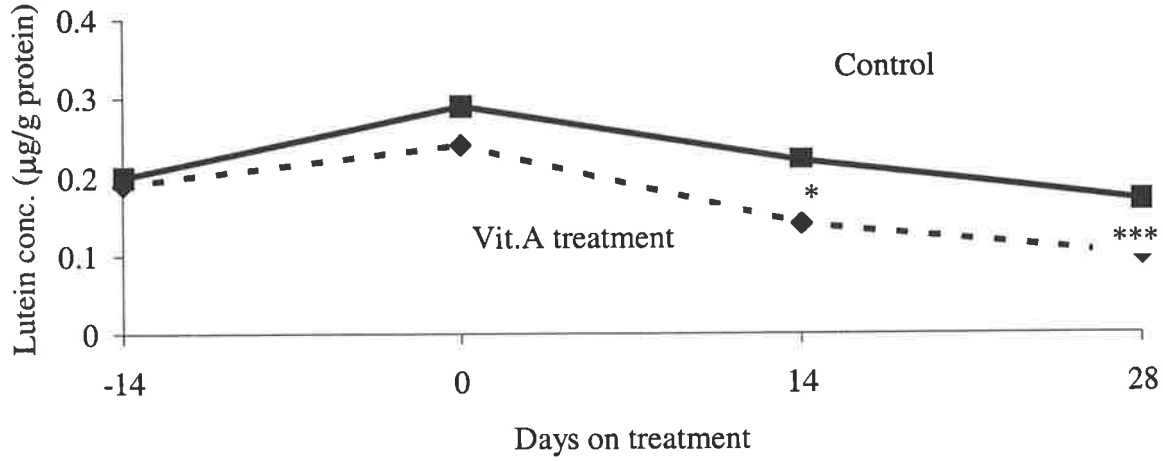


Figure 7.5. Influence of vitamin A supplementation on lutein concentration in blood plasma. * = P<0.05, *** = P<0.001.

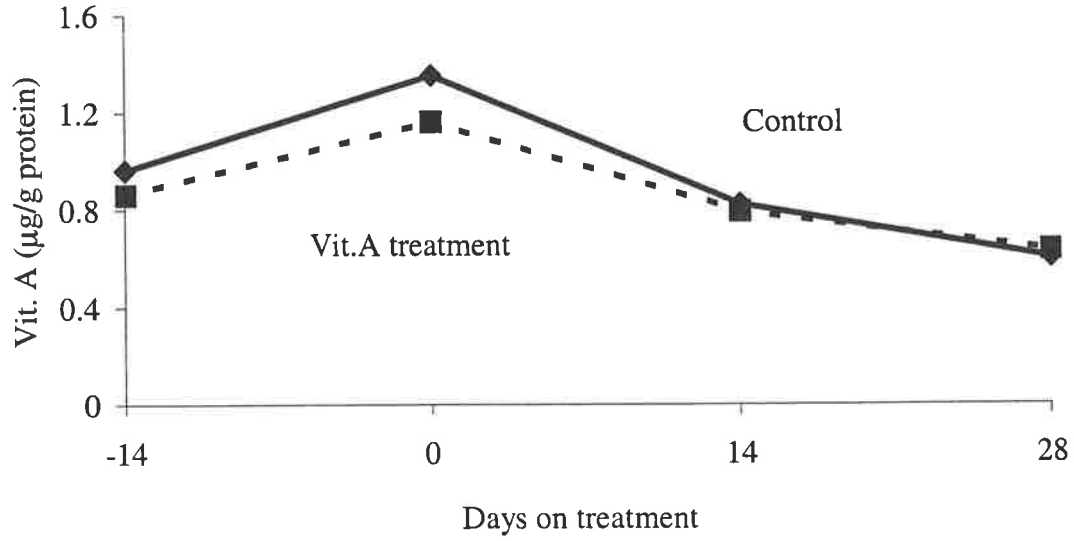


Figure 7.6. Plasma concentration of vitamin A in different vitamin A treatment groups.

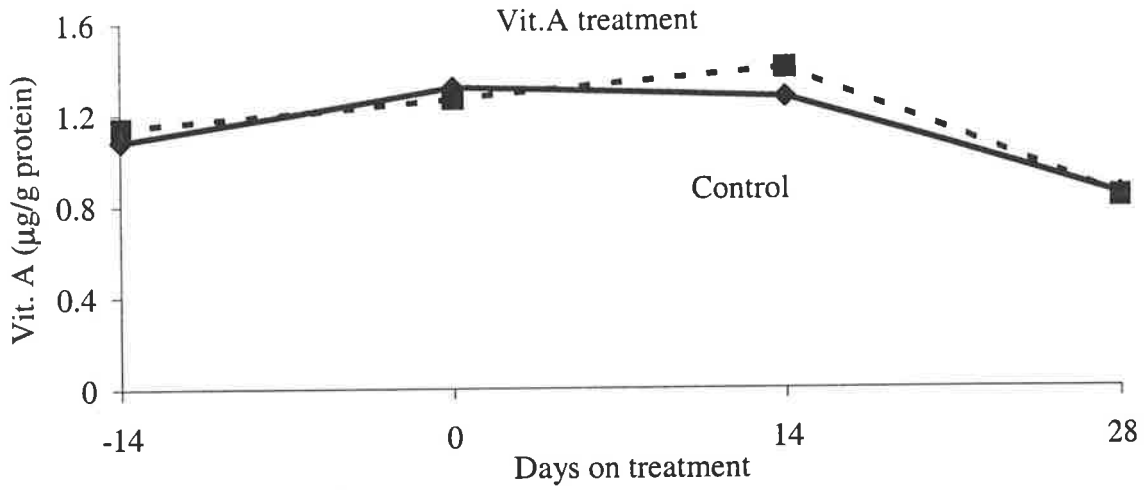


Figure 7.7. Chylomicron concentration of vitamin A in different vitamin A treatment groups.

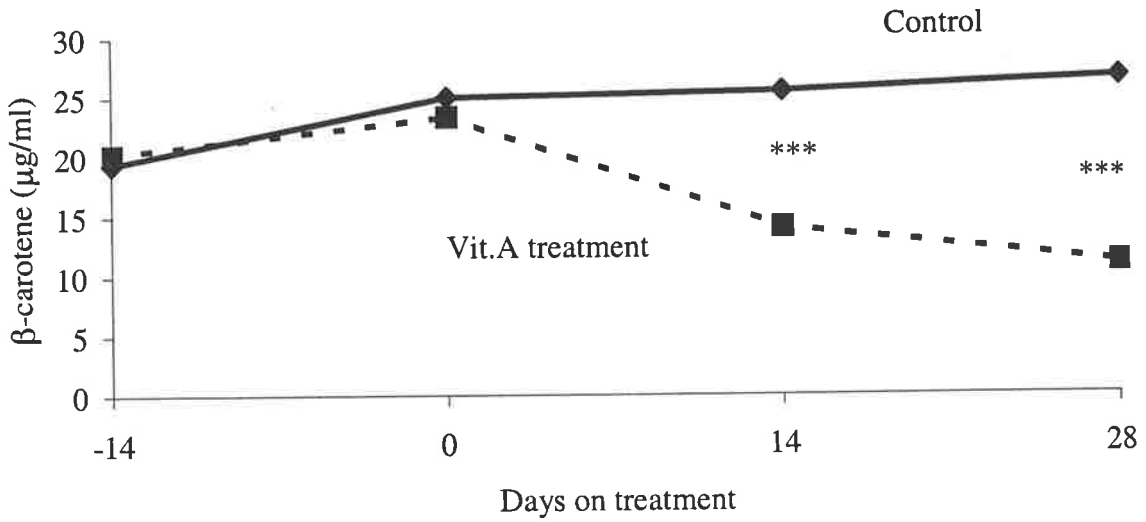


Figure 7.8. Influence of vitamin A supplementation on β-carotene concentration in chylomicrons. *** = P<0.001.

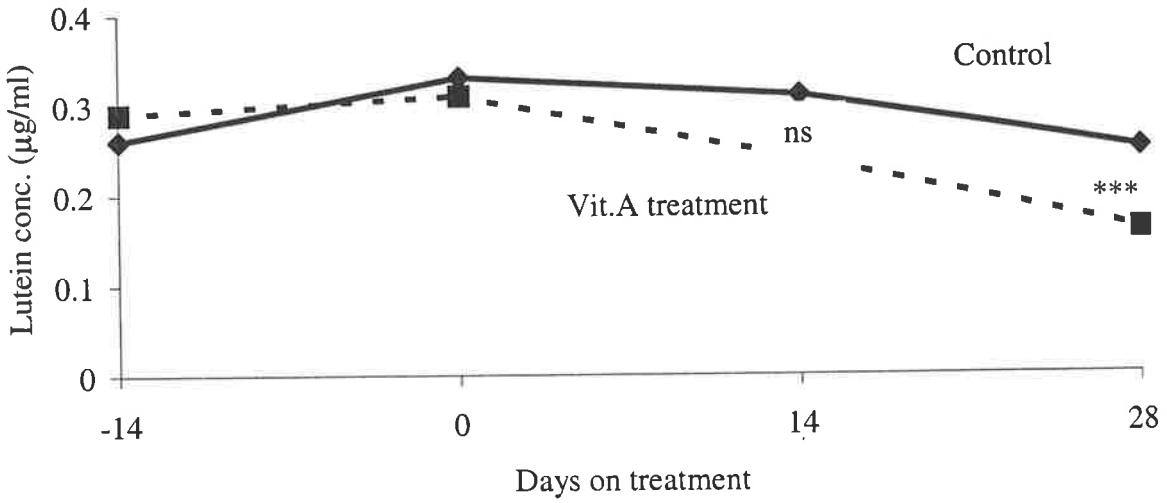


Figure 7.9. Influence of vitamin A supplementation on lutein concentration in chylomicrons. *** = P<0.001, ns = not significant (P>0.05).

The decline of β-carotene and lutein concentrations in blood plasma observed herein demonstrated that the supplementation with vitamin A affected the concentration of both carotenoids. Moreover, this effect of vitamin A on β-carotene and lutein was extended to chylomicrons which reflected the absorption of various nutrients from the small intestine and/or possible changes in the enterocytes. The lack of differences in concentration of vitamin A (retinol) in the chylomicrons and blood plasma in both groups and the significantly lower concentrations of β-carotene and lutein in the vitamin A treated group suggest an influence of vitamin A on the absorption of these two carotenoids from small intestine. However, to confirm such a conclusion, other forms of vitamin A need to be investigated. Retinal, and subsequently retinol and retinoic acid, can be produced by the enzyme β-carotene-15,15'-dioxygenase which converts provitamin A carotenoids in the intestinal mucosa (Crain et al., 1967; Larksman et al., 1993; Wang et al., 1994). The difference in the activity of the enzyme or the availability of the substrate affected by the vitamin A to lutein and β-carotene ratios may be of importance (Yang and Tume, 1993; Vliet et al., 1996). On the other hand, an effect of vitamin A on the absorption of lutein can be concluded as the lutein is not a provitamin A carotenoid.

7.2.2.2. Factors affecting carotenoid and vitamin A concentration in chylomicrons

Two major factors, breed and season, had an influence on total carotene concentration in chylomicrons of cattle. Jersey cows had a higher concentration of carotene than Limousin cows (23.8 ± 2.2 vs 15.1 ± 2.3μg/g protein, respectively; $P<0.01$). More carotene was present in chylomicrons collected in the winter (green pasture) than in the chylomicrons collected in summer (dry pasture) (24.8 ± 2.2 vs 14.1 ± 2.3μg/g protein, respectively; $P<0.001$). The difference between the two seasons was consistent for both breeds. However, in winter the Limousin cows, in spite of lower chylomicron carotene content, were not statistically different from Jersey cows (Table 7.2).

Contrary to blood plasma total carotene content, β-carotene in the chylomicrons was not significantly affected by the season (summer: 64 ± 14μg/g protein vs winter: 87 ± 13μg/g protein, respectively; $P<0.21$) and breed was the only factor differentiating Jersey and Limousin cows (Jersey: 101 ± 14μg/g protein vs Limousin: 50 ± 14μg/g protein; $P<0.01$). The concentration of β-carotene in both seasons was higher in Jersey cows. However, due to the large variability, it was statistically significant only in winter (Table 7.2).

Lutein, another predominant carotenoid in chylomicrons of cattle, did not differ significantly between seasons and breeds. A higher concentration in winter samples was not statistically different from the concentration in summer samples (summer: 4.3 ± 0.9μg/g protein vs winter: 5.4 ± 0.9μg/g protein, P<0.61). The Jersey cows had higher concentration of lutein than the Limousin cows and the difference just missed significance (Jersey: 6.0 ± 0.9μg/g protein vs Limousin: 3.7 ± 0.9μg/g protein, P<0.08).

Table 7.2. Breed by season interaction between carotenoids and retinol in chylomicrons and chylomicron morphology of Jersey and Limousin cows sampled in winter and summer.

Component	Summer			Winter		
	Jersey	Limousin	Sig.	Jersey	Limousin	Sig.
Total carotene	19.2±3.1	9.7±3.0	98%*	28.1±3.1	21.0±3.0	34%
β-Carotene	85.6±18.8	42.1±20.0	103%	116.3±19.1	58.1±18.2	100%*
Lutein	4.9±1.3	3.7±1.4	32%	7.2±1.3	3.6±1.2	100%
Retinol	14.2±1.7	14.9±1.7	-5%	11.0±1.7	9.4±1.6	17%
Chylomicron size	65.2±1.0	66.1±1.0	-1%	68.7±1.0	67.67±0.9	1%

Note: Sign.=significance, *=P<0.05, Summer–April sampling, Winter – November sampling, ns = not significant, results for carotenoid and retinol concentration expressed as μg/g of protein in chylomicrons, results for chylomicron size expressed in nm. The higher concentration of β-carotene than total carotenoids is due to different extraction conditions (ie, solvents) (see Materials and Methods).

There was no breed difference in retinol concentration in chylomicrons (Jersey: 12.6 ± 1.2μg/g protein vs Limousin: 12.1 ± 1.2μg/g protein). Season had an impact on vitamin A in chylomicrons by reducing the concentration significantly in winter (summer: 14.5 ± 1.2μg/g protein vs winter: 10.2 ± 1.2μg/g protein, P<0.01). The concentration of retinol was lower in winter, but reached a significantly different level only in Limousin cattle (Table 7.2).

7.2.2.3. Factors influencing morphology of chylomicrons

The size of the chylomicron particles did not vary between Jersey and Limousin cows even though there was a significant difference between these breeds in concentration of total

carotenoids or β-carotene. However, larger chylomicron particles were found in winter than in summer (68.17 ± 0.67nm and 65.65 ± 0.69nm, respectively; $P<0.01$). The seasonal difference was observed only in the Jersey cattle where the cows differed significantly. (Table 7.2).

7.2.2.4. Relationship between chylomicron carotenoids, fatty acids, vitamin A and particle size

There were a number of significant correlations between chylomicrons and various carotenoids, fat soluble nutrients and fatty acids (Table 7.3). Total carotene concentration was highly correlated with β-carotene, α-tocopherol and moderately correlated with lutein and retinol. The correlations were highly significant. Some chylomicron fatty acids were also correlated with carotenoids. A low but significant relationship was observed between total carotenoids and polyunsaturated fatty acids (PUFA) and 18:2 ω–3 fatty acids regardless whether the concentration of fatty acids was expressed as per total fatty acid or nmol/g protein. Moreover, moderate and positive correlations were observed with 16:0, 18:1 ω-9cis, 18:3 ω-3 fatty acids. β-Carotene was highly associated with lutein, retinol, α-tocopherol and moderately associated with 16:0, 17:0, 18:2, 18:1 ω-9, 18:3 ω–3, monounsaturated (MONO) and polyunsaturated (PUFA) fatty acids. Lutein was significantly correlated with retinol, 14:1 and 17:0 fatty acids. A negative correlation was found with ratios of monounsaturated (MONO) to saturated (SAT) and polyunsaturated (PUFA) fatty acids (Table 7.3). The only significant relationship of chylomicron size was noted with 15:0 and 18:3 ω-6 fatty acids when their concentration was expressed in nmol/g of protein. The correlation of chylomicron size with carotenoids and fat soluble vitamins was very low and not significant.

7.2.2.5. Discussion - absorption of carotenoids in cattle

A higher concentration of total carotene in chylomicrons of Jersey cows raised in the same environment as Limousin cows, demonstrated higher absorption of carotenoids in this breed of cattle or lower conversion rate of provitamin A carotenoids in the small intestine. Such differences in the chylomicrons can account for the breed differences in the concentration of carotenoids in blood plasma which have been reported in a number of studies (McGillvray, 1960; Morgan et al., 1969), and consequently, for breed differences in tissue carotenoid concentration (Hoppe et al., 1996).

Table 7.3. Relationship between morphology and fat soluble nutrients in cattle chylomicrons

Parameter correlated to:	Correlation	Significance
Total carotene:		
β-Carotene (μg/g protein	0.74	***
α-Tocopherol (μg/g protein)	0.71	**
Retinol (μg/g protein)	0.51	***
Lutein (μg/g protein)	0.53	***
18:2 fatty acid (% in total)	0.34	**
PUFA (% in total)	0.27	*
16:0 fatty acid (nmol/g protein)	0.25	*
18:1 ω-9 cis fatty acid (nmol/g protein)	0.33	**
18:3 ω-3 fatty acid (nmol/g protein)	0.27	*
PUFA (nmol/g protein)	0.20	*
β-Carotene:		
α-Tocopherol (μg/g protein)	0.63	*
Retinol (μg/g protein)	0.63	***
Lutein (μg/g protein)	0.60	***
18:3 ω-3 fatty acid (% in total)	0.30	*
16:0 fatty acid (nmol/g protein)	0.30	*
17:0 fatty acid (nmol/g protein)	0.28	*
18:1 ω-9 fatty acid (nmol/g protein)	0.41	***
18:2 fatty acid (nmol/g protein)	0.30	*
18:3 ω-3 fatty acid (nmol/g protein)	0.27	*
MONO (nmol/g protein)	0.34	*
PUFA (nmol/g protein)	0.28	*
Lutein :		
Retinol (μg/g protein)	0.72	***
14 :1 fatty acid (nmol/g protein)	0.31	*
17:0 fatty acid (nmol/g protein)	0.63	***
MONO/SAT (nmol/g protein)	-0.34	**
MONO/PUFA (nmol/g protein)	-0.32	**
Chylomicron size:		
Total carotene (μ/g protein)	0.05	ns
β-Carotene (μg/g protein)	-0.05	ns
Lutein (μg/g protein)	0.07	ns
α-Tocopherol (μg/g protein)	-0.16	ns
Retinol (μg/g protein)	0.11	ns
15:0 fatty acid (nmol/g protein)	0.25	*
18:1 ω-6 fatty acid (nmol/g protein)	0.24	*

Note: ***=P<0.001, **=P<0.01, *=P<0.05, ns=not statistically significant, MONO=monounsaturated fatty acids, SAT= saturated fatty acids, PUFA=polyunsaturated fatty acids,

β-Carotene and lutein are the two major carotenoids present in cattle tissues (Chapter 3). Lutein, unlike β-carotene, is not a provitamin A carotenoid in mammalian species (Olson, 1989). If β-carotene and lutein are absorbed in the small intestine by a similar mechanism (e.g. passive diffusion), then the lack of a breed difference in the concentration of lutein in

chylomicrons suggests that the breed difference in β-carotene concentration is due to differential cleavage by the dioxygenase enzyme in the two breeds. However, retinal is the primary metabolite of the intestinal enzyme β-carotene-15, 15'- dioxygenase (Goodman et al., 1967; Olson, 1989) and can be reduced to retinol (Fidge and Goodman, 1968) or oxidised to retinoic acid (Crain et al., 1967). The lack of a difference in the concentration of retinol between Jersey and Limousin chylomicrons indicated higher absorption of carotenoids in the Jersey cattle rather than cleavage differences. On the other hand, retinoic acid was not investigated in the herein study. If its production varied between Jersey and Limousin cattle then a difference in retinol concentration between these breeds may not have been observed.

Intestinal enterocytes are the most active site of carotene-15, 15'- dioxygenase in rat (During et al., 1996). The action of this cytosolic enzyme is influenced by a number of factors (Singh and Cama, 1974). The high levels of carotenoids in diet decreases the enzyme activity (Van Vliet et al., 1996). More carotene in winter diet resulted in more carotene in chylomicrons. This could have been caused by a higher absorption of β-carotene or lower conversion of β-carotene to retinol. Lower level of retinol in the chylomicrons during winter also suggests that high carotenoid diet reduced carotene-15, 15'- dioxygenase activity in cattle. However, different mechanisms may be suggested for different breeds of cattle as the Limousin cows had lower retinol content in winter whereas Jersey cows did not differ between the seasons. Breed differences in absorption and conversion of carotenoids have been reported by MacDowell (1989). He stated that Gurnsey and Jersey breeds readily absorb carotenoids while Holstein cattle are efficient converters of carotenoids to vitamin A.

Chylomicrons are complex particles consisting of various components influencing their size (Blomhoff et al., 1992). Significantly higher level of carotenoids in Jersey cows in winter may contribute to the larger size of chylomicrons in this season. However, the correlation between chylomicron size and carotenoids was very low and not statistically significant. Carotenoids may not contribute significantly to the size of chylomicrons. Green and Glickman (1981) reviewed intestinal lipoprotein metabolism and concluded that chylomicron size appears dependent on the rate of lipid absorption or degree of saturation of absorbed fatty acids. Borel et al., (1998) pointed out that the chain length of lipids influenced β-carotene concentration in chylomicrons. However, season was the only factor influencing the saturation of fatty acids in this study and only 15:0 and 18:3 ω-6 fatty acids were significantly correlated with chylomicron size.

7.2.3. Transport of carotene in cattle

High density lipoprotein (HDL) particles consist of various apoproteins, cholesteryl esters, and phospholipids which originate from liver and/or intestine (Babiak and Rudel, 1987). Some authors also have reported variable quantities of triacylglycerides in this lipoprotein fraction in cattle (Dryden et al., 1971; Jonas, 1972). HDL particles represent a non-homogenous fraction varying in size between 5.5–12.0nm in humans (Patsch and Gotto, Jr, 1987), 8.9-11.5nm in steers (Yang et al., 1992) and 9.0-16.0nm in lactating cows (Tall et al., 1981). HDL is the major carrier of carotenoids in cattle and transports about 80% of these pigments through the blood stream in steers (Yang et al., 1992), bulls, lactating (Schweigert et al., 1987) and non-lactating cows (Chapter 7).

7.2.3.1. Factors affecting carotenoid concentration in HDL and HDL morphology

Age, breed and season had an effect on total carotene concentration in HDL. Older cows had less carotene present in this lipoprotein fraction than younger cows ($P<0.01$) (Figure 7.10). The age effect was similar in these two breeds, as age by breed interaction was not statistically significant.

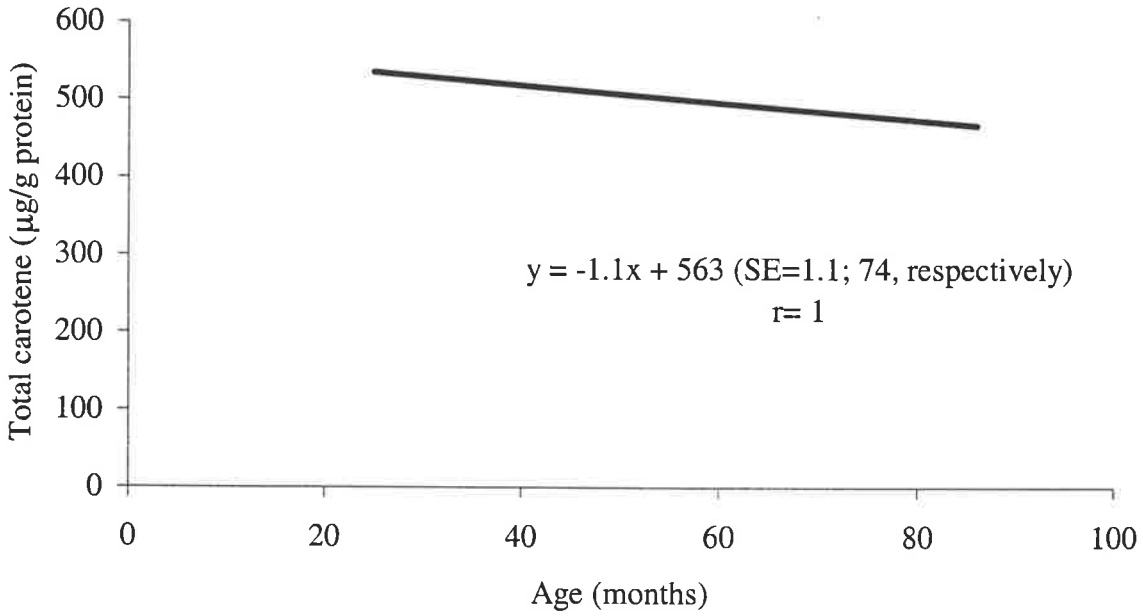


Figure 7.10. Age effect on carotene concentration in HDL blood fraction.

The concentration of carotene in Jersey HDL was significantly higher ($P<0.0001$) than in the Limousin cows (734.6 ± 33.3µg/g protein vs 426.64 ± 33.9µg/g protein, respectively). The difference between these breeds was consistent in summer and winter (255.69 ± 33.5µg/g protein in summer vs 905.6 ± 33.4µg/g protein in winter). Breed by season interaction was not statistically significant.

Season influenced size of HDL particles with larger particles appearing in winter (10.68 ± 0.14nm in winter vs 9.62 ± 0.15nm in summer). There was no significant difference between breeds and there was no breed by season interaction. However, a difference was observed between breeds when particle size was assigned to different diameter classes. In summer, the diameter of particle ranged from 8-11nm in both breeds (Figure 7.11.). However, the most abundant class in Jersey cows were particles between 10-11nm, whereas in Limousin cows, the particles between 9-10nm were the most common. In Jersey cows, the HDL size distribution was skewed, while in Limousin cows, the distribution was normal. When the mean of total carotene in each class was plotted against particle diameter in each class, the most carotene was present in 9-10nm class in Jersey cows (40.2%) and in 10-11nm class in Limousin cattle (35.6%).

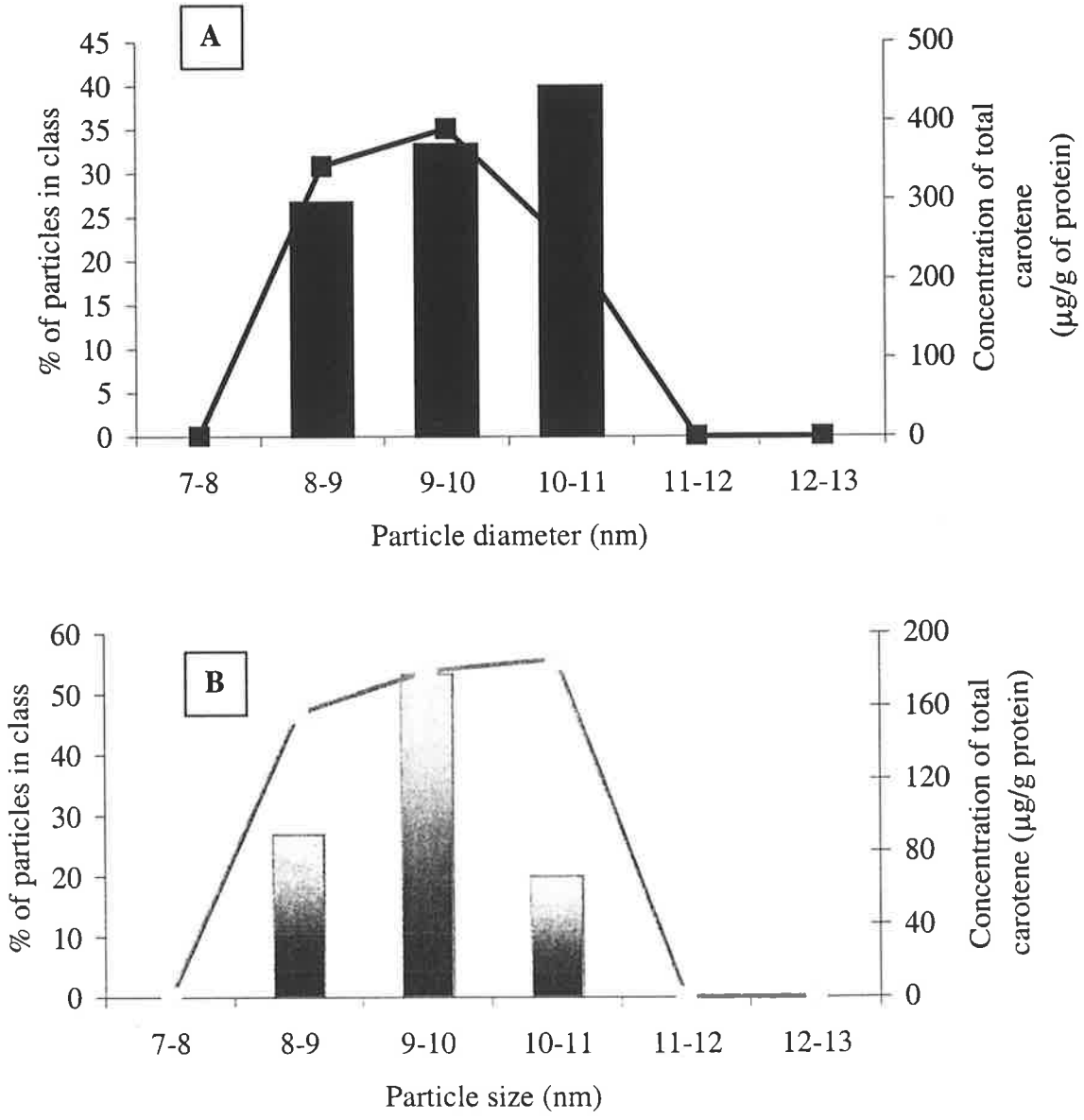


Figure 7.11. Distribution of HDL size and carotene in cows during summer. A) Jersey, B) Limousin, squares = carotene concentration.

In winter, larger HDL particles (11-12nm in diameter) were present in both breeds (Figure 7.12.). However, there were no small 8-9nm particles in Limousin cattle. The distribution of the diameter classes in this breed again was close to a shape of normal distribution. The Jersey cows had 6.7% particles in the smallest class and similar percentages in 10-11nm and 11-12nm classes. The distribution of carotenoids varied between particle classes. Jersey cows had the highest concentration in the particles ranging from 11-12nm in diameter. No difference was observed between the various classes in Limousin cattle.

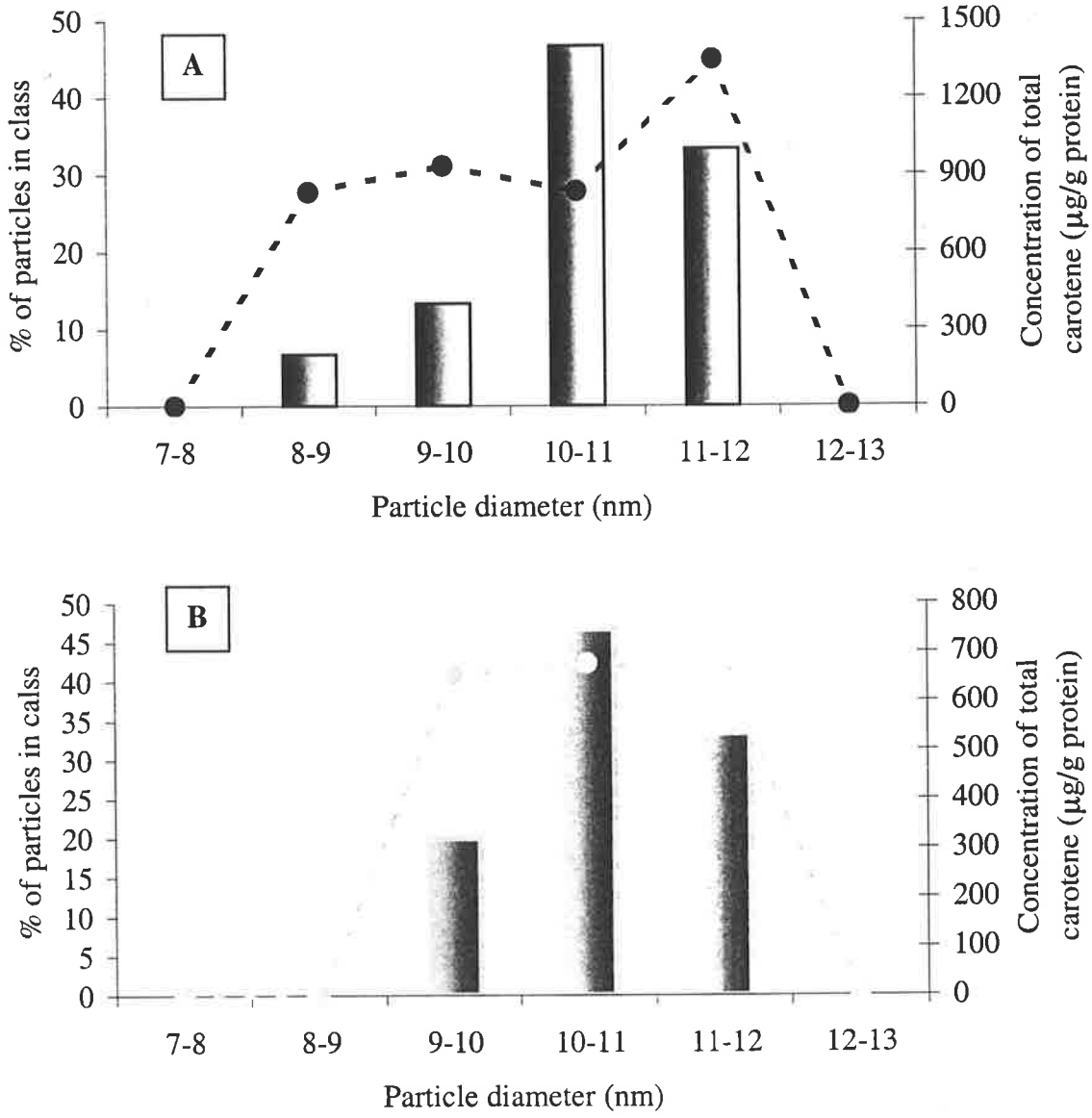


Figure 7.12. Distribution of HDL size and carotene in cows during winter. A) Jersey, B) Limousin, circles = carotene concentration.

Lack of breed by season interaction demonstrated that both breeds were affected in the same manner in each season. This permitted the data from summer and winter to be combined and the relationship between HDL particle size and carotene content in these breeds investigated. There was a different particle size pattern between these breeds with Jersey cows having the majority of particles between 10-11nm (Figure 7.13). Limousin cows had the majority of particles in two classes: 9-10nm (36.7%) and 10-11nm (33.3%) (Figure 7.13). The carotene concentration increased with particle size and reached its maximum in the largest particle class in both breeds.

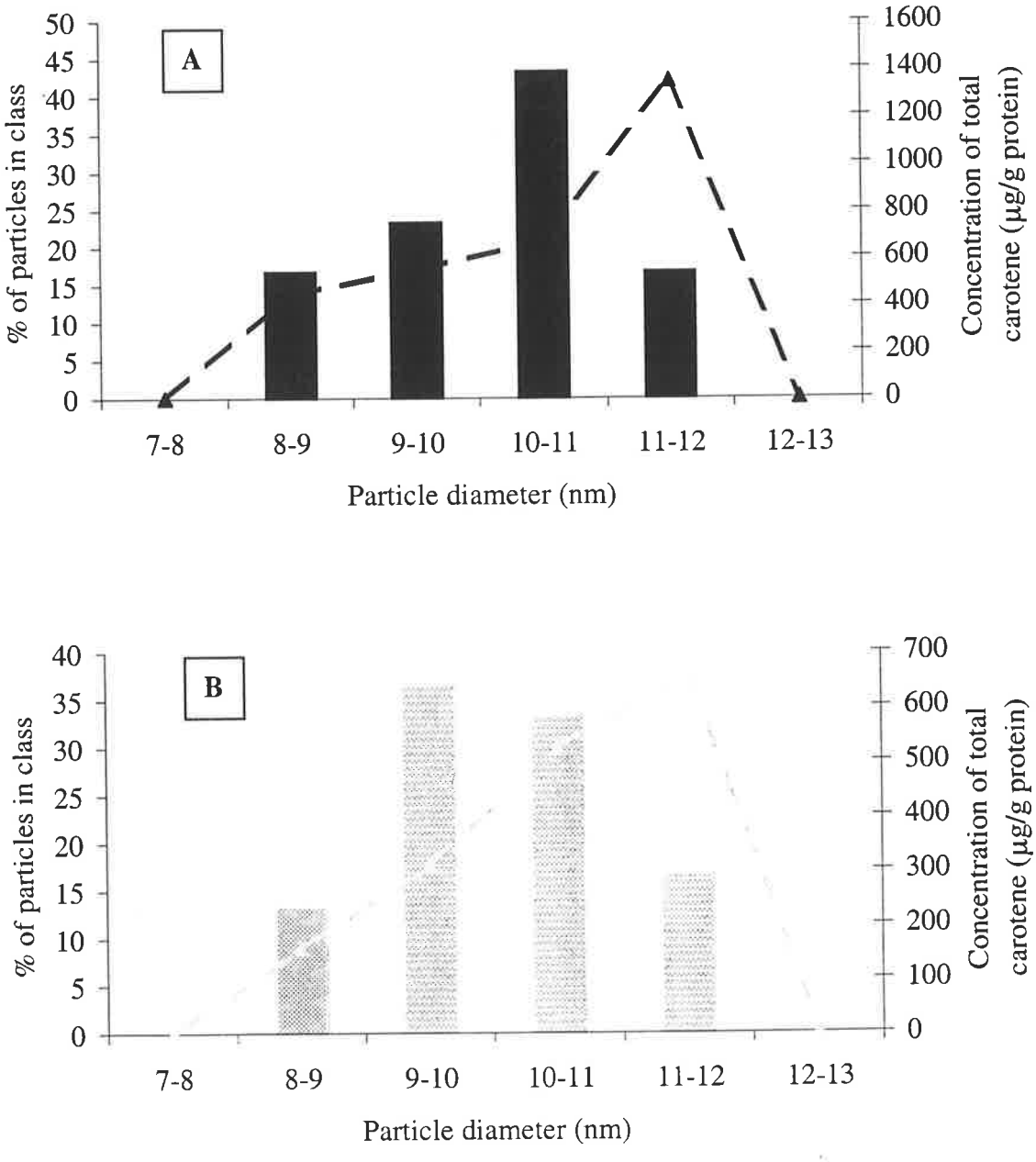


Figure 7.13. Distribution of HDL size and carotene in cows during summer & winter. A) Jersey, B) Limousin, triangles = carotene concentration.

7.2.3.2. Relationship between carotenoids, fatty acids and HDL particle size

A number of moderate and statistically significant correlations were observed between the concentration of total carotene in HDL fraction and various fatty acids (Table 7.4). Carotene was positively associated with linolenic acid (18:3 ω-3), a fatty acid of dietary origin. Another dietary fatty acid, linoleic acid (18:2), just missed the significance when expressed as proportion of total, but was statistically significant when expressed as nmol/g of protein. The correlations of these fatty acids were positive and moderate. Also a positive and moderate correlation was observed for di-homo-gamma-linolenic (20:3 ω-3) fatty acid. This was extended to a positive and low relationship with polyunsaturated fatty acids (PUFA), which just missed significance when reported as % in total but was significantly correlated as nmol/g of protein. The reverse relationship was observed between total carotene and oleic acid (18:1 ω-9) and monounsaturated fatty acids. The correlations were moderate. However, the ratio of monounsaturated and polyunsaturated to saturated fatty acids was positive and moderately correlated with carotene content in HDL lipoproteins.

Table 7.4. Relationship between HDL carotene, fatty acids and particle size.

Parameter	Correlation	Significance
Total carotene:		
18:3 ω-3 (% in total)	0.33	**
18:3 ω-3 (nmol/g protein)	0.27	*
18:2 (% in total)	0.23	ns
18:2 (nmol/g protein)	0.57	***
20:3 (% in total)	0.37	**
20:3 (nmol/g protein)	0.50	***
PUFA (% in total)	0.25	ns
PUFA (nmol/g protein)	0.54	***
18:1 ω-9 (% in total)	-0.27	**
MONO (% in total)	-0.27	*
Total carotene in plasma (μg/g protein)	0.55	***
β-carotene in plasma (μg/g protein)	0.23	ns
HDL particle diameter (nm)	0.22	ns

Note: ***=P<0.001, **=P<0.01, *=P<0.05, ns=not statistically significant, PUFA=polyunsaturated fatty acids, MONO=monounsaturated fatty acids.

A very strong and high relationship was found between carotene in HDL and total carotene in blood plasma (Table 7.4). The relation with β-carotene in plasma was also positive but missed statistical significance. Total carotenoids were also correlated with HDL particle size, however, just missed the significance at the 95% level. No other significant correlations were found with HDL diameter.

7.2.3.3. Discussion - transport of carotenoids in cattle

Transport of carotenoids in cattle blood occurs via the high density lipoproteins (HDL) which carry about 80% of this pigment in blood (Schwaigert et al., 1987). A positive correlation between HDL particle size and total carotene concentration in HDL suggested that carotenoids contribute significantly to the size of HDL particles. Yang et al., (1992) showed that a significant amount of β-carotene in cattle was associated with larger HDL particles which were visibly yellow. Ashes et al., (1984) also reported more β-carotene per unit weight in larger HDL particles. The size of the HDL particles was higher in winter when more carotenoids were available from pastures. In winter, there were also cows with average particle size between 11-12nm. No cows with these particle sizes were present in summer in both breeds. The distribution of carotene within the different particle diameter groups showed that more carotene was present among the Jersey and Limousin cows in the largest HDL particle size.

Breed differences in carotene concentration and lack of variation between Jersey and Limousin cows in HDL size can be explained by the distribution of these particles within each breed. In summer, the majority of Jersey cows had an average particle size between 10-11nm, whereas in Limousin cows, the 9-10nm class was the most abundant. In winter, the size of the highest diameter classes was identical in both breeds so the difference in concentration of carotene in this season must be caused by other factors. Tall (1981) suggested that interior of the very small HDL particles is more compact and not able to accumulate large amounts of carotenoids. In addition, HDL is not only a heterogenous fraction in terms of particle size, but also in terms of composition (Muller, 1987). For instance, the volume of the core of HDL_2 class in humans (particle size 9.5-12.0nm) is about three times of that of size of HDL_3, (particles size between 5.5-9.5nm) (Patsch and Gotto, 1987). Consequently, the larger particles can accumulate more carotenoids. Apoprotein E (ApoE), which plays an important role in receptor-mediated transport in the liver and other peripheral tissues, is predominantly a constituent of larger HDL particles (Muller, 1987). HDL particles rich in ApoE are also enriched in β-carotene (Brown and Frogoso, 1994). Breed differences in HDL particle size, ApoE presence and carotene availability can influence the clearance speed of carotenoids from blood by peripherial tissues.

7.2.4. Deposition of carotene in cattle adipose tissue

Adipose tissue is quantitatively the most important site of storage of fat-soluble nutrients (Parker, 1993). β-Carotene and lutein, which belong to this group, are the most abundant carotenoids present in cattle fat (Strachan et al., 1993). Their mechanism of uptake by the

adipose tissue is not fully understood and may be connected with adipocyte size and surface area if a passive diffusion process takes place. Therefore, the objective was to investigate adipose tissue cellularity in the two cattle breeds diverse in fat colour and evaluate the relationship between the concentration of carotene in blood and adipose tissue.

7.2.4.1. Breed differences in adipose tissue cellularity (experiment 3)

To determine the effect of breed of cattle on adipose tissue cellularity such morphological parameters as cell diameter, number, surface and volume were measured in Jersey and Limousin steers (Experiment 2). A significant difference was observed in all of these parameters (Table 7.5). The Jersey steers had more adipocytes per gram of fat and they were larger than the adipocytes of the Limousin steers. The surface area and volume estimates based on the diameter of adipocytes were also significantly higher in the Jersey steers. Such differences between the breeds were consistent even when three different anatomical locations were sampled. There was no difference in cell morphology between subcutaneous fat from ribs, rump and shoulder within a breed (Table 7.6).

Table 7.5. Breed differences in various morphological parameters of adipocytes.

Parameter	Jersey	Limousin	Percentage difference	Significance
Cell number	4.18±0.08	2.56±0.08	63%	***
Cell diameter	15.07±0.33	12.24±0.33	23%	***
Cell surface	30.43±1.41	12.22±1.41	149%	***
Cell volume	8.10±0.58	2.57±0.58	215%	***

Note: cell number= x 10^6 cells/g, cell diameter=μm, cell surface=μm^2, cell volume=μm^3
***=P<0.001, **=P<0.01, *=P<0.05

Table 7.6. Comparison of adipocyte morphology between various anatomical locations.

Parameter	Ribs	Rump	Shoulder
Cell number	3.39±0.09	3.38±0.09	3.34±0.09
Cell diameter	13.27±0.4	13.85±0.4	13.86±0.4
Cell surface	19.86±1.72	22.23±1.72	22.04±1.72
Cell volume	4.68±0.71	5.68±0.71	5.64±0.71

Note: cell number= x 10^6 cells/g, cell diameter=μm, cell surface=μm^2, cell volume=μm^3

Distribution of adipocytes, when assigned to different size classes, also varied between the breeds (Figure 7.14). Jersey steers did not possess small adipocytes between 10-12μm in diameter and the range of sizes varied from 12μm to 23μm. The distribution of adipocytes in the Jersey steers was broad and irregular with the most abundant classes of 12-13μm and 15-16μm. In contrast, the Limousin steers had smaller adipocytes varying from 10–16μm in diameter with the most abundant class of 11-12μm and a more compact, normal distribution.

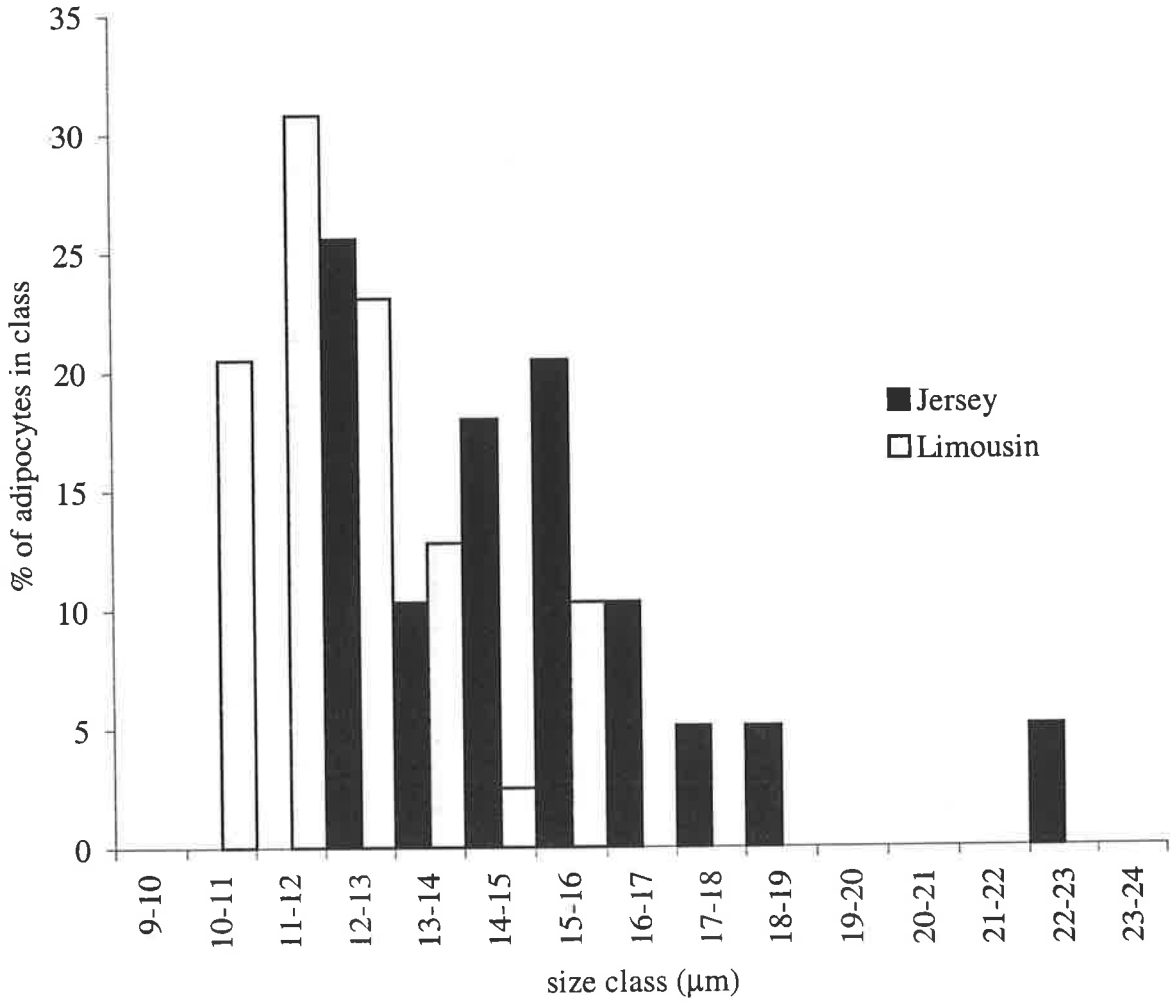


Figure 7.14. Breed difference in distribution of adipocyte cell size. The percentage of adipocytes per class in each breed was derived from 39 samples (13 animals in each breed and 3 sampling sites).

The concentration of total carotenoids differed between Jersey and Limousin steers whether expressed per gram of wet or dry fat (0.96 ± 0.03μg/g vs 0.33 ± 0.03μg/g in wet fat and 1.62 ± 0.06μg/g vs 0.68μg/g in dry fat, respectively). Breed by anatomical site interaction was not significant for wet and dry fat samples. Sire also did not have an impact on carotene content.

7.2.4.2.Correlation between carotenoids in adipose tissue, blood and blood lipoprotein fractions

A high correlation was observed between carotenoids in wet and carotenoids in dry fat (Table 7.7) even though the water content varied significantly between anatomical sites (Figure 7.15). Cell number was positively correlated with cell surface and just missed the significance with cell volume. Cell surface and volume were highly associated with cell diameter as expected.

Table 7.7. Correlation between adipocyte morphology and carotenoid.

Components	Correlation	Significance
Carotene in wet fat & carotene in dry fat	0.66	***
Cell number & cell surface	0.35	**
Cell surface & cell diameter	0.88	***
Cell diameter & cell volume	0.89	***
Cell surface & cell volume	0.98	***
Cell number & cell volume	0.22	P<0.06
Carotene in blood & carotene in dry fat	0.42	***
Carotene in wet fat & cell number	-0.13	ns
Carotene in dry fat & cell number	-0.19	ns
Carotene in wet fat & cell diameter	0.03	ns
Carotene in dry fat & cell diameter	0.06	ns

Note: cell number= x 10^6 cells/g, cell diameter=µm, cell diameter=µm, cell surface=μm^2, cell volume=μm^3, ***=P<0.001, **=P<0.01, *=P<0.05, carotene in fat=µg/g, carotene in blood=µg/ml

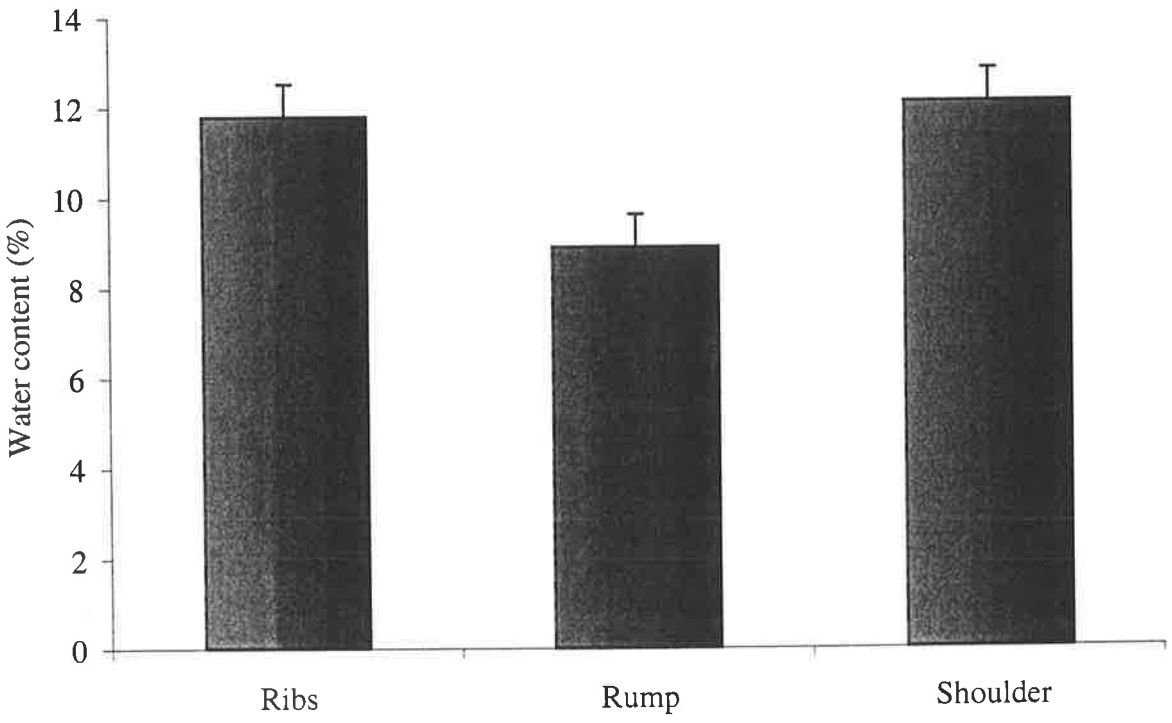


Figure 7.15. Comparison of water content in fat from different anatomical locations.

Correlations between carotenoids in fat and morphological parameters of fat cells were negative and not statistically significant. When expressed as total cell diameter and volume per gram of fat, the correlations with carotenoids were still not significant (data not presented). The only correlation which was significant for carotenoids was their association with the carotenoid level in blood. Total carotenoid content in adipose tissue expressed in μg per gram of fat was associated positively with carotenoid concentration in blood (μg/ml). This would allow the prediction of fat colour from blood carotene level. However, such a prediction can vary and may not be accurate as the changes in blood occur more rapidly than in the solid tissues. In the study of Seirer et al., (1992), blood plasma level of β-carotene declined from 2.6μg/ml at day 0 of the experiment to 0.3μg/ml after 34 days on grain diet with no further significant changes. The Jersey and Limousin steers in the experiment herein, which spent 170 days on low carotene diet, should have reached the minimum stable level of carotenoids in blood. However, the correlation between carotene in blood and adipose tissue before slaughter was moderate. A similar moderate correlation between subjective fat colour measurements at the AUS-MEAT site and β-carotene in blood was reported by Seirer et al., (1992). They concluded that β-carotene in plasma would not accurately predict fat colour of individual animals. The later study of Hayes et al., (1995) who found a poor phenotypic correlation (r=0.2) between fat colour and carotene concentration in plasma, also questioned the reliability of β-carotene concentrations in blood as a useful predictor of fat colour in the carcass. The previous work of Morgan et al., (1969) and Strachan et al., (1993) also raises questions.

No significant correlations were found between total carotene in biopsy fat and total carotene in blood lipoprotein fractions when expressed per gram of protein (data not presented here). Only low and positive relationships were found with total carotene in blood plasma and lutein in blood plasma, which were near significance ($P<0.09$). The only significant relationship found with lutein content in chylomicrons was low (r=0.26). This demonstrated that not only carotene concentration in blood plasma but also carotene concentration in various lipoprotein fractions is a poor predictor of fat colour in cattle.

7.2.4.3. Discussion - deposition of carotenoids in cattle fat

Various morphological parameters of adipocytes have been reported in different cattle breeds. However, there are no such reports available for Jersey and Limousin cattle. In this study the number of adipocytes in Jersey and Limousin steers was in accordance with reports of Prior (1983) and Dawson et al., (1993). However, lower numbers of adipocytes per gram of fat were reported by the others (Miller et al., 1991; May et al., 1994). Such a discrepancy in

reports could be due to experimental differences such as cattle breed used in the study, age of animals, as well as the techniques used for estimating the fat cell morphology. Hirsh and Gallian (1969) reported the inadequacy of DNA index for quantification of adipocytes. The large quantity of DNA in fat can be contributed by other cell types present within adipose tissue, and consequently, give inadequate estimates. Also the use of a Coulter counter for cell size and number estimates will cause variation since adipocytes smaller than 20-25μm are not counted. Consequently, the results reported as mean diameter can be higher than in studies where other techniques have been used.

The adipocytes from Jersey cattle were larger and there were more adipocytes per gram of fat than in the Limousin breed. This can be explained by differences in the matrix content (connective tissue, blood vessels etc) of adipose tissue between these breeds. Jersey and Limousin cattle in general had smaller cell diameters (Table 7.5) compared with those of other studies as estimated by microscopic measurements of cells (Proir, 1983). This also accounted for the smaller average cell volume and surface area. The deposition of carotenoids in adipose tissue is believed to occur by non-energy required transport and cell surface area may be a very important factors. Lack of significant correlations between carotene in wet or dry fat and size, volume, surface, total volume and total surface of cells suggest that a mechanism other than passive diffusion could be involved. It has been reported that large HDL particles which carry most of carotene in their cores, also contain apo E, an apoprotein involved in receptor transport. This may indicate that carotenoids can be transported via this route and cell size or surface may not affect this process. On the other hand, other mechanisms within the tissue such as conversion of carotenoids to vitamin A (Wang et al, 1991) or low level of carotenoids in this study can be responsible for the lack of such correlation.

A positive moderate correlation between cell surface, volume and cell number may suggest that these breeds differ in pre-adipocyte numbers (Table 7.7). However, it may be possible that both hyperplasia and hypertrophy occur in these breeds during the fattening period and these two processes are responsible for the increase of adipose tissue mass. The reports on this topic are contradictory, however, Plaas and Cryer (1980) have shown that potential cell division is present in subcutaneous fat from cattle of normal slaughter weight.

7.3. Summary

(i) The concentration of carotene in blood plasma in cattle was influenced by age, breed and season when cattle remained on pastures. Older cows had less carotene per gram of protein in

plasma but more when expressed per plasma volume. In spite of breed differences, season and age affected Jersey and Limousin cows in the same manner with regards to carotene content.

(ii) Distribution of carotene in blood of non-lactating cows was similar regardless of breed (Jersey vs Limousin), physiological stage of animal (lactating vs non-lactating), sex (steers, bulls, cows) and season (winter vs summer). Supplementation with vitamin A decreased concentration of β-carotene and lutein in blood plasma and chylomicrons. This demonstrated a decreasing effect of retinyl palmitate on the absorption of lutein and β-carotene or conversion ratio of β-carotene to vitamin A within intestinal enterocytes. Absorption or conversion of carotene was also influenced by breed and season.

(iii) Positive and significant correlations between carotene, fat soluble vitamins and exogenous fatty acids in blood lipoprotein fractions suggested their interaction during absorption and a possible crossover of metabolic pathways. The same factors influencing the plasma carotene concentration affected transport of carotene in HDL. Such an influence was not surprising as approximately 80% of plasma carotene was present in this blood fraction. Seasonal differences in carotene availability were demonstrated in HDL lipoprotein fraction which consisted of larger particles in winter. The contribution of carotene to the size of these particles was substantial. The morphology of chylomicron particles was affected by season with larger particles present in winter when more carotene was available. However, carotene did not contribute significantly to the chylomicron size.

(iv) The morphology of adipose tissue differed significantly between Jersey and Limousin cattle. The lack of a correlation between carotene and adipocyte size, volume and number suggests a mechanism other than passive diffusion for carotene entry into the fat cells. Only a moderate correlation between carotene in fat, blood and blood lipoprotein fractions confirmed that blood carotene level is not an accurate predictor of carotene status in adipose tissue.

Chapter 8

General discussion

8.0. Introduction

Carotenoids are a class of natural pigments that not only create fascinating, bright and intense colours but more importantly, play a critical role in various biological processes. They are frequently positively associated with human health (Chapter 1, Figure 1.5). Carotenoids and their metabolites act in photosynthesis as accessory light-harvesting and photoprotecting pigments (Britton, 1995), possess antioxidant properties (Krinsky, 1993), serve as vitamin A precursors (Underwood, 1984), have an effect on immune function (Chew, 1993) and modulate gene expression (Bendich, 1993). In humans, an increased intake of foods containing carotenoids is associated with a decreased risk of certain types of cancer (van Poppel and Goldbohm, 1995), a decreased incidence of age-related diseases (Snodderly, 1995), and a decreased risk of cardiovascular disease (Kohlmeier and Hastings, 1995). In other species, carotenoids are positively correlated with reproductive performance, production (Arechiga et al., 1998), and health (Chew et al., 1982). The accumulation of carotenoids in animal tissues used for human consumption is associated with better quality food.

In spite of the positive influence of carotenoids on health, some consumers dislike the visible appearance of the meat products containing high levels of carotenoids, especially the yellow colouration of fat. There are no reports concerning the degree to which carotenoids from meat products contribute to the total pool of carotenoids consumed by humans. However, the positive influence of carotenoids on human health conflicts with the demand of modern day consumers for white fat and has caused a significant reduction of profits from highly priced beef markets. It has been reported by Hayes et al. (1995) that in Australia, if yellow fat incidence was reduced 50%, over $18 million would be saved annually. A significant loss of profit due to yellow colour of fat has also been noted in the sheep industry (Kirton et al., 1975).

The metabolism of carotenoid in mammalian species is not fully understood. The majority of work on carotene metabolism has been confined to rats, gerbils, and pre-ruminant calves as models for human studies (Lee et al., 1999). The results of research in other species can only be applied to humans with caution as there are significant metabolic differences between the species used as models and those producing meat for human consumption (Olson, 1994). Therefore, understanding carotenoid metabolism in cattle is of importance as it would provide the knowledge to manipulate the animals and provide the best quality product to satisfy meat consumers.

8.1. Consumer perception of meat quality and non-genetic factors influencing meat quality and fat colour

The majority of meat consumers associate yellow fat colour with older animals. However, there are contradictory scientific reports regarding the association of yellower fat with older animals. An increasing and positive trend between age of cows and carotene concentration was observed herein but was not statistically significant (Figure 4.1). This trend was the same between breeds, as breed by age interaction was not significant. The lack of influence of age on fat colour was demonstrated in cows by Morgan et al. (1969). In contrast, other studies have shown a positive influence of age on fat colour (Walker et al., 1990).

A difference in fat colour was observed herein between weaners (~250 days of age) and cows, which was probably a consequence of the length of time on a high carotenoid diet (pasture). Even among weaners, the differences in fat colour between animals existed pre-slaughter. The pre-weaning geographical location significantly influenced this phenomenon (Chapter 4). Moreover, the length of time animals were on a carotenoid diet between birth and weaning had a significant effect as weaners born later in the year had lower fat carotene content and fat colour than those born earlier in the year (Chapter 5). This shows clearly that the accumulation of carotene in the early stage of life or the length of time on carotene diet between birth and weaning can influence the life-time performance.

On the other hand, a significant difference in total carotenoid content between pre-weaning groups did not affect the meat quality as the average subjective fat colour score of 0.28 ± 0.02 probably would not be noticed by the consumers and these carcasses could easily be accepted in Japanese or Korean markets. However, the data in this thesis demonstrate that the association of fat colour is not always related to old animals (Chapter 4). Moreover, there is no difference in meat quality (flavour, tenderness, and juiciness) between the yellow pigmented carcasses and normal coloured carcasses (Kirton et al., 1975). It has been suggested that the change of consumer's acceptance by education, advertising of "sunkissed lambs" (grass fed lambs with yellow fat) or "sunkissed beef" could not only solve the problem of yellow fat but may have a number of positive implications where human health is concerned. In the USA, a similar problem was solved successfully with yellow fatted chickens (Kirton et al., 1975).

Clearly, fat colour can be altered by environmental factors. Diet is the most important. Green pastures are an excellent source of carotenoids for cattle and sheep. New Zealand has a much longer pasture growing period than South Australia (Chapter 4). Rainfall, another factor

distinguishing different geographical locations, was very highly correlated with the concentration of carotenoids in fat. Yearly variation in rainfall can have a significant impact on fat colour. This was observed especially in cows, which varied highly in fat colour in different years (Figure 4.6).

As expected, the length of time in the feedlot had an impact on fat and meat carotenoid levels. Steers lot-fed for 140 days had lower carotenoid level in fat and meat than heifers, which were on feedlot for only 70 days (Figure 6.2).

Seasonal changes (winter vs summer) in carotenoid content in diet did not change fat colour but did change carotenoid levels in the blood (Chapter 4). This was probably due to the fact that changes in adipose tissue carotenoid concentrations occur much more slowly compared to blood changes and more time is required to notice a significant difference. Another explanation would be that the seasonal changes in the diet carotene concentration were not large enough to cause a significant reduction in fat colour.

Carotenoids are fat soluble components and to large extent are associated with adipose tissue. High and positive correlations were found between carotenoids in various fat depots indicating the similarity between all types of adipose tissue (Chapter 6). Since carotenoids are fat soluble, most are found in fat cells rather than in muscle cells. Hence, the carotene content of meat should be directly related to the fat content of the meat. The correlations between predicted fat content based on carotenoid concentration in muscle and extracted fat content from the muscle found in cows were extremely high (Chapter 6). This demonstrated that estimation of fat content based on carotenoid concentration in meat could predict the intramuscular fat content more precisely and more rapidly than solvent extraction methods. However, when repeated on animals of the same age, which spent 140 days on low carotene diet (feedlot), the correlations were only moderate. This indicates that the carotenoid level in fat is not a very good predictor of intramuscular fat content when the level of these compounds in lot-fed animals is low.

Consumer awareness of carotene concentration and its influence on carcass quality has necessitated the search for methods which would allow the prediction of fat colour at an early stage of the animal's life, and consequently, reduce the costs of feeding such animals knowing that they will not be suitable for highly profitable markets. One of the indicators considered for such a prediction was carotenoid level in blood. However, the correlation was not high as changes in blood carotenoid concentration are faster and many factors can affect

carotenoid concentration in blood plasma (Chapter 7). Low correlations between carotenoid status in blood plasma and other tissues have been reported by others (e.g. Hayes et al., 1995). These authors concluded that blood is a weak predictor of carotenoid status in cattle solid tissues. The prediction in this thesis was extended to various blood lipoprotein fractions but this was also poor. A slightly higher correlation (0.42) was observed between total carotenoid in plasma and total carotenoid in adipose tissue after feeding steers for 170 days on high grain diet. However, it was still not satisfactory (Chapter 7).

A high correlation between carotenoid content or fat colour at weaning (biopsy sample) and at slaughter, regardless of the number of days in the feedlot, showed that analysis of biopsy fat samples can be useful for selecting cattle to satisfy consumer and industry needs. Removing the animals which possess a greater potential to deposit carotenoid in their fat could reduce the high cost of maintaining these animals and direct them to less profitable markets (Chapter 5, Figure 5.4 and 5.5). However, this procedure would require biopsy sampling, which is time consuming, costly, and carries a risk of infection and reduced performance. On the other hand, if such a procedure provided useful information about further performance of the animals in relation to other important carcass traits, then early biopsy would be a useful option.

8.2. Genetic factors influencing fat colour

Data of the genetic factors concerning the influence of carotenoid on fat colour are very limited. There have been reports that have demonstrated the influence of breed on fat colour in sheep and strong evidence of the genetic basis for fat colour in this species (Baker et al., 1985). However, in cattle, this issue is still not resolved. Reports about different cattle breeds varying in fat colour have been published by many researchers (Morgan et al., 1969; Walker et al., 1990). Jersey and Limousin cattle in the study herein also were extremely different in many traits including fat colour and carotenoid concentration in fat, blood and meat (Chapter 4 and 7). Moreover, there was a statistically significant difference between pure breeds, F1 and reciprocal crosses between the Jersey and Limousin cattle. This clearly suggests that both genetic and environmental factors influenced fat colour. The data from feedlot experiments conducted by Strachan et al. (1993) supports this hypothesis. In their study, even lot feeding for a long period did not reduce the intensity of fat colour in certain animals. Some steers after 70 and 175 days on low carotene diet had fat colour scores as high as 6 and 4, respectively. A similar phenomenon was observed among animals used herein. Lot feeding for 250 days was not effective in reducing fat colour in approximately 4% of the population (Chapter 5). Additionally, large differences between the breeds in this study showed that

there was a significant influence of sire. The data from the South Australian and New Zealand cattle clearly suggest that genetic factors have a large influence on fat colour.

As reported by Morgan et al. (1969), crossbreeding of Jersey cattle with Friesian, Charolais and Hereford reduces fat colour with the F1 progeny being intermediate. The same trend appeared between Jersey and Limousin in this study. The F1 progeny were intermediate in fat colour in spite of the fact that they were derived from very divergent fat colour breeds (Figure 5.14). Moreover, the difference appeared not only between F1 and the parents, but also in the backcross progeny which were statistically different from the parents, the F1 and from each other.

The distribution of carotenoid concentration in adipose tissue between pure and crossbred cattle provided an interesting observation, which strongly emphasised the genetic component in fat colour of cattle. Pure Limousin and LJLL crosses had almost symmetric and uniform distributions whereas the distributions for the pure Jersey, F1 and LJJJ were tailed with irregular peaks. The differences in distributions were confirmed by a test statistic, which compared the shape of the distributions, steam-and-leaf plots, skewness and kurtosis. These results indicated that the distributions of carotenoid in fat of Limousin and LJLL were normal but the distributions were not normal for Jersey, F1, LJJJ (Chapter 5).

The animals with the highest fat carotenoid concentration were derived from parents where at least one of the parents was regarded as an "outlier" with high fat colour in parental population. More interestingly, almost all animals with high carotenoid content or fat biopsy colour score at weaning also scored high in AUS-MEAT® scale at slaughter, regardless of number of days on feedlot. The sire influence was statistically significant in New Zealand and South Australia where certain sires tended to produce yellow fatted progeny (Figure 4.4).

The heritability of fat colour, estimated on total carotenoid concentration in adipose tissue or fat colour score, was low (~6%) in average, but higher in Jersey than in Limousin cattle (Chapter 5). A low heritability based on fat colour of Brahman crosses was also reported by Hayes et al. (1995). Statistical analysis of genetic effects on fat colour demonstrated a significant influence of additive and epistatic effects, but no maternal or heterosis effects.

The above observations and analyses suggest a single major gene model for the mode of inheritance of fat colour in cattle. Limousin cattle can be regarded as a homozygous population for white fat (WW) and Jersey cattle would represent all genotypes, homozygotes for white fat (WW), heterozygotes for white/yellow fat (WY) and homozygotes for yellow fat (YY). The presence of yellow fat "outliers" suggests a major gene effect. Based on the ratio of yellow coloured progeny within the population and assuming that the Jersey cow population in the herein study is in a Hardy-Weinberg equilibrium, there could be an additive allele for yellow fat in the Jersey breed. Consequently, fat colour in cattle is influenced by both genetic and environmental factors and there may be a partially dominant gene having a major effect with lesser effects from the other genes. Higher heritability estimates for sires supports the conclusion drawn by Kirton et al. (1975) in sheep that culling rams/bulls for yellow fat can be effective in controlling the problem.

Completion of the Davis Gene Mapping Project will provide a sufficient amount of information to test the genetic model for fat colour in cattle using a segregation analysis computer package. However, the moderate gene frequency in the population and the resulting small number of homozygous progeny carrying the yellow fat colour gene may require further gene mapping to isolate the gene. To accomplish this, two heterozygotes for yellow fat colour should be crossed and the distribution of the yellow fat colour gene measured in F_2 progeny and genetically linked to microsatelite markers.

8.3. Metabolic pathway of carotene in cattle

Cattle cannot synthesize carotenoids *de novo* and depend on their influx from feed. The metabolic pathway for carotenoids begins with their intake from the diet and can be influenced by various environmental factors (Chapter 4) as well the genetic factors described above. The environmental factors can be well controlled if animals are stall-fed. The best example for the application of a controlled feeding with carotene is large scale lot feeding. The animals are fed a low carotene/high energy diet (grain) to improve their carcass yield, quality and reduce fat colour.

It is thought that carotenoids are not degraded in the rumen of ruminants and most of the carotene passes unchanged to the abomasum. However, contradictory reports exist about carotene destruction in the rumen. The anaerobic conditions in the rumen probably preclude the oxidation of β-carotene. This is supported by the work of Keating et al. (1964) who found no destruction of β-carotene in the rumen. On the other hand, other researchers have reported a loss of β-carotene in rumen fluid. For example, King et al. (1962) reported a 30% loss of β-

carotene and vitamin A during 9 hours of *in vitro* incubation of rumen fluid. Also Dawson and Hemington (1977) demonstrated a loss of β-carotene in the rumen of sheep although only in negligible amounts. A small loss of β-carotene in the rumen cannot be ruled out as some oxygen is swallowed during eating and may promote partial oxidation of carotene. Moreover, the long transit in the rumen could possibly bleach the plant pigments (Larsen et al., 1993). Plant lipoxygenases may be responsible for the bleaching of consumed carotenoids. Mora et al. (1999) suggested that carotenoids disappear probably by adhering to cellular contents and not by their direct destruction or by attack from the ruminal microorganisms. This process is independent of the animal species studied.

8.3.1. Absorption of carotenoids

The small intestine is probably the first major section of gastro-intestinal tract in which the most significant changes occur to carotene. Carotenoids appear to be absorbed in the small intestine by a mechanism involving passive diffusion (Hollander, 1981). Many factors influence carotene availability including the presence of lutein, canthaxantin and vitamin A. Vitamin A reduces the concentration of carotene in blood (Knight and Death, 1996). Herein both β-carotene and lutein decreased significantly after the first fortnight of supplementation with vitamin A whereas the concentration of vitamin A in blood was constant throughout the whole experiment (Figure 7.4, Figure 7.6). In the chylomicron fraction, there was a reduction in carotenoids and no change in concentration of retinol (Figure 7.8, Figure 7.9). This observation demonstrates that vitamin A suppresses the absorption of β-carotene and lutein or influences dioxygenase activity in the enterocytes. Lutein is not a precursor for vitamin A, and consequently, is not metabolized to retinol. The decreased level of lutein in chylomicrons after vitamin A supplementation suggests that this nutrient influences the absorption of lutein. Lutein also negatively affects β-carotene absorption when it is simultaneously administrated orally to humans, but has no effect on β-carotene cleavage (Van der Berg and Van Vliet, 1998).

The lower level of β-carotene in chylomicrons after the supplementation with vitamin A may not be simply explained by an effect on its absorption level. β-Carotene, as a strong provitamin A carotenoid, can also be converted in the enterocytes to a form of vitamin A. In the study of Villard and Bates (1986) as well as that of Van Vliet et al. (1996), intestinal β-carotene cleavage activity in rats was higher in vitamin A deficient rats than in those with a high intake of either vitamin A or β-carotene. Van Vliet et al. (1996) also observed that intestinal dioxygenase activity was 90% higher in the animals fed unsupplemented low

vitamin A diet than in animals fed unsupplemented high vitamin A diet. If this is the case in cattle, then the supplementation of vitamin A may affect the cleavage of β-carotene as well as absorption. However, such a suggestion has to be taken with caution, as there is species specificity in dioxygenase activity (Yang and Tume, 1993).

A difference in dioxygenase enzyme activity theoretically could cause the difference of carotenoid concentration in various cattle breeds. Jersey cows had higher level of β-carotene in chylomicrons than the Limousin cows (Table 7.2). In winter, when more carotenoid was available from the diet, the level of β-carotene was statistically higher in Jerseys and the retinol level was statistically lower in Limousin cows. This suggests that the high carotene influx influenced dioxygenase activity mostly in Limousin cows. The Jersey cows also had lower retinol level in winter though not statistically significant. During summer, when less carotenoid was available from the diet, there was no difference between these breeds in β-carotenoid and retinol concentrations (Table 7.2).

In order to test factors influencing absorption of carotenoids and differences in β-carotene-15,15'-dioxygenase activity in Jersey and Limousin cattle, an appropriate experiment should be designed to address these issues. At present, insufficient research has been done in this area and the experiment would require adaptation and modification of methods applied in other animal species. β-Carotene-15,15'-dioxygenase activity in cattle is lower than in other species and there is a large variation between animals (Yang and Tume, 1993). Therefore, such an experiment would require a larger number of animals and diverse treatment groups with various levels of carotenoids and fat soluble vitamins supplemented. Since β-carotene and vitamin A affect absorption of carotenoids and β-carotene concentration in blood (Chapter 4 and Chapter 7), the following experimental groups could be proposed: (i) control, (ii) Vitamin A (VitA) (2 million IU/head/day), (iii) β-carotene (BC) (1000mg/day/head), (iv) lutein (LUT) (500-1000mg/head/day), and a combination of the treatments: (v) VitA+BC, (vi) VitA+LUT, (vii) BC+LUT, (viii) VitA+BC+LUT. Such an approach would:

a) establish the effect of β-carotene, lutein and vitamin A on the absorption of carotenoids from small intestine and the interaction between these fat soluble nutrients,

b) investigate the variation in absorption in the small intestine,

c) determine the influence of carotenoids and vitamin A on dioxygenase activity in cattle. The monitoring of retinal and/or apocarotenals formation would answer the

question about the type of enzyme cleavage (central vs excentric) and the efficiency of conversion of carotenoids, and

d) examine the dioxygenase activity in various cattle breeds and the outliers in fat colour.

Vitamin A and β-carotene concentration are only two of the many factors influencing absorption and conversion of carotenoids in the small intestine. Other carotenoids may play an important role in this process. For example, canthaxantin is not a substrate for β-carotene dioxygenase but it affects the activity of provitamin A carotenoids in rats by direct interaction with the enzyme (Grolier et al., 1997). Also the difference between polar and non- polar carotenoids may be of importance. The solubility of β-carotene in biological emulsions is higher than the polar carotenoids and is increased when the chain-length of the triacylglyceride fatty acids decreases (Borel et al., 1996). Different distributions of polar and non-polar carotenoids within the fat droplets influences the absorption of carotenoids. This different behavior of polar and non-polar carotenoids in biological emulsions cannot be neglected.

8.3.2. Transport of carotenoids

While in blood, carotenoids are exclusively associated with lipoprotein fractions (Krinsky et al., 1958; Erdman et al., 1993), and in cattle, no carotene was present in the lipoprotein depleted serum (LPDS) (Chapter 7). Proportional distribution of carotenoids among lipoproteins remains similar regardless of the physiological state of the animal with ~80% of carotenoids being associated with high density lipoprotein fraction (HDL) (Schwaigert et al., 1987, Yang et al., 1992, Table 7.1). Different cattle breeds, such as Limousin and Jersey which are very diverse in carotene status in their tissues, still have ~80% of plasma carotenoids in the HDL fraction (Table 7.1). The higher influx of carotenoids from a winter diet did not influence the proportional distribution of carotenoids in either breed and ~80% was still transported in the HDL. However, a diet rich in carotenoids increased the size of the HDL particles (Table 7.4). This trend occured in both Limousin and Jersey cattle. The breeds did not differ in average HDL particle size, but there were significantly larger particles present in winter than in summer. Moreover, there was a positive correlation between the size of the HDL particles and total carotenoid concentration in HDL fraction (Table 7.4). This demonstrated that carotenoids contributed to the size of particles transporting these nutrients in the blood of cattle. Similar results were obtained by Ashes et al. (1984) from Friesian cows. They reported more carotene per unit weight in larger HDL particles. When cows were fed with protected lipid-rich diets containing large proportion of linoleic acid residues, they

observed higher percentage of large HDL particles. In the herein study, total carotenoid present in HDL fraction was also positively correlated with 18:3 and 18:2 fatty acids as well as with other polyunsaturated fatty acids (Table 7.4).

When investigating carotenoid distribution among different HDL particle classes, there was a breed difference in HDL size between Jersey and Limousin cows (Figure 7.12). Jersey cows had a higher percentage of large HDL particles than the Limousin cows. This could be one of the factors explaining breed differences in tissue carotenoid status between Jersey and Limousin cattle.

In the study of carotene metabolism in humans, Brown and Fragoso (1994) fractionated HDL into apo-E-rich and apo-E-poor sub-fractions, and found that the apo-E-rich sub-fraction was enriched also in β-carotene. Apo-E is one of the HDL apolipoproteins which plays an important role in receptor mediated transport in the liver and other peripheral tissues. If it is the case in cattle, then the apo-E-rich HDL particles which are also rich in β-carotene may explain the breed differences in carotene status in blood as well as in the other tissues.

Exchange of carotenoids between different lipoprotein fractions may take place as is the case with other lipoprotein components (Goodman et al., 1984). Chylomicrons, after transit through the lymphatic system, enter blood plasma and undergo various structural changes. Carotenoids are differently distributed within lipoprotein particles, which may affect their interaction. Polar carotenoids (lutein) are preferentially associated with the surface whereas apolar carotenoids (β-carotene) are preferentially solubilised in the core (Borel et al., 1996).

Such compartmentalisation of carotenoids may cause differences in the exchange between lipoproteins or cell membranes. As hypotethised by Borel et al. (1996), the polar carotenoids localized at the lipoprotein surface, can be spontaneously extracted and solubilised in the aqueous phase, while apolar carotenoids localised in the core require the production of the aqueous-soluble lipids to be transferred between triglyceride rich lipoproteins (TRL) and other lipoproteins or between TRLs and cell membranes. Such a mechanism for carotene exchange has also been proposed by Traber et al. (1994). They suggested that due to the high hydrophobicity of β-carotene, it does not readily exchange between lipoproteins. Also Romanchik et al. (1995) reported in humans that transfer of carotenoids among lipoprotein fractions did not occur in their experiments. Consequently, it can be speculated that the pool of carotenoids, especially β-carotene associated with the core of lipoproteins, may accumulate in the hepatic tissue and subsequently be incorporated into newly synthesized

HDL particles. However, this speculation is based on transport of carotene in humans only and it may not be the same in cattle as the major lipoprotein fraction carrying carotene in humans is LDL. It is also important to note that HDL particles presumably arise from various components secreted by the liver and/or intestine (Babiak and Rudel, 1987) and the transport of carotenoids cannot be separated from lipoprotein metabolism.

To resolve the issue of transport and exchange of carotenoids in cattle lipoproteins and the association of Apolipoprotein E with larger HDL particles, two major experiments can be proposed. Experiment 1 would involve the injection of radioactively labeled carotenoids and monitoring their distribution between lipoprotein fractions as well as between the core and surface of the lipoproteins at different times. Experiment 2 would require a separation of HDL particles into various size sub-fractions, the blockage of HDL receptors with antibodies and incubating adipose tissue with the HDL sub-fractions monitoring carotenoid concentration in the fat tissue.

8.3.3. Carotenoid deposition in adipose tissue

Little has been reported on the mechanisms of uptake of carotenoids from lipoproteins into peripheral tissues not only in cattle, but also in other mammalian species (Furr and Clark, 1997). Surprisingly, factors affecting the exchange of carotenoids within tissues and the mechanisms of mobilization of carotenoids from extrahepatic tissues back into plasma are unknown. Breed differences between Jersey and Limousin cattle in carotenoid concentration in adipose tissue and the lack of information about the mechanism of deposition of carotenoids in cattle fat suggested that the exploration of adipocyte morphological differences between these breeds might be useful. These two breeds differed significantly in adipose cell size and number as well as in cell surface and volume (Chapter 7).

A possible passive diffusion process would result in a proportional relationship between these morphological parameters of the cells and carotenoid content. However, the correlation between the morphology of fat cells and total carotene content was low and not statistically significant (Table 7.7). The lack of such correlation still cannot rule out the passive diffusion mechanism if other changes occur to carotene within adipose tissue. Metabolism of β-carotene to retinal has been shown to take place in such peripheral tissues as human adipose tissue, primate lung and kidney and bovine corpus luteum (Schweigert et al., 1988; Wang et al., 1991). As suggested by Wang (1994), the significance of this local tissue conversion of β-carotene to retinoic acid may be high considering the unstable nature of the water-soluble retinoic acid which cannot be stored and is cleared rapidly from body fluids.

Romanchik et al. (1995) incubated *in vitro* different human lipoprotein classes and found no transfer of carotene from one lipoprotein class to another. They concluded that the transfer between LDL and HDL could occur only via the transfer into and out of other tissues, not by direct exchange between the lipoprotein particles. Hence, a receptor mediated transport of carotenoids into adipose tissue may take place. Large HDL particles, which carry most of carotene in their cores, also contain apo E, an apoprotein involved in this receptor transport. The lack of exchange of carotenoids between lipoprotein fractions may direct the carotenoids to the liver or to extrahepatic tissues. Larger adipocytes with larger surface area may possess more receptor binding sites and absorb more carotene into the cells. This may in turn explain the difference in fat colour/carotene concentration between Jersey and Limousin cattle. Clearly, the mechanism of either passive diffusion or receptor binding needs more research so the deposition of β-carotene can be resolved.

The extension of the experiment 2 proposed in the section 8.5.2. where adipose tissue would be incubated with various HDL size sub-fractions and the measurement of β-carotene-15,15'-dioxygenase activity would answer the question how carotenoids are transported into the adipose tissue and could explain the lack of correlation between adipocyte morphology and concentration of carotenoids in fat.

8.4. Conclusion

The aim of the this thesis was to determine the genetic and non-genetic factors influencing carotenoid concentration in cattle tissues, to understand the mechanism of action of carotenoids in cattle and to investigate the practical implementation of carotene metabolism. Clear evidence has been presented that, regardless of breed, the same non-genetic factors affect carotenoid concentration in blood, fat and meat. Moreover, no difference in carotenoid status was reported between different anatomical locations within adipose tissue and between subcutaneous and intermuscular fat. Carotenoid concentration was strongly influenced by various environmental factors and the response of animals raised on high carotene diet differed from those raised on low carotene diets.

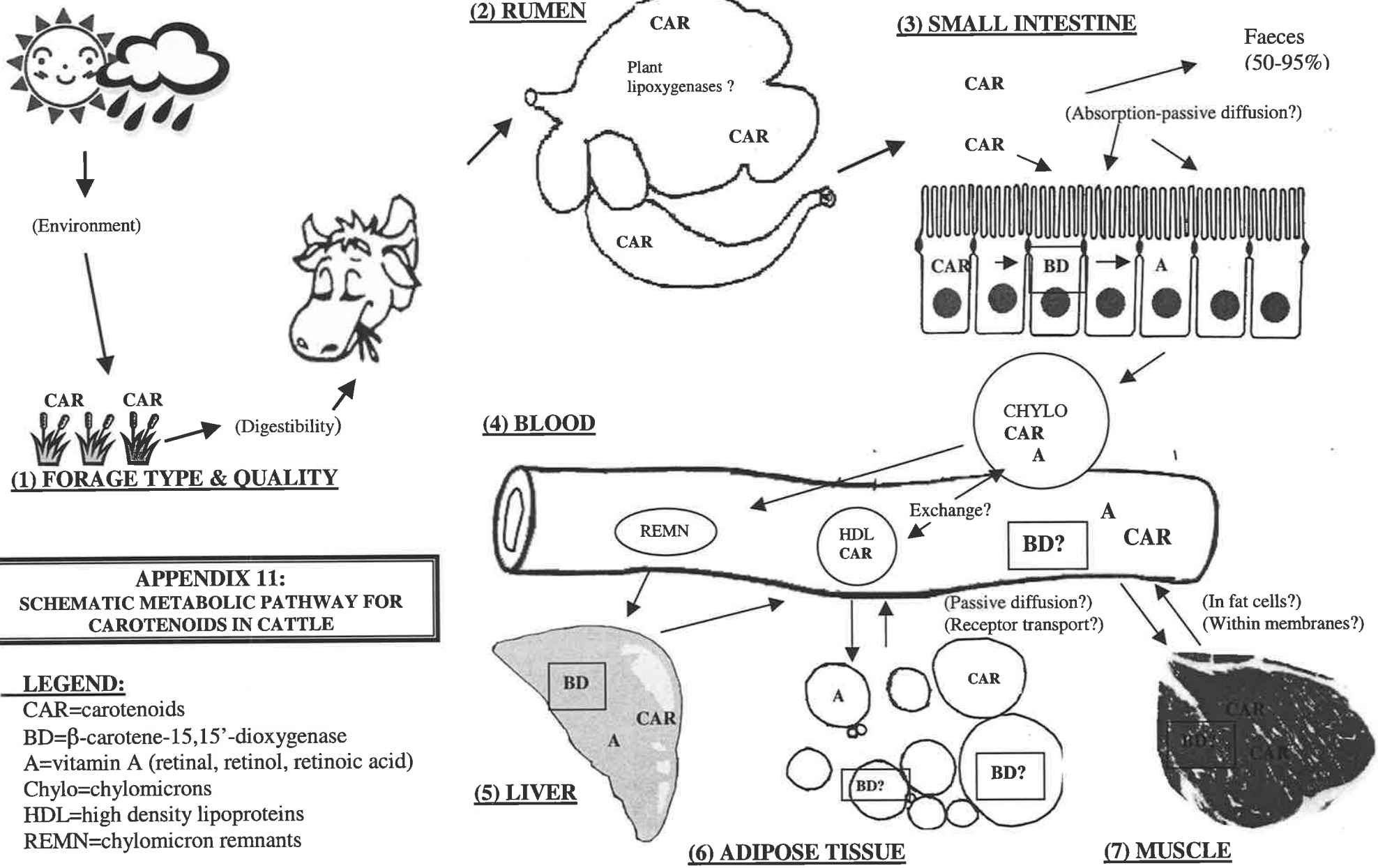
8.4.1. Model of β-carotene metabolism in cattle

From the work herein and the other studies, a model for carotenoid metabolism in cattle can be proposed (Figure 8.1). Carotenoids derive from feed and their concentration and the digestibility of the feed depends on type of diet, season, species of plant, dry matter content, rainfall, temperature etc. **(1)**. In the rumen **(2)**, carotenoids are not degradable, in general. However, negligible amounts may be oxidised due to the small quantity of oxygen swallowed or bleached by plant lipoxygenases if the transit through the rumen has been prolonged. The observed disappearance can occur by the incorporation of the carotenoids to the cellular content, not by their direct destruction or by the attack of the rumen microorganisms.

Carotenoids pass to the brush border membranes of the small intestine **(3)** and are absorbed in a non-energy requiring process, passive diffusion. A number of factors (such as the distribution of carotenoids within the micelles, interaction with other carotenoids, protein and fat content of the diet, etc.) influence the absorption of carotenoids. High levels of vitamin A suppresses the absorption of both lutein and β-carotene.

Within the enterocytes, provitamin A carotenoids are partially metabolised by central or excentric cleavage to vitamin A by the enzyme β-carotene-15,15'-dioxygenase. The activity of the enzyme depends also on many factors, and carotenoids or vitamin A act as depressants. From the enterocytes, the remaining carotenoids are partially re-secreted into the intestinal lumen as part of the enterocyte turnover process or are incorporated into chylomicrons and secreted via lymphatic ducts to the blood stream **(4)**. Lack of exchange of carotenoids between different lipoprotein fractions (especially β-carotene associated with the core of chylomicrons) indicates that β-carotene in the catabolised chylomicrons (chylomicron remnants) is delivered to the liver **(5)**.

In the liver, carotenoids are re-incorporated into newly formed large HDL particles and delivered to extrahepatic tissues without exchange between lipoprotein particles. Adipose tissue **(6)** absorbs carotenoids from the blood by passive diffusion or more likely receptor mediated transport. An action of β-carotene-15,15'-dioxygenase converts some provitamin carotenoids to vitamin A. In muscles **(7)**, the majority of carotene is associated with fat cells and only small amount is incorporated into muscle cell membranes. In total, the utilization of carotenoids from diets is low, variable and ranges from 5-50%.



APPENDIX 11:
SCHEMATIC METABOLIC PATHWAY FOR CAROTENOIDS IN CATTLE

LEGEND:

CAR=carotenoids
BD=β-carotene-15,15'-dioxygenase
A=vitamin A (retinal, retinol, retinoic acid)
Chylo=chylomicrons
HDL=high density lipoproteins
REMN=chylomicron remnants

8.4.2. Major gene controlling β-carotene metabolism

Based on the above physiological model, it is not likely that breed differences in carotenoid concentration between Jersey and Limousin cattle, raised in the same environment, were caused by dietary factors or conditions in the rumen. The difference in the animal size may influence the amount of feed consumed. However, it would be expected that the Limousin being a larger breed, would consume more carotene in total than the smaller Jerseys. This did not follow the concentration pattern of carotenoids in blood and solid tissues, as the concentration was lower in Limousin cattle.

The results herein and of others clearly indicate that carotenoid concentration in cattle adipose tissue has a genetic component. Breed distributions of carotenoid concentration in adipose tissue and pedigree analysis suggest that fat colour in cattle is controlled by a major additive gene with the allele for yellow fat (Y) at a moderate frequency in Jerseys.

The first most important step in the metabolic pathway for carotene in cattle, which could differentiate these breeds, is the activity of β-carotene-15,15'-dioxygenase in the enterocytes. The enzyme could be less active in Jersey cattle than Limousin cattle, and consequently, more pro-vitamin A carotenoids pass unchanged to the chylomicron fraction in this breed. This hypothesis is supported by significant breed differences in the concentration of total carotenoids (mainly β-carotene and lutein) in chylomicrons. Jersey cattle had a higher concentration of total carotenoids in this blood fraction. Moreover, this breed also differed significantly in the major pro-vitamin A carotenoid, β-carotene. The Jersey cows had higher concentration of this nutrient in chylomicrons. There was no breed difference in the other major non-provitamin A carotenoid, lutein, in chylomicrons.

Blood transport of carotenoids in both breeds had similar distributions with approximately 80% of these nutrients present in the HDL fraction, regardless of the season. In winter, HDL particles were larger partially as a result of the higher carotenoid concentration. More total carotenoids in the HDL of Jersey cattle could be due to lower conversion in the liver by the di-oxygenase enzyme. The dioxygenase activity has been detected in the liver in other species. If the higher level of carotene reduces the activity of this enzyme in cattle, then the lower level of carotene in the HDL of Limousin cattle can be caused also by the higher activity of dioxygenase in the liver of this breed and consequently, less carotene from the liver would be incorporated into the newly secreted HDL particles.

When HDL particles were assigned to different size classes, Jersey cattle had more larger particles with a higher content of carotene. The larger HDL particles contain apolipoprotein E which is synthesized in the liver and several extrahepatic tissues. The Apo E is recognized by Apo E receptors, which may mediate the catabolism of the largest and most mature HDL. More larger/mature HDL particles in Jersey cattle can mediate the transport to adipocytes, and consequently, more carotene would be transported and stored in Jersey than in Limousin fat. Larger adipocyte size in Jersey cattle (as well as the larger surface and volume) suggests that Jersey have more HDL receptors than the smaller cells of Limousin cattle if receptor mediated transport takes place. In case of a passive diffusion pathway, the surface of cells would also be of importance. The smaller adipocyte cell surface in Limousin cattle would allow less carotene to enter or exit in this breed. However, low and not significant correlations between adipocyte morphology and carotenoid concentration indicates that the breed difference in fat colour is unlikely to be caused by breed differences in adipocyte HDL receptors or passive diffusion process.

Finally, the activity of dioxygenase was reported in adipose tissue in other species (Wang et al, 1991). If this is the case in cattle, then the different level of adipose carotene in these two breeds may be a result of differences in dioxygenase activity in the adipose tissue. The metabolism of carotene in cattle consists of many complex stages where each could be influenced by a major gene and cause breed differences. However, if there is variation in the dioxygenase activity in the enterocytes, hepatic and adipose tissues in these two breeds, then the activity of this enzyme may be the major factor differentiating Jersey and Limousin cattle in carotene concentration in various tissues, and consequently, fat colour. Clearly, this hypothesis can now be tested (as described above) by examining the β-carotene-15,15'-dioxygenase activity in animals of different genotypes and by mapping the gene.

Appendixes

APPENDIX 1:

STANDARD PROCEDURE FOR BIOPSY FAT / MEAT SAMPLING

PROCEDURE:

(i) Restrain animal in the crush, clip the area of sampling, spray the sampling site with betadine, inject 5ml of lignocaine subcutaneously and make 3-5cm incision.

(ii) Use a pair of sterile forceps to hold the incised skin and remove approximately 5-10g of subcutaneous fat and meat using a power drill or scalpel.

(iii) Apply Topical Antibiotic (Apex Laboratories Pty Ltd.) into the wound and close the incision site with 3-5 sterile surgical metal staples.

(iv) Inject with Terramycin (10ml).

(v) Apply Stockholm tar on the site to prevent contact with dust and flies.

(vi) Place collected fat samples in a sieve and spray with water.

(vii) Estimate fat colour score subjectively on a 5-point scale (1-white to 5-very yellow).

(viii) Weigh fat/meat and divide into 2-3 sub-samples (1-2g).

(ix) Place fat/meat in 5ml labeled vials and freeze in liquid nitrogen.

(x) Remove samples to portable freezer.

(xi) Flush frozen samples with nitrogen gas and store in –20°C.

(xii). After approximately 2 weeks, when the wound is healed, remove staples.

MATERIALS:

Clippers, drill + sharp drill bits, cutting board, vial rack, surgical set (scalpels + forceps long and small), balance, tongs, beakers, sharps container, syringes, needles 18G and 23G, sieve, esky, sample book.

REAGENTS AND SOLUTIONS:

Liquid nitrogen, ethanol (99%), Betadine, Stockholm tar, dionised water (MQ standard), Terramycine, Lignocaine, Topical Antibiotic (Apex Laboratories Pty Ltd.), detergents, soap.

LITERATURE:

Malau-Aduli, A.E.O., Siebert, B.D., Pitchford, W.S., Bottema, C.D.K. (1997), Genetic variation in the fatty acid composition of cattle fat and muscle, *Proc. Aust. Assoc. Anim. Bred. Genet.*, **11,** 554-557.

APPENDIX 2:

STANDARD PROCEDURE FOR SEPARATION OF BLOOD PLASMA AND LIPOPROTEIN FRACTIONS

PROCEDURE:

(i) Centrifuge blood at 1500xg for 25 min. at 4°C

(ii) Pour 12 ml of blood plasma into the 13 ml Beckman centrifuge tube and overlay with 1 ml 1.006 g/ml KBr density gradient, filling the tube to the neck.

(iii) Seal tubes using metal cups and Beckman tube topper for quick seal and balance them with an accuracy of 10mg.

(iv) Centrifuge sample on the ultracentrifuge for 12 min. at 265,000xg (~60,000 rpm, Beckman L8 ultracentrifuge, 70.1 Ti rotor),

(v) Remove tubes from the centrifuge. Using a tube cutter cut the top of the tube and remove the top (chylomicron) fraction (~0.8-1.0 ml) which is distinguished from the residue by a narrow ring. Use Pasteur pipette and wash the top of the cut tubes to ensure complete removal of the chylomicron fraction.

(vi) Transfer the residue to another 13 ml Beckman centrifuge tube using a 12 ml syringe and 18g needle.

(vii) Overlay sample with 1.006g/ml density gradient as in step (ii) using 3 ml syringe and 23g needle, balance the tube and centrifuge for 6h 20min at 265,000xg.

(viii) When the centrifugation is completed, repeat step (v) removing the top VLDL fraction.

(ix) Transfer the residue to another Beckman 13 ml tube, add 0.0834g of KBr per 1.0ml to the residue and put on shaker for 10-15min until KBr is dissolved.

(x) Overlay the residue with resuspended KBr using 1.055g/ml density gradient (trying not to mix the gradient and the residue) and repeat step (iii).

(xi) Centrifuge sample for 11 hours at 148,500xg (~45,000rpm).

(xii) Repeat step (v) and remove LDL fraction.

(xiii) Weigh 0.2343g of KBr per 1.0ml of residue and put on a shaker for 10-15min.

(xiv) Take another 13ml Beckman centrifuge tube, pour in the residue with KBr, overlay with 1.19g/ml density gradient, seal, and balance the tube. Centrifuge for 18h30min at 148,500xg or for 13h40min at 265,000xg.

(xv) When the centrifugation is finished, remove the HDL fraction as described in previous steps making sure that the fraction is well washed from the cut top of the tube. All fractions can be stored in ependorff tubes at –20°C if analysed soon or at –70°C for longer storage.

REAGENTS AND SOLUTIONS:

KBr density solutions (1.006g/ml, 1.055g/ml, 1.19g/ml).

Preparation of density solutions:

Solutions with different densities are made from two solutions: 1.006g/ml and 1.35g/ml. 1.006g/ml solution is made by mixing NaCl (8.766g), NaN_3 (0.2g), and EDTA (0.375g) with distilled water (1L total). The 1.35g/ml solution is made mixing KBr (354g), NaCl (153g), NaN_3 (0.2g), and EDTA (0.375g) with distilled water (total volume 1L).

Solution of different densities are made according to the formula:

$$\text{Vol. of denser solution} = \frac{\text{Vol. Lower density solution (density required – density of lower density solution)}}{\text{(density of denser solution – density required)}}$$

LITERATURE:

Yang,A., Larsen, T.W., Tume, R.K. (1992), "Carotenoid and retinol concentrations in serum, adipose tissue and liver and carotenoid transport in sheep, goats and cattle", *Aust. J. Agric. Res.* **43:** 1809

Havel, R.J., Eder, H.A., Bragdon, A. (1955), "The distribution and chemical composition of ultracentrifugationally separated lipoproteins in human serum", *J. Clin. Invest.*, **34,** 1345.

APPENDIX 3:
EXTRACTION AND QUANTIFICATION OF CAROTENOIDS

PROCEDURE:

(i) Accurately measure the volume of a liquid sample or weight of a partially frozen sample.

(ii) Precipitate proteins of liquid samples (milk, blood) by addition of ethanol (1:5 v/v) and vortex for ~ 20sec. Thaw solid samples for 1-2 hours or overnight in room temperature.

(iii) Hydrolyse solid samples and milk at 65°C by adding KOH in ethanol (20% w/v).

(iv) Dilute sample after hydrolysis with 3ml of water, and extract carotene twice with organic solvents, shaking sample vigorously by hand for 20sec.

(v) Wash combined extracts with water, and transfer the organic fraction to another bottle.

(vi) Evaporate extracts to dryness under the heated nitrogen gas (~40°C).

(vii) Resuspend the residue in ethanol and centrifuge at 8400xg for 10min.

(viii) Determine total carotenoids on a spectrophotometer at 450nm using pure β-carotene for plotting a standard curve or separate and quantify individual carotenoids on HPLC (Chapter 3).

REMARKS:

- Use partially thawed solid samples (fat and meat) to more easily purify from connective tissue and to give more repeatable results.
- Use only glasswear for extraction as polypropylene reacts with organic solvents causing interference with spectrophotometric readings.
- If the fat is very white, increase the detectability of carotenoids by using ~0.3-0.5g samples with the same volumes of solvents as for 0.1-0.2g samples, or use less ethanol in resuspending the residue for spectrophotometry.
- The amount of 20% KOH in methanol does not have to be measured very precisely. Use a marked Pasteur pipette and bulb as KOH may damage the filter in automatic pipetters.
- After hydrolysis, add water and extract with the organic solvent. Wait about 15min and then perform the second extraction. If extracting a large number of samples, it is useful to work with 30-40 samples because when the first extraction is finished, the second group of samples is ready. Perform extractions carefully and do not lose extract, especially the first one which contains approximately 45-80% of carotenoids. It is better to include some of the water layer during the second extraction and later wash the combined extract with water (step iv) than to leave some organic solvent fraction.

- If a solid residue appears after hydrolysis with 20% KOH, rapidly tap the bottle a few times and the residue will disperse.
- To remove organic solvent extracts, the same Pasteur pipette can be used for about 20-30 samples as long as there is no solvent left in the pipette after transfers. To increase the speed of removal of the organic extracts, an automatic pipeter can be used with specially designed 10ml glass pipette.
- Two options are possible while removing extracts (step v), remove top layer or remove the bottom layer. The second option has an advantage in terms of reducing the number of bottles used for the extraction. However, contamination from the water fraction that remains may cause interference with spectrophotometric readings.
- Faulty lids used for extraction bottles can cause evaporation of the solvents. This can be crucial when samples are resuspended in ethanol or hexane for spectrophotometry.
- When measuring samples on the spectrophotometer (between 400-500nm), the absorption curve begins close to the baseline, rises to its maximum at ~450nm and then decreases to the baseline again. In some cases, the peak at ~450nm is not indicated on the spectrophotometer. If the absorption curve has a shape as described above, the absorption at 450nm can be used. This was confirmed by reading a number of samples which were prepared in triplicates. Some samples did not have the peak detected, but results based on the absorption at 450nm were identical as in samples where the peak was detected.
- To measure samples on the spectrophotometer wash the interior of the cuvette with 70% ethanol after each sample, invert the cuvette to dry, add a new sample, wipe the exterior of the cuvette with a soft tissue. If there are no small bubbles in the solution, smear on the outside of the cuvette, or milky appearence, the results should be satisfactory. In samples with low concentrations of carotene, the absorption curve should begin close to the baseline. If it does not, the sample may be contaminated, cuvette is dirty or there is not enough solution in the cuvette.
- If during muscle extraction, an emulsion in the interface of hexane and water appears, tap bottle slightly a few times to disperse the emulsion.

REAGENTS AND SOLUTIONS:

Ethanol (99%), diethyl ether, hexane (analytical grade), solution of 20% KOH in methanol.

LITERATURE:

Yang,A., Larsen, T.W., Tume, R.K. (1992), "Carotenoid and retinol concentrations in serum, adipose tissue and liver and carotenoid transport in sheep, goats and cattle", *Aust. J. Agric. Res.* **43:** 1809

APPENDIX 4:
QUANTITATIVE LIPID EXTRACTION
(Fat content in muscle)

PROCEDURE:

(i) Trim visible muscle fat immediately after collection of sample if possible or prior to analysis. Use a sharp kitchen knife or scalpel to obtain approximately 100g of extraneous fat-free muscle (only marbling fat should be obvious).

(ii) Chop and blend the muscle to an homogenous paste in a food processor (Brown Multi-quick Food processor, Model CA5 with Chopper attachment, or equivalent), avoiding separation of fat on the inside of the bowl.

(iii) Accurately weigh 1.0-1.5g of homogenised muscle into a 50ml polypropylene plastic tube (Cellstar® 50 ml PP-tubes, Lot. No. 99120195).

(iv) Add 10ml of methanol to the sample and homogenise the mixture in a stainless steel blender for approximately 1min. Then add 20ml of chloroform (containing 0.005% butylated hydroxytoluene (BHT)) and continue homogenising for a further 2min.

(v) Filter the mixture through a Whatman No. 1 filter paper (21.5mm) into a Buchner funnel under vacuum.

(vi) Resuspend the residue (peel the filter paper from the residue) in 30ml chloroform:methanol (2:1, v/v) and homogenise for further 2min.

(vii) Filter the mixture again and wash the residue with ~2 ml of chloroform. Larger samples (eg 5-10g) can be extracted with double quantities of methanol and chloroform (20ml and 40 ml) in a 100ml polypropylene measuring cylinder with a stainless steel homogeniser and the same filtering techniques as stated above).

(viii) Pour the combined filtrates into a measuring cylinder (100ml or larger for bigger samples) and add one quarter of the extract volume of aqueous 0.88% KCl.

(ix) Shake the mixture thoroughly and allow it to settle (approx. 5min.).

(x) Remove the upper layer by aspiration. Pour the bottom layer containing the purified lipid into an empty plastic tube. Rinse tube with chloroform and combine with extract.

(xi) Centrifuge briefly at 1300xg and remove water by aspiration.

(xii) Reduce the volume of the sample in a rotary evaporator or under a stream of nitrogen in a heating block at ~40°C. When many samples are being processed, place tubes containing the extracts in a fume hood and leave until the volume is reduced to 5-10ml.

(xiii) Pour the contents of each tube and chloroform washes into an accurately weighed culture tube keeping the volume to about 15ml.

(xiv) Dry the tubes under a stream of nitrogen gas in a heating block at~40°C.

(xv) Place the tubes in a dessicator containing active silica gel overnight before weighing again. The difference in weight is then expressed as '% fat' (wet weight) of the meat. It contains the intramuscular fat and the phospholipid content of the tissue membrane. It may contain some intermuscular fat as well.

REMARKS:

If transferring lipid extract to another tube or flask, rinse tube with chloroform and combine with lipid extract.

REAGENTS AND SOLUTIONS:

Chloroform and methanol, 'Analytical Reagent' grade, re-distilled from highly qualitative work.

LITERATURE:

Christie, W.W. (1989) Gas Chromatography and Lipids: a practical guide, The Oily Press, Ayr, Scotland.

APPENDIX 5:
DETERMINATION OF ADIPOCYTE SIZE AND NUMBER BY COLLAGENASE TREATMENT

PROCEDURE:

(i) Using freshly siliconised culture tubes, pour 0.5ml of Krebs-Ringer biocarbonate buffer (KRB) pH 7.4 with 4% albumin and collagenase (5mg/ml).

(ii) Cut ~50mg of fat from frozen adipose tissue sample. Wash with warm (37°C) isotonic (0.15M) saline to remove surface fat resulting from cell breakage during slicing and dry on filter paper.

(iii) Weigh tissue slices precisely and place in culture tubes containing 0.5ml of KRB buffer with collagenase.

(iv) Incubate tubes at 37°C for 2 ½ hours in a gently shaking incubator (60 cycles/min).

Determination of adipocyte number:

(v) When the incubation is completed and adipocytes are almost completely freed, gently stir the specimens. Immediately take 50µl aliquots of the cell suspension (using 50µl yellow tips) and place in a heamocytometer chamber. Keep the remaining cell suspension at 37°C.

(vi) Focus slide on the grid and count the number of adipocytes in each of 25 squares under a magnification of 200x. If the results vary between 2 readings, repeat step (v). Each large square should have an area of $1mm^2$ and a depth of 0.1mm and volume 10^{-4}ml.

(vii) Calculate cell number by taking the average of cell counts and multiply the result by 10^4 (number of cells in 1ml) and divide by 2 as 0.5ml of KRB buffer with collagenase was used for cell liberation. Express number of cells per 1g of fat by multiplying the result by 1000 and dividing by sample weight.

Determination of adipocyte size:

(viii) Stain cell suspension with 5% Sudan black in methanol (w/v) and keep for 2-5min in 37°C incubator shaking gently.

(ix) Take a 100µl aliquot of the suspension of stained cells and place on a siliconized glass slide.

(x) Examine 100 cells under the microscope (Olympus BH-2 with CMA-D1CE Sony camera adapter, Japan) equipped with a CCD Sony camera and monitor under a final magnification of 670x. Assign the cells into classes of 10µ diameter intervals based on the calibration marked on the camera monitor. (Calibration for cell sizing was performed on the screen of

the monitor using a calibrating slide. After focusing, the slide scale with 10μ intervals was marked on the monitor).

REMARKS:

- To assure that small adipocyte-like particles ranging between 10-20μ are fat cells and not fat droplets resulting from sample cutting, Leishman's dye was used. Nuclei and other cell organelles stained purple and demonstrated that all investigated objects were adipocytes.

- Staining adipocytes with Sudan black solution was not necessary as the adipocytes were clearly distinquishable on the monitor. The ethanol used in preparation of the Sudan black solution may cause rupture of larger adipocytes. A comparison was made between stained and not stained aliquots of the same cell suspension and no difference between the treatments was found.

MATERIALS:

Collagenase (Type I, Lot26H0174, Sigma Chemical CO., St Louis, USA)
Sudan Black solution
Leishman's stain
0.15 M NaCl

REAGENTS AND SOLUTIONS:

Preparation 5% Sudan Black in methanol

Resuspend 0.5g of Sudan Black in 70% ethanol and stir for ~30min. Filter solution through Whatman filter paper No 1, wrap the storage container with aluminium foil and store at room temperature.

Siliconisation procedure

Using dichlorodimethylsilane make 5% solution in chloroform or in hexane. Soak glasswear (culture tubes, slides) for 5-10min., rinse well with distilled water and dry in the air or in ~80°C oven.

Leishman's stain

Place Leishman powder (0.15g) in a mortar with methyl alcohol. Mix very thoroughly. Pour into a bottle and leave for seven days before using. Take 150μl and put into 3ml cell suspension. Incubate for ~10min. at 37°C.

Preparing 1L Krebs-Ringer biocarbonate buffer

The following components were used to make 1L of Krebs-Ringer buffer with pH of 7.4: NaCl (M=58.44, 118mM-6.896g), KCl (M=74.56, 4.7mM-0.35g), $MgSO_4 \cdot [7H_2O]$ (M=246.47, 1.2mM-0.26g), $CaCl_2$ (M=147.02, 2.5mM-0.368g), KH_2PO_4 (M=136.09, 1.2mM-0.163g), EDTA (M=372.24, 1mM-0.372g), HEPES (hydroxyethyl-piperazine-ethane-sulfonic acid 25mM which was prepared as 1M solution and 25ml/L was added), BSA (fraction V, 4%-40g, 800ml of ultra pure water was add to the components, the pH adjusted to 7.4 and the volume bring up to 1L with ultra pure water. The buffer was stirred ~2h and stored at 4°C.

LITERATURE:

Smith, U., Sjostrom, L., Bjorntorp, P. (1972) Comparison of two methods for determining human adipose cell size., *J. Lipid Res.* **13,** 822-824.

Bancroft, J.D., Cook, H.C. (1984) Manual of histological techniques., Churchill Livingstone, Edinburgh London Melbourne and New York.

Chayen, J., Bitensky, L. Practical Histochemistry., second edition, John Wiley and Sons, Chichester, New York, Brisbane, Toronto, Singapore.

Clayden, E.C. (1971) Practical section cutting and staining., Chrchil Livingstone, Edinburgh and London, London Group Limited, 5th edition.

APPENDIX 6:

DETERMINATION OF CHYLOMICRON AND HDL PARTICLE SIZE USING ELECTRON MICROSCOPY

PROCEDURE:

(i) Thaw chylomicron or HDL lipoprotein fraction at room temperature.

(ii) Place ~25µl of HDL in a 1.5ml ependorf tube and dilute with ~1ml of ultra pure water. Use chylomicron fraction without diluting.

(iii) Place a droplet of chylomicrons or diluted HDL onto the Formvar-carbon-coated grid, wait 15sec. then remove excess lipoprotein fluid with filter paper, and immediately add a droplet of 2% sodium phosphotungstate, pH 7.4.

(iv) Stain for 30-60sec. and remove excess stain.

(v) Using locking fine forceps, place the grid into the microscope specimen chamber (TEM 100, Philips) and examine under the magnification of 10,500x for chylomicrons and 92,000x for HDL.

(vi) Adjust the focus of the background to minimal granularity and save selected images on the computer. Repeat this step 5-10 times recording and saving the images from different parts of the grid.

Measurement of chylomicron and HDL particle size

Lack of appropriate equipment such as ocular micrometer or digitizing tablet necessitated the application of different techniques and equipment to measure chylomicron and HDL particle size. Images of the lipoproteins saved on the computer were opened in Adobe Photoshop, resized and the 100 chylomicron and 50 HDL particles were measured manually using a PV32 computer program.

REMARKS:

- Make sure that the lipoprotein sample on the grid does not dry prior to adding stain.
- The forceps should be rinsed in ethanol after each sample in order to avoid contamination from the previous sample, which tends to adhere to the tip of the forceps.
- Microscope settings: acceleration voltage of 80kV, small objective aperature (50µm), illumination reduced when scanning the grid to minimase radiation damage.

MATERIALS:

Standard Formvar-carbon-coated grids

2% sodium phosphotungstate

REAGENTS AND SOLUTIONS:

Preparing 2% Sodium phosphotungstate staining solution

Resuspend sodium phosphotungstate powder in distilled water (2:100, w/v). Bring pH of the solution to 7.4 using NaOH or KOH. After the final pH is established, filter the solution through a 0.45μm Nalgene filter (Nalge Co., Rochester, NY) and store in a clean glass bottle. If care is taken so that only clean pipettes are introduced into the stain bottle, the stain is usable for many months.

LITERATURE:

Forte, T.M., Nordhausen, R.W. (1986) Electron microscopy of negatively stained lipoproteins., *Methods in Enzymology,* **128,** 442-457.

APPENDIX 7:

DETERMINATION OF FATTY ACID PROFILES IN BLOOD PLASMA AND LIPOPROTEIN FRACTIONS BY GAS CHROMATOGRAPHY

PROCEDURE:

(i) Add 50µl of internal standard [nonadecanoid (C19) fatty acid], 100 µl blood plasma or lipoprotein fraction, 1ml of methanol, 2ml of chloroform to a 20ml culture tube and vortex for 30sec.

(ii) Add 0.5ml 0.1M HCl and vortex again for 30sec.

(iii) Centrifuge for 10min at 580xg.

(iv) Remove aqueous (top) layer by aspiration.

(v) Transfer as much as possible of the lower chloroform layer to another 20ml culture tube while avoiding the aqueous phase.

(vi) Evaporate the extract to dryness under a stream of nitrogen in a heating block at ~40°C.

(vii) Add approximately 1.5ml acidified (1% H_2SO_4) methanol, screw the cap on tightly, and heat the tubes in an oven at 100°C for 45min or at 60°C overnight.

(viii) Allow the sample to cool to room temperature, then add 3ml purified water and 5ml petroleum ether.

(ix) Vortex or shake the sample for 15sec., then transfer the upper ether layer to a clean, dry 20ml culture tube taking care not to include the aqueous layer.

(x) Add another 5ml of petroleum ether to the aqueous sample, repeat the extraction and remove of the ether layer.

(xi) Pool the extracts and evaporate the solvents with a stream of nitrogen on an heating block at ~40°C.

(xii) Optional: To remove pigments or complex lipids such as cholesterol; add 1.5ml of hexane (1 Pasteur pipette) to the dried esters. Dissolve the esters and transfer the solution to a small column containing Fluorosilicate (approximately 20mm in pasteur pipette). Elute the methyl esters with 2ml 10% diethyl ether in hexane, collect the eluate in clean culture tubes, and dry under pre-heated nitrogen.

(xiii) Dissolve the extracted methyl esters in 100µl of iso-octane (trimethyl pentane) and pipette solution into 1.5ml ependorf tubes before storing at 4°C (short term) or -20°C (long-term) prior to gas chromatography analysis.

REMARKS:

- Use greater volume of iso-octane for more concentrated samples (xiii).

REAGENTS AND SOLUTIONS:

Methanol (dried and distilled by re-fluxing A.R. methanol over calcium hydride for 1 hour before use and filtering through fine paper, storing in air-tight bottle), chloroform, 0.1M HCl, 1% H_2SO_4, petroleum ether (40-60°C b.pt.), iso-octane (trimethyl pentane). All solvents should be of analytical grade.

EQUIPMENT:

Hewlett Packard Gas Chromatograph, model 5890A, series II fitted with a capillary column (BPX70, SGE, Melbourne, Australia). Hydrogen gas used as a carrier.

LITERATURE:

Christie, W.W. (1989) Gas Chromatography and Lipids: a practical guide, The Oily Press, Ayr, Scotland.

APPENDIX 8:
QUANTIFICATION OF PROTEINS USING BRADFORD METHOD

The Bradford method quantifies the binding of a dye, Coomassie Brilliant Blue, to an unknown protein and comparing this binding to different concentrations of a standard protein, usually bovine serum albumin. It is designed to quantify 1 to 10μg protein. Protein determinations in the range of 10 to 100μg may be made by increasing the volume of the dye solution 5-fold and using larger tubes.

PROCEDURE:

(i) Aliquot duplicate amounts of 0.5mg/ml BSA (5, 10, 15, 20μl) into 8 Eppendorf microcentrifuge tubes and use 0.15 M NaCl to bring the volume in each to 100μl.

(ii) Aliquot 100 μl of 0.15 M NaCl into 2 Eppendorf tubes as blank controls.

(iii) Add 1ml Coomassie Brilliant Blue solution and vortex. Allow to stand 2min. at room temperature.

(iv) Determine the absorbance at 595 nm using a 1-cm pathlength microcuvette (1ml) and make a standard curve by plotting absorbance at 595nm versus protein concentration. Determine the absorbance for the unknown protein sample using the same procedure. Use the standard curve to determine the concentration of protein in the unknown sample.

REMARKS:

- If the unknown protein concentration is too high, dilute the protein, assay a smaller aliquot of the unknown, or generate another standard curve in a higher concentration range (e.g., 10 to 100).

MATERIALS: 0.5 mg/ml bovine serum albumin (BSA)
0.15 M NaCl
Coomassie Brilliant Blue solution

REAGENTS AND SOLUTIONS:

0.5 mg/ml bovine serum albumin (BSA)

The concentration of BSA is determined using the A_{280} = 6.6 for a 10mg/ml solution of BSA measured in a 1-cm pathlength cuvette (e.g., a 0.5mg/ml solution will have an A_{280} = 0.33).

Coomassie Brilliant Blue solution

In a 1-litre volumetric flask, dissolve 100mg Coomassie Brilliant Blue G-250 in 50ml of 95% ethanol. Add 100ml of 85% phosphoric acid. Bring to volume with water. Filter through Whatman No. 1 filter paper. Store at 4°C.

LITERATURE:

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding., *Anal. Biochem.* **72:** 248-254.

APPENDIX 9:

PEDIGREES FAT COLOUR "OUTLIERS" ANIMALS

Dam ID	Breed of dam	Progeny ID	Breed of progeny	Year of birth	Sire ID	Breed of sire	Carotene at weaning	Carotene at slaughter	Birth group
27	JJJJ	459	JJJJ	1995	**78**	JJJJ	-	1.6	95
		630	JJJJ	1996	**77**	JJJJ	**2.9**	**3.7**	106
		879	LJJJ	1997	368	LLJJ	1.4	-	117
32	JJJJ	350	-	1994	-	-	-	1.1	98
		707	LJJJ	1996	361	LLJJ	1.0	-	129
		941	LJJJ	1997	**398**	LLJJ	**3.2**	-	157
86	JJJJ	**310**	JJJJ	1994	**78**	JJJJ	-	**2.2**	89
		543	JJJJ	1995	**77**	JJJJ	1.5	1.2	121
		923	LJJJ	1997	**398**	LLJJ	0.9	-	146
87	JJJJ	**536**	JJJJ	1995	**77**	JJJJ	**1.9**	1.0	120
		736	LJJJ	1996	368	LLJJ	1.2	-	142
		800	LJJJ	1997	368	LLJJ	1.1	-	101
96	JJJJ	**588**	JJJJ	1995	*77*	JJJJ	**1.9**	2.0	153
		648	JJJJ	1996	*78*	JJJJ	**2.4**	**4.9**	111
107	JJJJ	317	LLJJ	1994	74	LLLL	0.8	-	90
		461	LLJJ	1995	75	LLLL	0.9	**2.3**	97
		618	LJJJ	1996	361	LLJJ	1.5	-	102
		818	LLJJ	1997	368	LLJJ	1.2	-	105
112	JJJJ	373	LLJJ	1994	74	LLLL	1.1	-	102
		691	JJJJ	1996	**77**	JJJJ	**2.4**	**4.0**	120
		781	LJJJ	1997	361	LLJJ	1.4	-	92
118	JJJJ	324	LLJJ	1994	75	LLLL	0.9	-	118
		535	JJJJ	1995	**77**	JJJJ	**2.4**	0.9	120
		661	LJJJ	1996	361	LLJJ	1.5	-	113
		888	LJJJ	1997	368	LLJJ	1.3	-	122
126	JJJJ	**457**	JJJJ	1995	**78**	JJJJ	1.2	**2.2**	94
		728	LJJJ	1996	352	LLJJ	0.6	1.6	138
		940	LJJJ	1997	368	LLJJ	1.0	-	126
193	JJJJ	**545**	JJJJ	1995	**77**	JJJJ	**2.0**	0.8	122
		672	LJJJ	1996	361	LLJJ	1.1	-	114
		843	LJJJ	1997	368	LLJJ	1.3	-	108
195	JJJJ	**557**	JJJJ	1995	**78**	JJJJ	**2.4**	**3.0**	126
		663	LJJJ	1996	**398**	LLJJ	1.0	-	113
		866	LJJJ	1997	361	LLJJ	1.5	-	112
206	JJJJ	**311**	JJJJ	1994	**78**	JJJJ	-	**2.5**	89
		480	LLJJ	1995	75	LLLL	0.8	0.4	107
		640	JJJJ	1996	**78**	JJJJ	1.8	**5.5**	109
221	JJJJ	**544**	JJJJ	1995	**78**	JJJJ	-	**2.7**	122
224	JJJJ	735	LJJJ	1996	361	LLJJ	1.1	-	142
		801	LJJJ	1997	**398**	LLJJ	**2.1**	-	101
226	JJJJ	**312**	JJJJ	1994	**77**	JJJJ	-	**2.2**	90
		634	LJJJ	1996	368	LLJJ	0.9	-	108
		887	LJJJ	1997	361	LLJJ	1.2	-	121

Dam ID	Breed of dam	Progeny ID	Breed of progeny	Year of birth	Sire ID	Breed of sire	Carotene at weaning	Carotene at slaughter	Birth group
269	JJJJ	**326**	JJJJ	1994	**78**	JJJJ	-	**2.5**	94
		463	LLJJ	1995	75	LLLL	1.0	1.4	100
		904	LJJJ	1997	368	LLJJ	0.9	-	127
273	JJJJ	**378**	LLJJ	1994	75	LLLL	-	**2.5**	103
		706	JJJJ	1996	**77**	JJJJ	**2.4**	**3.9**	129
274	JJJJ	565	JJJJ	1995	**77**	JJJJ	1.5	0.6	128
		623	JJJJ	1996	**78**	JJJJ	**1.9**	2.9	105
		873	LJJJ	1997	361	LLJJ	1.3	-	114
278	JJJJ	568	LLJJ	1995	74	LLLL	1.0	0.4	131
		621	LJJJ	1996	**398**	LLJJ	**3.8**	**3.5**	104
283	JJJJ	**370**	JJJJ	1994	**78**	JJJJ	-	**2.1**	101
		585	LLJJ	1995	74	LLLL	0.9	0.4	150
285	JJJJ	**328**	LLJJ	1994	75	LLLL	-	**3.1**	94
		561	LLJJ	1995	74	LLLL	0.9	1.2	127
		651	JJJJ	1996	**78**	JJJJ	**3.9**	**6.2**	111
		816	LJJJ	1997	368	LLJJ	0.9	-	105
292	JJJJ	**551**	JJJJ	1995	**77**	JJJJ	1.6	**2.3**	124
295	JJJJ	**560**	JJJJ	1995	**77**	JJJJ	**2.4**	1.2	127
		727	JJJJ	1996	**78**	JJJJ	1.2	1.8	137
		906	LJJJ	1997	**398**	LLJJ	0.7	-	128
302	JJJJ	**465**	JJJJ	1995	**78**	JJJJ	**2.1**	2.0	101
		737	JJJJ	1996	**77**	JJJJ	0.5	**2.4**	143
		919	LJJJ	1997	361	LLJJ	1.2	-	143
434	JJJJ	**701**	JJJJ	1996	**78**	JJJJ	1.5	**6.1**	127
		864	LJJJ	1997	**398**	LLJJ	**2.2**	-	112
602	JJJJ	**827**	LJJJ	1997	**398**	LLJJ	**2.4**	-	106

Note: carotene at weaning and slaughter expressed in μg/g fat, birth group relates to number of days from the beginning of the year to the day of birth, bold numbers represent progeny, sire and dam "outliers".

APPENDIX 10:
PUBLICATIONS

Refereed Conference Proceedings:

Kruk, ZA, Malau-Aduli, AEO, Thomson, AM, Siebert, BD, Pitchford, WS, Bottema, CDK (1997). Do breed and season affect β-carotene and fatty acid composition of muscle phospholipid in beef? *43rd Int. Cong. Meat Sci. Techn.*, 314-315.

Kruk, ZA, Malau-Aduli, AEO, Thomson, AM, Siebert, BD, Pitchford, WS, Bottema, CDK (1997). Are β-carotene and fatty acid composition related in cattle? *Proc. Aust. Assoc. Anim. Breed.Genet.* **12:** 278-282.

Kruk, ZA, Siebert, BD, Pitchford, WS, Bottema, CDK (1998). Relationship between carotenoids and intramuscular fat content in cattle. *44th Int. Cong. Meat Sci. Techn.*, 664-665.

Kruk, ZA, Malau-Aduli, AEO, Pitchford, WS, Bottema, CDK (1998). Genetics of fat colour in cattle. *Proc. 6th World Cong. Gen. Apl. Livestc Prod.*, 121-124.

Kruk, ZA, Pitchford, WS, Bottema, CDK (1999). Carotenoid concentration in different anatomical locations of subcutaneous fat in cattle. *45th Int. Cong. Meat Sci. Techn.*, 448-449.

Kruk, ZA, Pitchford, WS, Bottema, CDK (1999). Relationship between cattle fat colour at weaning and at slaughter. *45th Int. Cong. Meat Sci. Techn.*, 450-451.

Journal Publications:

Siebert, BD, Pitchford, WS, Kuchel, H, Kruk, ZA, Bottema, CDK (2000). The effect of β-carotene on desaturation of ruminant fat. *Asian-Aust. J. Anim. Sci.* **13:** suppl. 185-188.

Bibliography

Arechiga, CF, Staples, CR, McDowell, LR, Hansen, PJ (1998). Effect of timed insemination and supplemental β-carotene on reproduction and milk yield of dairy cows under heat stress. *J. Dairy Sci.* **81:** 390-402.

Ascarelli, I, Edelman, Z, Rosenberg, M, Folman, Y (1985). Effect of dietary carotene on fertility of high-yielding dairy cows. *Anim. Prod.* **40:** 195-207.

Ashes, JR, Burly, RW, Davenport, JB, Sidhu, GS (1982). Effects of dietary supplements of protected lipids on the concentration and transport of β-carotene and cholesterol in bovine blood and milk: unusual chromatographic behaviour of the high-density lipoprotein with high levels of β–carotene. *J. Dairy Res.* **49:** 39-49.

Ashes, JR, Burley, RW, Sidhu, GS, Sleigh, RW (1984). Effect of particle size and lipid composition of bovine blood HDL on its function as a carrier of β-carotene. *Bioch. Bioph. Acta,* **797:** 171-177.

AUS-MEAT (1990). Chiller Assessment. A Pictorial Guide. Australian Meat and Livestock Corporation: Woolloongabba, Qld.

Babiak, J, Rudel, LL (1987). Lipoproteins and arteriosclerosis In Clinical endocrinology and metabolism. vol.1 No.3, Bailliere Tindall, Sydney.

Baker, RL, Steine, T, Vabeno, AW, Breines, D (1985). The inheritance and incidence of yellow fat in Norwegian sheep. *Acta agric. Scand.* **35:** 389-397.

Barton, RA, Jones, CF, Donaldson, IL, Barnes, FR, Evans, DA, Clifford, HJ (1968). A comparison between Friesian, Jersey and Friesian-Jersey cross steers. *Proc. NZ Soc. Anim. Prod.* **28:** 180.

Barton, RA, Donaldson, JL, Barnes, FR, Jones, CF, Clifford, HJ (1994). Comparison of Friesian, Friesian-Jersey-cross, and Jersey steers in beef production. *NZ J. Agric. Res.* **37:** 51-58

Bauernfeind, JC, Brubacher, GB, Klaui, HM, Marusich, WL (1971). Use of carotenoids. *In* Carotenoids. Birkhauser, Basel, 743-770.

Bauernfeind, JC (1972). Carotenoids vitamin A precursors and analogs in foods and feeds. *J. Agr. Fod Chem.* **20(3):** 456-473.

Bendich, A, Olson, JA (1989). Biological actions of carotenoids. FASEB J. **3:**. 1927-1932.

Bendich, A (1993). Biological functions of dietary carotenoids. *In* Carotenoids in human health, ed. by LM Canfield, NI Krinsky, JA Olson, The New York Academy of Science, 61-67.

Bertram, JS (1993). Cancer prevention by carotenoids. Mechanistic studies in cultured cells. *In* Carotenoids in human health, ed. by LM Canfield, NI Krinsky, JA Olson, The New York Academy of Science, 177-191.

Bindas, EM, Gwazdauskas, FC, Aiello, R, Herbein, JH, McGilliard, ML, Polan, CE (1984). Reproductive and metabolic characteristics of dairy cattle supplemented with β-carotene. *J. Dairy Sci.* **67:** 1249-1255.

Block, E, Farmer, B, (1987). The status of β–carotene and vitamin A in Quebec Dairy herds; Factors affecting their status in cows and their effects on reproductive performance. *Can. J. Anim. Sci.* **67:** 775-788.

Blomhoff, R, Green, H, Green, JB, Berg, T, Norum, KR (1992). Vitamin A metabolism: new perspectives on absorption, transport, and storage. *Physiol. Rev.* **71(4):** 951-990.

Blomhoff, R (1994). Transport and metabolism of vitamin A. *Nutr. Rev.* **52(2):** (II) 13-23.

Borel, P, Grolier, P, Armand, M, Partier, A, Lafton, H, Lairon, D, Azais-Braesco, V (1996). Carotenoids in biological emulsions : solubility, surface-to-core distribution, and release from lipid droplets. *J. Lip. Res.* **37:** 250-261.

Borel, P, Tyssandier, V, Mekki, N, Grolier, P, Rochette, Y, Alexandre-Gouabau, MC, Lairon, D, Azais-Braesco, V (1998). Chylomicron beta-carotene and retinyl palmitate responses are dramatically diminished when men ingest beta-carotene with medium-chain rather than long-chain triglycerides. *J. Nutr.* **128(8):** 1361-1367

Bradford, MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:** 248-254.

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

Browne, GM (1993). Causes of rejection of beef carcasses prepared for the Japanese market. *Proc. Aust. Soc. Anim. Prod.* **19:** 91-93.

Brown, AJ, Fragoso, YD (1994). Tocopherols and carotenoids are differently distributed in subfractions of high-density lipoprotein. *Biochim Biophys Acta* **20:**373-376

Burton, GW, Ingold, KU (1984). β-Carotene: An unusual type of lipid antioxidant. *Science* **224:** 569-573.

Cazes, JP, Vallade, Ch, Quackebeke, EV (1990). Effect of concentrates on quality of subcutaneous adipose tissue of lamb carcasses. *World. Rev. Anim. Prod.* **25:** 55-62

Cetinkaya, N, Ozcan, H, (1991). Investigation of seasonal variations in cow serum retinol and β–carotene by HPLC method, *Comp. Biochem. Physiol.* 100A 1003.

Chanda, R (1950). The partition of carotenoids and vitamin A in the milk of cows and goats throughout lactation. *Proc. Bioch. Soc.* 399.

Chew, BP (1993). Role of carotenoids in the immune response. *J. Dairy Sci.* **76:** 2804-2811.

Chew, BP, Hollen, LL, Hillers, JK, Herlguson, ML (1982). Relationship between vitamin A and β-carotene in blood plasma and milk and mastitis in Holsteins. *J. Dairy Sci.* **65:** 2111-2118.

Christie, WW (1989). Gas Chromatography and Lipids: a practical guide. The Oily Press, Ayr, Scotland.

Coodley, GO, Nelson, HD, Loveless, MO, Folk, C (1993). β-Carotene in HIV infection. In carotenoids in human health. Ed. by LM Canfield, NI Krinsky, JA Olson. *Ann. NY Acad.Sci.* **691:** 277-278.

Crabtree, DV, Adler, AJ (1997). Is ß-carotene an antioxidant? *Med. Hypoth.* **48:** 183-187.

Craig, HB, Blumer, TN, Barrick, ER (1959). Effect of several combinations of grass and grain in the ration of beef steers on the fat colour characteristics of lean and fat. *J. Anim. Sci.* **18:** 241-248.

Crain, FD, Lotspeich, FJ, Krause, RF (1967). Biosynthesis of retinoic acid by intestinal enzymes of the rat. *J. Lip. Res.* **8:** 249-254

Crane, B, Clare, NT (1975). Nature of carotenoid pigments in yellow fat of sheep. *N.Z.J. Agric. Res.* **18:** 273-275.

Cundiff, LV, Dikeman, ME, Koch, RM, Crouse, JD, Gregory, KE (1988). Breeding for lean beef (Germ plasm evaluation program). *Beef Research Progress Report No.3,* United States Department of Agriculture, Agriculture Research Service, ARS-71, June.

Davison, AR, Rousseau, E, Dunn, B (1993). Putative anticarcinogenic actions of carotenoids: nutritional implications. *Can. J. Physiol. Pharmacol.* **7:** 732-745.

Dawson, RMC, Hemington, N, Hazlewood, GP (1977). On the role of higher plant and microbial lipases in the ruminal hydrolysis of grass lipids. *Br. J. Nutr.* **38:** 225-232.

Dawson, JM, Essex, CP, Walsh, A, Beevers, DE, Gill, M, Buttery, PJ (1993). Effect of fish-meal supplementation and β-agonist administration on adipose tissue metabolism in steers given silage. *J. Anim. Prod.* **57:** 397-406.

Dickerson, GE (1969). Experimental approaches in utilizing breed resources. *Anim. Breed. Abstr.* **37:** 191-202.

Dryden, FD, Marchello, JA, Adams, GH, Hale, WH (1971). Bovine serum lipids II. Lipoprotein quantitative and qualitative composition as influenced by added animal fat diets. *J. Anim. Sci.* **32:** 1016.

Duel, HJ, Halliday, N, Hallman, LF, Johnston, C, Miller, AJ (1941). The production of high vitamin A milk by diet *J.Nutr.* **22:** 303-313.

Duel, HJ, Hallman, LF, Johnston, C, Mattson, F (1942). The effect of a high vitamin intake on the blood and milk carotene of Holstein and Guernsey cows. *J.Nutr.* **23:** 567-579.

During, A, Nagao, A, Hoshino, C, Terao, J (1996). Assay of β-carotene-15, 15'-dioxygenase activity by reverse-phase high-pressure liquid chromatography. *Analit. Biochem.* **241:** 199-205.

El-Gorab, MI, Underwood, BA (1973). Solubilization of ß-carotene and retinol ino aqueous solutions of mixed micelles. *Bioch. Bioph. Acta* **306:** 58-66.

El-Gorab, MI, Underwood, BA, Loerch, JD (1975). The roles of bile salts in the uptake of β-carotene and retinol by rat everted gut sacs. *Biochim. Biophy. Acta,* **401:** 265-277.

Erdman, JWJr, Bierer, TL, Gugger, ET (1993). Absorption and transport of carotenoids. *Ann. N.Y. Acad. Sci.* **691:** 76-85.

Ferreri, LF, Elbein, RC (1982). Fractionation of plasma trigliceride-rich lipoproteins of the dairy cow: evidence of chylomicron-size particles. *J. Dairy Sci.* **65:** 1912-1920.

Fidge, NH, Goodman, DS (1968). The enzymatic reduction of retinal to retinol in rat intestine. *J. Biol. Chem.* **243:** 4372-4379

Field, RA, Ho, L, Russel, WC, Riley, ML, Murdoch, WJ, Van Kirk, EA, Ercanbrack, SK, Williams, FLJr (1989). Influence of age and testosterone levels on masculine development in rams. *J. Anim. Sci.* **67:** 2943-2949.

Folman, Y, Ascarelli, I, Herz, Z, Rosenberg, M, Davidson, M, Halevi, A (1979). Fertility of dairy heifers given a commercial diet free of β-carotene. *Br. J. Nutr.* **41:** 353-359.

Forrest, RJ (1981). Effect of high concentrate feeding on the carcass quality and fat coloration of grass-reared steers. *Can. J. Anim. Sci.,* **61:** 575-580.

Forte, TM, Nordhausen, RW (1986). Electron microscopy of negatively stained lipoproteins. *Methods in Enzymology,* **128:** 442-457.

Frank, HA (1993). Physical and chemical properties of carotenoids. *In* Carotenoids in human health, ed. by LM Canfield, NI Krinsky, JA Olson, The New York Academy of Science. 1-9.

Furr, HC, Clark, RM (1997). Intestinal absorption and tissue distribution of carotenoids. *J. Nutr. Biochem.* **8:** 364-377.

Gaunt, GM, Ferrier, GR, Seirer, RC, Kenney, PA (1994). Relationship between objective and subjective measurements of beef fat colour. *Proc. Aust. Soc. Anim. Prod.* **20.** 351.

Gawienowski, AM, Stacewicz-Sapuncakis, M, Longley, R (1974). Biosynthesis of retinol in bovine corpus luteum. *J. Lipid Res.* **15:** 375-379.

Gaziano, JM, Hennekens, CH (1993). The role of beta-carotene in the prevention of cardiovascular disease. In Carotenoids in human health. *Ann. NY Acad. Sci.* **691:** 148-155.

Goodman, DS, Huang, HS, Kanai, M, Shiratori, T (1967). The enzymatic conversion of all-*trans* β-carotene into retinol. *J. Biol. Chem.* **242(15):** 3543-3554.

Goodman, DS, Blaner, W (1984). Biosynthesis, absorption, and hepatic metabolism of retinol. *In* The retinoids ed. by MB Sporn, AB Roberts, DS Goodman, Academic Press, INC., Sydney, vol 2.

Grolier, P, Duszka, C, Borel, P, Alexandre-Gouabau, M, Azais-Braesco, V (1997). In vitro and in vivo inhibition of β-carotene dioxygenase activity by canthaxanthin in rat intestine. *Arch. Bioch. Biop.* **348 (2):** 233-238.

Green, PHR, Glickman, RM (1981). Intestinal lipoprotein metabolism. *J. Lipid Res.* **22:** 1153-1173.

Hasegawa, T (1993). Anti-stress effect of β-carotene. In Carotenoids in human health. Ed. by LM Canfield, NI Krinsky, JA Olson. *Ann. NY Acad. Sci.* **691:** 281-283.

Havel, RJ, Eder, HA, Bradgon, JH (1955). The distribution and chemical composition of ultra centrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345-1353.

Hayes, BJ, Burrow, HM, Hunter, RA (1995). Genetic and environmental factors affecting fat colour in carcasses of beef cattle grazing at pasture. *Proc. Aust. Assoc. Anim. Breed. Genet.* **11:** 550-553.

Hebuterne, X, Wang, X, Johnson, EJ, Krinsky, N.I (1995). Intestinal absorption and metabolism of 9-cis-ß-carotene in vivo: biosynthesis of 9-cis-retinoic acid. J. *Lipid Res.* **36:** 1264-1273.

Hebuterne, X, Wang, X, Smith, DEH, Tang, G, Russel, RM (1996). In vivo biosynthesis of retinoic acid from β-carotene involves an excentric cleavage pathway in ferret intestine. *J. Lip. Res.*, **37:** 482-492.

Hidiroglou, N, McDowell. LR, Boning, A (1986). Liquid chromatographic determination of carotenes in cattle serum and liver. *J. Vit. Nutr. Res.* **56,** 339-344.

Hill, F (1962). Biochemistry of xanthophyll pigmentation in sheep fat. *Nature*, June, **194:** 865-866.

Hirsh, J, Gallian, E (1969). Methods for the determination of adipose cell size in man and animals. *J. Lip. Res.* **9:** 110-119.

Hollander, DI (1981). Intestinal absorption of vitamins A, E, D, and K. *J. Lab. Clin. Med.* **97:** 449-462.

Hopkins, DL, (1993). Variation of carcass parameters in beef for Japan. *Proc. Aust. Soc. Prod.* **19:** 94.

Hoppe, P, Chew, BP, Safer, A, Stegemann, I, Biesalski, HK (1996). Dietary β-carotene elevates plasma steady-state and tissue concentrations of β-carotene and enhances vitamin A balance in preruminant calves. *J. Nutr.* **126:** 202-208

Huber, T (1988). Vitamins in ruminant nutrition. *In* The ruminant animal digestive physiology and nutrition, ed. by D.C. Church, A Reston book Prentice Hall, Englewood Clifs, New Jersey, 313-325.

Isler, O (1971). Carotenoids. Birkhauser Verlag, Basel.

IUPAC Commision on Nomenclature of Organic Chemistry and the IUPAC-IUB Commision on Biochemical Nomenclature. (1975). Nomenclature of carotenoids (Rules Approved 1974). *Pure Appl. Chem.* **41:** 407-431.

Jackson, PS (1981). A note on a possible association between plasma β–carotene levels and conception rate in a group of winter-housed dairy cattle. *Anim. Prod.* **32:** 109-111.

Jonas, A (1972). Physiochemical properties of bovine serum high density lipoprotein. *J. Biol. Chem.* **247:** 7767.

Karijord, O (1978). Correlation between the content of carotenoids in depot fat and in plasma of sheep. *Acta Agric. Scand.* **28:** 355-359.

Keating, EK, Hale, WH, Farris, HJr (1964). *In vitro* degradation of vitamin A and carotene by rumen liquor. *J. Anim. Sci.* **23:** 111-117.

Kerrer, P, Jucker, E (1950). Carotenoids. Elsevier Publishing Company, INC. New York.

Khachik, F, Beecher, GR, Whittaker, NF (1986). Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* **34:** 603-616.

Khachik, F., Beecher, GR, Goli, MB, Lusby, WR, Daith, CE (1992). Separation and quantification of carotenoids in human plasma. *Methods Enzymol.* **213:** 205-219.

King, TB, Lohman, TG, Smith, GS, (1962). Evidence of rumeno-reticular losses of vitamin A and carotene. *J. Anim. Sci.* **21:** 1002 (abs.).

Kirton, AH, Crane, B, Paterson, DJ, Clare, NT (1975). Yellow fat in lambs caused by carotenoid pigmentation. *N.Z. J. Agic. Res.* **18:** 865-866.

Knight, TW, Daeth, AF (1996). Effects of vitamin A supplementation on plasma carotenoid concentrations and fat colour of steers in a feedlot. *Proc. Aust. Soc. Anim. Prod.* **21:** 131-134.

Kohlmeier, L, Hastings, SB (1995). Epidemiologic evidence of a role of carotenoids in cardiovascular disease prevention. *Am. J. Clin. Nutr.* **62 (suppl):** 1370S-1376S.

Krinsky, NI, Cornwell, DG, Oncley, JL (1958). The transport of vitamin A and carotenoids in human plasma *Arch. Biochem. Biophys.* **73:** 233-246.

Krinsky, NI (1993). Actions of carotenoids in biological systems. *Annu. Rev. Nutr.* **13:** 561-587.

Kruggel, WG, Field, RA, Miller, GJ, Horton, KM, Busboom, JR (1982). Influence of sex and diet on lutein in lamb fat. *J. Anim. Sci.* **54(5)** 970-975.

Kruk, ZA., Siebert, BD, Pitchford, WS, Bottema, CDK (1998). Relationship between carotenoids and intramuscular fat content in cattle. *Proc. 44th ICOMST* **2:** 664-665.

Lakshman, MR, Chitua, O (1992). Carotenoid-protein complexes. *In* Methods in Enzymology, ed. Lester Packer, Academic Press, INC., Sydney. **214:** 74-86.

Larksman, MR, Johnson, LH, Okoh, Ch, Attlesey, M, Mychkovsky, I, Bhagavan, HM (1993). Conversion of all trans β-carotene to retinal by an enzyme from the intestinal mucosa of human neonates. *J. Nutr. Biochem.* **4:** 659-663

Larsen, TW, Yang, A, Tume, RK, (1993). The in vitro destruction of rumen fluid carotenoids by plant lipoxygenases. *Biochem. Mol. Biol. Int.* **30(2):** 197-207.

Lee, CM, Boileau, AC, Boileau, TWM., Williams, AW, Swanson, KS, Heintz, KA, Erdman, JW, Jr (1999). Review of animal models in carotenoid research. *J. Nutr.* **129:** 2271-2277.

Liaaen-Jensen, S (1971). Isolation, reactions. *In* Carotenoids. Birkhauser, Basel, 61-188.

Liebler, DC (1993). Antioxidant reactions of carotenoids. *In* Carotenoids in human health, ed. LM Canfield, NI Krinsky, JA Olson, The New York Academy of Science, 20-31.

Malau-Aduli, AEO, Siebert, BD, Pitchford, WS, Bottema, CDK (1995). Genetic variation in the fatty acid composition of cattle fat and muscle. *Proc. Aust. Assoc. Anim. Breed. Genet.* **11:** 554-557

Malau-Aduli, AEO, Siebert, BD, Bottema, CDK, Pitchford, WS, (1997). Genotype and site differences in the fatty acid composition of muscle phospholipids in cattle. *Proc. Assoc. Advmt. Anim. Bred. Genet.* **12:** 580-584.

May, SG, Savell, JW, Lunt, DK, Wilson, JJ, Laurenz, JC, Smith, SB (1994). Evidence for preadipocyte proliferation during culture of subcutaneous and intramuscular adipose tissue from Angus and Wagyu crossbred steers. *J. Anim. Sci.* **72:** 3110-3117

McCaughey, WP, Cliplef, RL (1996). Carcass and organoleptic charactersistics of meat from steers grazed on alfalfa/grass pastures and finished on grain. *Can. J. Anim.Sci.* **76:** 149-152.

McDowell, LR (1989). Vitamins in Animal Nutrition. Academic Press, INC., Sydney, 1054.

McGillivray, WA (1960) Carotenoids and vitamin A in the blood of the bovine. *N.Z. J. Agric. Res.* **3:** 934-949.

McGillvray, WA (1961). The role of carotene and vitamin A in animal feeding. *World Rev. Nutr. & Dietetics,* **2:** 135-155.

Miller, WK, Small, MD (1987). Structure of triglyceride-rich lipoproteins: an analysis of core and surface phases. In *Plasma Lipoproteins* ed. AM Gotto, Jr, ELSEVIER, NY.

Miller, MF, Cross, HR, Lunt, DK, Smith, SB (1991). Lipogenesis in acute and 48-hour cultures of Bovine intramuscular and subcutaneous adipose tissue explants. *J. Anim. Sci.* **69:** 162-170

Mora, O, Romano, JL, Gonzales, E, Ruiz, FJ, Shimada, A (1999). In vitro and in situ disappearance of beta-carotene and lutein from lucerne (Medicago stiva) hay in bovine and caprine ruminal fluids. *J. Sci. Food and Agreic.* **79(2):** 273-276.

Morgan, JHL, Pickering, FS, Everitt, GC (1969). Some factors affecting yellow fat colour in cattle. *Proc. NZ. Soc. Anim. Prod.* **28:** 164-175.

Muller, NE (1987). High-density lipoprotein: A major risk factor for coronary atherosclerosis. In *Bailliere's Clinical Endocrinology and Metabolism* **1(3):** 603-607

Nagao, A, During, A, Hoshino, Ch, Terao, J, Olson, JA (1996). Stoichiometric conversion of all *trans*-β-carotene to retinol by pig intestinal extract. *Arch. Biochem. Bioph.,* **328:** 57-63.

Nakama, S, Tanaka, M, Nakahashi, S (1986). Measurement of serum β-carotene by high performance liquid chromatography in dairy cows. *J. Japan Vet. Med. Assoc.* **39:** 355-358.

Olson, JA (1986). Physiological and metabolic basis of major signs of vitamin A deficiency. *In* Vitamin A deficiency and control. (ed.) JCBauerfeind, Academic Press, INC., Sydney, 19-67.

Olson, JA, (1989). Biological actions of carotenoids. *J. Nutr.* **119:** 94-95.

Olson, JA (1989). Provitamin A function of carotenoids: the conversion of β-carotene into vitamin A. *J. Nutr.* **119:** 105-108.

Olson, JA (1994). Absorption, transport, and metabolism of carotenoids in humans. *Pure & Appl. Chem.* **66 (5):** 1011-1016.

Olson, JA, Krinsky, NI (1995). Introduction: The colorful, fascinating world of the carotenoids: important physiologic modulators. *FASEB Journal* **9:** 1547-1550.

Palozza, P, Krinsky, NI (1992). Antioxidant effect of carotenoids *in vivo* and *in vitro*: an overview. *In* Methods in Enzymology, ed. Lester Packer, Academic Press, INC., Sydney. 403-420.

Parker, RS (1993). Analysis of carotenoids in human plasma and tissues. *Methods in Enzymol.***214:** 86-93

Parker, RS (1996). Absorption, metabolism, and transport of carotenoids. *FASEB J.* **10:** 542-551.

Parker, RS, Swanson, JE, Marmor, B, Goodman, KJ, Spielman, AB, Brenna, JT, Viereck, SM, Canfield, WK (1993). Study of β-carotene metabolism in humans using ^{13}C-β–carotene and high precision isotope ratio mass spectrometry. *In* Carotenoids in human health, ed. LM Canfield, NI Krinsky, JA Olson, The New York Academy of Science, 86-95.

Patsch, JR, Gotto, Jr, AM (1987). Metabolism of high density lipoproteins In Plasma Lipoproteins Ed. A.M. Gotto Jr., Elsevier Science Publishers B.V.,

Patterson, DSP (1965). Plasma carotenoids and fat mobilisation in stall-fed cattle. *Nature,* June, 1069.

Payne, E, Twist, OJ (1966). Yellow pigmentation in a sheep. *Aust. J. Sci.* **29(5):** 140-141.

Pfander, H (1992). Carotenoids: an overview. In *Methods in Enzymology.* **213** ed. by Lester Packer, Academic Press, INC., Sydney 3-13.

Pitt, GAJ (1971). Vitamin A. *In* Carotenoids. Birkhauser, Basel, 717-742.

Plaas, HA, Cryer, A (1980). The isolation and characterisation of a proposed adipocyte precursor cell type from bovine subcutaneous white adipose tissue. *J. Dev. Physiol.* **2(5):** 275-289

Poor, CL, Bierer, TL, Merchen, NR, Faney, GC, Erdman, JW (1993). The accumulation of α-carotene and β–carotene in serum and tissues of preruminant calves fed raw and steamed carrot slurries. *J. Nutr.* **123(7):** 1296-1304.

Prior, RL (1983). Lipogenesis and adipose tissue cellularity in steers switched from alfalfa hay to high concentrate diets. *J. Anim. Sci.* **56(2):** 483-492.

Raphael, BC, Dimick, PS, Puppione, DL (1973). Electrophoretic characterisation of bovine serum lipoproteins through gestation and lactation. *J. Dairy Sci.* **56:** 1411-1414.

Redlich, CA, Grauber, JN, Van Bennekum, AM, Clever, SL, Ponn, RB, Blaner, WS (1996). Characterisation of carotenoid, vitamin A, and α-tocopherol levels in human lung tissue and pulmonary macrophages. *Am. J. Respir. Crit. Care Med.* **154:** 1436-1443.

Romanchik, JE, Morel, DW, Harrison, EH (1995). Distribution of carotenoids and a-tocopherol among lipoproteins do not change when human plasma is incubated in vitro. *J. Nutr.* **125:** 2610-2617.

SAS Institute, Inc. (1989). SAS user's guide. Ver. 5.04, Cary, NC.

SAS Institute, Inc. (1996). SAS user's guide. Ver. 6.03, Cary, NC.

Schmitz, HH, Poor, CL, Wellman, RB, Erdman, JW (1991). Concentration of selected carotenoids and vitamin A in human liver, kidney and lung tissues. *J. Nutr.* **121:** 1613-1621.

Schweigert, FJ, Rambeck, WA, Zuker, H (1987). Transport of β-carotene by the serum lipoproteins in cattle. *J. Anim. Physiol. Anim. Nutr.* **57,** 162-167.

Schweigert, FJ, Wierich, M, Rambeck, WA, Zucker, H (1988). Carotene cleavage activity in bovine ovarian follicles. *Theriogenology* **30:** 923-930.

Scita, G (1992). Stability of β-carotene under different laboratory conditions. *Methods Enzymol.* **213:** 175-185.

Seirer, R.C, Gaunt, GM, Thatcher, LP (1992). Final Report to Meat Research Corporation. Dep. Agric., Rutherglen Research Institute, 11-17.

Shemeis, AR, Liboriussen, T, Bech Abdersen, B, Abdallah, OY (1994). Changes in carcass and meat traits of Danish Friesian cull cows with the increase of their age and body composition. *Meat Sci.* **37:** 161-167.

Shepard, J, Packard, CJ (1987). Lipid transport through the plasma: the metabolic basis of hyperlipidemia. *In* Bailliere's Clinical Endocrinology and Metabolism, Bailliere Tindal, Sydney, vol. 1, no.3, 495-511.

Siebert, BD, Deland, MP, Pitchford, WS (1996). Breed differences in the fatty acid composition of subcutaneous and intramuscular lipid of early and late maturing, grain-finished cattle. *Aust. J. Agric. Res.* **47:** 943-952.

Singh, H, Cama, HR (1974). Enzymatic cleavage of carotenoids. *Biochim. Biophys. Acta,* **370:** 49-61.

Singh, H, Cama, HR (1975). Metabolism of carotenoids. *J. Scien. Ind. Res.* **34:** 219-230.

Smith, U, Sjostrom, L, Bjorntorp, P (1972). Comparison of two methods for determining human adipose cell size. *J. Lipid Res.* **13:** 822-824.

Snodderly, DM (1995). Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* **62 (suppl):** 1448S-1461S

Stahl, W, Sies, H (1993). Physical Quenching of singlet oxygen and *cis-trans* isomerization of carotenoids. *In* Carotenoids in human health, ed. by L.M. Canfield, NI, Krinsky, JA, Olson, The New York Academy of Science, 10-19.

Strachan, DB, Yang, A, Dillon, RD (1993). Effect of grain feeding on fat colour and other carcass characteristics in previously grass-fed *Bos indicus* steers. *Aust. J. Exp. Agric.* **33:** 269-273.

Strzalka, K, Gruszecki, WI (1994). Effect of β–carotene on structural and dynamic properties of model phosphatidylcholine membranes. I. An EPR spin label study. *Biochim. Biophys. Acta,* **1194:** 139-142.

Subczynski, WK, Markowska, E, Sielewiesiuk, J (1991). Effect of polar carotenoids on the oxygenated diffusion-concentration product in lipid bilayers. An ESR spin label study. *Biochim. Biophys. Acta* **903:** 132-141.

Tall, AR, Puppione, DL, Kunitake, ST, Atkinson, D, Small, DM, Waugh, D (1981). Organization of the core lipids of high density lipoproteins in the lactating bovine. *J. Biol. Chem.* **256:** 170-174.

Thatcher, AJ, Lee, CM, Erdman, JWJr (1998). Tissue stores of β-carotene are not conserved for later use as a source of vitamin A during compromised vitamin A status in Mongolian Gerbils (Meriones unguiculatus). *J. Nutr.* **128 (7):** 1179-1185.

Townsend, SJ, Siebert, BD, Pitchford, WS (1997). Variation in milk fat content and fatty acid composition of Jersey and Friesian cattle. *Proc. Assoc. Advmt. Breed. Genet.* **12:** 283-286.

Traber, MG, Diamond, SR, Lane, JC, Brody, RI, Kayden, HJ (1994). β-Carotene transport in human lipoproteins. Comparisons with α-tocopherol. *Lipids,* **29(10):** 665-669.

Tucker, RE, Mitchell, GEJr, Little, CO (1967). Absorption of labelled carotene from the large intestine of sheep. *J. Anim. Sci.* **26:** 225-226 (abs.).

Underwood, BA (1984). Vitamin A in animal and human nutrition. *In* The retinoids. (ed.) MB Sporn, AB Roberts, DS Goodman, Academic press, INC., Sydney, **1:** 281-392.

Walker, PJ, Warner, RD, Winfield, CG (1990). Sources of variation in subcutaneous fat colour of beef carcasses. *Proc. Aust. Soc. Anim. Prod.* **18:** 416-419.

Wang, XD, Tang, GW, Fox, JG, Krinsky, NI, Russell, RM (1991). Enzymatic conversion of beta-carotene into beta-apocarotenals and retinoids by human, monkey, ferret, and rat tissues. *Arch. Biochem. Biophys.* **285:** 8-16.

Wang, XD (1994). Review: absorption and metabolism of beta-carotene. *J. Am. Coll. Nutr.* **134:** 314-325.

Wang, X, Krinsky, NI, Benotti, PN, Russel, RM (1994). Biosynthesis of 9-cis-retinoic acid from 9-cis-β-carotene in human intestinal mucosa in vitro. **313:** 150-155.

Weddon, BCL (1971). Stereochemistry. *In* Carotenoids. Birkhauser, Basel, 267-324.

Wing, JM (1969). Effect of source and season on apparent digestibility of carotene in forage by cattle. *J. Dairy Sc.* **52(4):** 479-483.

Wolf, G, Phil, D (1995). The enzymatic cleavage of β–carotene: still controversial. *Nutr. Rev.* **53:** 134-137.

Van der Berg, H, Van Vliet, T (1998). Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl ester responses in the triacylglycerol-rich lipoprotein fraction of men. *Am. J. Clin. Nutr.* **68:** 82-89.

Van Poppel, G, Goldbohm, RA (1995). Epidemiological evidence for β-carotene and cancer prevention. *Am. J. Clin. Nutr.* **62 (suppl):** 1393S-1402S.

Van Steveninck, J, De Goeij, AFPM (1973). Determination of vitamin A in blood plasma of patients with carotenaemia. *Clin. Chim. Acta,* **49:** 61-64.

Van Vliet, T, Van Vlissingen, MF, Van Schaik, F, Van Den Berg, H (1996). β-Carotene absorption and cleavage in rat is affected by the vitamin A concentration of the diet. *J. Nutr.* **126:** 49-508.

Villard, L, Bates, CJ (1986). Carotene dioxygenase (EC 1.13.11.21) activity in rat intestine: effects of vitamin A deficiency and of pregnancy. *Br. J. Nutr.* **56:** 115-122.

Visser, FR, Blair, KA (1991). β-Carotene content of twelve pasture crops measured over one dairy season. *NZDRI Report No. FV91R11,* New Zealand Dairy Research Institute, Palmerston North.

Yamanushi, T, Igarashi, O (1995) The mobilization and tissue distribution of β-carotene in the rat by the venous injection method. *J. Nutr. Sci. Vitam.* **41:** 169-177.

Yang, A, Larsen, TW, Tume, RK (1992). Carotenoid and retinol concentrations in serum, adipose tissue and liver and carotenoid transport in sheep, goats and cattle, *Aust. J. Agric. Res.* **43:** 1809

Yang, A, McLennan, SR, Armstrong, J, Larsen, TW, Shaw, FD, Tume, RK (1993). Effect of short-term grain feeding on bovine body-fat colour: a cautionary note. *Aust. J. Agric. Res.* **44:** 215-220.

Yang, A, Tume, RK (1993). A comparison of β-carotene-splitting activity isolated from intestinal mucosa of pasture-grazed sheep, goats and cattle. *Biochem. Molec. Biol. Inter.* **30:** 209-217

Yang, CS, Lee, M (1987). Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies. *J. Nutr.Growth & Canc.* **4:** 19-27.

Zhou, GH, Yang, A, Tume, RK (1993). A relationship between fat colour and fatty acid composition. *Meat Sci.* **35:** 205-212.

Ziegler, RG (1993). Carotenoids, cancer, and clinical trials. *In* Carotenoids in human health ed by L.M. Canfield, NI, Krinsky, JA, Olson, The New York Academy of Science, 110-119.