Genomic approach to understanding variation in bovine fat colour

Presented by

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Declaration

I declare that this thesis is a record of original work and contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Rugang Tian except where as stated in chapter 3, some sequencing (*BCO2* gene) was completed as part of a research project for a Masters by Coursework degree at the University of Adelaide in 2006. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

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Dedications

I dedicate this work to my parents and my wife, Yuan Li, for their great support with love during the period of this study.

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Abstract

Subcutaneous fat is important not only in the live animal but also in the carcass, as it prevents the rapid chilling of the underlying muscle tissues, thereby reducing weight loss during chilling. However, beef with yellow fat is considered undesirable by consumers in most European and Asian markets. Beta-carotene is the major carotenoid deposited in adipose tissue, which results in the yellowness. Genes involved in the metabolism of β-carotene in the cattle are likely to regulate beef fat colour. Therefore, DNA variants in candidate genes related to β-carotene metabolism were examined for association with beef fat colour.

Based on their location in fat colour quantitative trait loci (QTL) and function in the metabolism of β-carotene, *ALDH8A1*, *APOM*, *BCMO1*, *BCO2*, *RARA*, *RDHE2*, *PPARGC1A* and *SCARB1* were chosen as candidate genes. One hundred eleven (111) DNA variants were identified from the direct sequencing of 3 F1 sires for these eight genes, of which, 27 DNA single nucleotide polymorphisms (SNPs) were selected for association studies (3-5 SNPs per gene). Most of these genotyped SNPs and their interactions were associated with fat colour related traits (biopsy fat colour (Fc-bio), carcass fat colour (Fc-car), beta-carotene concentration (Bc-bio)), although the size of the effects was relatively low for many of the variants. However, among the DNA variants, a nonsense mutation in the *BCO2* gene (*BCO2* W80X) accounted for a large proportion (12-16%) of the total SNP variation in fat colour related traits in Jersey-Limousin backcross progeny. Validation of this SNP in other independent herds (Group 2-7) confirmed the *BCO2* W80X genotype has a large effect on beef fat colour and milk colour. The individual genotypic effects of *RDHE2* SNP2 and SNP3 were also large.

However, these effects were greater in the New Zealand abattoir samples than from pedigreed Jersey-Limousin backcross progeny, amounting to 8-17% of the variance in one population. There was a significant interaction between the *BCO2* W80X and the *RDHE2* SNP2, which accounted for 1.8% of the total SNP variance in milk fat colour in a New Zealand Holstein cow population, and 4.0% of the total SNP variance in carcass fat colour in New Zealand Jersey-Limousin backcross progeny.

In addition to the individual SNP effects, the effects of the haplotypes formed for each gene were also investigated. Only haplotypes of *BCMO1*, *PPARGC1A*, *RDHE2* and *SCARB1* genes had effects on beef fat colour.

The most likely pathways involved in the beef fat colour were clarified. The association studies showed that the *BCMO1*, *BCO2*, *RARA*, *RDHE2*, *PPARGC1A* and *ALDH8A1* genes and their interactions account for a large proportion of the variation in beef fat colour. These genes have roles involving retinol or retinoic acid synthesis. Therefore, the retinol/retinoic acid synthesis pathway appears to be the most important in terms of the contribution to the β-carotene concentration in adipose tissue. The effects of APOM and SCARB1 indicate that transportation of β-carotene is also important in the regulation of the β-carotene concentration in fat.

Differences in the expression of *BCMO1*, *BCO2* and *RDHE2* genes were investigated. The *RDHE2* gene mRNA transcript level was significantly different between yellow fat and white fat samples. The gene expression of *BCO2* was highly correlated with β-carotene concentration. The results further support the role of *BCO2* in cleaving β-carotene eccentrically and the association of *RDHE2* with β-carotene concentration.

The results also indicate that the control of the retinol/retinoic acid pathway at the gene expression level is important for the β-carotene concentration in subcutaneous adipose tissue and consequently, for beef fat colour.

The study conducted herein contributes to the understanding of the metabolism of carotenoids and their numerous derivatives. BCO2-mediated conversion of β-carotene to vitamin A is confirmed in cattle. Epistatic effects accounted for much of the beef fat colour and β-carotene concentration variation. DNA variants that have a large influence on fat colour, such as the *BCO2* W80X, can be used in marker selection systems to rapidly reduce the incidence of yellow fat colour in beef.

Chapter 1

Literature Review

1.1 Introduction

Subcutaneous fat is important in both live animals and carcasses. The function of this fat depot includes acting as an energy deposit (that is, energy storage), being protective padding and providing insulation against body temperature losses (Thompson *et al.*, 1983). Also, carcass fat plays an important role in meat quality. Carcasses are susceptible to cold shortening and weight losses during chilling. Surface fat prevents the rapid chilling of the underlying muscle tissues, thereby reduces weight loss and the likelihood of cold shortening. However, if the beef fat is yellow, then this is considered undesirable by consumers in most European and Asian markets. Presumably, this is because beef with yellow fat is perceived as being from old or diseased animals (Morgan and Everitt, 1969). β-carotene is the major carotenoid deposited in adipose tissue and milk fat, which results in the yellowness (Palmer and Eckles, 1914, Morgan and Everitt, 1969). Conventional breeding methodologies are limited by the lack of phenotypic progeny data for traits like beef fat colour. There has been increasing emphasis on the development of molecular genetic tools, such as DNA variants or polymorphisms for marker-assisted selection, in order to improve traits that lack progeny trait data (Ibeagha-Awemu *et al.*, 2008). Over the past decade, candidate genes have been identified with possible association with these traits and may explain the reported quantitative trait loci (QTL) for the traits. As β-carotene is the major contributor to the yellow fat in beef, obvious candidate genes to improve fat colour would be the enzymes and proteins required for the metabolism and transport of β-carotene.

1.2 Fat colour and market specification

Australia is one of the world's most efficient producers of cattle and the world's second

largest beef exporter. The value of total beef and veal exports in 2009-2010 was $4.1 billion (Meat and Livestock Australia, 2010). The high quality of Australian beef is critical in order to maintain or increase the share of the international beef trade. Meat texture, tenderness, juiciness, meat colour, fat colour and flavour are the most important beef quality criteria (Egan *et al.*, 2001). As one of the assessment criteria, the quality of fat can markedly affect the carcass value at the wholesale level. The fat quality attributes of colour, hardness and texture are important for specific markets. Beef fat with yellow or dark colour affects the carcass grade, and results in a lower price as consumers in Mediterranean Europe, North-East Asia and United States prefer 'white' or 'pale' adipose tissue. It has been reported by Hayes (1995) that in Australia, based on a downgrading of 10% of the export carcasses due to yellow fat, a reduction in incidence of yellow fat by 50% would yield an annual saving of $9.2 million for Australian beef producers.

1.3 Factors affecting fat colour

Beef fat colour is affected by many factors. These include both extrinsic factors (non-genetic), such as nutrition, environment and management, as well as intrinsic factors (genetic), such as breed, gender, age at slaughter and fatness.

1.3.1 Non-genetic factors

1.3.1.1 Diet

Diet is the major factor to influence fat colour in cattle. Pasture represents the largest component of ruminant diets in most countries, and is usually rich in carotenoid pigments. When cattle graze green pasture, the carotenoids are deposited in the adipose

tissue and cause the fat colour to became yellow (Morgan and Everitt, 1969). The carotene in forage plants is generally most abundant in fresh pasture (Figure 1-1). Hay, silage and so called “concentrated feedstuffs” (which are usually either whole grain cereals or formulated concentrates containing specified proportions of individual ingredients) have relatively low levels of carotenoids. Carotenoid concentration in the sun-dried forages is decreased dramatically when rain occurs during haymaking (Park *et al.*, 1983). This carotenoid loss is mainly due to solar radiation because the carotenoids are destroyed when they are exposed to UV rays (Cardinault *et al.*, 2004) or due to the oxidation during in-barn hay storage because of the presence of oxygen (Bruhn and Oliver, 1978).

Walsh *et al*. (2008) reported that when feeding steers five contrasting diets, carcass fat yellowness tended to decrease in the order of grass silage> whole crop wheat silage > ad libitum concentrates > maize silage > alkalage. Alkalage is produced by preserving whole-crop (grain + stem + leaves) cereals using a process of ammoniation. It was also reported that steers finished on pasture have more yellow carcass fat than their counterparts of equivalent carcass weight finished on maize silage plus concentrates, both at 24 hours and 7 days post-mortem (Varela *et al.*, 2004). In Japan, Japanese Black steers fed roughage containing very little β-carotene have very low levels of yellow colour in the intramuscular fat and subcutaneous fat (Muramoto *et al.*, 2003). In general, feeding diets low in carotenoids tends to produce less yellow fat colour than diets containing green forage.

Feed	β-carotene (mg/kgDM)	Lutein (mg/kgDM)
Forages		
Fresh green legumes and grasses, immature (wet basis)	33–88[a]; 200–700[b]; 136–384[c]; 294–384[d]; 409, 134[e]	806, 356[e]
Legume silage (wet basis)	11–44[a]	NR
Maize and sorghum silages, medium to good green colour (wet basis)	4 – 22[a]; 9.1–18[f]	NR
Whole crop wheat silages, with/without urea application	NR	NR
Non-legume hays, including timothy, cereal and prairie hays, well-cured, good green colour	20–31[a]	NR
Non-legume hays, average quality, bleached, some green colour	9–18[a]	NR
Legume hays, including alfalfa, very quickly cured with minimum sun exposure, bright green, leafy	77–88[a]	NR
Cereals		
Barley	11–63.5[g], 10–12[h]	6–10[h]
Wheat	4- 9[ij]	NR
Root Crops/Miscellaneous		
Peas	3[k]	NR
Potatoes	Trace[k]	NR
Turnip	0.2[k]	NR
Carrot	125[k]	NR
Curly kale	32[k]	NR

NR: not reported.
[a] Adapted from McDowell (2000), reported as mg carotene/kg.
[b] Coultate (1996).
[c] Bauernfeind et al. (1981), perennial ryegrass *Lolium perenne*.
[d] Bauernfeind et al. (1981), white clover, *Trifolium repens*.
[e] Knight et al. (1996b), values for November, December.
[f] Nehring and Hoffmann (1967).
[g] Alvarez, Martín, and Martín (1999).
[h] Knight et al. (1996b).
[i] Kumar, Kaswan, and Madan (1995) and Mahal et al. (1998).
[j] Kumar et al. (1995) and Mahal et al. (1998).
[k] Food Standards Agency (2002), results are mean values and are given as mg carotene/kg.

Figure 1-1 β-carotene and lutein content of forages, cereals and root crops used in cattle diets
(from Dunne *et al*., (2009))

1.3.1.2 Season

Fat colour also can be influenced by season as β-carotene concentration fluctuates in pastures. For example, in British forage, β-carotene levels reached a maximum value during mid-season, with levels dropping in both the spring and autumn (Forrest, 1981). Consequently, there is a higher intake and deposition of carotene in cattle adipose tissues in the wet season. Hayes *et al.* (1995) also reported that in Australia, animals slaughtered in the dry season following the wet season had more yellow fat than those slaughtered in the wet season following the dry season in a study using several breeds, which included Brahman (B), Hereford (H) x Shorthorn (S), Brahman x HS (BX), Africander x HS (AX) and reciprocal AX x BX (AXBX) ($P<0.01$). However, Kruk (2001) reported there was no significant difference between summer and winter for total carotenoid content in the fat (3.36 ± 0.17μg/g in summer and 3.18 ± 0.66μg/g in winter) or subjective fat colour score (2.24 ± 0.17 in summer and 2.41 ± 0.17 in winter) in Jersey and Limousin cows. The lack of a difference could be a result from a slow turnover of carotenoids in the adipose tissue or the marginal difference of β-carotene concentration in the green feed between two seasons. Alternatively, season was confounded with age in this study, as old cows were all slaughtered in the dry season and only young animals were slaughtered in the wet season.

1.3.1.3 Management strategies

One approach to reduce the fat colour of grass-fed cattle is to finish them on a grain-based (low carotenoid) diet for several months. The length of finishing period is of critical importance to beef producers since this impacts on the profitability of beef production. Generally for the beef industry, it is recommended that 90 days of grain feeding before slaughter is required to reduce the yellow colour of carcass fat (Miller,

2002). However, the length of the finishing period and the type of finishing diets that is required varies. Several authors has demonstrated that it only took 28 days to reduce carotenoid concentration and fat colour score significantly in subcutaneous fat of cattle, which were fed a non-grass based diet before slaughter (Forrest, 1981, Knight *et al.*, 2001, Dunne *et al.*, 2006). In contrast, in other studies, there was no decrease in fat yellowness even after 56 days of feeding with a low carotenoid ration (Yang *et al.*, 1993, Morris *et al.*, 1997). The failure to decrease fat yellowness after 28 days could result from a difference of fat deposition in those studies. It is reported that steers fed a low carotenoid ration had a lower fat depth, perinephric and pelvic fat weight than the pasture-fed controls, although the plasma β-carotene concentration decreased to low values in the steers (Morris *et al.*, 1997). Similarly, in the study by Yang *et al*. (1993), steers fed a low carotenoid diet for 56 days had low irregular growth rates suggesting that they had little increase in fat deposits. The fat deposition effect on duration for the finishing period was further supported by Knight's study (2001). In this experiment, 24 to 26-month-old steers in 3 groups (n = 13 per group) were fed a ration of maize grain and pasture silage, containing 33 mg β-carotene/kg dry matter. The ration was fed to steers for 63 days with the aim of gaining weight rapidly, gaining weight slowly, or losing weight. A fourth group of steers (n = 9) was slaughtered at the beginning of the experiment as the control. The growth rates of the steers were 1.72, 0.71, and –0.03 kg/head/day for those gaining weight rapidly, slowly and losing weight, respectively. Steers gaining weight rapidly tended to have heavier and fatter carcasses than other groups. The yellowness and carotenoid concentrations of subcutaneous fat of the steers gaining weight rapidly were significantly lower than the control group or those cattle losing weight ($P < 0.05$).

1.3.1.4 Age

The colour of intensity of fat is related to animal age. It has been observed that older culled cows have higher fat colour scores than younger animals. The ability of cattle to turnover carotenoids in the adipose tissue depends on age (Shemeis *et al.*, 1994, Hayes *et al.*, 1995). Buchanan-Smith and Mandell (1994) observed no yellow subcutaneous fat when younger animals (under 2 years of age) were fed a wide range of forage diets, including pasture. However, if the concentration of carotenoids is extremely high, no difference in fat colour was observed between young and old cows (Morgan and Everitt, 1969, Kruk, 2001). The contradictory reports could be explained by the fact that 1) different breeds of cattle were studied, 2) samples in different body sites were assessed (carcass *vs.* biopsy sample), 3) there were different criteria for the dividing age groups, and 4) the ranges of β-carotene concentration were different. This influence of age may be apparent only if the concentration of carotenoids in fat or the fat colour scores are relatively low.

1.3.1.5 Sex

There are contradictory reports about the influence of sex on fat colouration. Higher fat colour scores were found in female than male cattle from British breeds, Brahmans and Brahman cross-breeds (Hayes *et al.*, 1995). Reynoso *et al.* (2004) reported that in crossbreds of Zebu, Brown Swiss, Charolais and Brahman from two tropical regions of Mexico, the content of β-carotene in adipose tissue in female animals (42 μg/100 g) was greater than that in males (26 μg/100 g). However, no difference was observed between heifers and steers in other studies (Morgan and Everitt, 1969, Kruk, 2001). This difference in these reports again could be due to confounding breeds, age and total carotenoid concentrations in the adipose tissue. It is unclear why there would be a

difference between female and male animals other than the fact that female cattle deposit fat more readily than male cattle (Mcintyre *et al.*, 2009).

1.3.1.6 Growth rate

Dilution of carotenoids can render the adipose tissue less yellow. Generally, cattle on grain-fed diets accrete more adipose tissue than cattle of a similar age fed grass or forage. Consequently, the carotenoids are more dilute in the adipose tissue. Pitchford *et al.* (2002) reported that fat colour scores were reduced from 3.0 to 0.5 (~83% decrease) in a short period of grain feeding (often around 70 days). Because the P8 fat depth increased from 8.7 to 12.9 mm and total β-carotene remain the same during this time, the β-carotene concentration fell from 2.325 to 1.125 μg/g of fat (~52% decrease). This indicates grain feeding can result in a large improvement in fat colour score in a short period of time by dilution of previous accumulated β-carotene in the fat tissue, rather than by turnover of accumulated β-carotene in fat tissue. In addition, it has been shown that the growth rate, which influences fat deposition, has an impact on beef fat colour. Knight *et al.* (2001) demonstrated that steers, which have poor growth rates, are unlikely to have a reduced fat yellowness despite feeding a ration with a low carotenoid concentration. Gazzola *et al.* (2001) showed that castration alters the portioning of food energy to fat and muscle growth, and subsequently, produces a suitable fat cover in the carcass. Bulls castrated at 18 months have whiter fat than bulls castrated at 9 months. In their experiment, the late castrated cattle did not accumulate fat during the first growth phase on green feed, whereas the early-castrated bulls did fat accumulation in the first season of green feed. It was assumed that both groups of cattle should have accumulated similar carotene levels and fat during the second growth period on grain feed. Thus, the difference in fat colour is presumably a result of differential

accumulation of β-carotene during the first growth period which was affected by the time of castration.

1.3.2 Genetic factors

In addition to the impact of environmental factors, the variation in fat colour or carotenoid concentration in fat between different breeds, as well as between sires within a breed in the same environment, suggests genetic factors also affect fat colour and carotenoid concentration in fat.

Most studies have confirmed that fat colour in cattle can be influenced by breed. Generally, the dairy breeds have more yellow fat colour than the beef breeds. Walker (1990) compared subcutaneous fat colour for cattle which were slaughtered at 30 months of age over 5 consecutive years among five beef breeds (Angus, Beef Shorthorn, Galloway, Hereford and Red Poll), and four dairy breeds (Friesian, Milking Shorthorn, Ayrshire and Jersey). The beef breeds had significantly ($P<0.01$) more carcasses with white fat than the dairy breeds. Among all these breeds, the Jersey had the highest fat colour score. According to Hayes *et al*. (1995), the variation in the intensity of fat colour between breeds (Brahman (B), Hereford x Shorthorn (HS), Brahman x HS (BX), Africander x HS (AX) and reciprocal AX x BX crosses) is large, which indicates that fat colour in cattle probably is under genetic control. Data from crosses between Hereford cows and sires from seven breeds (Jersey, Wagyu, Angus, Hereford, South Devon, Limousin, and Belgian Blue) also indicated that Jersey-sired progeny has higher fat colour scores than progeny of other breeds (Pitchford *et al.*, 2002). A study conducted by Kruk *et al*. (1998) showed that Jersey cattle have higher heritability estimates for subcutaneous fat carotenoid concentration than either Jersey x Limousin

crosses or pure Limousin cattle. Therefore, the authors suggested that there is a major gene(s) involved in accumulation of β-carotene in the Jersey (dairy breed) that results in increased yellow fat colour.

Studies indicate that yellow fat colour is under genetic control in sheep as well. The heritability of yellow fat colour in sheep was estimated to be 0.18, based on half-sib analysis of 33 sires (Kirton *et al.*, 1975). It was also suggested that a homozygous recessive genotype causes yellow fat colour in sheep (Baker *et al.*, 1985). However, in cattle, the heritability estimates are relatively higher. It has been reported that the heritability of yellow fat score was 0.32 (SE 0.11) in a study which included 585 pedigreed animals across four years. Because there were 131 sires from 6 breeds represented in the data though, this estimate should be regarded as tentative (Hayes *et al.*, 1995). Pitchford *et al.* (2002) also reported an estimated heritability for fat colour of 0.33 (SE 0.06). However, most of the genetic variation came from the Jersey cross calves because when the Jersey cross animals were removed from the data set, then the heritability decreased to 0.16. Nevertheless, all the heritability estimates are sufficiently high to suggest that fat colour can be improved by genetic means.

Unfortunately, there is no consistent correlation between fat colour or carotenoid concentration in subcutaneous fat and serum carotenoid concentration. The phenotypic correlation between fat colour score and β-carotene concentration in plasma was positive but low with r =0.20 in one study (Hayes *et al.*, 1995), although a moderate correlation (r = 0.49) was reported in another study (Strachan *et al.*, 1993). In contrast, subcutaneous fat carotenoid concentration was highly correlated with the subjective fat colour score (r=0.61) and objective assessment of fat colour readings (r = 0.73),

respectively (Strachan *et al.*, 1993). Furthermore, unlike carotenoid concentration changes in adipose tissue, plasma carotenoid concentration decreases much more rapidly when animals are removed from pasture (Yang *et al.*, 1993, Knight *et al.*, 1994). The dynamic rate of turnover of β-carotene in blood and the relationship with β-carotene concentration in the adipose tissue is still not clear. Nevertheless, the β-carotene concentration in plasma is not a useful predictor of fat colour in the carcass.

In summary, beef fat colour is directly associated with β-carotene concentration. Environmental factors, such as management, diet, season, age and sex, influence beef fat colour. In addition, growth rate can alter fat depths and subsequently, impact carotenoid concentration in adipose tissues. As well, the variation in fat colour or carotenoid concentration in fat between different cattle breeds and within a breed indicates fat colour is controlled by genetic factors. Major genes involved in the accumulation of β-carotene in the fat of Jersey cattle has been suggested but not yet investigated.

Quantitative trait loci (QTL) mapping can help to dissect the complexity of beef fat colour genetics by identifying candidate genomic regions affecting the trait variation. Unfortunately, quantitative trait loci have not been routinely mapped for beef fat colour with one exception. A genome scan has been performed in Jersey-Limousin backcross progeny from Australia and New Zealand to map QTL for diverse 34 traits, which included fat colour related traits (Esmailizadeh, 2006). In that study, 14 suggestive QTL segregating for fat colour traits on 11 chromosomes were detected by interval mapping. However, there is no knowledge of the responsible gene(s) for these identified QTL or their allelic variation and modes of action underlying fat colour variation.

1.4 Carotenoids and fat colour

In the context of the yellow fat colour, carotenoids play the major role. In order to identify the genetic basis of fat colour, it is important to understand the metabolism of carotenoids. Carotenoids are a group of chemical compounds found in plants. These include the xanthophylls, carotene and lycopene, which are responsible for yellow, orange and red colouring, respectively. Despite the large variety of carotenoids in plants, most quantitatively important in fat are β-carotene and lutein. There are many different isomeric forms of carotenes. All-*trans* β-carotene is the isomer most commonly found in forage crops (Kalac and Mcdonald, 1981) and is the main circulating carotenoid in cattle (Yang *et al.,* 1992).

Among the 600 carotenoids found in nature, β-carotene is one of the most potent vitamin A precursors and is also a physiological antioxidant (Lejeune *et al.*, 2000). As only microorganisms and plants can synthesize carotenoids, carotenoids in animal tissues are derived strictly from the feed. The β-carotene concentration in adipose tissue is determined by the nature and amount in the diet as well as by the rate of transfer to the adipose tissue. The different transport steps (including rumen digestion, intestinal absorption, transfer to tissues via the blood, and individual tissue metabolism) influence carotenoid deposition in the mammary gland and adipose tissue (Parker, 1996).

1.4.1 Carotenoid function

One of the main functions of carotenoids is to serve as precursors of vitamin A. Vitamin A is known to be essential for mammals. The functions of vitamin A include a role in visual processes, maintenance of epithelial surfaces, bone growth, reproduction, immunological responses, disease control, plus embryonic growth and development

(Blomhoff and Blomhoff, 2006). The plant-derived carotenoids displaying provitamin-A activity must be first absorbed, delivered to the site of metabolism within the body, and then cleaved to form one or two functional retinoid molecules (vitamin A) to become biologically active. There are three forms of vitamin A, which include retinal, retinol, and retinoic acid, and represent the aldehyde form, the alcohol form and the oxidized form of vitamin A, respectively. Retinal, a precursor of retinol, is a necessary structural component of the light-sensitive pigment, rhodopsin, which is found within the rod and cone cells of the retina. Retinoic acid is a potent developmental signal that acts through gene regulation (Chambon, 1996). As such, it is crucially important for development, cell differentiation, cancer prevention, plus membrane and skin protection (Biesalski *et al.*, 2007).

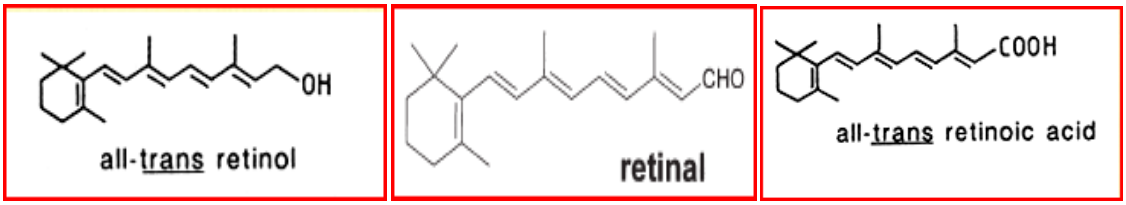


Figure 1-2 Three forms of vitamin A

Carotenoids also have functions other than provitamin A activity. Because carotenoids quench singlet oxygen or free radical formation, they are important in treating photo sensitization. Other non-provitamin A carotenoids (such as lutein, zeaxanthin and lycopene) and untransformed β-carotene have anti-oxidative properties and protect mammalian tissues from oxidative damage (Moise *et al.*, 2005).

Beta-carotene also plays a role in delaying the onset of LDL oxidation by desaturating saturated fatty acids (an oxidative process) (Lapointe *et al.*, 2006). In a study with cattle

breeds that had either yellow fat or white fat, e.g., Channel Island breeds (Jersey) vs. European breeds (Limousin), animals with the higher β-carotene in their fat had higher mono-unsaturated fatty acid levels than the cattle with less β-carotene in their fat even when fed the same quantity of β-carotene (Siebert *et al.*, 2003).

1.4.2 Ruminal digestion

Different absorption efficiencies of β-carotene occur in ruminants and non-ruminants. In humans, many factors affect the efficiency of β-carotene release from the food or supplement matrix, such as heating, blending, and finely chopping the food before consumption (Deming and Erdman, 1999). After the ingestion of food, β-carotene is released by gastric hydrolysis of dietary lipids and proteins. However, the extent of release and the physical-chemical state of the carotenoids in the stomach is not known (Yeum and Russell, 2002). Before absorption in ruminants, such as cattle, the first step is degradation of the vegetable matrix to release carotenoids into the rumen liquid phase (Mora *et al.*, 1999). The reported carotenoid degradation by microorganisms in the rumen is quite controversial. Dawson and Hemington (1974) reported that there is no degradation in the whole sheep rumen. In cattle, moderate degradation (10–34%) was found in *in vitro* studies when β-carotene was incubated in bovine rumen fluid for 9 to16 hours (King *et al.*, 1962, Keating *et al.*, 1964, Mora *et al.*, 1999). However, in other *in situ* studies, higher β-carotene degradation rates were reported of 40–55% by King *et al*. (1962) and 57-91% by Mora (1999). The varying results are most likely attributable to the form in which the carotenoids were delivered. Degradation rates are usually higher when carotenoids were supplied as purified products than when provided in forage (Noziere *et al.*, 2006a). Moreover, the loss of β-carotene may result from plant lipoxygenase bleaching (Larsen *et al.*, 1993) or adherence to cellular components (Mora

et al., 1999).

1.4.3 Carotenoid absorption

Having passed through the rumen, β-carotene is solubilized into mixed lipid micelles with bile components and hydrolysates of dietary lipids in the lumen of the small intestine. Transfer of carotenoids from emulsion to micelles could be the limiting step for intestinal carotenoid absorption (Deming and Erdman, 1999).

Carotenoids were assumed to be absorbed in the small intestine via passive diffusion, similar to that of cholesterol and the products of triacylglycerol lipolysis (Parker, 1996). However, differences among animal species or parts of the intestine involved in carotenoid absorption suggest an active mechanism may occur. This was recently supported by cell culture studies. The results showed that saturation of β-carotene transport in Caco-2 cells occurred at concentrations (>15 μM) higher than "physiological" concentrations. Also, the intestinal absorption of carotenoids was facilitated by the participation of a specific epithelial transporter scavenger receptor B1 (SR-B1).

Other factors can affect carotene absorption, such as the presence of lutein, vitamin A, vitamin E and fatty acids. There is an interaction between β-carotene and α-tocopherol (the dominant isomeric form of vitamin E) with regard to absorption from the small intestine into the bloodstream. The effects of pasture-feeding alone or with vitamin E supplementation on tissue levels of anti-oxidants were evaluated in a study conducted by Daly *et al.* (1999). In this study, 32 Hereford cross steers were divided into four groups of eight animals each. The treatments were pasture supplemented with and

without vitamin E and grain-fed with or without vitamin E. The pasture-fed cattle without vitamin E supplementation had a higher concentration of α-tocopherol but a low concentration of β-carotene in liver than the pasture-fed cattle with vitamin E. In contrast to the liver, both groups had similar concentrations of α-tocopherol in fat. However, the concentration of β-carotene was higher in the pasture-fed cattle without vitamin E supplementation. Furthermore, a similar factorial experiment was carried out by Yang *et al.* (2002) to compare pasture and grain-fed cattle that were either unsupplemented or supplemented with vitamin E. Plasma β-carotene concentrations were the highest in the unsupplemented pasture-fed group in all groups. These interactions may occur because both β-carotene and vitamin E are lipid soluble and are structurally quite similar. Consequently, they compete for incorporation into micelles for absorption. Alternatively, there may be the competition for absorption sites in blood lipoproteins. Competition in the deposition in the adipose tissue may also occur as it has been deserved that the high intake of one fat soluble vitamin can interfere with the uptake or utilisation of other fat-soluble vitamins (Pellett *et al.*, 1994).

Knight *et al.* (1996) hypothesized that daily feeding of vitamin A could reduce the carotenoid concentration in fat and fat colour based on the fact that vitamin A supplementation increases the catabolism of absorbed carotenoids. However, in their experiment, despite decreasing the plasma carotenoid concentration, vitamin A supplementation failed to reduce the adipose tissue colour and carotenoid concentration. Similarly, in another study (Knight and Death, 1999), treatment with vitamin A again failed to reduce the colour of fat in pasture-fed and feedlot Angus and Simmental crossbred steers even with increasing the dosage of vitamin A.

Absorption of β-carotene is increased when cattle are fed a diet rich in polyunsaturated fatty acids (PUFA) (French *et al.*, 2000). Interestingly, there are also cattle breed differences in absorption of carotene. The Guernsey and Jersey breeds absorb carotene more readily than other breeds (Mcdowell, 2000).

The conversion of β-carotene to vitamin A occurs mainly in the intestinal wall by means of a proposed intestinal wall enzyme complex called the carotenase system. There is an efficiency difference of β-carotene cleavage in different species. In rodents, β-carotene is entirely cleaved to retinal, leaving no intact β-carotene in circulation. In humans, the majority of absorbed β-carotene (60–70%) is believed to be converted directly to retinal after absorption, while the remainder is absorbed intact and deposited in the liver and adipose tissues (Parker, 1996). In cattle, not all of the ingested β-carotene is absorbed in the intestine and transformed into vitamin A. The surplus is incorporated into chylomicrons and processed in the Golgi complex of intestinal enterocytes. Then, chylomicrons leave the intestinal enterocytes through exocytosis and are transported into the plasma (Shepherd and Packard, 1987).

1.4.4 Carotenoid transportation

The hydrophobic character of β-carotene results in the β-carotene being exclusively present in the lipid core of different lipoprotein particles during transport in the blood. Chylomicrons are responsible for the transport of carotenoids from the intestinal mucosa in the bloodstream to the various tissues, including the liver. In humans, low-density lipoproteins (LDL) carry 58-73% of total serum carotenoids between the liver and peripheral tissues (Clevidence and Bieri, 1993). However, in cattle, high-density lipoproteins (HDL) are the richest fraction of β-carotene and account for 77-83% of the

total carotenoids (Yang *et al.*, 1992). Interestingly, a study conducted by Kruk (2001) showed different breeds have different HDL particle sizes. Jersey cows have a higher percentage of large HDL particles than the Limousin cows. This may explain some of the breed differences in tissue carotenoid status between Jerseys and other cattle.

1.4.5 Carotenoid deposition

The surplus of β-carotene is deposited in the peripheral tissues including the liver, subcutaneous adipose and intramuscular fat. Carotenoid availability to the mammary gland and other tissues is mainly controlled by the liver, which regulates their distribution between biliary recycling, conversion into vitamin A, mobilization of carotenoid stores and secretion as part of liver VLDL (Noziere *et al.*, 2006a).

It is still not clear how carotenoids are deposited in adipose tissue in cattle. It may occur via passive diffusion or receptor mediated transport. In addition, it is still not known whether the carotene accompanies fat deposition or is incorporated into existing deposits of fat. It has been suggested that β-carotene in cattle can be mobilized independently of lipids when the dietary supply of carotene is reduced (Noziere *et al.*, 2006b). However, Knight *et al.* (2001) suggested that carotenoids are not easily removed from fat once deposited. When cattle are fed a low carotenoid ration, the carotene remains in the fat despite blood plasma carotenoid concentrations decreasing to very low levels. Also as discussed above, Pitchford *et al.* (2002) showed that fat colour scores decreased by 83% in a short period of grain feeding. However, this decrease was the consequence of diluting the previous accumulated β-carotene in the adipose tissue because of the accretion of more fat, rather than from the turnover of accumulated β-carotene in the adipose tissue.

In summary, all these steps can control carotenoid transfer from the diet to fat tissues. However, the knowledge of carotenoid flux, transport and metabolism within and among tissues such as intestinal wall, liver, and adipose tissues is insufficient to fully understand the regulation of fat colour.

1.4.6 Enzymes in metabolism of β-carotene

No mammalian species has the capability of *de novo* vitamin A synthesis. Vitamin A is derived from dietary provitamin A carotenoids. Among those carotenoids displaying provitamin A activity, β-carotene is the most important nutrient for animals (Yeum and Russell, 2002). A number of enzymes are involved in cleavage of β-carotene, synthesis of vitamin A and other derivatives.

1.4.6.1 β-carotene cleavage enzymes

There are two possible mechanisms to convert β-carotene oxidatively to vitamin A derivatives in animals: central cleavage and eccentric cleavage. Central cleavage (Figure 1-3 (1) is the predominant pathway in which the enzyme *β, β*-carotene 15, 15'-oxygenase (BCMO1) cleaves β-carotene symmetrically at the 15-15' double bond to yield retinal, a direct precursor of retinol and subsequently, retinoic acid (Wyss *et al.*, 2000, Redmond *et al.*, 2001). In *Drosophila*, symmetric cleavage of β-carotene is the only pathway as only the BCMO1 enzyme is found (Von Lintig and Wyss, 2001). In vertebrates, there has been a long controversy over symmetric versus asymmetric cleavage of β-carotene in the biosynthesis of vitamin A derivatives. With the molecular cloning and characterization of the β-carotene, β, β-carotene-9', 10'-oxygenase (*BCO2*) gene from mouse, it was confirmed that asymmetric oxidative cleavage of β-carotene

exists (Kiefer *et al.*, 2001). This enzyme specifically acts at the 9’ 10’-double bond of β-carotene, resulting in the formation of β-ionone and two molecules of β-apocarotenal with different chain lengths. In addition to cleavage at 9’ 10’-double bond of β-carotene, the existence of an additional enzymatic eccentric pathway at random cleavage positions has been proposed (Glover and Redfearn, 1954, Sharma *et al.*, 1976, Hebuterne *et al.*, 1996), however, no specific enzyme has been discovered for the proposed eccentric cleavage.

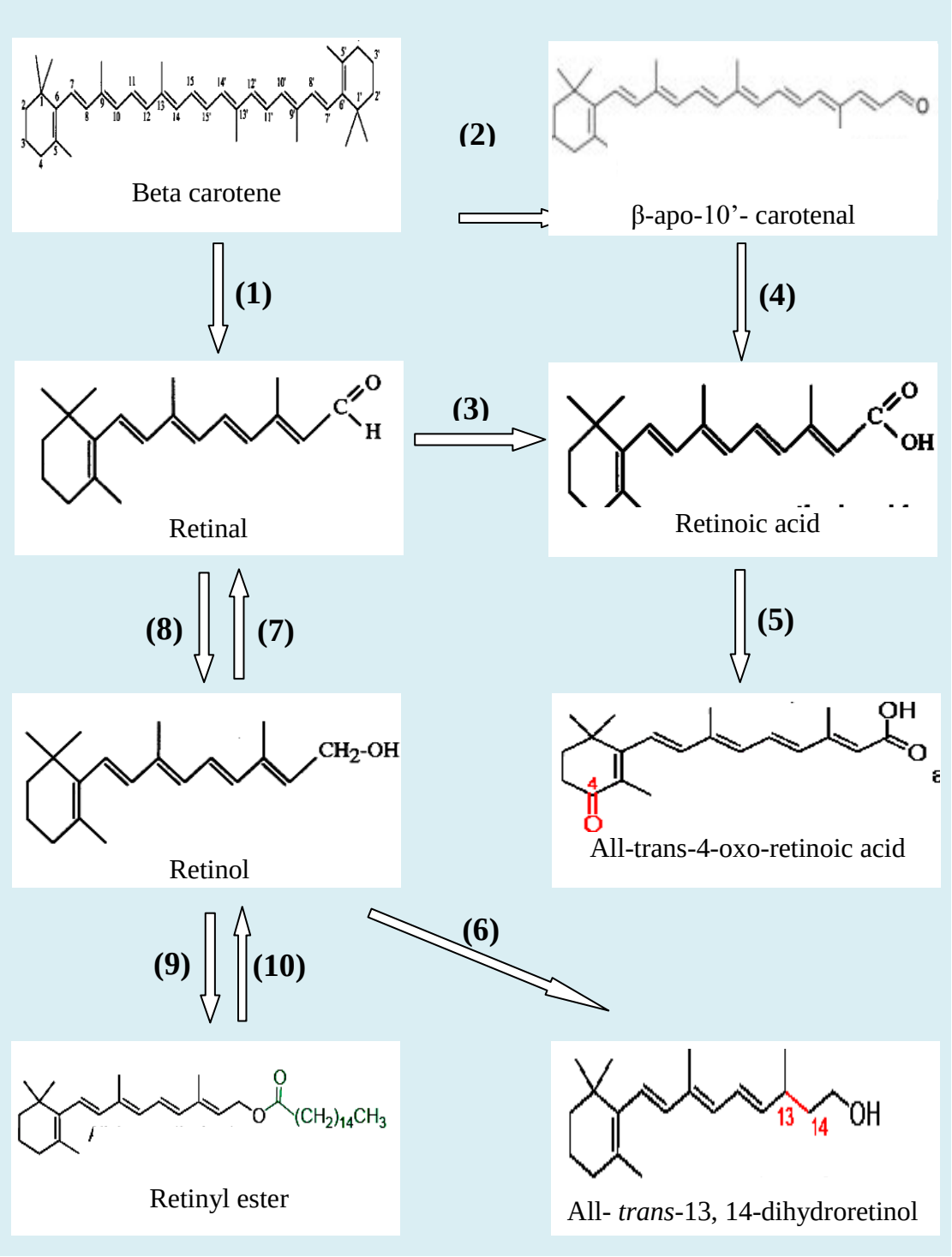


Figure 1-3 Schematic overview of β-carotene metabolism

(1) Symmetric cleavage of β-carotene by BCMO1. **(2)** Asymmetric cleavage of β-carotene. BCO2 is identified to catalyze the cleavage of β, β-carotene at the 9', 10' double bond. **(3)** Formation of retinoic acid by Cytosolic RALDHs (RALDH1, RALDH2, and RALDH3). **(4)** β-apocarotenals are shortened by a mechanism similar to β-oxidation of fatty acids to form retinoic acid. **(5)** Cytochrome P450 enzymes CYP26A1, CYP26B1 and CYP26C1 **(6)** Retinol saturase. **(7)** Some microsomal members of shot-chain alcohol dehydrogenase/reductase (SDR) superfamily (RDHs) (e.g. RDHE2) and alcohol dehydrogenases (ADHs). **(8)** Retinal reductase. **(9)** lecithin:retinol acyltransferase. **(10)** Retinyl ester hydrolases.

β, β-carotene 15, 15'-oxygenase

β, β-carotene 15, 15'-oxygenase (BCMO1) is formally known as a dioxygenase. However, more work has demonstrated that this enzyme should be termed *β, β*-carotene 15, 15'-monooxygenase because the central cleavage of β-carotene follows a monooxygenase mechanism (Figure 1-3 (1)) (Leuenberger *et al.*, 2001). BCMO1 has been biochemically characterized in various species, such as human, mouse, rat, chicken, ferret, rabbit, pig and guinea pig. Its activity and gene expression has been detected in several tissues including the intestinal mucosa, liver, kidney, lung, rain, testis, and adipose tissue of various vertebrate species (During *et al.*, 1998, Wyss *et al.*, 2000, Paik *et al.*, 2001, Lindqvist and Andersson, 2002, Takitani *et al.*, 2006, Zaripheh *et al.*, 2006, Lindqvist *et al.*, 2007). BCMO1 enzyme activity can be significantly inhibited by various ferrous iron chelators (such as α-phenanthroline and α, α'-dipyridyl and ethylenediaminetetraacetic acid) (Lindqvist and Andersson, 2002) and sulfhydryl-binding compounds (Wyss, 2004).

BCMO1 was characterized as a hydrophilic, non-membrane-bound protein by expressing cloned *Drosophila melanogaster* BCMO1 in *Escherichia coli* (Von Lintig and Vogt, 2000). Lindqvist and Andersson (2002) demonstrated the cytosolic localization of the native human BCMO1. Because BCMO1 is a cytosolic enzyme, it requires specific binding proteins to transport the carotenoid substrate and retinoid products, which are highly lipophilic compounds. Three cellular retinoid-binding proteins have been demonstrated to bind to all-*trans*-, 13-*cis*-, and 9-*cis*-retinol in mice (Vogel *et al.*, 2001). However, cellular retinoid-binding proteins failed to interact with murine BCMO1 *in vitro* (Paik *et al.*, 2001). Hence, it remains to be elucidated whether BCMO1 interacts with a certain subset of proteins involved in retinoid metabolism.

Such proteins may control the metabolic flow of the primary cleavage product retinal, either to retinol formation or retinoic acid formation for retinoid-signalling (Von Lintig *et al.*, 2005).

Many factors can influence BCMO1 enzymatic conversion of β-carotene to vitamin A. High fat intake can increase the post-absorptive conversion of β-carotene to vitamin A in the liver of gerbils (Deming *et al.*, 2000), and in the liver, lung, kidney, adipose tissue and testis of ferrets (Lakshman *et al.*, 1996). The quantity and quality of dietary protein and vitamin A levels in the diet also influences BCMO1 enzyme activity. Protein-deficient ferrets showed lower vitamin A levels in liver (2-fold), lung (3-fold), and adipose tissue (1.8-fold) than those fed with high protein diets (Lakshman *et al.*, 1996). Rats showed significantly higher intestinal BCMO1 enzyme activity when they were fed a protein-sufficient diet (Hosotani and Kitagawa, 2005). Moreover, BCMO1 activity is enhanced by vitamin A deficiency, copper depletion, and fructose feeding (During *et al.*, 2000, Hosotani and Kitagawa, 2005).

Unlike vitamin A itself, high-dose supplementation of β-carotene in humans causes no hypervitaminosis, indicating that β-carotene cleavage to vitamin A by BCMO1 is tightly regulated. BCMO1 activity is likely controlled at the transcriptional level by transcription factors. Recently, Boulanger *et al.* (2003) reported a peroxisomal proliferator response element in the promoter region of the *BCMO1* gene in mice. Site-directed mutagenesis and gel shift experiments demonstrated that this peroxisomal proliferator response element was essential for *BCMO1* promoter specificity and binds to PPARγ specifically. PPARγ is a member of the steroid hormone receptor superfamily and is activated by a variety of chemical compounds and natural ligands (Desvergne

and Wahli, 1999). In addition, retinoic acid strongly inhibits intestinal *BCMO1* mRNA expression and down-regulates BCMO1 enzyme activity up to 90% in rats and chickens. This transcriptional level regulation of the enzyme BCMO1 can be explained by the identification of retinoic acid response elements (RARE) in the promoter of the mouse *BCMO1* gene (Bachmann *et al.*, 2002). These sites are recognized by retinoic acid receptors which comprise ligand-dependent transcription factors that regulate gene expression. Moreover, the gut-specific homeodomain transcription factor, intestine specific homeobox (ISX), also has been shown to repress the intestinal expression of *BCMO1*. The intestine specific homeobox acts as a retinoic acid gatekeeper that controls vitamin A production by repressing expression of the *SCARB1* and *BCMO1* genes. Consequently, intestinal lipid absorption and retinoid production is controlled (Seino *et al.*, 2008).

β, β-carotene-9', 10'-dioxygenase

In addition to the symmetric cleavage of β-carotene, β, β-carotene-9', 10'-oxygenase (BCO2), which catalyzes the cleavage of β, β-carotene at the 9', 10' double bond, represents an eccentrically cleaving carotene oxygenase (Figure 1-3 (2)). *BCO2* shows strong mRNA expression in both the liver and skin of chickens (Eriksson *et al.*, 2008). Using RT-PCR analysis, high steady-state mouse *BCO2* mRNA levels were detected in the small intestine, liver, kidney and testis. Low levels were found in spleen, brain, heart and lung (Kiefer *et al.*, 2001). In humans, *BCO2* mRNA has been also demonstrated in skeletal muscle (Von Lintig and Vogt, 2004).

Sequence analyses of the *BCO2* gene in mouse (Von Lintig and Vogt, 2004) revealed that the cDNA encodes a protein of 532 amino acids, and the deduced amino acid

sequence shares ~40% identity with the mouse *BCMO1*. Besides successful cloning in mouse, Kiefer *et al.* (2001) cloned and sequenced the corresponding full-length cDNA encoding *BCO2* in humans and zebrafish. The human cDNA encodes a protein of 556 amino acids, whereas the cDNA isolated from the zebrafish encodes a protein of 549 amino acids. The deduced amino acid sequences shared 72% and 49% identity, respectively, to the mouse *BCO2*. Sequence comparison revealed that the different vertebrate oxygenase family members have a high degree of similarity to each other. Moreover, six histidine residues within the amino acid sequence (presumably involved in the binding of the co-factor Fe^{2+}) and a particularly well conserved family signature in the C-terminus are found in all these oxygenases. Therefore, these vertebrate enzymes probably evolved from a common ancestor (Kiefer *et al.*, 2001).

The role of the BCO2 enzyme is not fully known. Hessel *et al.* (2007) tested a hypothesis that BCO2 acts as an alternative pathway for vitamin A formation from provitamin A in a mouse knock-out model for *BCMO1* (*BCMO1*$^{-/-}$). Unchanged *BCO2* mRNA expression levels in the visceral adipose tissue of the knock-out animals suggested that enzymatic eccentric cleavage is not an alternative pathway for conversion of the bulk of β-carotene accumulated in BCMO1 deficiency. In addition, the BCO2 enzyme is expressed in some human tissues and cell types that are not sensitive to vitamin A deficiency and where no BCMO1 activity has been detected. Lindqvist (2005) suggested the BCO2 enzyme may be involved in metabolism of carotenoids, possibly to assist in the production of biologically active metabolites (such as β-ionone and β-apocarotenals) rather than retinoic acid synthesis. However, Kiefer *et al.* (2001) proposed that eccentric cleavage is an alternative pathway for the formation of retinoic acid and may contribute to retinoic acid homeostasis of the body, certain

tissues or cells.

Besides cleavage of β-carotene, it has been shown the recombinant ferret BCO2 also catalyzes *cis*-lycopene isomers effectively but not all-*trans*-lycopene at the 9', 10'-double bond. The expression of *BCO2* in the lungs of ferrets can be up-regulated compared with the control ferrets by lycopene supplementation (Hebuterne *et al.*, 1996). Similarly, by feeding lycopene to rats, *BCO2* mRNA expression was moderately decreased in the small intestine compared with the non-supplemented animals, although the *BCO2* mRNA is expressed at a relatively low degree in the small intestine (Zaripheh *et al.*, 2006). Taken together, much further work needs to be done to fully understand the exact physiological functions of BCO2.

1.4.6.2 Other enzymes

Enzymes of retinal biosynthesis

Retinal, also called retinaldehyde or vitamin A aldehyde, is one of the many forms of vitamin A. In vertebrates, 11-*cis*-retinaldehyde is essential for visual processes (Oberhauser *et al.*, 2008) and regulation of lipid metabolism. Each provitamin carotenoid can be processed more or less efficiently in numerous cell types (mainly in enterocytes and hepatocytes) (Borel *et al.*, 2005) to yield two molecules of retinal by oxidative cleavage via the BCMO1 enzyme (Figure 1-3 (1)). Besides production from cleavage of β-carotene, retinal can be produced by the oxidation of retinol, which is the first step in the retinoic acid synthetic pathway (Figure1-3 (7)). Three enzyme families are involved in retinal formation from retinol: namely, the alcohol dehydrogenases (ADH) (class I, III and IV) (Duester *et al.*, 2003), the short chain dehydrogenases (SDR) (especially, the retinol dehydrogenases) (Duester, 2000) and the cytochrome P450s

(CYP1A1, CYP1A2, CYP1B1, CYP2D6, CYP3A4 and CYP3A5) (Chen *et al.*, 2000).

Enzymes of retinol biosynthesis

Retinol represents the most abundant retinoid in blood. Retinal produced from the cleavage of β-carotene can be reduced to retinol. Several retinal reductases are able to cause this reduction (Figure1-3 (8)). However, detailed enzyme studies still need to be conducted (Li and Tso, 2003). Identification of the enzyme retinol saturase in the mouse provided evidence that all-*trans*-retinol can be converted to all-*trans*-13, 14-dihydroretinol (Figure1-3 (6)), which is a peroxisome proliferator activated receptor PPARγ target (Moise *et al.*, 2004). Retinol saturase is required for adipocyte differentiation in the 3T3-L1 cell culture model. It was also proposed that dihydroretinoids, produced by retinol saturase, control the physiological processes that influence PPARγ activity and regulate lipid accumulation in mice (Moise *et al.*, 2010).

Metabolism of retinoic acid

Retinoic acid is a major regulator controlling a wide range of biological processes, including embryonic development, cell differentiation, immunity, metabolic control, reproduction, and maintenance of adult tissues (Von Lintig, 2010). Retinoic acid regulates expression of retinoid-responsive genes via interactions at specific DNA control regions (Bachmann *et al.*, 2002). Retinoic acid is a ligand of two superfamilies of nuclear hormone receptors, the retinoic acid receptors (RAR) and the retinoid X receptors (RXRs) (Chambon, 1996). The active receptor complex is a RAR/RXR heterodimer that binds DNA regulatory sequences and regulates gene transcription in response to retinoic acid binding.

All-*trans*-retinal can be oxidized to all-*trans*-retinoic acid by three cytosolic aldehyde dehydrogenases (RALDH1, RALDH2, and RALDH3) (Figure1-3 (3)), (Von Lintig *et al.*, 2005). Besides oxidation of all-*trans*-retinol, 9-*cis* retinal can be oxidized to 9-*cis*-retinoic acid by the enzyme RALDH4, which is expressed in liver and kidney in mice. This enzyme has been shown to be two-orders of magnitude more active *in vitro* with 9-*cis* retinal than with all-*trans* retinal (Lin *et al.*, 2003).

In addition to the formation of retinoic acid from retinal as the initial product of symmetric β-carotene cleavage, retinoic acid could be directly formed from long-chain apocarotenoic acids in cell-free homogenates. Long-chain apocarotenoic acids (>C20) are shortened to retinoic acid in a stepwise process, which is most probably mechanistically related to the β-oxidation of fatty acids (Wang *et al.*, 1996).

The retinoic acid levels in cells and tissues are important for retinoic acid function. Retinoic acid has potent biological activity even when present in very small quantities. As these are irreversible reactions, the conversion to retinoic acid must be tightly controlled. Some members of the cytochrome P450 enzyme family (CYP26A1, CYP26B1 and CYP26C1) (Figure1-3 (5)) detect the retinoic acid level and regulate the oxidative metabolism of all-*trans*-retinoic acid accordingly by the catabolism of retinoic acid to polar metabolites, including 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy retinoic acid, 5,6-epoxy retinoic acid, and 5,8-epoxy retinoic acid (White *et al.*, 2000, Lampen *et al.*, 2001, Taimi *et al.*, 2004). It has been also shown that these metabolites may be more active than all-*trans*-retinoic acid in certain systems. For example, 4-oxo- retinoic acid is more effective in the modulation of positional specification in early embryos and in the transcriptional induction of the development

genes *Hoxb-4* and *Hoxb-9* (Pijnappel *et al.*, 1993).

Retinyl esters formation and hydrolysis

Retinyl esters represent both a major storage form of vitamin A and possible intermediates of the visual cycle (Batten *et al.*, 2004, Imanishi *et al.*, 2004). In enterocytes, absorbed all-*trans*-retinol retinol is bound to a specific binding protein called cellular retinol-binding protein type II (CRBP-II), which solubilizes the fat-soluble retinol to protect retinol from degradation. Bound to CRBP-II, retinol is esterified with long chain fatty acids (mainly palmitate) through the actions of the enzyme lecithin:retinol acyltransferase (LRAT) (Figure1-3 (9)) (Ruiz *et al.*, 1999). Besides LRAT, another enzyme, acyl-CoA:retinol acyltransferase (ARAT), is believed to participate in retinyl ester formation in intestine, mammary gland, and adipose tissues based on studies in LRAT-deficient mice (Randolph *et al.*, 1991). Retinyl ester hydrolases catalyzes the reverse reaction to produce retinol (Figure 1-3 (10)). One of the hydrolases (cAMP-dependent lipase) catalyzes retinyl esters in adipose tissues. It has been suggested the combination of cAMP-dependent lipases and retinol binding proteins could be an alternative source of blood retinol (Wei *et al.*, 1997).

1.5 Summary

In summary, the amount of adipose tissue accumulated during grain feeding and the carotenoid content of the diet are critical factors in determining carcass fat colour. In vertebrates, β-carotene is derived from the diet and serves as a major vitamin A precursor. After being released from food, β-carotene passes through the rumen and is absorbed in the small intestine. The surplus is incorporated into chylomicrons in the plasma and is delivered to the liver, adipose tissue and muscles. The literature reviewed

above suggests that the intestinal digestion and absorption of β-carotene is a highly complex process in which a number of enzymes and other proteins participate. In the metabolism of β-carotene, two oxygenases (BCMO1 and BCO2) carry out the initial cleavage to form retinoids. Retinoids can down-regulate BCMO1 and other enzymes involved in catalysing the chemical modification of apocarotenoids (Figure 1-3). As β-carotene is the major yellow pigment deposited in cattle adipose fat tissue, all of the proteins and enzymes involved in the transport and metabolism of β-carotene are potential candidate genes for controlling beef fat colour.

The main aim of this research project was to examine these potential candidate genes which are located in the QTL for beef fat colour traits and determine their involvement of fat colour. In doing so, the biological pathways involved in fat colour should be clarified. It was hypothesised that candidate genes involved in conversion of β-carotene to vitamin A, carotenoid transportation and fat deposition are likely regulate beef fat colour. Thus, the DNA variants in of candidate genes within the pathways were examined for association with beef fat colour. Any associated DNA variants may be used for selection against yellow fat in cattle and may provide more insight into β-carotene metabolism in mammals.

Chapter 2

General methods and materials

2.1 Animal resources

The cattle used in this project were categorised into seven groups. The phenotyping and genotyping of animals in Group 1, 3 and 7 were conducted in Australia, whereas in Group 2, 4, 5 and 6, the phenotypes and genotypes of animals were provided by AgResearch.

Group 1 and Group 2: Animals in these two groups were from the Davies Gene Mapping Project herd. The herd was a double-backcross design using two extreme *Bos taurus* breeds, namely the Jersey dairy breed (J) and Limousin beef breed (L). These two breeds were chosen because of significant differences in many economic traits, including meat yield, carcass composition, fat colour, marbling and body size (Cundiff *et al.*, 1986). This herd was established to study the inheritance of important cattle traits and to map major genes controlling these traits. Purebred Jersey and purebred Limousin cattle were crossed to produce three pairs of half brothers F1 progeny (LJ). One of each half-sire pair of the six F1 sires were mated in Australia and the other in New Zealand, and their progeny were raised in two environments (Australia and New Zealand). This allowed genotype by environment interactions to be tested for traits measured in both environments. The three F1 bulls were randomly chosen and mated to the purebred Jersey or Limousin cows in Australia to generate Group 1, three-quarter Jersey (LJJJ) or three-quarter Limousin progeny (LJLL) (Figure 2-1). The other three F1 bulls were half sibs of the Australian bulls and also mated to purebred Jersey or Limousin cows in New Zealand to produce Group 2 backcross progeny.

Group 1 comprised 366 experimental backcross calves (205 XJ and 161 XL) born over the three years (1996-1998) and reared with their dams in the one location (Martindale,

Mintaro, South Australia). Calves were born in autumn (March-May, average 26th April) and were maintained under the same management system and grazed on pastures throughout year with some supplement of hay from January to June. The calves were weaned at 250 days and grown out on pasture until approximately 28 months of age. Then, animals were finished on grain in a feedlot for at least 6 months as part of an intensive feed efficiency trial and were slaughtered at the end of lot feeding.

Group 2 comprised 262 calves (162 XJ and 100 XL) born in spring 1996, and another 156 were born in spring 1997 (102 XJ and 54 XL). They were grown out on pasture in New Zealand without grain concentrates and slaughtered at 24-28 months of age.

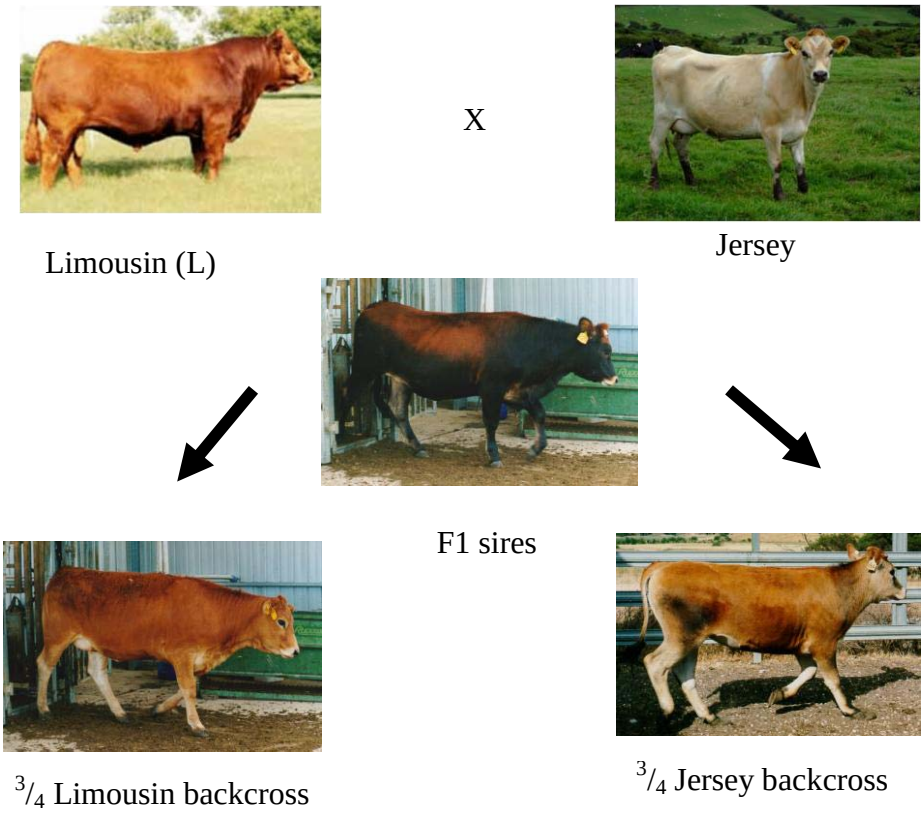


Figure 2-1 Backcross design of the Davies Gene Mapping Herd

Group 3: This Group included 119 Jersey dams used to generate the Jersey-Limousin backcross mapping progeny in Australia. Animals varied in age from 2-9 years. Fat biopsy samples were taken in January after being on spring fed on green pastures.

Group 4: Animals in this group were from a milk QTL study carried out by AgResearch in 2000 and 2001 (Morris *et al.*, 2007). Approximately 4100 Holstein-Friesian-sired cows were from 21 commercial New Zealand dairy herds. All cows were daughters of 11 Friesian sires that were used widely in New Zealand to supply semen for artificial insemination. The cows ranged in age from 2 to 11 years. Approximately 590 animals with milk colour data were genotyped in this project.

Group 5: Yellow fat samples were collected from 265 cattle slaughtered at two local Hamilton abattoirs in New Zealand over a 6-month period beginning in November 2006, and a further 44 samples from animals with white fat were collected at the same time from similar cattle and were treated as 'controls'.

Group 6: Milk from 192 Jersey-sired cows of known pedigree was collected by AgResearch staff in August 2007 from a single Jersey herd.

Group 7: Fat and liver samples were collected from 119 random cattle fat slaughtered at local Hamilton abattoirs in New Zealand in March 2009. These samples were used for RNA, DNA and β-carotene extraction.

2.2 Phenotypic data collection

Phenotypic data in Groups 1, 2 and 3 were obtained from a previous study and detailed

procedures for sample collection and sample analysis have been described by Kruk (2001). Generally, biopsy subcutaneous fat samples were obtained at weaning from Groups 1 and 2 (approximately 250 days of age). Fat samples from Group1 in 1996 were obtained from the loin eye muscle (M. longissimus dorsi) area between 12^{th}-13^{th} rib sites, whereas in 1997 and 1998, the fat samples were obtained from the base of tail. The biopsy samples from Group 2 (data from AgResearch) and 3 were from the tail. For the carcass samples, fat was dissected from the eye muscle area between 12^{th}-13^{th} rib sites after slaughter within 5-10 minutes or from the chillier within 24 hours. For Groups 1, 3 and 7, β-carotene in the fat was extracted using method described by Yang *et al.* (1992) with the modification described by Kruk (2001) (Appendix 1.1). High pressure liquid chromatography was used for the detection and quantification of β-carotene in fat. The fat colour scores of the biopsy adipose tissue samples were determined immediately after removal from the animal and rinsing with water. The intensity of the colour was expressed in a 5-point scale where a score of 1 was assigned to very white and score of 5 to extremely yellow fat. Scores of 2 and 4 represented fat more white and more yellow respectively, and 3 was the intermediate score. The carcass sample fat colour was also assessed but on a 10-point (0-white, to 9-very yellow) scale by trained chiller assessors using standard colour chips (AUS-MEAT, 2005) (Figure 2-2) after the carcasses were in the chiller for 16 hours. Biopsy fat colour score and biopsy β-carotene concentration in Group 2 was assessed by AgResearch and the method was same as for Group 1. No detailed experimental colour scoring of Group 2 back-cross carcasses was carried out by AgResearch in New Zealand, but any carcasses that were downgraded by the carcass grader because of fat colour were assigned a score of 1, with all others scored as 0.

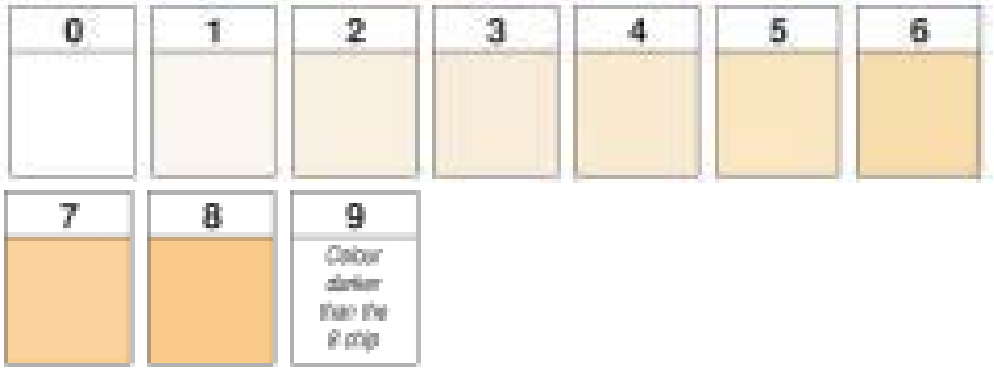


Figure 2-2 AUS-MEAT standard colour chips
0-white, to 9-very yellow.

Phenotyping data for Group 4, 5 and 6 of animals were provided by AgResearch. In Groups 4 and 6, only milk colour was analysed and was assessed by spectrophotometry (absorbance at 450 nm). In Group 5, fat was extracted from fat samples and assayed for fat colour by spectrophotometry (absorbance at 450 nm). A visual score of yellowness was also recorded (1 = white to 5 = very yellow). In Group 7, β-carotene in the fat was extracted using the methods as for Group 1 and the samples were scored as being either white or yellow (Appendix 1.1).

2.3 DNA preparation

Cattle blood was collected in ACD containers for Groups 1, 2 and 3. Blood was mixed thoroughly to prevent clotting and stored at -20^{0}C for long term storage. Genomic DNA was extracted by phenol/chloroform extraction method and stored in 1ml TE buffer (pH 8) (Appendix 2.1). DNA concentration and purity were estimated using a Shimadzu UV-160 spectrophotometer with 0.5ml quartz glass cuvettes. Measurement of optical density (OD) of samples was performed at 260 nm and 280 nm against a dd H_2O blank. An OD 260:280 ratio of 1.6-2.0 was sufficient quality for PCR amplification. DNA in Groups 4 and 6 was extracted from blood samples as described by Morris *et al*. (2007).

The DNA in Group 5 was extracted from the meat samples and the liver samples in Group 7 were used for DNA extraction. Genomic DNA for these two groups was extracted using TRI Reagent® DNA Isolation kit according to the manufacturer's protocols (*Applied Biosystems*) (Appendix 1.5).

2.4 Candidate gene selection

Previous genomic scans for QTL have given primary localization of 21 QTL affecting beef fat colour in cattle (Appendix 7) (Esmailizadeh, 2006). Comparative mapping was used to identify the candidate genes from human database as the bovine genome sequence was not well annotated at the beginning of the project. The microsatellites markers for QTL were firstly aligned on the cattle linkage and radiation hybrid maps available on http://www.thearkdb.org, http://www.marc.usda.gov/, and http://www.animalgenome.org/cattle/maps/COMRAD/ (accessed in September 2007). Then, the equivalent human regions were located with Bovine-Human Comparative Map (http://www.animalgenome.org/cattle/maps/RHMap3/). Information derived from the human genome using the human Ensembl (assembly GRCh37, http://www.ensembl.org) and NCBI's MAPVIEWER genome databases (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9913&query=) was used to provide candidate genes for the mapped QTL in cattle.

2.5 Single nucleotide polymorphisms experiments

2.5.1 Primer design and selection

Candidate gene genomic sequences were based on the Ensembl bovine database (Btau3.1 www.ensembl.org). Primers used for PCR amplification were derived from the

sequence of the exon-flanking intronic regions as well as from the 5'- and 3'-untranslated regions. Bovine PCR primers were designed using the PRIMER3 program (Rozen and Skaletsky, 2000). The selection of primers was based on the following criteria: 1) 18-25 bp in length and 2) 40-60% GC content. Selected primers were re-checked for the second structure by the OLIGO6 program (Molecular Biology Insights). Primer hairpins were avoided to prevent false priming during amplification. The difference in the annealing temperature for forward and reverse primer was minimized to less than 1ºC by deleting the first or last one or two nucleotides. Primers were synthesized by GeneWorks (Adelaide, Australia).

2.5.2 Optimization of PCR conditions

The PCR conditions were optimised for each exon and untranslated regions so that a single, specific product was amplified. A standard 25 µl reaction was used for PCR amplification (Table 2-1 and Table 2-2). Either *AmpliTaq Gold* (*Applied Biosystems*) or *Kapa Taq* DNA polymerase was used. 50 or 100ng DNA template was from 3 sires (361, 368 and 398).

Table 2-1 PCR reagent concentrations for *AmpliTaq Gold* DNA polymerase

PCR components	Volume µl	Concentration in the reaction
10xbuffer containing 20mM Tris-HCL (pH 8.4), 50 mM KCL, Magnesium chloride free	2.5	1x
dNTP (1.25 mM each of dTTP, dGTP dATP and dCTP)	2.5	0.125mM each
Forward primer (2.5, 5 µM) (*Geneworks*)	1	0.1, 0.2 µM
Reverse primer (2.5, 5 µM) (*Geneworks*)	1	0.1, 0.2 µM
Magnesium chloride (25 mM)	1.5, 2, 2.5,3	1.5, 2, 2.5, 3 mM
AmpliTaq Gold DNA polymerase (0.5 U) (*Applied Biosystems*)	1	0.02U
Template DNA (50,100 ng)	1	2, 4ng
PCR graded water to a final volume of 25 µl		

Table 2-2 PCR reagent concentrations for *Kapa* DNA polymerase

PCR components	Volume µl	Concentration in the reaction
10xbuffer A containing 1.5mM Magnesium chloride at final concentration	2.5	1x
dNTP (1.25 mM each of dTTP, dGTP dATP and dCTP)	2.5	0.5mM each
Forward primer (2.5, 5 µM) (*Geneworks*)	1	0.1, 0.2 µM
Reverse primer (2.5, 5 µM) (*Geneworks*)	1	0.1, 0.2 µM
Kapa DNA polymerase (0.5 U) (*Geneworks*)	1	0.02U
Template DNA (50,100 ng)	1	2, 4ng
PCR graded water to a final volume of 25 µl		

Reactions were performed at different concentrations of primers and magnesium in order to optimise amplification. PCR conditions, which produced clear and specific bands, were chosen for the future amplification. A Palm Cycler (*Corbett Research*) was used for the amplifications. Three touchdown programs with 3 different ranges of annealing temperatures or gradient programs were utilized in order to find the most suitable PCR conditions.

Touchdown program:

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 1 minute, T_A (70 – 60°C / 65 – 55°C / 60 – 50°C, where Ta started at 70°C, 65°C and 60°C, respectively and decreased 1°C x 10 cycles, until Ta reached 60°C, 55°C, 50°C, respectively) for 1 minute, 72ºC for 1 minute (24 replicates)

Step 3: 72ºC for 10 minutes

Gradient program:

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 1 minute, 70 ºC ~50 ºC for 1 minute (gradient), 72ºC for 1 minute (35 replicates)

Step 3: 72ºC for 10 minutes

4-6 µl of PCR products were separated by a gel electrophoresis in a 2% gel in 1x TAE (Appendix 2.2) buffer for 40-55 min at 110V depending on the expected PCR product size. The PCR products were sized against a pGEM marker ladder cut with *HinfI, RsaI* and *SinI* (*Promega*). Ethidium bromide (1mg/ml) or Sybr Gold (1mg/ml) was used to stain the gel for 15 min. Gel was then visualised under UV illumination (312nm) using a Gel-Documentation 1000 System (*Biorad*). The images were captured by Molecular Analyst software (*Biorad*).

2.5.3 Automated cycle sequencing of PCR products

After a single PCR product was identified from the gel image, PCR products were purified by Ultra Clean PCR Clean-up Kit (*MoBio*) following the manufacturer's protocol (Appendix 1.2) to remove any remaining contaminants (e.g. primers, magnesium, buffer and dNTPs). After the purification, 2 µl of post-purified PCR products were checked by gel electrophoresis for concentration and purity.

Once purified, the products were air-dried at 37ºC for 30 minutes to concentrate the amplicon. Then the concentration of the DNA was measured using a spectrophotometer ND-100 (*NanoDrop*). Each amplicon was sequenced in both the forward and reverse direction. Sequencing was performed as recommended by the manufacturer's BigDye

terminator cycle sequencing protocol (*PE Applied Biosystems*). 10 µl reactions were used for sequencing each PCR sample and contained 1-3 µl H_2O, 2 µl Big Dye Terminator (*ABI*), 1 µl glycogen (*Roche*), 1 µl 25 µM primers and 3-5 µl (30-100ng) DNA. The reactions were performed in a Corbett Palm-Cycler in 0.2 µl tubes (*Axygen*). The following conditions were used for sequencing:

Step 1: 95ºC for 5 seconds

Step 2: 96ºC for 30 seconds, 50 ºC for 15 seconds, 60ºC, 4 minutes (24 replicates)

Step 3: 4ºC hold

The sequencing reaction products were precipitated using 75% isopropanol according to the manufacturer's protocol (*PE Applied Biosystems*) (Appendix 1.3). The pellets were air-dried at 37ºC for 2 hours and sent to the Institute of Medical and Veterinary Science, Adelaide, South Australia for automated sequencing on an AB 373 or ABI 3700 DNA Sequencher (*PE Applied Biosystems*).

2.5.4 SNP discovery

The sequencing of candidate genes was conducted on three Australian crossbreed F1 half-sib sires (Limousin x Jersey). Sequences of each amplicon in both forward and reverse direction were aligned with the Sequencher^{TM} software package (*Gene Codes Corporation*). Sequences obtained were compared with sequences from the bovine genome Ensembl database (http://www.ensembl.org/Bos_taurus/Info/Index) to verify that the sequence of the amplified product was the specific target gene. Alignment of the 3 sires' genomic sequence data allowed the discovery of sequence variants. If sequence variants (usually single nucleotide polymorphisms or SNPs) were discovered, they were verified, first from the sequence in the opposite direction, and then, by

sequencing the PCR product from the grandparents of the sires to confirm Mendelian inheritance. [Note: The sequencing of the *BCO2* gene was done earlier as part of a Masters Coursework research project.]

2.5.5 Genotyping

Genotypes for Groups 2, 4, 5 and 6 were generated using a Sequenom iPLEX® Gold mass extend chemistry and the data were provided by AgResearch. Genotyping was performed using three methods for Groups 1, 3 and 7. PCR-RFLP was used for the SNP *BCO2* W80X, while high resolution melting (HRM) technique and the Illumina system (DPI Victoria) were used for the other SNPs.

2.5.5.1 Genotyping by PCR-RFLP

The SNP *BCO2* W80X was genotyped by PCR-RFLP. The presence of the G or A allele was distinguished by digesting a 525 bp PCR product with the *Bsrs*I restriction enzyme. The first *Bsrs*I restriction site was located at the base 13 position of the PCR product if the mutation is present. In addition, there was a second *Bsrs*I restriction site (base 329 position from the start of PCR product) present within all amplicons, and this was used as a positive control for the restriction digestion. PCR was performed using following primers that flanked the SNP site.

Forward: 5'-AACCCATCCCACTTCCTTATCT-3',

Reverse: 5'-GTAGAAACTTGCTCCTATATGTCAC-3'

PCR was performed in 25µl reaction, which consisted of 2.5µl Gold buffer with no MgCl2 (Applied Biosystems), 2.5µl 1.25mM dNTPs, 1µl 2.5µM each forward primer and reverse primer (Geneworks), 2.5µl 2.5mM MgCl2, 100ng of DNA and 0.5U AmpliTaq DNA polymerase (Applied Biosystems). Amplification was under the

following conditions:

Step 1: 95ºC for 5 minutes

Step 2: 95ºC for 1 minute, T_A (60 – 50°C where Ta started at 60°C and decrease 1°C x 10 repeats, until Ta reached 50°C x 24 replicates) for 1 minute, 72ºC for 1 minute

Step 3: 72ºC for 10 minutes

10µl of the PCR product were digested with 10 U of *BsrsI* at 65ºC for 2.5 hours according to the conditions recommended by the manufacturer (*Promega*). Restriction DNA fragments were separated by electrophoresis in a 2% agarose gel in TAE buffer and stained with Sybr Gold (*Invitrogen*) for 20 minutes.

2.5.5.2 Genotyping by High Resolution Melting (HRM)

HRM genotyping was conducted with Rotor-Gene 6000 (*Corbett*) machine. The Rotor-Gene 6000 was used to perform PCR, melting and fluorescence detection in a single run. The volume of HRM reactions was 20 µl in total. The reactions contained 10 µl commercial pre-mixed reagents Sensimix (*Quantace*), 1 µl of 2.5 µM each forward and reverse primers (*Geneworks or Sigma*), 0.8 µl EvaGreen dye (*Biotium Inc*), 2.2 µl water and 5 µl of 10 ng/ µl DNA template.

The PCR and HRM was conducted using the following program:

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 45 seconds, T_A for 45 seconds (T_A in Appendix 3.1), 72ºC for 45 seconds (acquiring to cycling A on green) (35 replicates)

Step 3: 72ºC for 10 minutes

Step 4: 60ºC for 1 minute

Step 5: high resolution melting (65ºC-95ºC raised by 0.1 ºC each step)

The genotype assignments were acquired using the Rotor-Gene 6000 Series Software (*Qiagen*) and Rotor-Gene ScreenClust HRM® Software (*Qiagen*).

2.6 Real-time PCR

2.6.1 Tissue collection

For group 7, 119 liver samples were collected 15–30 minutes after the cows were slaughtered at local Hamilton abattoirs in New Zealand in March 2009. Tissue samples were preserved using RNAlater solution (*Applied Biosystems*).

2.6.2 RNA isolation

RNA was extracted from the liver following the manufacturer's protocol (TRI Reagent® RNA Isolation, *Applied Biosystems*). Briefly, 50-100mg of liver were homogenised in 0.8ml of TRI Reagent® (*Applied Biosystems*) using an Ultra-Turrax® T-25 basic homogeniser (*Rose Scientific*). Samples were centrifuged at 12,000*g* for 10 minutes at 4ºC and the supernatant were transferred to a fresh tube. 800µl supernatant was mixed with 160 µl of chloroform (*SigmaAldridge*) and phase separated by centrifugation at 12,000*g* for 15 minutes at 4 ºC. The upper aqueous layer was placed in a new tube containing 400 µl isopropanol and incubated 10 minutes at room temperature. Then the samples were centrifuged at 12,000*g* for 8 minutes at 4ºC, and the supernatant was discarded. 0.8 ml of 75% ethanol was added and the tube centrifuged at 7,500*g* for 5 minutes. The ethanol was removed and the RNA pellet was air dried. RNA was dissolved in 0.3 ml DEPC-treated water. 20 µl total RNA was treated for genomic DNA contamination using Ambion DNase1 (*Applied Biosystems*) following manufactures' protocol (Appendix 1.4). 2µl of clean DNase treated RNA was analysed using a

NanoDrop (*Biolab, VIC, Australia)* for both quantity (260nm) and quality (260:280nm ratio). The RNA integrity was evaluated via electrophoresis. 2 µl RNA was added to 2µl formamide loading buffer (Appendix 2.3) and separated on a 1% agarose (*Progen*) gel at 70V for 30 minutes and stained with ethidium bromide. The integrity of the 28S and 18S ribosomal bands of the RNA preparation was assessed with UV illumination using a Gel Doc 1000 (*Biorad*).

2.6.3 cDNA production

For each sample, the total RNA concentration was diluted to 120ng/µl. Total RNA was converted to cDNA with oligo-dT primers and a Superscript III kit (*Invitrogen, Australia*) following the manufacturers' protocol. Briefly, approximately 1 µg of total RNA was mixed with 1 µl of oligo $(dT)_{20}$ (50 µM), 1µl of dNTPs (10 mM each) and up to 10µl of DEPC-treated water, and incubated at 65 ºC for 5 minutes. Samples were placed in an ice bath for 5 minutes, and then 2 µl of 10X RT First-Strand buffer, 2 µl of DTT (0.1 M), 1 µl of RNase inhibitor (40 U/µl) and 1 µl of Superscript III reverse transcriptase (200 U/µl) were added. Tubes were incubated at 50 ºC for 50 minutes and at 85 ºC for 5 minutes. The reactions were chilled on ice for 5 minutes before they were collected by brief centrifugation. 1 µl of RNase H was added to each tube and incubated for 20 minutes at 37°C. cDNA reactions were diluted with nuclease free 10mM TRIS pH 8.0 (Appendix 2.4), aliquoted into single use volumes in 96-well plates, sealed and snap frozen at -80 ºC.

2.6.4 Real-time PCR conditions

The relative expression of *BCO2, BCMO1, RDHE2, ACTB2, RPLPO, TBP* and *UCHIL15* mRNA transcripts were measured by quantitative real time reverse

transcription-PCR (qRT-PCR) using KAPA SYBR® FAST qPCR Kit (*Geneworks, Australia*) in the Rotor-Gene 6000 Real-Time PCR Detection System (*Corbett*). Each cDNA was used to optimise PCR cycling conditions. Reactions were performed in 200 µl tubes (*Axygen*). 20µl reaction consisted of 10 µl KAPA SYBR® FAST qPCR pre-mix, 2.5 µl (2.5 µM) forward and 2.5 µl (2.5 µM) reverse primers (Appendix 3.3) (*Geneworks, Australia*) and 5 µl cDNA. The PCR was conducted using the following program:

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 45 seconds, T_A for 45 seconds (gradient 65-50ºC), 72ºC for 45 seconds (acquiring to cycling A on green) (35 replicates)

Step 3: 72ºC for 5 minutes

Step 4: melting (65ºC-95ºC raised by 1 ºC each step)

A melting curve was constructed to verify that only one gene-specific peak was present and to ensure that primer dimers were completely absent. PCR products were visualised by agarose gel electrophoresis as described in section 2.5.2 The real-time PCR efficiency of each run was calculated with a 6-point 6-fold dilution series of cDNA, which was used to construct a relative standard curve. The efficiencies ranged between 89% and 105%. Every run also included a no-template control. The qPCR mRNA expression measurement of each sample was performed in triplicate.

2.7 Statistical analysis

2.7.1 Animal Group 1:

Animal model would take account of other polygenetic component better than a sire model. However, the sire model of analysis is enough for the purpose of this study, and

the cost of genotyping in dams would be higher if animal model were used for analysis.

Association analyses were tested by using Genstat version 9.2. There were 7 linear models used in the association analyses for Group 1.

Model 1: This model was used to determine the effects of cohort, breed and sire on fat colour related traits.

$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$

Where:

Y_{ijk} is the response variable

μ is the overall mean

α_i is the effect of i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of j^{th} breed of dam (Jersey or Limousin)

γ_k is the effect of k^{th} sire (three sires)

ε_{ijk} is the residual effect

Model 2: The model (Unbalanced Analysis of Variance) fitted cohort, breed, sire and the SNP genotype to analyse the effects of the individual SNPs on the fat colour related traits.

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \theta_l + \eta(x_{ijkl} - \bar{x}) + \varepsilon_{ijkl}$

Where:

Y_{ijkl} is the response variable

μ is the overall mean

α_i is the effect of i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of j^{th} breed (Jersey or Limousin)

γ_k is the effect of k^{th} sire (three sires)

θ_l is the effect the l^{th} SNP genotype (3 levels)

η is the effect of the covariate (when fat depth as a covariate is used)

ε_{ijkl} is the residual effect

Model 3: The model (Unbalanced Analysis of Variance) fitted cohort, breed, sire, *BCO2* W80X genotype and the SNP genotype to analyse the effects of the individual SNPs on the phenotypic traits taking the *BCO2* W80X genotype into account.

$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \theta_l + \lambda_m + \varepsilon_{ijklm}$

Where:

Y_{ijklm} is the response variable

μ is the overall mean

α_i is the effect of the i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of the j^{th} breed of dam (Jersey or Limousin)

γ_k is the effect of the k^{th} sire (three sires)

θ_l is the effect the l^{th} *BCO2* W80X genotype (GG, GA and AA)

λ_m is the effect of the m^{th} SNP genotype (3 levels)

ε_{ijklm} is the residual effect

Model 4: The additive and dominance effects were estimated using a general linear model (*Genstat*) by including these effects as variates. For the additive effect, -1, 0 and 1 were used to code for each genotype, whereas for the dominance effect, 0 and 1 were used to code for homozygous and heterozygous animals, respectively. Cohort, breed, sire, *BCO2* W80X genotype, additive and dominance effects were fitted in the model.

$Y_{ijklmn} = \mu + \alpha_i + \beta_j + \gamma_k + \lambda_l + bX_m + bX_n + \varepsilon_{ijklmn}$

Where:

Y_{ijklmn} is the response variable

μ is the overall mean

α_i is the effect of i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of j^{th} breed of dam (Jersey or Limousin)

γ_k is the effect of k^{th} sire (three sires)

θ_l is the effect the l^{th} *BCO2* W80X genotype (GG, GA and AA)

bX_m is the regression coefficient of the additive effect (-1, 0 and 1)

bX_n is the regression coefficient of the dominance effect (0 and 1)

ε_{ijklmn} is the residual effect

Model 5: Interactions between each SNP genotype were examined by fitting cohort, breed, sire, *BCO2* W80X genotype and each pair of SNP genotypes and their interactions in the model. Because the lower number of *BCO2* W80X AA genotype, there was not enough data for each category if 3-way interactions were tested, only two-way interactions were tested in the model 5:

$$Y_{ijklmn} = \mu + \alpha_i + \beta_j + \gamma_k + \theta_l + \lambda_m + \delta_n + (\lambda\delta)_{mn} + \varepsilon_{ijklmn}$$

Where:

Y_{ijklmn} is the response variable

μ is the overall mean

α_i is the effect of i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of j^{th} breed (Jersey or Limousin)

γ_k is the effect of k^{th} sire (three sires)

θ_l is the effect the l^{th} *BCO2* W80X genotype (GG, GA and AA)

λ_m is the effect of m^{th} SNP A genotype (3 levels)

δ_n is the effects of n^{th} SNP B genotype (3 levels)

$(\lambda\delta)_{mn}$ is the interaction between m^{th} SNP A genotype and n^{th} SNP B

ε_{ijklmn} is the residual effect

Model 6: The model fitted cohort, breed, sire, *BCO2* W80X genotype and the each haplotype to analyse the effects of the haplotype on the fat colour related traits.

$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \theta_l + \varphi_m + \varepsilon_{ijklm}$

Where:

Y_{ijklm} is the response variable

μ is the overall mean

α_i is the effect of i^{th} cohort (3years x 2sex = six levels)

β_j is the effect of j^{th} breed (Jersey or Limousin)

γ_k is the effect of k^{th} sire (three sires)

θ_l is the effect the l^{th} *BCO2* W80X genotype (GG, GA and AA)

φ_m is the effect of m^{th} haplotype (levels depending on specific gene)

ε_{ijklm} is the residual effect

Model 7: In the model 7, cohort, breed and sire were fitted as fixed effects, and all investigated DNA variants were fitted as random effects in order to measure the size of effect for each SNP. Variation of each observed SNP was measured by sum of squares for each SNP divided by all SNP sum of squares plus residual sum of squares.

2.7.2 Groups 2 to 6 :

The model for Group 2 included breed, cohort (sexes and 2 years of birth), sire and genotype as fix effects. For the carcass fat colour score (scored “0 or “1”), slaughter

group was also included as a fixed effect. For Group 3, year of sampling and genotype were included as fixed effects and age as a covariate in the model. Some cows had very high β-carotene values, so the data were log-transformed before analysis. This transformation was not necessary for the fat colour score data. The model in Group 4 was sire (five levels), herd (21 levels), management group (42 levels - range of 1–4 management groups per herd), cow age (2–8 + years), breed (three levels – pure-, ¾- and ½- Holstein-Friesian), genotype and the covariate was "Days in Milk". For Group 5, the abattoir account little variance in the model, therefore, it was not included in the model and genotype was the only fixed effect in the model. For Group 6, cow age (3–6 + years) and genotype were fitted as fixed effects.

2.7.3 Group 7:

The samples from this group were used for the gene expression studies.

Model 8 The model fitted fat colour group to compare the means of the mRNA levels for *BCMO1, BCO2* and *RDHE2.*

$Y_{ij=} \mu + \alpha_i + \varepsilon_{ij}$

Where:

Y_{ij} is the response variable

μ is the overall mean

α_i is the effect of i^{th} fat colour group (2 levels)

ε_{ij} is the residual effect

Model 9 The model fitted the genotype of the 2 major genes (*BCO2* W80X or *RDHE* SNP2) to compare the means of mRNA levels for *BCMO1, BCO2 and RDHE2* and the means of β-carotene concentration for each genotype.

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

Where:

Y_{ij} is the response variable

μ is the overall mean

α_i is the effect of i^{th} genotype (*BCO2* W80X or *RDHE* SNP2, 3 levels)

ε_{ij} is the residual effect

Model 10 The general linear model was used to determine the relationship between the mRNA levels of each gene (*BCMO1, BCO2 and RDHE2*) and the β-carotene concentration.

$Y = a + bX$

Where:

Y is the β-carotene concentration

a is the constant

b is the slope

X is the effect of mRNA levels for *BCMO1, BCO2 or RDHE2*

Chapter 3

Candidate gene selection

3.1 Introduction

The aim of animal breeding is to improve farm animals genetically by exploiting the phenotypic variation of a trait that is caused by the underlying genes. Quantitative trait loci (QTL) mapping provides a powerful approach to identify the functional pathways of complex traits. QTL mapping is defined as a process to localize chromosome regions harbouring genetic variants that affect a continuously distributed, polygenic phenotype (Dipetrillo *et al.*, 2005).

Publications on the QTL for traits from cattle have increased dramatically over the past few years. There have been 5207 cattle QTLs created into the Cattle QTLdb from 294 publications representing 378 different cattle traits (Hu *et al.*, 2010). However, only a few linkage studies have been published for traits related to fat colour in cattle. The use of linear regression interval mapping to detect QTL in structured outbred populations is a useful first step, providing information about important QTL. Esmailizadeh (2006) conducted a interval mapping study in Jersey-Limousin backcross progeny from Australia and New Zealand (Groups 1 and 2 described in Chapter 2) to map quantitative trait loci (QTL) for diverse 34 traits, which included fat colour related traits. Six crossbred sires and their progeny were genotyped for 189 informative microsatellite markers covering the 29 bovine autosomes. Interval mapping detected 14 suggestive QTL segregating for fat colour traits on 11 chromosomes (Table 3-1). Besides these QTL detected by interval mapping, more QTL were identified by multiple-trait multiple marker analysis and the joint analysis of multiple families in the two countries, which improved the detection of QTL (Table 3-2). Among the QTL detected by the two different analytical models, only one QTL was found to overlap, which was at 14cM on BTA14 and affected the fat colour score of biopsy samples.

A large number of candidate genes are usually embedded in a given QTL. Identification of the genes and the DNA sequence variants that contribute to relatively minor phenotypic differences in beef fat colour is a difficult task, as information on the genes likely to affect fat colour is limited. In spite that the bovine genome has 19,139 protein-coding genes and 31,599 gene transcripts, the bovine genes were still not well annotated (http://www.ensembl.org/Bos_taurus/Info/StatsTable?db=core, accessed November 2007). Therefore, in addition to the directed selection of candidate genes from the bovine genome sequence database, a comparative genomics approach provided another strategy to identify potential candidates. The essentially complete version of the human genome sequence still provides a foundation and reference for facilitating genome mapping in other mammals (Jiang *et al.*, 2002). Fortunately, detailed human/bovine comparative maps have been developed (Hayes *et al.*, 2003), which increases the possibility of exploiting the bovine genome sequence. The comparative mapping between the human and bovine genome provided a "first guess" for genes that may control fat colour for those genes that had not been annotated at the time. This comparative strategy included a comparative structural genomics approach and a comparative functional genomics approach in which both the position of genes within a QTL was considered as well identifying the genomic location of all enzymes and proteins known to be involved in β-carotene metabolism and vitamin A synthesis. The expected locations of these genes in the bovine genome were then examined for overlap with any of the detected QTL.

Table 3-1 QTL detected by interval mapping for fat colour traits
(from Esmailizadeh, 2006)

NOTE:
This table is included on page 57
of the print copy of the thesis held in
the University of Adelaide Library.

Table 3-2 QTL detected by multiple-trait multiple marker for fat colour traits
(from Esmailizadeh, 2006)

3.2 Methods

Because the human genome sequence was better annotated than the bovine genome, particularly at the beginning of the project, candidate genes were chosen based on the QTL for fat colour in cattle and comparative mapping between the cattle and human genome maps. Information on the QTL for fat colour and β-carotene concentration in subcutaneous fat in cattle was provided by Esmailizadeh (2006). The QTL intervals taken were approximately 25 cM either side of the significant QTL peaks. The human genome regions orthologous to these QTL intervals were obtained from the location of the flanking QTL markers (that is, the most tightly-linked marker on each side of a QTL) in the whole genome cattle radiation hybrid panel (http://www.animalgenome.org/cattle/maps/RHMap3/) and the location of the human map (Table 3-3). The candidate genes in these orthologous regions were selected from the human genome sequence in the National Centre for Biotechnology information database (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606) and Ensembl database (www.ensembl.org).

DNA variants were discovered by sequencing the genomic DNA of the three Davies gene mapping sires. Sequencing was performed as recommended by the manufacturer's BigDye terminator cycle sequencing protocol (*PE Applied Biosystems*). Sequences of each amplicon in both forward and reverse direction were aligned with the Sequencher^{TM} software package (*Gene Codes Corporation*) in order to identify the DNA variants.

3.3 Results

3.3.1 Candidate gene selection

Based on the comparative maps among human and cattle, candidate genes were selected for the regions of interest. The 21 QTL for β-carotene concentration in subcutaneous fat, fat colour score of biopsy and carcass samples were located on 18 chromosomes (Table 3-1 and Table 3-2). No obvious candidate genes related carotenoid metabolism in the human orthologous regions were found for the QTL on BTA 11, 12, 24 and 28. In total, 17 candidate genes, which are involved in metabolism of β-carotene and fatty acids, were identified in the orthologous regions in the human genome (Table 3-3). Of these, 7 candidate genes were selected based on their function in carotenoid metabolism for sequencing to identify DNA variants. Although BCMO1was not located in any of the identified QTL, it was also selected for study because of its important role in catalysing β-carotene cleavage into vitamin A (Goodman and Huang, 1965).

3.3.2 SNP identification and selection for association studies

In order to study the association between the candidate genes and the traits of interest, polymorphisms in each gene were found. The bovine candidate gene sequence information was obtained by accessing the sequence databases of NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=nucleotide), and Bovine Ensembl genome database (http://www.ensembl.org). Eight candidate genes were surveyed by directly sequencing the genomic DNA from the three gene mapping sires of Group 1. A total of 111 DNA variants were found in these 8 genes (Appendix 4). Most of the SNPs were located in the intronic regions (Table 3-4). Twenty nine were located in the 5’ or 3’ flanking regions, and 17 in the coding regions. Only *RARA* and

BCO2 were found to contain 3 and 1 DNA variants in the 3' untranslated region separately. *RDHE2* had the highest number of SNPs in exons, whereas no exonic SNPs were identified in *SCARB1*.

Of those identified DNA variants, 27 SNPs were selected for further study (Table 3-5). Eleven SNPs were located in the coding regions, which included three synonymous variants and seven non-synonymous amino acid substitutions. Notably, of the seven non-synonymous SNPs, one SNP in *BCO2* exon 3 causes the substitution of a stop codon (encoded by the A allele) for tryptophan[80] (encoded by the G allele).

After the polymorphisms were identified in the candidate genes, those SNPs selected for association studies were further sequenced using the grandparent DNA to verify Mendelian inheritance and to assist in the formation of the sire haplotypes.

Table 3-3 QTL on BTA and orthologous regions in human genomes and putative candidate genes

BTA	Trait	Possible QTL interval position[1] (cM)	Orthologous region in human[2] (Mb)	Candidate gene (HGNC symbol)[3]	Gene location in human (Mb)	Gene full name
1	Fc-bio	31-81	I3: 77-126; 188-198	ADH5P2	79.9	alcohol dehydrogenase 5 (class III), chi polypeptide, pseudogene 2
2	Bc-bio	47-97	I2: 114-121; 135-146, 192-225			
4	Bc-bio	0-37	I7: 90-100			
6	Fc-bio	24-74	I4: 21-56; 89-102	PPARGC1A	23.9	peroxisome proliferative activated receptor, gamma, coactivator 1, alpha
	Fc-car	32-82				
	Fc-bio	45-95	I4: 89-111	ADH6	100.1	alcohol dehydrogenase 6 (class V)
				ADH1A	100.2	alcohol dehydrogenase1A(classI), α polypeptide
				ADH1B	100.2	alcohol dehydrogenase 1B (class I), β polypeptide
				ADH1C	100.2	alcohol dehydrogenase 1C (class I), γ polypeptide
				ADH7	100.3	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
8	Fc-car	21-73	I9: 2-36			
9	Fc-car	50-100	I6: 88-101 132-160	ALDH8A1	135.2	aldehyde dehydrogenase 8 family, member A1
11	Fc-bio	90-130	I2: 10-20, I9: 188-198			
12	Fc-car	50-100	I13: 19-30, 76-98			
	Bc-bio	67-117	I13: 76-113			
14	Fc-bio	0-50	I8: 12-15, 49-65	RDHE2	57	short chain dehydrogenase/62eductases family 16C, member 5
15	Fc-bio	24-74	I11: 71-74, 110-121	BCO2	112	β-carotene oxygenase 2
16	Bc-bio	26-76	I1: 1-183, 211-224	RXRG	169.5	retinoid X receptor, gamma
17	Bc-bio	56-106	I4: 158-160; I12: 107-131; I22: 18.3-29	SCARB1	125.3	Scavenger receptor class B member 1
19	Fc-bio	59.2	I17: 43-50	MED1	39.4	mediator complex subunit 1
	Bc-bio			RARA	40.6	retinoic acid receptor alpha
22	Fc-bio	82.9	I6: 50-60	PPARG	54.7	peroxisome proliferative activated receptor, gamma
	Bc-bio					
23	Fc-bio	42.4	I6: 25-37	APOM	24.1	apolipoprotein M
	Bc-bio					
24	Fc-bio	8,1	I18: 60-72			
	Bc-bio					
27	Fc-bio	10	I4: 180-190	ALDH1A3	11.7	aldehyde dehydrogenase family 1, subfamily A3
	Bc-bio					
28	Fc-bio	24.7	I10: 58-70			
	Bc-bio					

[1]Bovine genetic map. [2]Human genome map. [3]HGNC symbol: official HUGO Gene Nomenclature Committee symbol.

Table 3-4 DNA variants of the candidate genes

Gene	Total # DNA variants	Types of DNA variants			
		5' and 3'flank	5' and 3'UTR	Exon	Intron
ALDH8A1	17	5		3	9
APOM	14	8	-	1	5
BCMO1	17	8	-	1	8
BCO2	10	3	1	1	5
RARA	13	3	3	2	2
RDHE2	9	1	-	5	3
PPARGC1A	14		-	4	10
SCARB1	17	1	-	-	16

Table 3-5 Genotyped DNA variants

SNP name	DNA variant	Location
ALDH8A1 SNP10	G/A (glycine^{135}→aspartic acid^{135})	Exon 3
ALDH8A1 SNP15	T/C (threonine^{240}→ methionine^{240})	Exon 5
ALDH8A1 SNP16	A/G (isoleucine^{330}→ valine^{330})	Exon 6
APOM SNP2	A/G	5'flanking
APOM SNP6	A/G	5'flanking
APOM SNP7	T/G	Intron 1-2
BCMO1 SNP4	A/C	5'flanking
BCMO1 SNP7	C/T	5'flanking
BCMO1 SNP8	G/C	5'flanking
BCMO1 SNP13	C/T	Intron 9-10
**BCO2* SNP1	C/T	Intron 3-4
**BCO2* SNP2	G/T	Intron 3-4
**BCO2* SNP3	G/T	Intron 4-5
**BCO2* W80X	G/A (tryptophan^{80}→stop)	Exon 3
RARA SNP1	T/G	5'flanking
RARA SNP3	A/G	5'flanking
RARA SNP7	C/T (asparagine^{133}→asparagine^{133})	Exon 3
RDHE2 SNP2	T/C (valine^{6}→ alanine^{6})	Exon 1
RDHE2 SNP3	C/T (alanine^{33}→valine^{33})	Exon 1
RDHE2 SNP4	G/T (threonine^{94}→threonine^{94})	Exon 1
PPARGC1A SNP4	G/A	Intron 2-3
PPARGC1A SNP9	T/C (histidine^{403}→histidine^{403})	Exon 8
PPARGC1A SNP10	G/A (alanine^{437}→threonine^{437})	Exon 8
PPARGC1A SNP12	C/T (proline^{616}→leucine^{616})	Exon 9
SCARB1 SNP1	G/C	5'flanking
SCARB1 SNP2	G/A	Intron 1-2
SCARB1 SNP16	G/C	Intron 11-12

* Variants were discovered earlier in a Coursework Master research project.

3.3.3 Genotype and allele frequencies

The allele and genotype frequencies were determined for the genotyped SNPs (Group 1, Table 3-6). The lowest frequency SNP was *RDHE2* SNP4 allele A (p = 0.1), followed by *RARA* SNP3 allele G (p = 0.12). *RDHE2* SNP2 alleles were segregating with equal frequency (p = 0.5) in the population. Due to low minor allele frequencies for *APOM* SNP 6, *BCO2* SNP W80X, *RARA* SNP3, *RDHE2* SNP4 and *PPARGC1A* SNP10, the genotype frequency for the rare homozygote was below 1%.

Table 3-6 Genotypic and allelic frequencies of SNPs

	Genotype	No. of animal	Genotype frequency	Allele	Allele frequency
ALDH8A1 SNP10	AA	27	0.07	A	0.33
	GA	187	0.51	G	0.67
	GG	151	0.41		
ALDH8A1 SNP15	CC	8	0.02	C	0.20
	CT	127	0.35	T	0.80
	TT	230	0.63		
ALDH8A1 SNP16	AA	159	0.44	A	0.70
	AG	191	0.52	G	0.30
	GG	15	0.04		
APOM SNP2	AA	267	0.73	A	0.86
	GA	92	0.25	G	0.14
	GG	6	0.02		
APOM SNP6	AA	284	0.78	A	0.88
	GA	78	0.21	G	0.12
	GG	3	0.01		
APOM SNP7	GG	132	0.36	G	0.59
	GT	169	0.46	T	0.41
	TT	64	0.18		
BCMO1 SNP4	AA	279	0.76	A	0.87
	CA	79	0.22	C	0.13
	CC	7	0.02		
BCMO1 SNP7	CC	212	0.58	C	0.78
	CT	142	0.39	T	0.22
	TT	11	0.03		
BCMO1 SNP8	CC	9	0.02	C	0.20
	CG	131	0.36	G	0.80
	GG	225	0.62		
BCMO1 SNP13	CC	141	0.39	C	0.62
	CT	171	0.47	T	0.38
	TT	53	0.15		
BCO2 SNP1	AA	8	0.02	A	0.13
	AG	80	0.22	G	0.87
	GG	275	0.76		
BCO2 SNP2	AA	7	0.02	A	0.13
	AC	82	0.23	C	0.87
	CC	271	0.75		
BCO2 SNP3	CC	265	0.79	C	0.88
	CG	65	0.19	G	0.12
	GG	7	0.02		
BCO2 W80X	AA	4	0.01	A	0.13
	AG	86	0.24	G	0.87
	GG	272	0.75		
RARA SNP1	GG	47	0.13	G	0.40
	GT	194	0.53	T	0.60
	TT	123	0.34		

Table 3-6 continued.

	Genotype	No. of animal	Genotype frequency	Allele	Allele frequency
RARA SNP3	AA	284	0.78	A	0.89
	AG	79	0.22	G	0.11
	GG	2	0.01		
RARA SNP7	CC	190	0.52	C	0.72
	CT	147	0.40	T	0.28
	TT	28	0.08		
RDHE2 SNP2	TT	98	0.27	T	0.50
	TC	164	0.45	C	0.50
	CC	101	0.28		
RDHE2 SNP3	AA	16	0.04	A	0.23
	AG	132	0.36	G	0.77
	GG	216	0.59		
RDHE2 SNP4	AA	1	0.00	A	0.10
	AC	71	0.20	C	0.90
	CC	291	0.80		
PPARGC1A SNP4	AA	14	0.04	A	0.26
	GA	160	0.44	G	0.74
	GG	191	0.52		
PPARGC1A SNP9	CC	27	0.07	C	0.27
	CT	140	0.38	T	0.73
	TT	198	0.54		
PPARGC1A SNP10	AA	4	0.01	A	0.12
	GA	81	0.22	G	0.88
	GG	280	0.77		
PPARGC1A SNP12	CC	115	0.32	C	0.62
	CT	221	0.61	T	0.38
	TT	29	0.08		
SCARB1 SNP1	CC	119	0.33	C	0.57
	GC	176	0.48	G	0.43
	GG	70	0.19		
SCARB1 SNP2	AA	37	0.10	A	0.32
	GA	162	0.44	G	0.68
	GG	166	0.45		
SCARB1 SNP16	CC	13	0.04	C	0.19
	GC	110	0.30	G	0.81
	GG	242	0.66		

3.4 Discussion:

Because the human genome sequence was better annotated than bovine genome particularly at the beginning of the project, candidate genes were chosen based on the QTL for fat colour in cattle and comparative mapping between the cattle and human genome maps. With the recent annotation developments of bovine genome sequence, particularly the release of BTA 4.0, more bovine genes could be accessed from bovine public genome database. Therefore, the selected candidate genes were continually confirmed for their location using the Ensembl Bovine Genome Database (http://www.ensembl.org). Identification of the candidate genes (and hence, the corresponding underlying causal mutation) was hampered by two factors: 1) the limited knowledge of the genetics of beef fat colour and 2) interruption in the synteny conservation between the human and bovine genomes prevented completely accurate alignment. As a consequence of these problems, the candidate gene search herein cannot be considered exhaustive. Firstly, since the major contribution of yellow fat colour is deposition of β-carotene, only genes involved in the metabolism of β-carotene were investigated. Secondly, as broad QTL regions were considered, candidate genes at syntenic breakpoints or unannotated genes from the bovine genome may have been overlooked.

Eight suitable candidate genes were chosen based on their positional, physiological and comparative aspects for genotyping and subsequent investigation of associations with beef fat colour. From the identified QTL, although the QTL on BTA 15 had a relatively low F-value of 7.3, β-carotene oxygenase 2 gene (*BCO2*) had the greatest potential as a positional candidate gene for this QTL because of the function of BCO2 in the metabolism of β-carotene. Kiefer *et al.* (2001) provided the first evidence that the

BCO2 enzyme plays a role in catalysing the eccentric cleavage of β-carotene. Therefore, it was conceivable that genetic variation in the BCO2 gene on BTA 15 affects β-carotene concentration and consequently, impacts fat colour.

The other major pathway for β-carotene cleavage is the symmetric cleavage by BCMO1. The *BCMO1* gene was not found in any of the QTL interval regions. However, this gene plays the critical role of cleaving β-carotene symmetrically in the rate limiting step for vitamin A synthesis from β-carotene, and the gene for *BCMO1* is tightly controlled by transcriptional factors like PPARγ and RXR. Therefore, this gene was also considered as a potential candidate gene.

Peroxisome proliferator-activated receptor γ (PPARγ), one of nuclear hormone receptors, is a master regulator of adipogenesis (Macdougald and Lane, 1995). A peroxisomal proliferator response element (PPRE) was identified in the promoter region of the mouse *BCMO1* gene (Boulanger *et al.*, 2003). The PPRE region specifically binds peroxisome proliferator-activated receptor-γ and subsequently, regulates transcriptional expression of the *BCMO1* gene. The PPARγ coactivator 1 (PPARGC1A) is the PPARγ main coactivator and binding ligand (Larrouy *et al.*, 1999). It has been found this coactivator plays a critical role in fat metabolism and coordinating the process of metabolic adaptation in liver, fat tissue and muscle (Puigserver and Spiegelman, 2003). Therefore, *PPARGC1A* was a potential positional candidate gene for the QTL on BTA 6.

In addition to PPRE, a retinoic acid response element (RARE) has been also identified in the promoter region of the *BCMO1*gene (Bachmann *et al.*, 2002). This RARE site is

recognised by the retinoic acid complex. This complex is either formed by retinoic acid and retinoic acid receptor or formed by retinoic acid and one of the retinoid X receptors (RXRα, RXRβ and RXRγ). Thus, *BCMO1* gene expression can be regulated by retinoic acid at the transcriptional level. Consequently, the *RARA* gene, which functions as a heterodimer with RXR, was a candidate for the QTL effects on BTA 19.

Other enzymes, which contribute to the retinoic acid formation, also are potential candidates for effects on beef fat colour. In vertebrate tissues, retinoic acid can be formed from retinol or retinal oxidation as catalysed by alcohol and aldehyde dehydrogenases, respectively. RDHE2, as a member of the short-chain alcohol dehydrogenase/reductases (SDR) families, is involved in the oxidation of all-*trans* retinol to all-*trans* retinal. This oxidation is first and rate-limiting step in the retinoic acid synthetic pathway from retinol (Duester, 2000). It should be noted that the name of *RDHE2* gene in the bovine gene database (http://www.ensembl.org) currently refers to the wrong gene. There are the *RDHE2* (*SDR16C6*, accession No: ENSBTAP00000048107) and *RDHE2 similar* genes (*SDR16C5*, accession No: ENSBTAP00000024714) in the bovine gene database (Appendix 8.1). These two bovine gene names are reversed compared to the homologous human *RDHE2* gene (*SDR16C5*). Comparative analysis was conducted between amino acid sequences of the two bovine *RDHE2* genes (*RDHE2* and *RDHE2 similar*) and the human *RDHE2* gene (Appendix 8.2). The amino acid sequences were aligned using ClustalW Multiple program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). There are 3 human *RDHE2* transcripts (*SDR16C5-001*, *SDR16C5-002* and *SDR16C5-003*) (Appendix 8.1) in Ensembl database. All of these three human *RDHE2* transcripts had higher homologies to the bovine *RDHE2 similar* gene (*SDR16C5*) than to the bovine *RDHE2* gene

(*SDR16C6*) (Appendix 8.3). This indicates that there is a mistake in the current annotation of the current bovine genome sequence (BTA4.0) and the bovine *RDHE2 similar* gene (*SDR16C5*) is the actual "*RDHE2*" gene and should be the sequence used for analyses herein. Therefore, the correct annotation name "*RDHE2*" is used herein but refers to the bovine *SDR16C5* sequence in BTA4.0 (accession No: ENSBTAP00000024714).

The second pathway for retinoic acid synthesis is catalysed by the aldehyde dehydrogenase (ALDH) family and is an irreversible conversion. ALDH8A1 encodes the protein that plays a critical role in converting 9-*cis*-retinal into 9-*cis*-retinoic acid, the retinoid X receptor ligand. It was the first known aldehyde dehydrogenase to show a preference for 9-*cis*-retinal relative to all-*trans*-retinal and has a higher activity with 9-*cis*-retinal than with all-*trans*-retinal (Lin and Napoli, 2000). Due to the regulation of *BCMO1* by retinoic acid, the dehydrogenase genes *ALDH8A1* and *RDHE2* were both logical candidate genes for the beef fat colour QTL on BTA 9 and BTA 14, respectively.

The remaining candidate genes are involved in the transportation of β-carotene. Chylomicrons are responsible for the transport of carotenoids from the intestinal mucosa to the bloodstream. However, the high-density lipoproteins (HDL) in cattle are the richest fraction of β-carotene and account for 77-83% of the total carotenoids (Yang *et al.*, 1992). The recently discovered apolipoprotein M (APOM) is a plasma protein of the lipocalin family associated with the lipoproteins, mainly HDL (Nielsen *et al.*, 2009). Changes in plasma APOM concentration can cause quantitative and qualitative changes in human HDL. Therefore, the hypothesis was that concentration of β-carotene may be altered by a change of plasma APOM concentration in cattle HDL.

Scavenger receptor class B, type I (SCARB1) was reported to facilitate β-carotene cellular uptake in *Drosophila* (Kiefer *et al.*, 2002). In addition, an *in vitro* study provided the evidence that β-carotene transport can be inhibited by anti-SCARB1 in Caco-2 cells (During *et al.*, 2005). Therefore, the *APOM* and *SCARB1* genes, which could affect the transportation of β-carotene, represent potential candidates for the QTL on BTA 23 and BTA 17, respectively.

In summary, *ALDH8A1*, *APOM*, *BCMO1*, *BCO2*, *RARA*, *RDHE2*, *PPARGC1A* and *SCARB1* were chosen as candidate genes based on their location in fat colour QTL and their function in the metabolism of β-carotene. These eight candidate genes were sequenced in the three gene mapping sires (sires of Group 1 progeny) for the promoter, 5' UTR, 3' UTR, each exon and their flanking regions. SNPs might be associated with functional significance (coding, intronic, promoter, *etc*.) by affecting amino acid composition, mRNA stability, gene expression, or alternative splicing (Duan *et al.*, 2003). Twenty seven out of the 111 identified DNA variants were selected for further study (3-5 SNPs per gene) (Table 3-5). 40% of these selected DNA variants were in coding regions. The remaining variants in the introns or flanking regions were selected based on a preliminary study of grandparental haplotypes for each candidate gene to avoid linkage disequilibrium. As the alleles of one polymorphic marker would not be a surrogate for a specific allele of another marker (Brookes, 1999), these selected SNPs should provide more information in beef fat colour association studies.

***Note**: Some results in this chapter (as indicated in the Tables) were part of a research project in fulfilment of the requirements for my Masters of Agricultural Biotechnology by Coursework at the University of Adelaide in 2006. These included the sequencing of the bovine *BCO2* gene and identification of the variants therein. The genotyping and analyses of the *BCO2* SNPs in Groups 1-7 were completed as part of my PhD research described in Chapters 4-7 and published (Tian *et al.*, 2010).

Chapter 4

Association between candidate gene SNPs and fat colour traits

4.1 Introduction

Compared with other types of DNA markers, single nucleotide polymorphisms (SNPs) are attractive because they are abundant, have low mutation rates and are easily adapted to high throughput methods of genotypic analysis. Based on the important role of β-carotene in beef fat colour, a number of candidate genes involved in the metabolism of β-carotene were selected for genotyping (Chapter 3). The objective of this study was to explore the possible association of selected single nucleotide polymorphisms (SNPs) in these bovine candidate genes with fat colour related traits in the Davies Limousin x Jersey backcross progeny (Group 1).

4.2 Materials and methods

The animals and methods used in this chapter were as described in Chapter 2. The animals used in this analysis were derived from Group 1, the Davies gene mapping Limousin x Jersey backcross progeny. Twenty seven SNPs in eight candidate genes were investigated in the association studies with 3 traits (biopsy fat colour score, carcass fat colour score and β-carotene concentration in the fat). For the statistical analysis, as described in Chapter 2. Model 1 was used to show the effects of cohort, breed and sire on fat colour related traits; Model 2 was used to analyse the effects of the individual SNPs on the fat colour related traits by fitting cohort, breed, sire and the SNP genotype; Model 3 fitted cohort, breed, sire, the *BCO2* W80X genotype and the SNP genotype to analyse the effects of the SNPs on the phenotypic traits taking the *BCO2* W80X mutation into account; Model 4 was used to estimate the additive and dominance effects of the SNPs, as cohort, breed, sire, *BCO2* W80X genotype, additive and dominance effects were fitted in the model.

For QTL mapping, linkage of each trait to markers on the autosomes was tested using the QTL Express software package (Seaton *et al.*, 2002) (http://qtl.cap.edu.ac.uk, accessed 3rd November 2007). QTL Express was replaced by GridQTL in 2011, so QTL also were detected using this web service (http://www.gridqtl.org.uk/ accessed 3rd April 2011). The same model to detect QTL was used in both cases. The analysis was a multimarker approach for interval mapping in the half-sib families, as described by Haley and Knott (1992). Three steps were applied to detect QTL. Firstly, informative marker alleles from the sires were identified to determine which allele the progeny inherited. Each marker was informative for at least for one sire. Secondly, the probability of the individual progeny inheriting either allele 1 or 2 from their sire was calculated. Then, these probabilities were combined, providing coefficients upon which the phenotypic data were regressed.

A single QTL model was fitted for each trait. The least squares regression model was used for the QTL mapping. Model Q-1 was fitted with cohort (combination of sex and year, six levels), breed of dam (Jersey or Limousin), and sire (3 levels). To evaluate the association of the SNP genotype with the QTL, the SNP genotype was fitted in the Model Q-2. If the SNP is causal or in linkage disequilibrium with the QTL, it is expected to absorb a large proportion of the QTL effect and decrease the QTL significance.

$$Y_{ijlm} = \mu_i + \alpha_j + \beta_l + b_i x_{ij} + e_{ijlm} \qquad \text{(Model Q-1)}$$

$$Y_{ijklm} = \mu_i + \alpha_j + \beta_l + b_i x_{ij} + \theta_k + e_{ijklm} \qquad \text{(Model Q- 2)}$$

where

μ is the mean of sire family,

α_j is effect of the j^{th} breed of dam (Jersey or Limousin),

β_l is effect of the l^{th} cohort (combination of sex and year, six levels),

$b_i x_{ij}$ b_i is the allele substitution effect of the QTL within family I, x_{ij} is the probability that animal m inherited the (arbitrarily assigned) first haplotype of sire I,

θ_k is effect of the k^{th} candidate gene genotype , and

e_{ijklm} is the residual effect.

Within every half-sib family, a QTL was fitted at 1-cM intervals along the chromosome. The 5% genome-wise significance threshold was used as a threshold for suggestive linkage. F values ≥4 and F values ≥7 were regarded as above the threshold for statistically inferring the potential position of a QTL for the across three sire family analysis and the single sire family analysis, respectively (Churchill and Doerge, 1994). Confidence intervals for QTL positions were estimated by generating 1000 bootstrap resamples (Churchill and Doerge, 1994). Permutation and bootstrap analysis were performed using QTL Express or GridQTL.

4.3 Results

4.3.1 Cohort, breed, and sire effects

In order to discover the effects of cohort, breed of dam and sire families on fat colour related traits, studies were performed with a model that included the effects of cohort, breed and sire (Model 1, section 2-7). The least squares means with standard errors from the model were calculated for cohort, breed, and sire effects of the fat colour related traits (Table 4-1, Figure 4-1, Figure 4-2 and Figure 4-3). Breed had a highly significant effect on all traits. The Jersey backcross progeny had higher β-carotene

concentrations and fat colour scores than the Limousin backcross progeny. There was a significant effect of cohort (combination of year and sex) on all traits. The fat in the animals born in 1998 was more yellow and contained higher β-carotene concentrations than the animals born in 1996 and 1997. Sire also had significant effects on all traits with exception of the fat colour score from the biopsy samples.

Table 4-1 Least squares means with standard errors for cohort, breed, and sire

Trait[1]	Cohort[2]						Breed[3]		Sire[4]		
	96H	**96S**	**97H**	**97S**	**98H**	**98S**	**XJ**	**XL**	**361**	**368**	**398**
Bc-bio	0.98	0.83	0.95	0.93	1.49	1.52	1.23	1.04	1.11	1.07	1.28
	±0.07	±0.09	±0.06	±0.05	±0.06	±0.05	±0.03	±0.04	±0.04	±0.04	±0.04
			P<0.001				**P<0.001**		**P=0.002**		
Fc-bio	2.02	1.96	1.88	1.96	2.18	2.13	2.23	1.76	1.95	2.04	2.10
	±0.08	±0.11	±0.07	±0.06	±0.07	±0.07	±0.07	±0.07	±0.05	±0.05	±0.05
			P=0.04				**P<0.001**		P=0.13		
Fc-car	0.89	0.70	0.71	1.92	2.98	2.59	2.25	1.21	1.62	1.71	2.04
	±0.14	±0.18	±0.12	±0.11	±0.12	±0.11	±0.07	±0.07	±0.09	±0.08	±0.09
			P<0.001				**P<0.001**		**P=0.003**		

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-9); Fc-bio, fat colour score of biopsy samples (scale 1-5). [2]**Cohort**: combination of year (96, 97 and 98) with sex (heifer and steer). [3]**Breed**: XJ, Jersey backcross; XL, Limousin backcross. [4]**Sire**: 3 sire families (361, 368 and 398)

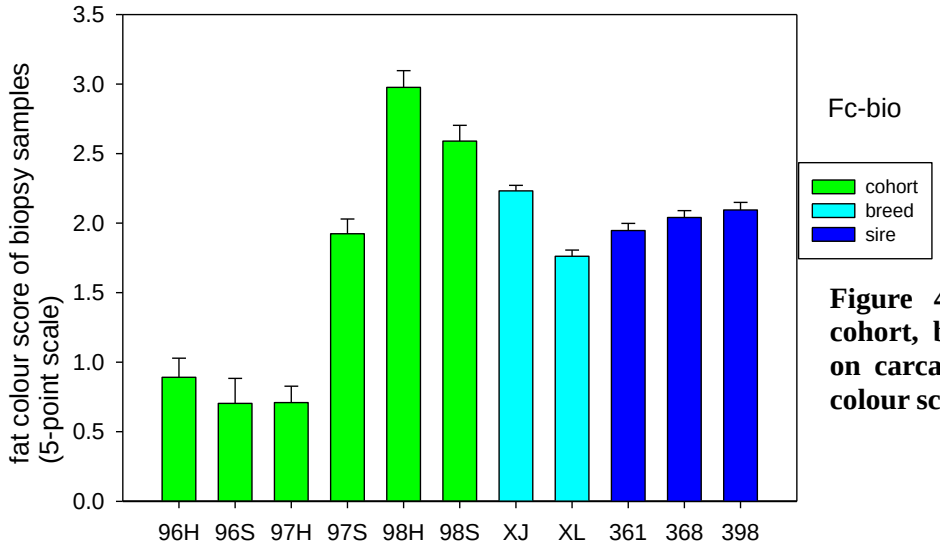


Figure 4-2 Effects of cohort, breed and sire on carcass samples fat colour score

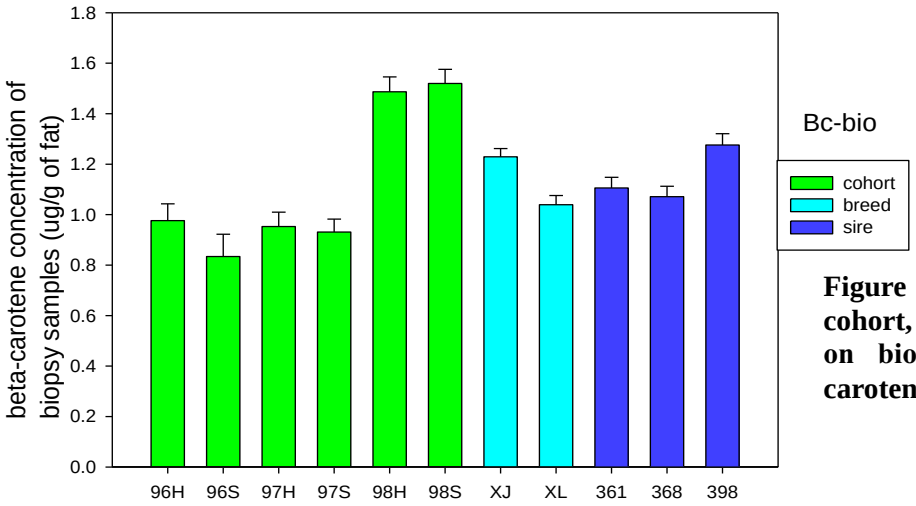


Figure 4-1 Effects of cohort, breed and sire on biopsy sample β-carotene concentration

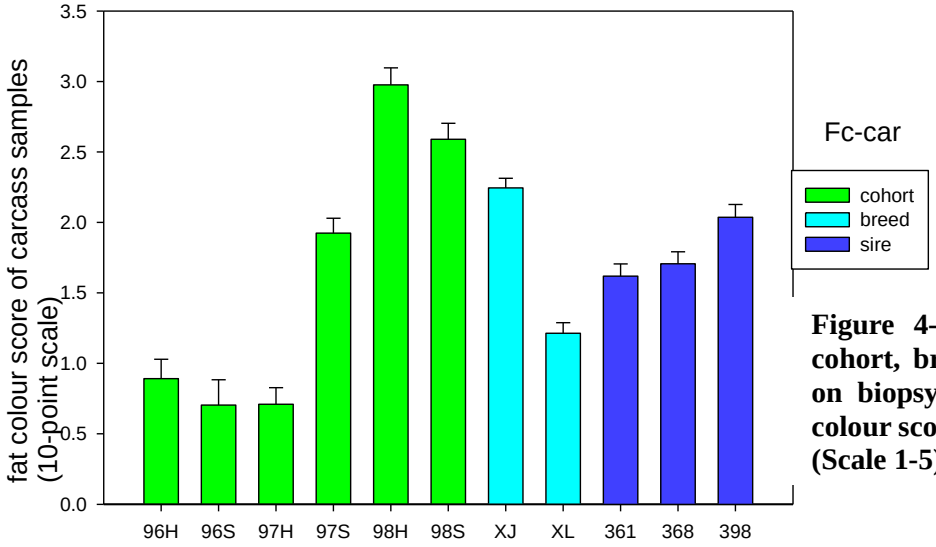


Figure 4-3 Effects of cohort, breed and sire on biopsy samples fat colour score (Scale 1-5)

4.3.2 Individual SNP genotype effects

In order to discover the genes associated with fat colour related traits, the SNP genotype effects were determined with a model (Model 2) that included cohort, breed, sire and individual SNP genotype (Table 4-2 and Table 4-3). At least one SNP for each candidate gene was near the significance threshold ($P<0.05$) with the exception of *RARA*.

For the *ALDH8A1* gene SNP, *ALDH8A1*SNP15, the C allele was associated with a lower carcass fat colour score than the T allele (Table 4-2). The estimated carcass fat colour score difference between the homozygous genotypes was 0.64 on a 5-point scale. The *APOM* SNP2 was significantly associated with carcass fat colour score. Animals with the GA genotype had 15.9 and 12.4% higher fat colour scores than animals with AA or GG genotypes ($P<0.05$), respectively. Similarly, animals with the heterozygous genotype for *BCMO1* SNP4 had significantly higher β-carotene concentrations than those with either homozygous genotype. A highly significant relationship also existed between the *PPARGC1A* SNP12 genotype and all traits. The animals with the TT genotype tended to have yellower fat and higher β-carotene concentrations (Table 4-2). For the *SCARB1*gene, only the *SCARB1* SNP1 was associated with fat colour score of biopsy samples, cattle with the CC genotype had the highest biopsy fat colour scores. Most importantly, the SNP *BCO2* W80X had highly a significant effect on all fat colour traits. In terms of the proportion of variation in fat colour related traits, the fraction of the phenotypic variance explained by a single SNP varied between 0% and 16% (Table 4-3). Most of the SNPs explained less than 2% variation in the fat colour traits. However, the BCO2 W80X contributed half (12-15%) of the total SNP variance for the fat colour related traits. In total, the SNP effects accounted for 25-29% of the phenotypic variance in the mapping progeny (Table 4-3).

Table 4-2 Association of candidate gene SNPs with fat colour traits

		Bc-bio[1]			Fc-bio[1]			Fc-car[1]		
	Genotype	**mean**	**se**	**P-value**	**mean**	**se**	**P-value**	**mean**	**se**	**P-value**
ALDH8A1 SNP10	AA	1.06	0.09	0.52	2.02	0.11	0.72	1.92	0.19	0.20
	GA	1.14	0.03		2.00	0.04		1.69	0.07	
	GG	1.17	0.04		2.05	0.05		1.86	0.08	
ALDH8A1 SNP15	CC	1.11	0.16	0.69	2.32	0.19	0.30	1.10	0.36	**0.03(*)**
	CT	1.11	0.05		2.03	0.06		1.92	0.07	
	TT	1.16	0.03		2.01	0.04		1.74	0.06	
ALDH8A1 SNP16	AA	1.18	0.04	0.68	2.08	0.04	**0.09†**	1.85	0.08	0.32
	AG	1.13	0.03		1.97	0.04		1.69	0.07	
	GG	1.08	0.12		2.20	0.14		2.03	0.25	
APOM SNP2	AA	1.14	0.03	0.75	2.03	0.04	0.92	1.69	0.06	**0.05(*)**
	GA	1.16	0.05		2.01	0.07		2.01	0.11	
	GG	1.01	0.20		1.96	0.24		1.80	0.40	
APOM SNP6	AA	1.13	0.03	0.72	2.02	0.04	0.91	1.77	0.06	0.27
	GA	1.19	0.06		2.05	0.07		1.82	0.12	
	GG	1.15	0.27		2.05	0.33		0.73	0.68	
APOM SNP7	GG	1.17	0.04	0.53	2.04	0.05	0.44	1.76	0.09	0.24
	GT	1.15	0.04		2.05	0.04		1.85	0.07	
	TT	1.09	0.06		1.94	0.07		1.61	0.12	
BCMO1 SNP4	AA	1.11	0.03	**0.01 (**)**	2.03	0.04	0.90	1.75	0.06	0.72
	CA	1.30	0.06		2.01	0.07		1.83	0.12	
	CC	1.00	0.18		2.11	0.22		2.01	0.40	
*BCMO1*SNP7	CC	1.14	0.03	0.92	2.02	0.04	0.70	1.73	0.07	0.53
	CT	1.16	0.04		2.05	0.05		1.82	0.08	
	TT	1.11	0.15		1.91	0.18		2.01	0.30	
BCMO1 SNP8	CC	0.91	0.16	**0.07†**	2.09	0.17	0.63	2.19	0.37	0.51
	CG	1.21	0.04		1.99	0.05		1.76	0.09	
	GG	1.12	0.03		2.04	0.04		1.77	0.07	
BCMO1 SNP13	CC	1.18	0.04	0.63	2.00	0.05	0.68	1.84	0.09	0.53
	CT	1.13	0.04		2.05	0.04		1.76	0.08	
	TT	1.11	0.07		1.99	0.08		1.64	0.14	
BCO2 SNP1	AA	0.94	0.17	**0.10†**	1.61	0.21	**0.01(**)**	1.48	0.35	**0.10†**
	AG	1.05	0.06		1.88	0.07		1.56	0.12	
	GG	1.18	0.03		2.08	0.04		1.84	0.06	
BCO2 SNP2	AA	1.20	0.18	0.67	1.79	0.22	**0.08†**	1.82	0.37	0.19
	AC	1.18	0.06		2.14	0.07		1.96	0.12	
	CC	1.13	0.03		1.99	0.04		1.71	0.06	
BCO2 SNP3	CC	1.12	0.03	0.83	1.98	0.03	0.27	1.68	0.05	0.72
	CG	1.08	0.05		2.02	0.06		1.79	0.12	
	GG	1.12	0.16		1.71	0.19		1.71	0.33	
BCO2 W80X	AA	2.67	0.23	**<0.001(***)**	4.14	0.27	**<0.001(***)**	4.82	0.46	**<0.001(***)**
	AG	1.17	0.05		2.14	0.06		1.98	0.11	
	GG	1.12	0.03		1.96	0.03		1.68	0.06	

Table 4-2 continued.

		Bc-bio[1]			Fc-bio[1]			Fc-car[1]		
	Genotype	**mean**	**se**	**P-value**	**mean**	**se**	**P-value**	**mean**	**se**	**P-value**
RARA SNP1	GG	1.12	0.07	0.94	2.03	0.09	0.94	1.84	0.15	0.23
	GT	1.15	0.04		2.01	0.04		1.85	0.07	
	TT	1.16	0.05		2.04	0.06		1.63	0.10	
RARA SNP3	AA	1.16	0.03	0.75	2.00	0.04	0.56	1.78	0.06	0.95
	AG	1.11	0.06		2.10	0.08		1.78	0.13	
	GG	0.99	0.34		2.21	0.41		1.56	0.68	
RARA SNP7	CC	1.16	0.03	0.12	2.06	0.04	**0.06†**	1.77	0.07	0.61
	CT	1.16	0.04		2.03	0.05		1.81	0.08	
	TT	0.95	0.10		1.78	0.12		1.60	0.20	
RDHE2 SNP2	TT	1.04	0.05	**0.06†**	2.07	0.07	0.52	1.65	0.11	**0.06†**
	TC	1.15	0.04		1.99	0.04		1.73	0.08	
	CC	1.24	0.05		2.04	0.06		2.02	0.11	
RDHE2 SNP3	AA	1.04	0.13	0.52	2.02	0.16	0.54	1.94	0.28	0.16
	AG	1.11	0.04		1.97	0.05		1.91	0.09	
	GG	1.17	0.03		2.05	0.04		1.68	0.07	
RDHE2 SNP4	AA	1.77	0.46	0.19	2.68	0.57	0.50	2.84	0.95	0.50
	AC	1.21	0.06		2.01	0.08		1.81	0.13	
	CC	1.12	0.03		2.02	0.03		1.75	0.06	
PPARGC1A SNP4	AA	1.19	0.13	0.94	2.20	0.16	0.26	1.44	0.26	**0.09†**
	GA	1.15	0.04		1.98	0.05		1.68	0.08	
	GG	1.14	0.04		2.05	0.04		1.88	0.07	
PPARGC1A SNP9	CC	1.12	0.10	0.54	2.08	0.12	0.22	2.13	0.20	0.17
	CT	1.11	0.04		1.96	0.05		1.78	0.08	
	TT	1.17	0.04		2.07	0.04		1.72	0.07	
PPARGC1A SNP10	AA	1.33	0.24	0.47	2.03	0.29	0.20	2.00	0.49	0.88
	GA	1.10	0.06		1.91	0.07		1.76	0.12	
	GG	1.16	0.03		2.06	0.04		1.78	0.06	
PPARGC1A SNP12	CC	1.12	0.05	**0.005 (**)**	2.09	0.06	**<0.001(***)**	1.77	0.10	**0.01 (**)**
	CT	1.12	0.03		1.95	0.04		1.71	0.07	
	TT	1.43	0.09		2.35	0.11		2.29	0.19	
SCARB1 SNP1	CC	1.16	0.05	0.72	2.11	0.06	**0.04(*)**	1.88	0.10	0.45
	GC	1.15	0.04		1.95	0.04		1.72	0.07	
	GG	1.10	0.06		2.09	0.07		1.74	0.13	
SCARB1 SNP2	AA	1.13	0.09	0.34	2.09	0.11	0.79	1.69	0.19	0.67
	GA	1.19	0.04		2.02	0.05		1.82	0.08	
	GG	1.10	0.05		2.01	0.05		1.74	0.09	
SCARB1 SNP16	CC	1.04	0.14	0.37	1.93	0.16	0.59	1.68	0.28	0.93
	GC	1.10	0.05		1.98	0.06		1.79	0.10	
	GG	1.17	0.03		2.05	0.04		1.77	0.06	

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-9); Fc-bio, fat colour score of biopsy samples (scale 1-5). *** P<0.001; **P<0.01; *P<0.05; †P<0.1. **Boldface**= effects with P values < 0.1

Table 4-3 SNP size of effect on fat colour traits as percentage of total SNP variation (%)

	Bc-bio[1]	**Fc-bio**[1]	**Fc-car**[1]
cohort[2]	24.74 ***	2.92 *	39.81 ***
breed[2]	3.05 ***	13.86 ***	13.82 ***
sire[2]	2.56 ***	0.91	1.75 ***
Individual SNP[3]	**Bc-bio**	**Fc-bio**	**Fc-car**
ALDH8A1 SNP10	0.62	0.26	1.11
ALDH8A1 SNP15	0.54	0.91	**1.48 ***
ALDH8A1 SNP16	1.09	**3.35 *****	0.00
APOM SNP2	0.11	0.31	1.03
APOM SNP6	0.12	0.08	0.92
APOM SNP7	0.56	0.73	0.91
BCMO1 SNP4	**1.90 ***	0.13	0.04
BCMO1 SNP7	0.14	0.23	0.62
BCMO1 SNP8	0.38	0.57	0.46
BCMO1 SNP13	0.12	0.18	0.17
BCO2 W80X	**12.01*****	**15.78 *****	**12.14 *****
PPARCG1A SNP4	0.05	0.73	1.07
PPARCG1A SNP9	0.15	0.56	0.77
PPARCG1A SNP10	0.46	0.74	0.82
PPARCG1A SNP12	**1.62 ***	**1.35 †**	0.94
RARA SNP1	0.04	0.15	0.78
RARA SNP 3	0.08	0.16	0.14
RARA SNP7	0.52	0.62	0.00
RDHE2 SNP2	**1.54 ***	0.33	**1.79 ***
RDHE2 SNP3	0.90	0.13	0.67
RDHE2 SNP4	0.43	0.34	0.80
SCARB1 SNP1	0.94	0.69	0.19
SCARB1 SNP2	0.79	**1.14 †**	0.80
SCARB1 SNP16	0.61	0.29	0.21
Total of SNP size effect[4]	25.72	29.76	27.84

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-9); Fc-bio, fat colour score of biopsy samples (scale 1-5).
[2]Calculation: cohort, breed and sire % = (sum of squares of cohort, breed or sire/ total sum of squares)*100
[3]Calculation: Individual SNP% = (sum of squares of SNP or interaction/ total sum of squares excluding sum of squares of cohort, breed of dam, and sire)*100
[4]Calculation: total of SNP size effect% = sum of individual SNP size effect.
† P<0.1,* P<0.05, ** P<0.01, ***P<0.001. **Boldface**= effects with P values < 0.1.

4.3.3 SNP additive and dominance effects

The additive and dominance effects were estimated by using Model 4 (as described in section 2-7). Eighteen SNPs had significant or nearly significant dominance and/or additive effects on the fat colour related traits (Table 4-4). Eight SNPs had a significant dominant effect ($P<0.05$) on fat colour traits. Estimations using the predicted trait values showed that dominant effects ranged from -0.12 to 0.78 μg/g of fat, -0.17 to 1.09 score, and -0.22 to 1.57 score in biopsy fat β-carotene concentration, carcass fat colour score and biopsy fat colour score, respectively. In addition, five SNPs were significant for additive effects on the fat colour traits. Only one SNP, *RDHE2* SNP2, had a negative additive effect on biopsy fat β-carotene concentration, reducing β-carotene concentration by 0.12 μg/g of fat. There was no significant additive effect on biopsy fat colour except for the SNP *BCO2* W80X. For the carcass fat colour score, both the *PPARGC1A* SNP4 and *RDHE2* SNP2 were associated with a negative additive effect by -0.21 and -0.22 on a 10-point scale, respectively, whereas the *BCO2* SNP2 had a significant additive association, increasing the fat colour score by 0.1 (10-point scale).

Notably, the SNP *BCO2* W80X had a highly significant effect on all the fat colour traits. The A allele of *BCO2* W80X mutation had an additive effect of 1.09 (±0.14) and 1.57 (±0.23) on biopsy fat colour scores (5-point scale) and carcass fat colour scores (10-point scale), respectively (Table 4-4). For β-carotene concentration, the *BCO2* W80X A allele was related to a highly significant increase in β-carotene concentration by 0.78 (±0.12) μg/g of fat. The dominance effect of the *BCO2* W80X A allele at the locus was negative for all 3 traits. The remaining SNPs in *BCO2* had relatively small additive and/or dominant effects for the fat colour traits (Table 4-4).

Table 4-4 Additive and dominance effects for significant trait association

	Bc-biop[1]		Fc-biop[1]		Fc-car[1]	
Marker	Type of effect[2]	Effect (SE)	Type of effect	Effect (SE)	Type of effect	Effect (SE)
BCO2 W80X	A	**0.78 (0.12) ****	A	**1.09 (0.14) *****	A	**1.57 (0.23) *****
	D	**-0.72 (0.12) *****	D	**-0.92 (0.14) *****	D	**-1.26 (0.25) *****
ALDH8A1 SNP10						
*ALDH8A1*SNP15					D	**0.48 (0.18) ****
*ALDH8A1*SNP16	A	0.09 (0.06) †	D	-0.01 (0.07) †		
APOM SNP2					A	-0.13 (0.19) †
APOM SNP6					D	**0.65 (0.33) ***
APOM SNP7					D	0.18 (0.1) †
BCMO1 SNP4	A	0.06 (0.09) †				
	D	**0.23 (0.1) ***				
BCMO1 SNP7						
BCMO1 SNP8	D	**0.19 (0.09) ***				
BCMO1 SNP13						
BCO2 SNP1						
BCO2 SNP2			D	**0.27 (0.12) ***	A	**0.1 (0.18) ***
BCO2 SNP3						
PPARCG1A SNP4					A	**-0.21 (0.13) ***
PPARCG1A SNP9					A	0.24 (0.1) †
PPARCG1A SNP10			A	-0.03 (0.13) †		
PPARCG1A SNP12			D	**-0.17 (0.07) ***		
RARA SNP1						
RARA SNP 3						
RARA SNP7			A	0.11 (0.06) †		
RDHE2 SNP2	A	**-0.12 (0.04) ****	D	-0.10 (0.06) †	A	**-0.22 (0.08) ****
RDHE2 SNP3					A	**0.25 (0.14) ****
RDHE2 SNP4	A	0.34 (0.22) †				
SCARB1 SNP1			D	**-0.11 (0.06) ***		
SCARB1 SNP2						
SCARB1 SNP16						

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-910); Fc-bio, fat colour score of biopsy samples (scale 1-5). [2]**Type of effect**: A (additive); D (dominant). † P<0.1; *P<0.05; ** P<0.01; ***P<0.001. **Boldface**= effects with P values < 0.1.

4.3.4 Non-candidate gene SNP associations

In addition to the SNPs genotyped in this study, data from a total of 134 SNPs in 69 genes from other projects were available for analysis. These SNPs were analysed simultaneously for association with fat colour traits using Model 7 (described in section 2-7). There were 45 SNPs that had a non-zero variance when fitted as additive effects, each with relatively small effects (Table 4-5). These SNPs accounted for 1-22% of total SNP variance. Only follistatin-like 1 (FSTL1_2), uridine monophosphate synthetase (UMPS_1), glucose/fructose transporter 5 (glut562) and insulin-like growth factor I (IGF1SNP2) and sucrase-isomaltase (SI_3) genotypes explained more than 5% of total SNP variance for the fat colour related traits.

Table 4-5 Additional gene SNP variance as percentage of total SNP variance (%)

SNP name	Fc-cam[1]	Bc-bio[1]	Fc-bio[1]
agtr1		0.78	
agtr2		1.75	
ahsg2		1.69	
ampk2		1.53	
APM11	1.41		
ATP6V1E2_1		2.49	1.17
cath1			0.58
EDG1UTR		1.45	1.16
ESR11A	1.44		
ESR12			0.76
foll2		4.14	
FST5	3.88	3.54	
FSTL1_2		10.23	
FSTL11		3.10	
GHR_3	8.36	1.35	
glut562		7.19	
GYG1_1	2.97		
GYG1_2	4.44		
IGF1SNP2			4.89
IGF2	2.95		
il1			1.73
il12			0.83
il2		0.74	
LPL_1		0.54	
NCOA72		1.02	
NCOA74	0.73		
Nocicep_1	2.23		
OBE243		0.75	
OBE604			0.68
PA2748			2.88
PI3K_1	3.73		
PI3K_2			0.99
PI3KR135	3.01		0.95
PKCI_1		4.84	
plpr2	1.65		0.95
pomc2			0.59
POUF_2		3.85	
pouf1		0.66	
SCDEex51	2.16		
SI_3	5.49		
SI_4			1.16
SLC2A2_1			2.23
Sst	0.75		
TEK_1			2.32
tek2		2.95	1.54
UMPS_1		7.65	
umps1			0.58

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-9); Fc-bio, fat colour score of biopsy samples (scale 1-5). Numbers = non-zero variances as a percentage of total SNP variance.

4.3.5 *BCO2* W80X effects on significance of other SNPs

Given that the *BCO2* W80X mutant accounted for a large proportion of the variation in fat colour related traits herein, this SNP genotype was fitted in the model for comparison to the original model. The inclusion of the *BCO2* W80X genotype in the model (Model 3, section 2-7) affected the variance of many SNPs (Table 4-6). With the *BCO2* W80X genotype in the model, four SNPs were no longer significant (*APOM* SNP2, *BCO2* SNP1, *PPARGC1A* SNP12 and *SCARB1* SNP1) (P>0.05), but four new SNPs were then significant or close to significant (*RDHE2* SNP2, *RDHE2* SNP3, *BCO2* SNP2 and *PPARGC1A* SNP9).

Table 4-6 Comparison of individual SNP significance P values when *BCO2* W80X was excluded or included in the model for fat colour traits in Group 1 animals

SNP name	P value of the traits					
	Bc-bio		Fc-bio		Fc-car	
	no	yes	no	yes	no	yes
ALDH8A1 SNP10	0.52	0.30	0.72	0.58	0.20	0.18
ALDH8A1 SNP15	0.69	0.73	0.30	0.25	**0.03**[b]	**0.02**[b]
ALDH8A1 SNP16	0.68	0.21	**0.09**	**0.09**	0.32	0.22
APOM SNP2	0.75	0.78	0.92	036	**0.05**[a]	0.23[a]
APOM SNP6	0.72	0.68	0.91	0.89	0.27	0.15
APOM SNP7	0.53	0.65	0.44	0.48	0.24	0.17
BCMO1 SNP4	**0.01**	**0.01**	0.90	0.73	0.72	0.88
BCMO1 SNP7	0.92	0.92	0.70	0.88	0.53	0.48
BCMO1 SNP8	**0.07**	**0.09**	0.63	0.39	0.51	0.48
BCMO1 SNP13	0.63	0.78	0.68	0.58	0.53	0.93
BCO2 SNP1	**0.10**	**0.53**	**0.01**[a]	0.36[a]	**0.10**[a]	**0.93**[a]
BCO2 SNP2	0.67	0.43	**0.08**[b]	**0.02**[b]	0.19[b]	0.05[b]
BCO2 SNP3	0.83	0.87	**0.27**	**0.22**	0.72	0.48
BCO2 W80X	**<.001**	**-**	**<.001**	-	**<.001**	-
RARA SNP1	0.94	0.84	0.93	0.86	0.23	0.29
RARA SNP3	0.75	0.55	0.56	0.68	0.95	0.92
RARA SNP7	0.12	0.29	**0.09**[a]	0.16[a]	0.61	0.93
RDHE2 SNP2	0.06[b]	**0.01**[b]	0.52	0.23	0.06[b]	**0.01**[b]
RDHE2 SNP3	0.59	0.91	0.54	0.52	**0.08**[b]	**0.02**[b]
RDHE2 SNP4	0.20	0.11	0.50	0.41	0.5	0.37
PPARGC1A SNP4	0.94	0.89	0.26	0.31	**0.09**[a]	0.12[a]
PPARGC1A SNP9	0.54	0.70	0.22	0.28	0.17[b]	**0.07**[b]
PPARGC1A SNP10	0.47	0.79	0.20	0.17	0.88	0.90
PPARGC1A SNP12	**0.005**[a]	0.13[a]	**<.001**[a]	0.04[a]	**0.01**[a]	0.29[a]
SCARB1 SNP1	0.72	0.50	**0.04**[a]	**0.14**[a]	0.45	0.80
SCARB1 SNP2	0.34	0.23	0.79	0.67	0.67	0.49
SCARB1 SNP16	0.37	0.50	0.59	0.82	0.93	0.69

Boldface= effects with P values < 0.1.
[a]P value change to non-significant level (P>0.05), [b]P value change to significant level (P<0.05)

4.3.6 QTL analysis

The SNPs *ALDH8A1* SNP16, *BCO2* W80X, *PPARGC1A* SNP12 and *RDHE2* SNP2 accounted for a large proportion of the variation in the fat colour related traits. Additional analyses were conducted to determine whether any of these SNPs represented the previously identified QTL (Appendix 7). The SNP genotypes for these genes were included as fixed effects in QTL regression models. If one of the SNP represents a QTL for that trait, or is in linkage disequilibrium with the quantitative trait nucleotide (QTN), the SNP is expected to decrease the residual variance by taking the SNP marker variance into the account for that QTL. Two models were employed in QTL mapping. In the Model Q-1, cohort, breed and sire were fitted as factors. In the Model Q-2, in addition to these factors, each of these SNP genotypes was included as a factor. Both across family and individual family linkage analyses were conducted.

The putative QTL across the 3 Australian Jersey-Limousin backcross families was mapped for each chromosome for the location with the highest test statistic and the parameter estimates were determined (Appendix 7.4). From the combined sire family analysis, if no additional SNP genotypes were included in the model, only 5 QTL were identified across the genome for fat colour (Table 4-7). These include 2 QTL (BTA 11 and 15) affecting biopsy fat colour and 3 QTL (BTA 4, 9 and 15) affecting β-carotene concentration. No QTL was detected for carcass fat colour (Table 4-7).

For the individual sire analysis, there also were 5 QTL, including 3 QTL for biopsy fat colour (BTA 6, 14 and 15) and 2 QTL for biopsy β-carotene concentration (BTA 16 and 17) (Table 4-8). The only common QTL in the 2 analyses was for biopsy fat colour score on BTA 15.

Table 4-7 Major SNP effects on QTL across sire families

Chromosome number	Traits	without major SNP effects		with BCO2 W80X		with ALDH8A1 SNP16		with PPARGC1A SNP12		with RDHE2 SNP2	
		QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value
1	Fc-bio[1]	25	2.07	0	**4.36**						
3	Bc-bio[2]	77	3.49	77	1.85						
4	Bc-bio	2	**4.64**	5	3.37						
9	Bc-bio	47	**4.26**	52	3.81	32	**3.93**				
10	Fc-bio	50	2.73	54	3.83						
10	Bc-bio	60	2.97	43	**4.13**						
11	Fc-bio	87	**5.31**	110	3.56			87	3.81		
14	Fc-bio	0	3.86	0	**4.00**	0	3.15			20	**5.38**
15	Fc-bio	16	**6.49**	83	3.71	15	**7.68**				
15	Bc-bio	5	**4.6**	11	2.19			9	**3.87**		
20	Fc-car[3]	17	3.45	17	**4.00**						
22	Fc-bio	66	2.53	67	3.72						
24	Fc-bio	41	3.27	40	**4.22**			44	**4.15**		

[1]Fc-bio, fat colour score of biopsy samples (scale 1-5); [2]Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); [3]Fc-car, fat colour score of carcass samples (scale 0-9). **Boldface**= effects with P values < 0.05

Table 4-8 Major SNP effects on QTL for individual sire families

Chromosome number	Traits	sire family	without major SNP effects		with *BCO2* W80X		with ALDH8A1 SNP16		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
			QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value	QTL position (cM)	F value
1	Fc-bio[1]	398	23	2.47	0	**6.72**						
6	Fc-bio	361	46	**9.03**					46	**8.74**		
6	Fc-bio	368	68	**7.96**					60	**10.45**		
7	Bc-bio[2]	368	46	5.58	43	**7.21**						
8	Bc-bio	398	0	3.65	0	**7.33**						
9	Fc-car[3]	368	73	**6.41**			72	2.86			76	**8.83**
14	Fc-bio	361	0	**15.45**			0	**14.47**			0	**22.16**
15	Fc-bio	398	21	**6.74**								
15	Fc-bio	398	45	**7.23**	89	4.25	46	**8.75**	22	5.04	22	5.04
16	Bc-bio	361	51	**7.64**			51	**8.21**			51	**8.87**
16	Bc-bio	368	85	**6.94**			82	**6.51**	85	6.18	78	**7.81**
17	Bc-bio	368	70	**7.53**	70	**6.83**			67	**8.27**	70	4.95
20	Bc-bio	368	28	5.43	28	**6.53**	28	**7.09**				
24	Fc-car	368	48	**6.82**	48	5.95	48	**8.27**			48	**7.32**

[1]Fc-bio, fat colour score of biopsy samples (scale 1-5); [2]Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); [3]Fc-car, fat colour score of carcass samples (scale 0-9). **Boldface**= effects with P values < 0.05

4.3.6.1 BCO2 W80X effects on QTL

The *BCO2* gene is located at 20.74 Mb (19 cM) on the bovine chromosome 15. Two fat colour QTL on BTA 15 were observed at 16 cM and 45 cM from the across family analysis without any SNP genotype in the model (Figure 4-4). Including the *BCO2* W80X genotype as a fixed effect in the linkage analysis for all the traits of interest resulted in the F-values decreasing for a number of the QTL in the across family analysis (Table 4-7). As expected, a dramatic decrease occurred on BTA 15 for biopsy fat colour (Figure 4-4). The F-value decreased below the threshold for both the QTL at 16 cM and the QTL at 45 cM on BTA 15. Because the *BCO2* gene is located at 19 cM, this analysis provides support for the *BCO2* W80X being the QTN within this region.

From the individual family QTL analysis, two biopsy fat colour QTL were also identified on BTA 15 at 21 cM (F = 6.74) and 45 cM (F = 7.23) (Table 4-8) when SNPs were excluded in the model (Figure 4-5). Again, both of these QTL on BTA 15 were influenced by the effect of *BCO2* W80X genotype, as the QTL were no longer significant with this SNP in the model.

As the presence of the *BCO2* W80X genotype in the model reduced the residual variance, more significant QTL could be detected. Three additional QTL from across family analysis were identified for fat colour traits (Table 4-7). These new significant QTL were located on BTA 1 (F = 4.36), BTA 10 (F = 4.13) and BTA 24 (F = 4.22) (P< 0.05), with the QTL peaks observed at the 0 cM, 43 cM, and 48 cM, respectively. In addition, the F-values of the QTL on BTA 14, BTA 20 and BTA 22 increased slightly, so these QTL were nearly significant or significant. Furthermore, individual family analysis indicated that 3 new QTL were identified with the *BCO2* W80X genotype

included as a fixed effect. One QTL for biopsy fat colour was discovered at 0 cM on BTA 1 (F = 6.72) as observed in the across family analyses. However, there were also two new QTL for β-carotene concentration located at 43 cM and 0 cM on BTA7 (F = 7.21) and BTA 8 (F = 7.33), respectively.

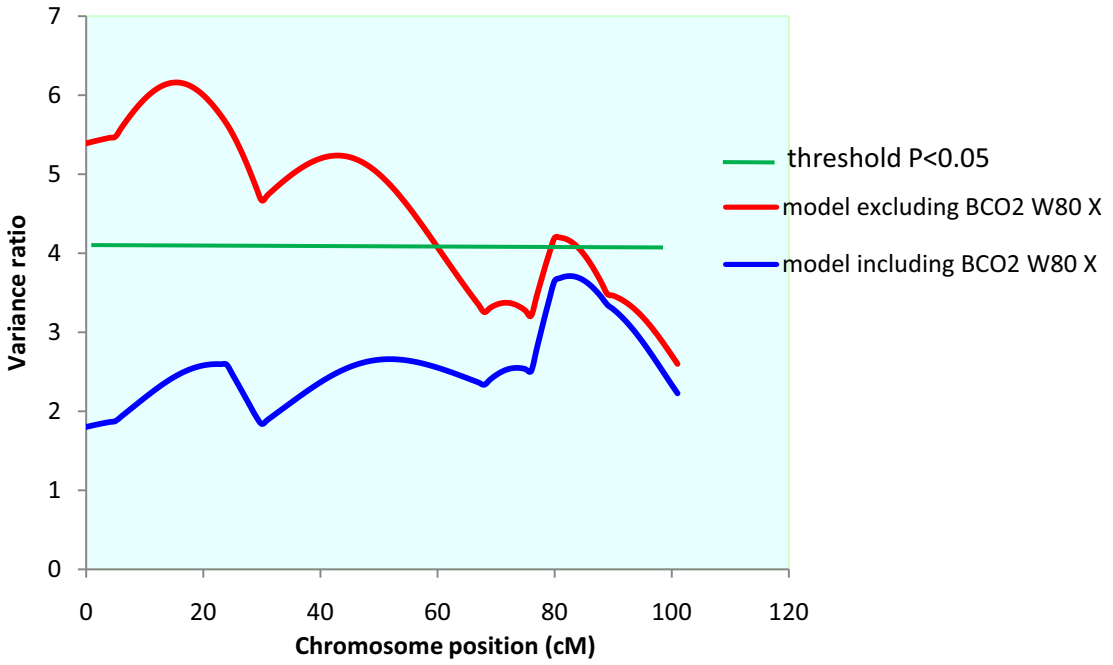


Figure 4-4 BCO2 W80X effects on BTA 15 QTL across families

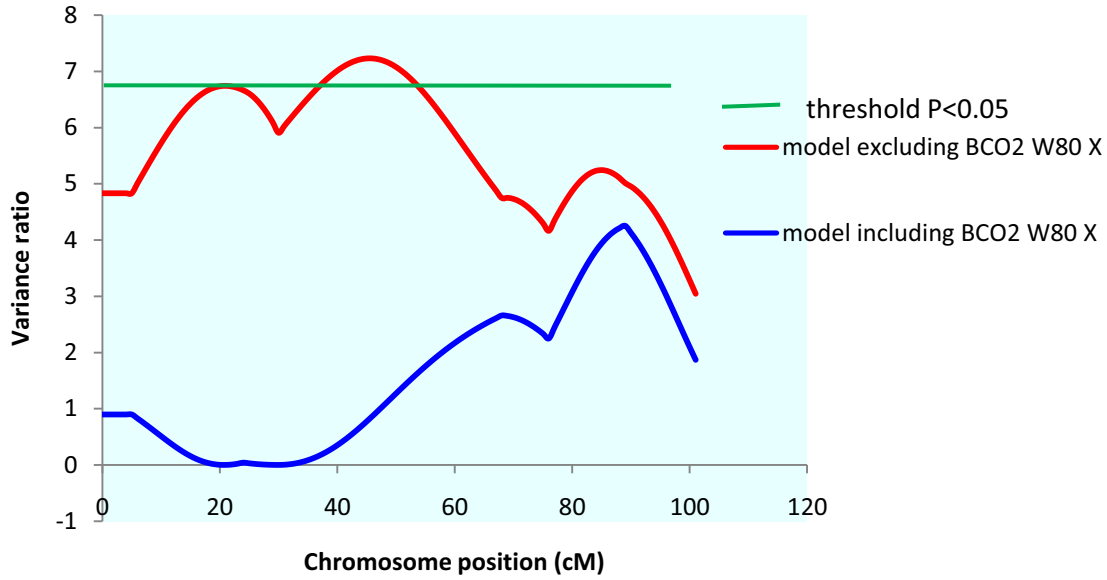


Figure 4-5 *BCO2* W80X effects on BTA 15 QTL for sire 398 family

4.3.6.2 ALDH8A1 SNP16 effects on QTL

The *ALDH8A1*gene is located at 75.20 Mb (75 cM) on bovine chromosome 9. The addition of the *ALDH8A1* SNP16 genotype in the model had an effect on both QTL from the across family and individual sire family mapping analyses (Table 4-7, Table 4-8). For chromosome 9, where the *ALDH8A1* gene is located, the most likely detected QTL location for β-carotene concentration shifted from 47 cM to 32 cM with the *F*-value reduced to 3.93 in the across family analysis. The F value of new QTL at 32 cM was just below the significance threshold level (Figure 4-6). However, a larger change was observed from the individual family analysis on the carcass fat colour. The significance of the QTL at 72 cM on BTA 9 decreased significantly from an *F* value of 6.41 to 2.86. This F value was well below significance level (Figure 4-7). Since the *ALDH8A1* gene was located at 75 cM, this provides support for the *ALDH8A1* SNP16 being the QTN or is in linkage disequilibrium with the QTN within this region.

When the *ALDH8A1* SNP16 genotype was taken into account, QTL on other chromosomes were also affected. For biopsy fat colour, a small decrease in the highest *F* value for chromosome 14 was observed for both the across family analysis (3.86 to 3.15) and the individual family analysis (15.45 to 14.47). The QTL location on BTA 14 from both analyses were not changed and mapped at 0 cm. In contrast, the effect of *ALDH8A1* SNP16 increased the *F*-value slightly from 6.49 to 7.68 on chromosome 15 with no change of QTL position for the across family analysis. In addition, the *F*-value on chromosome 15 increased from 7.23 to 8.75 with no change of QTL position for the individual family analysis.

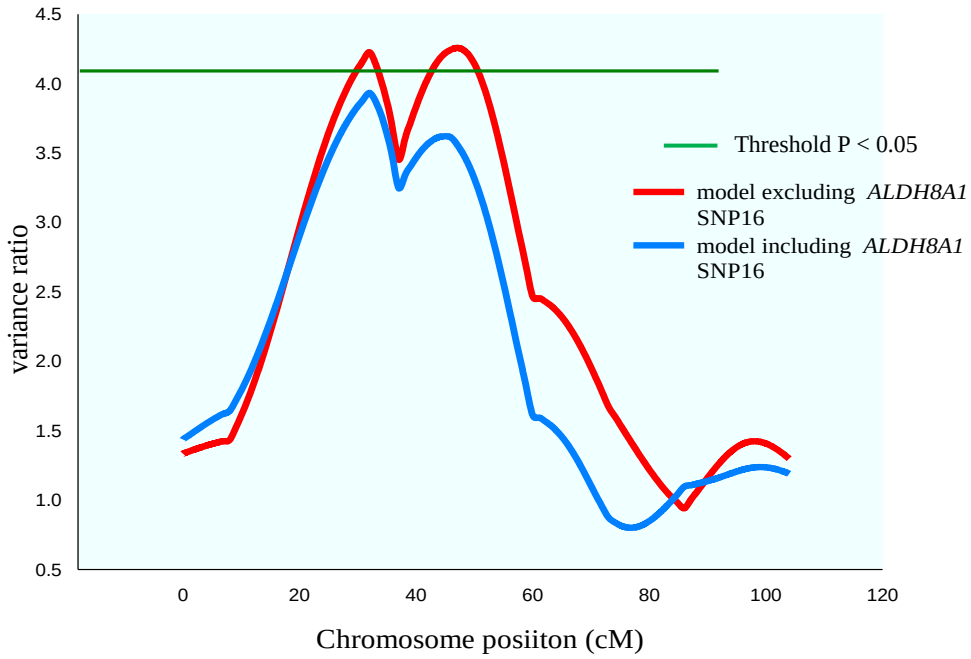


Figure 4-6 *ALDH8A1* SNP16 effect on BTA 9 QTL across families

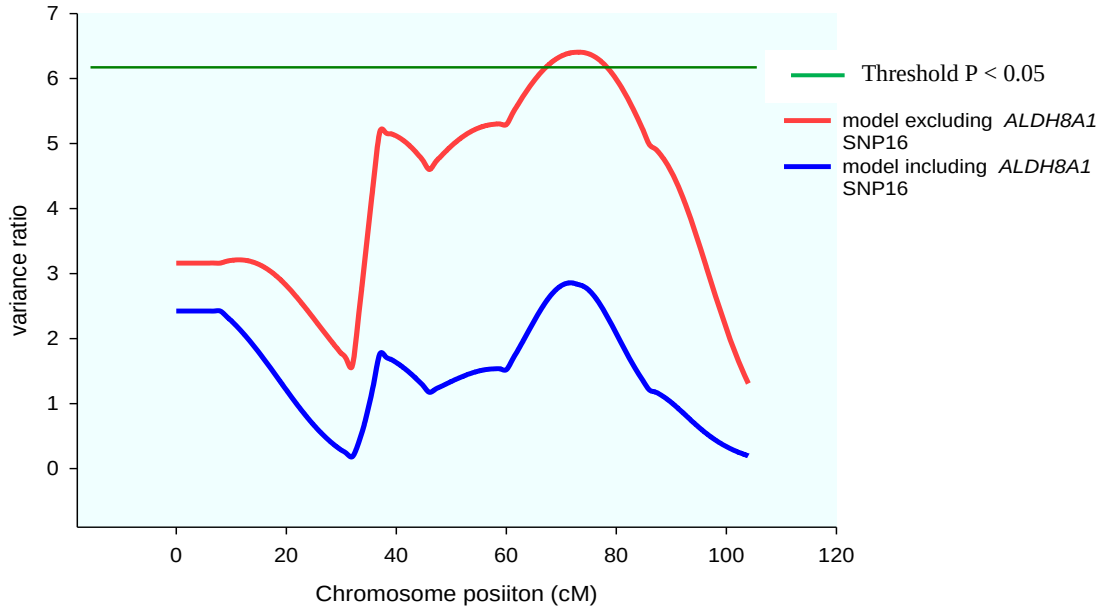


Figure 4-7 *ALDH8A1* SNP16 effect on BTA 9 QTL for sire 368 family

For the β-carotene concentration, no change was observed from the across family analysis for the *ALDH8A1* SNP16. A slight opposite change in direction was detected for chromosome 16 for the different sire families in the individual analysis with the *ALDH8A1* SNP16 genotype in the model. In the sire 361 family analysis, the *F*- value increased slightly from 7.64 to 8.21 at 51 cM. However, in the sire 368 family analysis, the *F*-value decreased slightly from 6.94 to 6.51 with a very minor change in QTL location from 85 cM to 82 cM. In addition, a new QTL was identified at 28 cM on BTA 20 (*F* = 7.09) for β-carotene concentration from the individual family analysis.

For carcass fat colour, inclusion of the *ALDH8A1* SNP16 genotype in the model had no effect on the across family analysis. However, for individual analysis, apart from the effect on BTA 9, inclusion of the *ALDH8A1* SNP16 genotype in the model increased the *F* value from 6.82 to 8.27 for the existing QTL on BTA 24 at 48 cM (Table 4-8).

4.3.6.3 PPARGC1A SNP12 effects on QTL

The *PPARGC1A* gene is located at 47 cM on BTA 6. From the across family analysis, no significant QTL for any fat colour traits was observed on BTA 6 when the *PPARGC1A* SNP12 genotype was either excluded (Figure 4-8) or included (Figure 4-9) in the model. However, the QTL on other chromosomes were influenced when the *PPARGC1A* SNP12 genotype was fitted in the model (Table 4-7). For biopsy fat colour score, the QTL at 87 cm on BTA 11 changed from significant (*F* = 5.31) to indicative (*F* = 3.81). A new significant QTL (*F* = 4.15) was identified for biopsy fat colour at 44 cM on BTA 24. For β-carotene concentration, the QTL at on BTA 15 changed marginally from significant (*F* = 4.60) to indicative (*F* = 3.87). The maximum of the QTL peak on this chromosome only shifted slightly from 5cM to 9cM.

In the individual family analysis, a QTL for biopsy fat colour was identified at 46 cM on BTA6 in the sire 361 family. However, inclusion of the *PPARGC1A* SNP12 genotype in the model only resulted in a minor decrease of *F* value of the QTL at 46 cm from 9.03 to 8.74 (Figure 4-10), which indicates that the *PPARGC1A* SNP12 is unlikely to be the QTN for this QTL.

For biopsy fat colour score, the *PPARGC1A* SNP12 genotype had opposite effects on the QTL on BTA 6 for different sire families. The *F* value of the QTL at 46 cm on BTA 6 decreased slightly from 9.03 to 8.74 for the sire 361 family. However, for sire 368 family, the *F*-value of the QTL on BTA 6 increased from 7.96 to 10.45 with a minor change of QTL location from 68 cM to 60 cM. This suggests that the QTN is in opposite phase to the *PPARGC1A* SNP12 in the 2 sire families.

In addition, the QTL on BTA 15 for biopsy fat colour was no longer significant (*F* = 5.04 from 7.23) when the *PPARGC1A* SNP12 genotype was included in the model similar to the results from the across family analysis. For β-carotene concentration, the effect of the *PPARGC1A* SNP12 genotype was only observed in the sire 368 family. The QTL on BTA 16 was less significant (*F* = 6.18 from 6.94). In contrast, the *F*-value of the QTL on BTA 17 increased slightly from 7.53 to 8.27 with a very minor QTL location change from 70 cM to 67 cM.

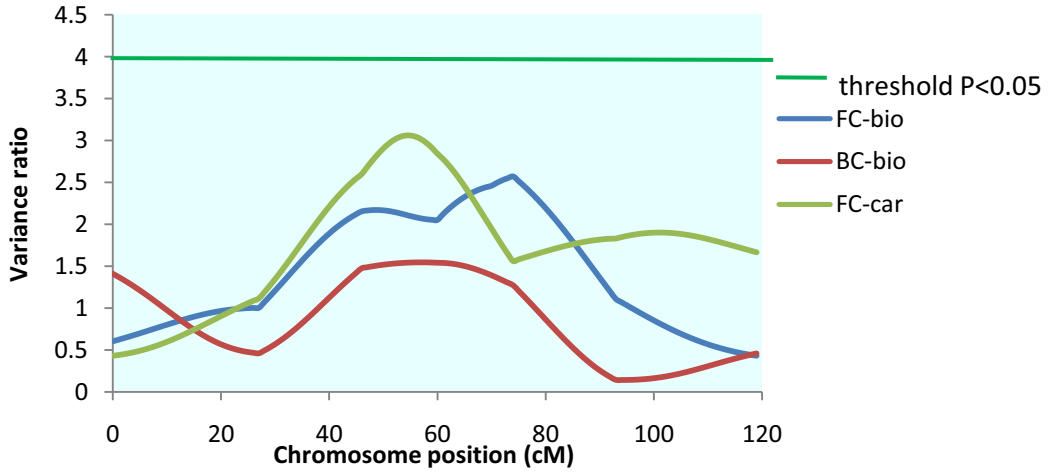


Figure 4-8 BTA 6 QTL across families without *PPARGC1A* SNP12 in the model

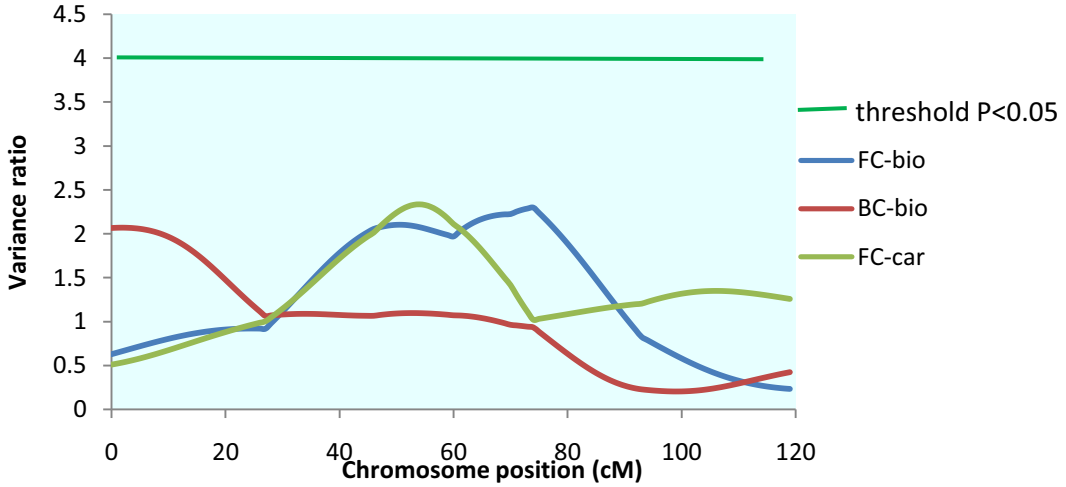


Figure 4-9 BTA 6 QTL across families with *PPARGC1A* SNP12 in the model

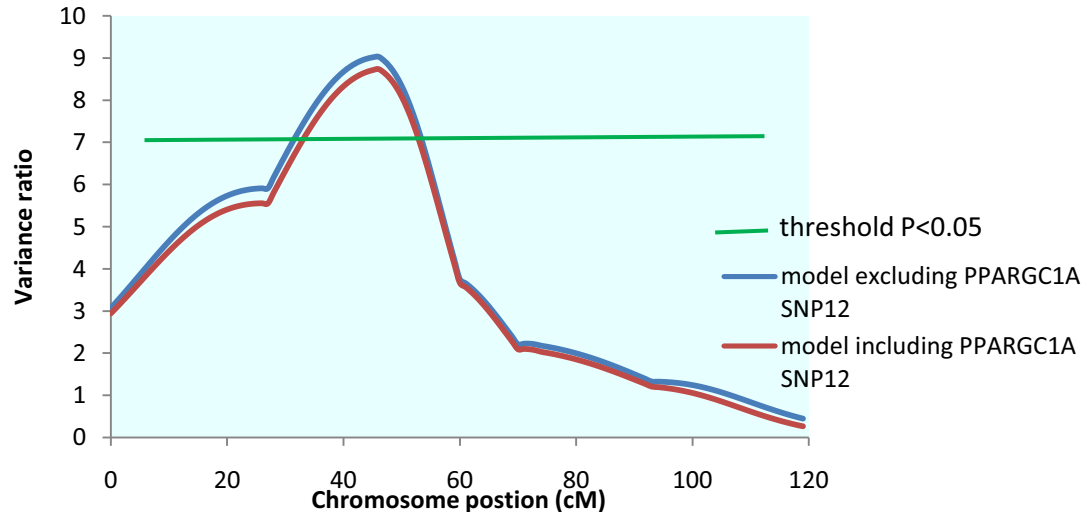


Figure 4-10 *PPARGC1A* SNP12 effect on BTA 6 QTL in sire 361 family

4.3.6.4 RDHE2 SNP2 effects on QTL

The *RDHE2* gene is located at 25 cM (23 Mb) on BTA 14. From the across family analysis, the inclusion of the *RDHE2* SNP2 genotype in the QTL analysis caused only small changes in the results. No effect was observed in β-carotene concentration and carcass fat colour QTL. Interestingly, a marginal biopsy fat colour QTL on BTA 14 became statistically significant with the inclusion of the *RDHE2* SNP2 genotype or both *RDHE2* SNPs (SNP2 and SNP3) genotype. The *F*-value for the QTL on BTA 14 increased to 5.38. The QTL location also shifted from 0 cM to 20cM on this chromosome (Table 4-7) (Figure 4-11). In contrast to *RDHE2* SNP2 genotype effect on the biopsy fat colour QTL of BTA 14, the inclusion of only the *RDHE2* SNP3 genotype in the QTL analysis decreased *F*-value for the biopsy fat colour QTL from 3.89 to 3.12 at 0cM on this chromosome (Figure 4-13).

From the individual family analysis, if the *RDHE2* SNP2 genotype or both *RDHE2* SNPs (SNP2 and SNP3) genotype were included in the QTL analysis, one marginal QTL became significant in the sire 368 family at 76 cM on BTA 9 for carcass fat colour (Table 4-8). In addition, the *F*-values of other existing QTL increased on BTA 14, BTA 16 and BTA 24. Notably, similar to results from the across family analysis, the *F*-value for one QTL affecting biopsy fat colour in sire 361 family at the centromeric end of the chromosome 14, where the *RDHE2* gene is located, increased from 15.45 to 22.16 (Figure 4-12). However, the inclusion of only the *RDHE2* SNP3 genotype in the QTL analysis just decreased the *F*-value marginally for the biopsy fat colour QTL from 15.45 to 14.83 at 0cM on this chromosome (Figure 4-14).This surprising result indicates that the gene *RDHE2* is not likely to be the gene underlying this QTL on BTA 14. Instead, *RDHE2* may have an epistatic effect on this QTL.

Similar to the *PPARGC1A* SNP12 effect on the QTL analysis, 2 QTL were no longer significant due to the effect of *RDHE2* SNP2 genotype. One was the BTA 15 QTL segregating in the sire 398 family for biopsy fat colour. The other was the BTA 17 QTL segregating in the sire 368 family for fat β-carotene concentration.

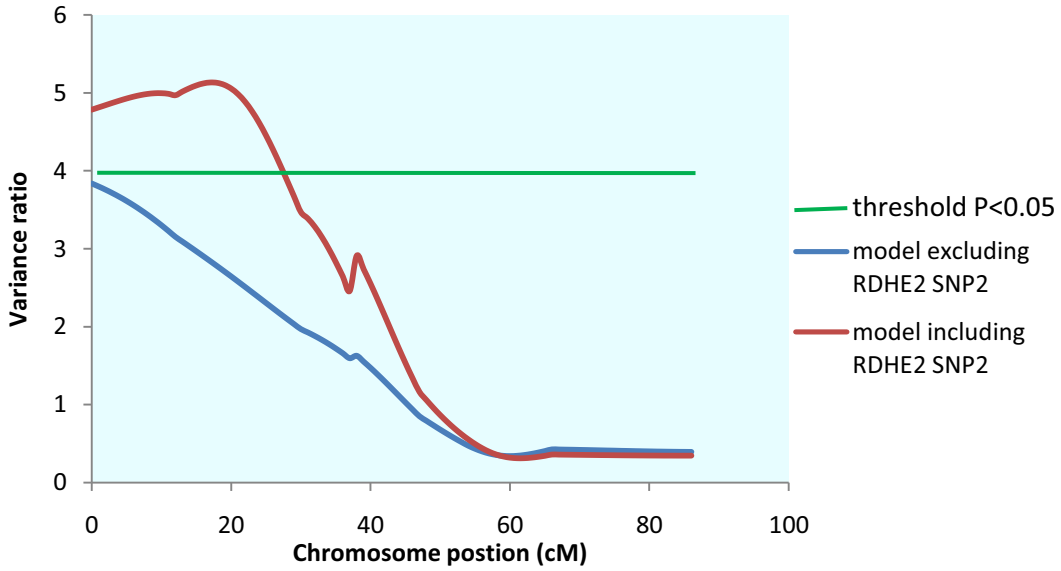


Figure 4-11 *RDHE2* SNP2 effect on BTA 14 QTL across families

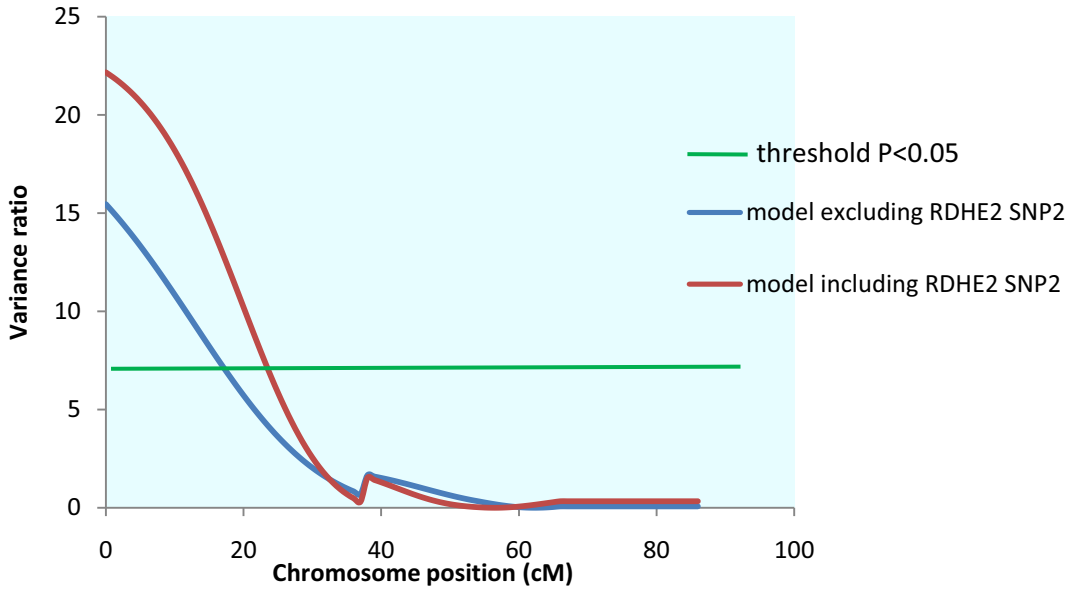


Figure 4-12 *RDHE2* SNP2 effect on BTA 14 QTL in sire 361 family

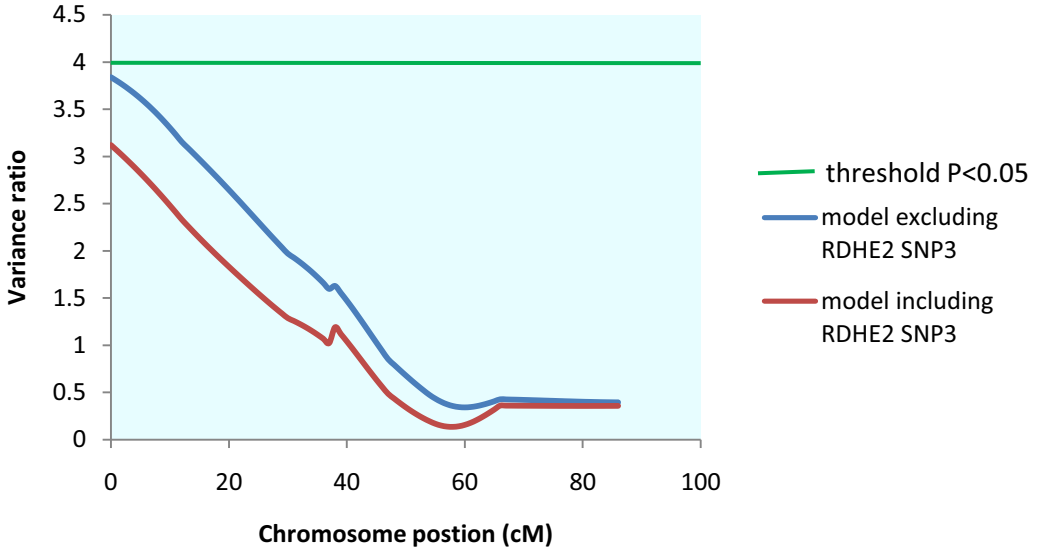


Figure 4-13 RDHE2 SNP3 effect on BTA 14 QTL across families

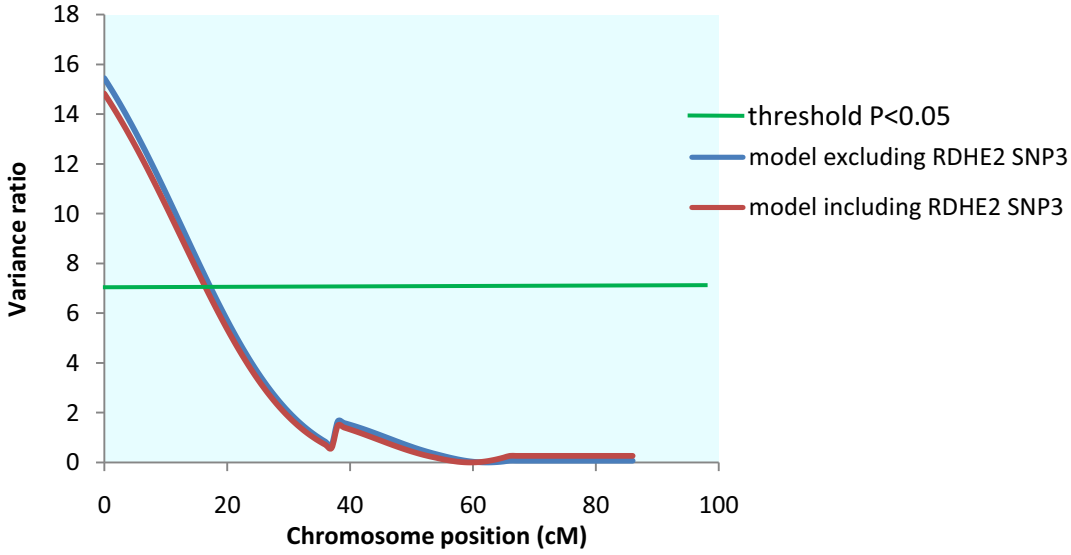


Figure 4-14 RDHE2 SNP3 effect on BTA 14 QTL in sire 361 family

4.4 Discussion

In the study herein, twenty-seven DNA variants in eight candidate genes were investigated in association studies of beef fat colour traits in the Australian cattle mapping progeny (Group 1). Some SNPs accounted for a large amount of the variance in beef fat colour. To test whether these SNPs represent the QTN underlying the QTL, additional QTL analyses with these SNPs were performed.

4.4.1 Breed effects

Dairy breeds, in general, have more yellow carcass fat than beef breeds (Morgan and Everitt, 1969). Herein, a two-generation resource population with two phenotypically divergent breeds, Jersey (J, dairy breed) and Limousin (L, beef breed) was used. Three first–cross (X) Jersey x Limousin half-brothers were mated to both pure Jersey and pure Limousin dams, generating Limousin backcross (XL) progeny and Jersey backcross (XJ). Breed was the main factor affecting fat colour score and β-carotene concentration (Table 4-3), with both being higher in the Jersey backcross progeny than in the Limousin backcross progeny (Figure 4-1, Figure 4-2 and Figure 4-3). This is in agreement with the observation by Walker (1990) that dairy breeds have subcutaneous fat which is more yellow than British or European beef breeds. In addition, Barton and Pleasants (1993) showed that the Jersey breed had more carcasses with yellow fat than beef breeds and other dairy breeds in a study in which they compared steers raised on pasture and slaughtered at 30 months of age from 7 breeds (Beef Shorthorn, Galloway, Angus, Ayrshire, Friesian, Milking Shorthorn and Jersey). Dunne *et al.* (2004) also reported that progeny of New Zealand Friesian origin had higher fat colour carcass scores than either Friesian progeny of Dutch/North American parentage or Belgian Blue-Holstein Friesian crosses. In these experiments, the Jersey genes contributed no

more than 2/16ths of the genetic make-up of the New Zealand Friesian cattle. Nevertheless, the authors suggested that even this marginal input of Jersey genes in the New Zealand Friesian progeny could have contributed to their yellower carcass fat. Herein, the large difference between the Jersey and Limousin breeds for fat colour scores and β-carotene concentration in subcutaneous fat suggests that specific gene(s) contribute to the incidence of higher accumulation of β-carotene in the fat and the yellow fat allele(s) would be from the Jersey breed. Therefore, one would predict the causative allele frequency would be lower in the XL backcross than the XJ backcross herein, and the number of the homozygotes in XL progeny would be minimal (Table 4-9).

Table 4-9 SNP allele frequency in XL and XJ backcross

SNP	Allele[1]	Allele frequency of breed		Additive Effect		
		XJ	XL	Bc-biop[2]	Fc-biop[3]	Fc-car[4]
ALDH8A1 SNP10	G	0.64	0.70			
ALDH8A1 SNP15	C	0.19	0.20			
ALDH8A1 SNP16	A	0.68	0.72	0.09 (0.06) †		
APOM SNP2	A	0.87	0.84			-0.13 (0.19) †
APOM SNP6	**A**	0.89	0.88			0.57 (0.33) †
APOM SNP7	G	0.65	0.52			
BCMO1 SNP4	**A**	0.16	0.10	0.06 (0.09) †		
BCMO1 SNP7	C	0.83	0.71			
BCMO1 SNP8	C	0.19	0.22			
BCMO1 SNP13	C	0.52	0.75			
BCO2 SNP1	A	0.12	0.15			
BCO2 SNP2	A	0.13	0.14			0.1 (0.18) *
BCO2 SNP3	C	0.88	0.88			
BCO2 W80X	**A**	0.16	0.10	0.78 (0.12) **	1.09 (0.14) ***	1.57 (0.23) ***
PPARCG1A SNP4	**A**	0.24	0.28			-0.21 (0.13) *
PPARCG1A SNP9	**T**	0.84	0.81			0.24 (0.1) †
PPARCG1A SNP10	**A**	0.11	0.14		-0.03 (0.13) †	
PPARCG1A SNP12	C	0.52	0.75			
RARA SNP1	G	0.36	0.43			
RARA SNP 3	A	0.88	0.89			
RARA SNP7	**C**	0.75	0.69		0.11 (0.06) †	
RDHE2 SNP2	**A**	0.29	0.75	-0.12 (0.04) **		-0.22 (0.08) **
RDHE2 SNP3	A	0.33	0.09			0.25 (0.14) **
RDHE2 SNP4	**A**	0.11	0.09	0.34 (0.22) †		
SCRB1 SNP1	C	0.61	0.52			
SCRB1 SNP2	A	0.32	0.33			
SCRB1 SNP16	C	0.17	0.21			

[1]**Allele with boldface** = higher frequency in XJ backcross with positive additive effects or lower frequency in XL backcross with negative additive effects. [2]Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); [3]Fc-bio, fat colour score of biopsy samples (scale 1-5); [4]Fc-car, fat colour score of carcass samples (scale 0-9). † P<0.1; *P<0.05; ** P<0.01; ***P<0.001.

4.4.2 Multiple and individual SNP testing

In this study, the SNP effects on fat colour related traits were tested by using both an individual and a multiple SNP model. For some SNPs, the significance level changed dramatically depending upon the model used. For example, for *BCMO1* SNP8, *SCARB1* SNP1, *APOM* SNP2 and *PPARCG1A* SNP12, the genotype effects were significant or nearly significant on the trait when only one SNP was tested in a model (model 2, section 2.7). However, these SNP genotype effects (*BCMO1* SNP8, *SCARB1* SNP1, *APOM* SNP2 and *PPARCG1A* SNP12, Table 4-3) were no longer significant when all SNPs were tested simultaneously in the model (model 7, section 2.7). This is most likely because the null hypothesis, under the condition that the polymorphism is truly associated with the phenotype, is rejected with the different threshold level. Traditionally, when only 1 or a few SNPs are tested, a significance level of $P<0.05$ is applied. For studies in which many hypotheses are tested, the nominal significance level chosen for a study dictates the proportion of all of the reported tests that are found to be significant, even when none of the hypotheses are true (Lunetta, 2008). Therefore, some SNPs may no longer be significant in a test which includes a large number of hypotheses (e.g., SNP associations) because some hypotheses are falsely rejected. Consequently, there are two options for association studies of a single phenotype and multiple SNPs (Lunetta, 2008). First, the most efficient way to limit the number of tests is to perform a single test per SNP. Second, the number of tests should be limited by performing a test of association between the phenotype and haplotypes or multilocus genotypes rather than each SNP individually.

4.4.3 Non-coding polymorphisms

Several non-exonic SNPs were associated with fat colour traits in this study. There are

at least two plausible reasons to explain these associations. Firstly, as the population used in this study were backcross progeny, non-exonic SNPs could easily be in linkage disequilibrium with the functional allele. Secondly, non-exonic variants can play a role in regulating the expression level of a gene. For example, a nucleotide substitution in intron 3 of the insulin-like growth factor-2 gene has been shown to be as a causative variant for QTL affecting muscle growth, fat deposition, and heart size in pigs (Van Laere *et al.*, 2003). Those SNPs in the 5-flanking regions of genes, intronic and 3-untranslated regions could be located in regulatory elements or microRNA sites which result in gene expression differences, and thereby, affect beef fat colour.

4.4.4 Functional significance of candidate genes

In this study, significant ($P \leq 0.05$) or indicative ($P \leq 0.10$) phenotypic associations were detected between SNPs in the eight candidate genes and fat colour traits. These genotype-phenotype associations reported are supported by the biological functions of the candidate gene proteins.

4.4.4.1 ALDH8A1

ALDH8A1, also known as *RALDH4* or *ALDH12*, encodes an aldehyde dehydrogenase (ALDH) that converts 9-*cis*-retinal into 9-*cis*-retinoic acid, the retinoid X receptor ligand. The gene for *ALDH8A1* is located at 75.20 Mb on the bovine chromosome 9 in the identified confidence interval of the fat colour QTL (66.95Mb- 76.75Mb) on BTA 9 (Table 3-3). The *ALDH8A1* SNP 15 in exon 5 is a T/C substitution, causing threonine^{244} to methionine^{244} non-conservative amino acid substitution in the protein. Carcass fat colour was significantly associated with this SNP. The fat colour score of the homozygous CC cattle was 74.5% and 58.2% lower than that of CT and TT cattle, respectively. Furthermore, there was a significant dominance effect with the estimated

0.48 ±0.18 allelic substitution effect of T allele. *ALDH8A1* may affect the fat colour by being involved with 9-*cis*-retinoic acid biosynthesis from 9-*cis*-retinal where the protein with the methionine^{244} substitution results in less yellow fat.

4.4.4.2 APOM

The *APOM* gene is located at 27.44Mb on bovine chromosome 23, and is within the identified QTL interval (27.41Mb-27,45Mb) for fat colour. APOM is a apolipoprotein present mostly in the high-density lipoprotein in blood plasma, with a small proportion present low-density lipoproteins in human (Luo *et al.*, 2004). It is also found in other lipoprotein classes, including chylomicrons. However, the role of APOM is in cattle is unknown. Because both chylomicrons and high-density lipoproteins are responsible for the transport of carotenoids in cattle (Yang *et al.*, 1992), it is hypothesised herein that the cattle APOM is involved in the transport of β-carotene. An *in vivo* study using genetically modified mouse models revealed that *APOM* is regulated by transcription factors that are also linked with hepatic glucose, lipid and lipoprotein metabolism (Christoffersen *et al.*, 2008). Furthermore, an association study has shown that the G2289C polymorphism in porcine *APOM* is significantly associated with several fat deposition traits including backfat thickness at the shoulder, thorax-waist, backfat and buttock and the average backfat thickness over shoulder, thorax-waist and buttock (Pan *et al.*, 2010). Considering the location of the *APOM* gene in a fat colour QTL and the potential involvement of *APOM* in fat deposition traits, *APOM* was selected as a positional candidate gene. The *APOM* SNP 2 in 5'flanking region of the gene is A/G substitution. Significant differences were found for different *APOM* SNP 2 genotypes for carcass fat colour. Therefore, the results support the concept that *APOM* may affect fat colour by the selective transportation of β-carotene into the adipose tissue

accompanied with fat deposition.

4.4.4.3 RARA

The location of the *RARA* gene is at 41.82Mb on bovine chromosome 19, which is located in another fat colour QTL interval (37.06Mb-43.06Mb). The retinoic acid receptor alpha (*RARA*) is one of three retinoic acid receptors. The retinoic acid receptors bind retinoid X receptors and as heterodimers, have biological effects ranging from morphogenesis and organogenesis, to cell growth, differentiation and apoptosis (Germain *et al.*, 2006). BCMO1, a key enzyme for catalysing the conversion of β-carotene to retinal, is negatively regulated by retinoic acid at the transcriptional level (Takitani *et al.*, 2006), and RARA appears to be involved with this regulation. This is further supported by evidence that treatment with a RARA antagonist increases BCMO1 activity in rats and chickens (Bachmann *et al.*, 2002). However, only a synonymous substitution *RARA* SNP7 showed weak association (P=0.09) with biopsy fat colour. This suggests the *RARA* may affect beef fat colour, perhaps by the regulation of *BCMO1* gene expression, but the effect is not large.

4.4.4.4 RDHE2

The *RDHE2* gene is located at 25 cM (23 Mb) on BTA 14. As a candidate gene for the QTL on this chromosome, the *RDHE2* gene showed a small effect on β-carotene concentration (P=0.06) and carcass fat colour (P=0.06). The SNP in *RDHE2* (*RDHE2* SNP2) is a T/C non-synonymous substitution, which results in a conservative amino acid change from valine^{6} to alanine^{6}. RDHE2 is a member of the short-chain alcohol dehydrogenase/reductase (SDR) family that can catalyse the first and rate-limiting step that generates retinaldehyde from retinol (Matsuzaka *et al.*, 2002). A study by Lee

(2009) provided the first evidence that RDHE2 functions as a retinol dehydrogenase in humans. Thus, one possibility for the *RDHE2* effect on fat colour traits is through its function in retinoic acid synthesis, which may consequently affect β-carotene concentration. However, in addition to the cytoplasmic dehydrogenase activity of RDHE2, it has been also shown both *in vivo* and *in vitro* that this protein is recruited to the profilaggrin promoter. This recruitment is directly linked to cytoplasmic and nuclear functions in the human embryonic kidney 293 cells and Chinese hamster ovary cells (Markova *et al.*, 2006). So, another possible explanation for the association between fat colour and *RDHE2* is its function in the nucleus as a transcriptional repressor.

4.4.4.5 PPARGC1A

The *PPARGC1A* gene encodes peroxisome proliferator-activated receptor gamma coactivator 1-alpha protein, which plays a critical role in several complex pathways of glucose, fat, energy metabolism and adipogenesis. It is a coactivator of a subset of oxidative phosphorylation genes that control glucose and lipid transportation and oxidation, skeletal muscle fibre type and mitochondrial biogenesis (Picard *et al.*, 2002). Studies of the *PPARGC1A* gene in both human and livestock reveal a significant role in lipid metabolism. It is a transcriptionally unregulated metabolic gene in the adipose tissue and skeletal muscle of patients with type 2 diabetes (Carey *et al.*, 2006). SNPs in exon 8 of the porcine *PPARGC1A* gene are associated with feed conversion ratio, abdominal fat content and backfat thickness (Stachowiak *et al.*, 2007). Also, *PPARGC1A* has been proposed as a positional candidate gene for the QTL on bovine chromosome 6 for milk production traits, including milk fat synthesis (Kuhn *et al.*, 1999). Numerous polymorphisms have been identified in the bovine *PPARGC1A* gene. A significant association between an SNP in intron 9 of the *PPARGC1A* gene and milk

fat yield was observed in a major dairy cattle population (Weikard *et al.*, 2005). Two SNPs were identified within bovine *PPARGC1A* exon 8 in their study. The first SNP is a G/A transition (SNP 1181), which causes a non-conservative amino acid change of serine^{364} to asparagine^{364}. The second SNP is a T/A transversion (SNP 1299), which is silent resulting in a synonymous change (CAC/CAT, histidine^{403}). As SNP 1181 is a non-synonymous change, only this SNP was further investigated by Soria *et al* (2009) in an association study with several traits including live body weight, gain in backfat thickness, kidney fat weight, kidney fat percentage, Warner–Bratzler shear force at 7 days post-mortem, intramuscular fat percentage and meat colour. However, there was no association with any of these traits and the SNP 1181 (Soria *et al.*, 2009). In the study herein, 14 variants were identified in *PPARGC1A.* The *PPARGC1A* SNP12 in exon 9 causes a non-conservative amino acid change of proline^{616} to leucine^{616} and this substitution showed strong significant effects on all three fat colour traits. The TT homozygous animals had the most yellow fat colour and the highest β-carotene concentration in the fat. However, the genetic variation for biopsy fat colour accounted by this SNP was relative low (1.69%). The PPARGC1A effects on fat colour could be a result of this protein's regulation of the *BCMO1* gene at the transcriptional level.

4.4.4.6 SCARB1

SCARB1 (scavenger receptor class B type I, also denoted as SR-BI), belongs to the family scavenger receptors class B, and is essential for β-carotene absorption. Provitamin A (carotenoid) and vitamin A studies in *SCARB1*-deficient mice and cell lines indicate that the role of SCARB1 in carotenoid absorption is well conserved in mammals (Duncan *et al.*, 2002, During *et al.*, 2005, During and Harrison, 2007). *SCARB1* is expressed at relatively high levels in adipose and steroidogenic tissues

(Terpstra *et al.*, 2000). The QTL for β-carotene concentration on BTA 17 was at 81cM, which is approximately at 63.94Mb in the bovine genome sequence near the bovine *SCARB1* gene (53.94Mb). The *SCARB1* SNP1 in the 5'flanking region showed a significant association with biopsy fat colour. This receptor may affect fat colour via its involvement in the uptake of high density lipoproteins, which is the major source of β-carotene in cattle tissues.

4.4.4.7 BCMO1

β,β-Carotene 15,15'-monooxygenase-1 (*BCMO1*) is the key enzyme in vitamin A metabolism in mammals. It cleaves β-carotene symmetrically to produce two molecules of retinal (Goodman and Huang, 1965). The BCMO1 enzyme activity is relatively lower in bovine tissues compared to other ruminant species, including the duodenal mucosa (Mora *et al.*, 2000), liver, plasma and subcutaneous fat (Yang *et al.*, 1992). This may explain why cattle store much more β-carotene in adipose tissues than other species. Morales (2007) conducted a study that compared the expression and activity of BCMO1 in the duodenum and liver of cattle with pigmented or non-pigmented fat. No enzyme activity difference was found in the duodenum between non-pigmented and pigmented animals, but higher enzyme activity ($P = 0.004$) was found in the liver of pigmented animals. Despite this higher activity, the level was not sufficient to prevent the storage of β-carotene in adipose tissues.

The *BCMO1* gene is located on bovine chromosome 18, where surprisingly, no significant fat colour QTL was found herein or in Esmailizadeh (2006). In the present study, seventeen SNPs were identified in the *BCMO1* gene, of which four SNPs were further analysed. The *BCMO1* SNP4 in the 5'flanking region was highly associated

with β-carotene concentration in fat (P<0.01). There is other evidence of genetic variants in the human *BCMO1* gene affecting β-carotene absorption and conversion. Park *et al*. (2008) demonstrated that the human *BCMO1* gene mutation at T381L produces a more enzymatically active BCMO1 protein with 75% increased catalytic activity for β-carotene. Another two common polymorphisms (R267S and A379V) in the human *BCMO1* gene occur at high frequencies and also alter β-carotene metabolism (Leung *et al.*, 2009). Interestingly, the authors showed in an *in vivo* study that 267S/379V double-mutant individuals have a reduced ability to convert β-carotene to vitamin A derivatives. In addition, the G allele at the polymorphism rs6564851 in the putative human *BCMO1* promoter region has been identified to be associated with higher plasma β-carotene levels (P<0.001) (Ferrucci *et al.*, 2009). The findings herein suggest that the A allele of *BCMO1* SNP4, or an allele in strong linkage disequilibrium, is associated with reduced BCMO1 activity, causing higher circulating levels of unconverted β-carotene in cattle similar to the results from the human studies.

4.4.4.8 BCO2

Beta-carotene oxygenase 2 (*BCO2*) catalyzes the asymmetrical oxidative cleavage of β-carotene into β-apo-10'-carotenal (C27) and β-ionone (C13) (Kiefer *et al.*, 2001). Mutations in the *BCO2* gene have a significant impact on the carotenoid metabolism in several species. In domestic chicken, yellow skin was shown to be caused by one or more *cis*-acting and tissue specific regulatory mutation(s) that inhibit mRNA expression of *BCO2* in the skin (Eriksson *et al.*, 2008). In sheep, a nonsense mutation (c.196C>T) in *BCO2* introduces a stop codon at amino acid position 66 and is strongly associated with the yellow fat phenotype (Vage and Boman, 2010).

The same nonsense mutation *BCO2* W80X found herein to affect adipose fat colour

(Tian *et al.*, 2010) was shown to be the QTN for a QTL discovered for β-carotene level in milk. The mutation increases the level of β-carotene in milk and serum (Berry *et al.*, 2009). Herein, the SNP was found to have a highly significant effect on β-carotene concentration, as well as biopsy and carcass fat colour score traits (P<0.001). The SNP accounted for a large proportion of the genetic variation in these traits (12.0% to 15.8%). The frequency of the A allele of the *BCO2* W80X SNP was relatively low in the backcross population with only four homozygous animals found. No homozygous animals were present in the Limousin backcross progeny as this allele only occurs in the Jerseys (Table 3-6).

4.4.4.9 Other non-candidate genes

To investigate other important pathways controlling beef fat colour besides of the beta-carotene pathway, the non-candidate gene SNPs from other projects in our SNP database were also included in the analysis. Based on the association analyses from the other non-candidate gene SNPs, a total of 47 SNPs in 34 genes had non-zero variances (Table 4-5). In terms of the proportion of variation in fat colour traits, most of these SNPs accounted for very little total SNP effect, ranging from 1%-10%. Of the 47 SNPs, only FSTL1_2 (10%) GHR_3 (8%) UMPS_1 (8%) and glut562 (7%) explained more than 5% of the total SNP effect. The *FSTL1* (follistatin-like 1) encodes a protein with similarity to follistatin, an activin-binding protein. It modulates the action of specific growth factors on cell proliferation and differentiation (Rosenberg *et al.*, 2006). *GHR* (growth hormone receptor) is a receptor for pituitary gland growth hormone involved in regulating postnatal body growth (White *et al.*, 2007). *UMPS* (uridine monophosphate synthetase) is responsible for mRNA turnover and uridine nucleotide synthesis (Suchi *et al.*, 1989). Glut (Glucose transporter 2, SLC2A2) is involved in regulation of glucose

transfer across the plasma membrane of hepatocytes and uptake of glucose by beta cells (Guillam *et al.*, 1997). None of the known functions of these proteins are directly related to β-carotene metabolism. However, Gazzola *et al.* (2001) showed that castration alters the portioning of food energy to fat and muscle growth, and subsequently, produces a suitable fat cover in the carcass and this affects the fat colour. Therefore, these genes may affect the growth rate, which in turn, may influence fat deposition and thus, impact beef fat colour. Other possible explanations are that these genes may be in linkage disequilibrium with the actual causative variant or there are other important but unknown pathways controlling beef fat colour.

4.4.5 Additional SNP effects on QTL

Four SNPs with large effects on fat colour were tested for their effects on the previously identified QTL. Theoretically, the inclusion of the SNP genotype as a fixed factor in the general linear model should reduce the residual variance by accounting for other genetic effects (Knott *et al.*, 1998). This should cause a large decrease in significance of a QTL if the SNP is the causative allele for that QTL or in linkage disequilibrium with the causative allele. As the candidate genes on BTA 9 and BTA 15, the variants in the *ALDH8A1* and *BCO2* genes, as expected, decreased the significance of the QTL on these chromosomes below the threshold level. More new significant QTL were also identified for fat colour related traits on BTA 1, BTA 8, BTA 10 and BTA 24 as also would be expected since more of the variance has been explained. However, the variant in the *PPARGC1A* gene, which represented the candidate gene on BTA 6, failed to eliminate the QTL on BTA 6 for any of the fat colour related traits. This indicates that since the *PPARGC1A* SNP12 did not explain the observed QTL effects on BTA 6, there is probably another QTN underlying this QTL effect.

The SNPs in the *RDHE2* gene had differing effects on the QTL on BTA 14, where the *RDHE2* gene is located. The significance of biopsy fat colour QTL on BTA 14 was increased and location shifted when the *RDHE2* SNP2 was fitted in the model, perhaps indicating an epistatic effect with a nearby gene. In contrast, the significance of this QTL was decreased by fitting the *RDHE2* SNP3 in the model. However, the lack of an individual effect for *RDHE2* SNP3 on biopsy fat colour in the cattle mapping progeny implies that this SNP is not QTN underlying QTL. Instead, the results suggest that this SNP may be in linkage disequilibrium with QTN underlying the QTL on BTA14.

Chapter 5

Interactions between candidate gene SNPs

5.1 Introduction

Analysis of interactions between SNPs within candidate genes is an obvious extension of association studies. Analysis of SNP-SNP interactions within a gene and haplotype analyses provide additional information to investigate the association between beef fat colour and candidate genes, particularly if the SNP in the candidate gene is not causative SNP. Also, gene-gene interactions or epistasis is likely to be a ubiquitous component of the genetic architecture of complex traits. The effects of epistasis can dictate the functional outcomes over the independent effects of any given individual gene. Although most of the individual SNPs showed small effects on fat colour traits, the genetic effect of the combinations of functionally relevant SNPs may additively contribute to increased beef fat colour. Therefore, analytical models that incorporated gene interactions were employed to examine the effect of gene-gene interaction or epistasis between genes as well as interactions within genes.

5.2 Materials and methods

The samples used in this study were derived from Group 1, the Davies gene mapping Limousin x Jersey progeny (described in section 2.1). For the statistical analysis, Model 5 was used to examine within gene and between gene interactions by fitting cohort, breed, sire, *BCO2* W80X genotype with each pair of SNP genotypes and their interaction (described section 2.7). Model 6 was used to analyse the haplotype effects on the fat colour related traits by fitting cohort, breed, sire, *BCO2* W80X genotype and each gene haplotype (described in section 2.7).

5.3 Results

5.3.1 Interaction effects between SNPs within genes

Interaction effects on fat colour related traits between the SNPs within each gene were tested with Model 5 (described in section 2.7) (Table 5-1). This test is valuable for determining if haplotypes are likely to provide additional information. Forming the haplotypes, may be more informative if the individual SNP is not the causative SNP. For β-carotene concentration, the interaction between SNP 15 and SNP 16 within the *ALDH8A1* gene and the interaction between SNP 3 and SNP 4 within the *RDHE2* gene were found to be significantly associated. Only the SNP 6 and SNP 7 interaction within *APOM* gene was found to affect biopsy fat colour significantly. The interaction of SNP 7 and SNP 13 within the *BCMO1* gene was the only one which affected biopsy fat colour significantly. There were no other interactions within genes that significantly affected carcass fat colour although the effect of the *PPARGC1A* SNP 9 and SNP 10 interaction was nearly significant.

Table 5-1 Interaction effects between SNPs within genes

Gene	SNP	Bc-bio	Fc-bio	Fc-car
ALDH8A1	SNP10*SNP15	0.67	0.58	0.56
	SNP10*SNP16	0.36	0.4	0.16
	SNP15*SNP16	**0.02***	0.13	**0.07†**
	SNP10*SNP15*SNP16	-	-	-
APOM	SNP2*SNP6	0.16	0.61	0.76
	SNP2*SNP7	0.17	0.32	0.4
	SNP6*SNP7	0.49	**0.03 ***	0.69
	SNP2*SNP6*SNP7	-	-	-
BCMO1	SNP4*SNP7	0.67	0.99	0.34
	SNP4*SNP8	0.18	**0.08†**	0.12
	SNP4*SNP13	0.36	0.86	0.47
	SNP7*SNP8	0.95	0.95	0.77
	SNP7*SNP13	0.11	**0.04***	0.29
	SNP8*SNP13	**0.08†**	0.59	0.71
	SNP4*SNP7*SNP8	-	-	-
	SNP4*SNP7*SNP13	0.74	0.68	-
	SNP4*SNP8*SNP13	-	-	-
	SNP7*SNP8*SNP13	-	-	-
	SNP4*SNP7*SNP8*SNP13	-	-	-
PPARGC1A	SNP4*SNP9	0.17	0.65	0.83
	SNP4*SNP10	0.82	0.36	0.17
	SNP4*SNP12	0.29	0.11	0.14
	SNP9*SNP10	0.69	0.74	**0.06†**
	SNP9*SNP12	0.32	0.41	0.71
	SNP10*SNP12	0.37	0.7	0.93
	SNP4*SNP9*SNP10	0.88	0.78	0.31
	SNP4*SNP9*SNP12	-	-	-
	SNP4*SNP10*SNP12	-	-	-
	SNP9*SNP10*SNP12	-	-	-
	SNP4*SNP9*SNP10*SNP12	-	-	-
RARA	SNP1*SNP3	0.54	0.16	0.8
	SNP1*SNP7	0.43	0.59	0.57
	SNP3*SNP7	0.93	0.95	0.86
	SNP1*SNP3*SNP7	0.19	0.61	0.33
RDHE2	SNP2*SNP3	**0.07†**	0.56	0.11
	SNP2*SNP4	0.59	0.34	0.58
	SNP3*SNP4	**0.02***	0.32	0.86
	SNP2*SNP3*SNP4	-	-	-
SCARB1	SNP1*SNP2	0.23	0.21	0.62
	SNP1**SNP16	0.79	0.18	0.82
	SNP2*SNP16	0.72	0.24	0.62
	SNP1*SNP2*SNP16	-	-	-

† P<0.1,* P<0.05, ** P<0.01, ***P<0.001. **Boldface**= effects with P values < 0.1. – No mean is provided due to no animals with all combination genotypes.

5.3.2 Haplotype associations

As the population used in this project was double back-cross design, the haplotypes could be predicted based on the family structure. In addition to examining the single SNP effect on fat colour traits and their interactions within the genes, overall tests of association were also performed on the haplotypes for each gene in order to further explore the relationship among SNPs in the candidate genes. The haplotypes were formed using the program *PHASE* 1.0 (Pirinen *et al.*, 2008) for the genotyped SNPs in each gene (Appendix 5), and diplotypes (diploid haplotype combinations in a gene) were constructed based on the haplotype information.

The haplotype analysis was performed with Model 6 (as described in section 2.7). No association between any gene diplotype and carcass fat colour score was detected (Table 5-2). For β-carotene concentration, only the *RDHE2* diplotype had a highly significant effect on this trait ($P < 0.008$). There were 11 diplotypes formed for *RDHE2*, but 2 combinations (1/7, 3/3) with low frequencies (<1%) had to be excluded herein from the haplotype association analysis. The individuals with diplotype 2/4 had a significantly higher β-carotene concentration than the other diplotypes, whereas the animals with the diplotype 2/2 had the lowest β-carotene concentration (0.94±0.1 μg/g of fat). In addition to *RDHE2*, the *BCMO1* diplotype effect was nearly significant for β-carotene concentration ($P = 0.08$). The diplotype 2/4 animals had the highest value (1.47±0.1 μg/g of fat) for β-carotene concentration.

Biopsy fat colour score was significantly affected by both the *PPARGC1A* and *SCARB1* diplotypes. A total of 16 and 10 diplotypes were found in *PPARGC1A* and *SCARB1*, respectively. Animals with 3/3 and 3/4 in *PPARGC1A* and *SCARB1*, respectively, tended to have increased biopsy fat colour scores compared with the other diplotypes.

Table 5-2 Diplotype association with significance and trait means

		Bc-bio[1]		Fc-bio[1]		Fc-car[1]	
Gene	**No. of animals**	**Prediction**	**se**	**Prediction**	**se**	**Prediction**	**se**
ALDH8A1		P=0.838		P=0.125		P=0.161	
1/1	4	1.00	0.23	2.38	0.27	0.89	0.47
1/2	16	0.99	0.11	1.79	0.14	1.92	0.23
1/3	105	1.17	0.05	2.06	0.06	1.96	0.10
1/4	6	1.08	0.19	2.13	0.22	1.54	0.38
1/5	4	1.23	0.23	2.23	0.28	1.40	0.55
2/2	15	1.07	0.12	2.26	0.14	1.98	0.25
2/3	159	1.14	0.04	2.00	0.04	1.66	0.07
2/4	11	1.05	0.14	1.72	0.16	1.98	0.29
3/3	42	1.19	0.09	2.04	0.10	1.69	0.18
APOM		P=0.495		P=0.685		P=0.319	
1/1	35	1.08	0.08	1.97	0.10	1.58	0.17
1/2	26	1.08	0.10	1.90	0.11	1.73	0.19
3/1	89	1.09	0.05	2.05	0.06	1.81	0.11
3/2	42	1.27	0.08	2.15	0.10	1.80	0.17
3/3	70	1.23	0.06	2.01	0.07	1.55	0.13
4/1	30	1.12	0.09	1.90	0.11	2.01	0.19
4/2	8	1.38	0.17	1.93	0.20	2.04	0.34
4/3	54	1.11	0.07	2.07	0.08	2.03	0.14
4/4	6	0.97	0.20	1.95	0.24	1.82	0.40
BCMO1		**P=0.078†**		P=0.845		P=0.836	
1/1	48	1.08	0.08	2.03	0.09	1.69	0.16
1/2	29	0.99	0.10	1.98	0.12	1.74	0.21
1/3	26	1.18	0.10	1.96	0.12	1.62	0.21
1/4	48	1.13	0.07	2.04	0.09	1.60	0.14
1/5	64	1.18	0.07	2.16	0.08	1.96	0.14
2/4	8	1.47	0.17	2.20	0.21	1.66	0.36
2/5	14	1.12	0.14	1.97	0.17	1.73	0.28
3/3	5	0.92	0.22	2.30	0.26	2.37	0.49
3/4	22	1.42	0.11	2.02	0.13	1.84	0.22
3/5	25	1.29	0.11	2.02	0.13	1.72	0.22
4/4	16	1.12	0.13	2.06	0.15	1.90	0.26
4/5	36	1.04	0.09	1.93	0.10	1.71	0.18
5/5	11	1.09	0.15	1.91	0.18	2.03	0.31
PPARGC1A		P=0.408		**P=0.037 ***		P=0.114	
1/1	4	1.31	0.24	2.04	0.29	2.07	0.49
1/2	10	1.19	0.16	1.79	0.19	1.77	0.31
1/3	38	1.08	0.09	1.86	0.10	1.92	0.18
1/4	13	1.14	0.14	2.22	0.17	2.22	0.28
1/6	9	1.02	0.17	1.84	0.20	1.80	0.35
2/2	10	1.00	0.15	2.15	0.18	1.32	0.30
2/3	93	1.15	0.05	1.92	0.06	1.63	0.11
2/4	17	1.10	0.12	2.06	0.14	1.84	0.24
2/6	25	1.18	0.10	2.26	0.12	1.87	0.19
2/8	5	0.99	0.22	1.82	0.26	0.78	0.43
3/3	29	1.42	0.09	2.39	0.11	2.24	0.19
3/4	48	1.07	0.08	2.01	0.09	1.69	0.16
3/6	33	1.10	0.09	2.04	0.10	1.79	0.17
3/7	6	1.29	0.20	1.91	0.23	1.62	0.39
4/4	6	1.05	0.20	2.09	0.24	1.88	0.40
4/6	7	1.05	0.19	2.27	0.22	2.20	0.40

Table 5-2 continued

		Bc-bio[1]		Fc-bio[1]		Fc-car[1]	
Gene	**No. of animals**	**Prediction**	**se**	**Prediction**	**Gene**	**No. of animals**	**Prediction**
RARA		P=0.734		P=0.52		P=0.857	
1/2	17	1.01	0.13	1.98	0.15	1.88	0.25
1/3	34	1.16	0.09	2.20	0.11	1.86	0.19
1/4	25	1.12	0.10	2.12	0.12	1.91	0.21
2/2	22	1.21	0.10	2.10	0.13	1.86	0.22
2/3	62	1.20	0.06	2.01	0.07	1.85	0.13
2/4	67	1.14	0.06	1.98	0.07	1.90	0.12
2/6	4	1.09	0.24	2.22	0.29	2.17	0.49
3/3	53	1.15	0.07	2.07	0.08	1.61	0.14
3/4	48	1.22	0.07	2.03	0.09	1.64	0.15
4/4	22	0.99	0.11	1.79	0.14	1.62	0.24
4/6	5	0.90	0.22	1.59	0.26	1.20	0.50
RDHE2		**P=0.008 ****		P=0.622		P=0.231	
1/1	97	1.06	0.06	2.08	0.07	1.64	0.12
1/2	62	1.27	0.06	2.04	0.08	1.74	0.13
1/3	29	1.19	0.10	2.07	0.12	1.57	0.20
1/4	72	1.03	0.06	1.89	0.07	1.78	0.12
2/2	13	0.94	0.14	1.94	0.17	1.69	0.28
2/3	13	1.27	0.13	1.96	0.17	1.55	0.28
2/4	30	1.34	0.09	2.14	0.12	2.18	0.20
3/4	28	1.19	0.09	1.96	0.12	2.13	0.19
4/4	14	1.08	0.14	1.97	0.17	2.16	0.31
SCARB1		P=0.126		**P=0.021 ***		P=0.496	
1/1	119	1.14	0.05	2.09	0.06	1.86	0.11
1/2	76	1.13	0.06	1.96	0.07	1.80	0.11
1/3	60	1.29	0.07	2.00	0.08	1.79	0.13
1/4	40	1.03	0.08	1.84	0.10	1.46	0.17
2/2	13	1.10	0.14	1.96	0.17	1.69	0.28
2/3	17	1.16	0.12	2.13	0.15	1.72	0.26
2/4	17	1.06	0.12	1.98	0.15	1.79	0.25
3/3	7	1.22	0.18	2.21	0.22	1.59	0.38
3/4	9	1.38	0.16	2.62	0.19	2.30	0.34
4/4	7	0.73	0.18	1.75	0.22	1.37	0.38
BCO2		P=0.995		P=0.147		P=0.663	
1/1	128	1.12	0.04	1.96	0.05	1.58	0.09
1/2	49	1.08	0.07	2.05	0.08	1.80	0.14
1/3	51	1.08	0.06	1.91	0.07	1.64	0.13
1/4	64	1.13	0.06	2.03	0.07	1.91	0.13
2/2	7	1.14	0.16	1.71	0.19	1.70	0.34
2/3	15	1.11	0.11	1.91	0.13	1.75	0.24
3/3	8	1.04	0.15	1.77	0.18	1.78	0.33
3/4	10	1.11	0.15	2.31	0.17	1.88	0.31

[1]**Trait**: Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); Fc-car, fat colour score of carcass samples (scale 0-9); Fc-bio, fat colour score of biopsy samples (scale 1-5). † P<0.1,* P<0.05, ** P<0.01, ***P<0.001. **Boldface**= effects with P values < 0.1

5.3.3 Epistatic interactions of major SNPs

In order to test for epistatic effects between genes, the degree and significance of interactions between the SNPs that accounted for largest variation in the fat colour traits (*BCO2* W80X, *ALDH8A1* SNP15, *ALDH8A1* SNP16, *BCMO1* SNP4, *PPARCG1A* SNP12, *RDHE2* SNP2 and *SCARB1* SNP2) and all the other SNPs was examined (Table 5-3). The proportion of the total SNP variation accounted by the SNP interaction varied between 1% and 6%. Interestingly, besides the *BCO2* W80X variant, the *PPARGC1A* SNP12 showed the largest number of interaction effects on all three traits. The interaction between *PPARGC1A* SNP12 and *BCMO1* SNP7 was highly significant for β-carotene concentration and accounted for 4.8% of the variation. However, the interaction between *PPARGC1A* SNP12 and *BCO2* W80X accounted for the largest percentage of the variation (6.0%) (Table 5-3). Animals with the TT genotype of *PPARGC1A* SNP12 and the AA genotype of *BCO2* W80X had up to 198% higher adipose β-carotene concentration than the other genotype combinations (Figure 5-1). For the biopsy fat colour, the interaction between *PPARGC1A* SNP12 and *SCARB1* SNP1 accounted for 4.6% of the variation (Table 5-3). The combinations of the *PPARGC1A* SNP12 TT genotype with the *SCARB1* SNP1 two homozygous genotypes had higher biopsy fat colour scores than the other combinations (Figure 5-2). The interaction between *PPARGC1A* SNP12 and *RDHE2* SNP3 contributed 2.5% to the total sum of squares for carcass fat colour (Table 5-3). The animals with the combination of the AA genotype of the *RDHE2* SNP3 and the TT genotype of fat *PPARGC1A* SNP12 also had yellower fat than the other animals (Figure 5-3).

Table 5-3 Size of interaction effects between SNPs as percentage of total SNP variation (%)

	BCO2 W80X			*ALDH8A1* SNP15			*ALDH8A1* SNP16			*BCMO1* SNP4			*PPARCG1A* SNP12			*RDHE2* SNP2			*SCARB1* SNP2		
	Bc-bio[1]	Fc-bio[2]	Fc-car[3]	Bc-bio	Fc-bio	Fc-car	Bc-bio	Fc-bio	Fc-car	Bc-bio	Fc-bio	Fc-car	Bc-bio	Fc-bio	Fc-car	Bc-bio	Fc-bio	Fc-car	Bc-bio	Fc-bio	Fc-car
***ALDH8A1* SNP10**	3.1*	3.3**												2.2†	2.0†		2.2†				
***ALDH8A1* SNP15**		1.3*																			
***ALDH8A1* SNP16**	3.4**	2.2*												3.7**							
***APOM* SNP2**									1.7†												
***APOM* SNP6**																		2.1*			
***APOM* SNP7**									2.4†												
***BCMO1* SNP4**																					
***BCMO1* SNP7**					1.8*								4.8***								
***BCMO1* SNP8**	1.6†										1.2†										
***BCMO1* SNP13**	1.8†						2.6*	4.2***					2.2†	2.2†							
***PPARCG1A* SNP4**			1.2†					2.7**									2.0†				
***PPARCG1A* SNP9**	2.9**				1.9†			1.9*			0.9†						3.1*				
***PPARCG1A* SNP10**			1.4*															2.9*			1.9†
***PPARCG1A* SNP12**	6.0***		1.7†					3.7 **			2.1†										
***RARA* SNP1**	2.9**								2.4*							2.21†					
***RARA* SNP 3**																2.73*					
***RARA* SNP7**	2.0*	1.6†						1.5†							2.2†		2.3*				
***RDHE2* SNP2**	3.4**			2.2†																	
***RDHE2* SNP3**	1.6†		2.3*					1.9*							2.5*					2.9**	
***RDHE2* SNP4**													1.3†								
***SCARB1* SNP1**											1.6†		2.9*	4.6***							
***SCARB1* SNP2**			1.9†																		
***SCARB1* SNP16**	1.3*		0.8†																		

[1]Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); [2]Fc-bio, fat colour score of biopsy samples (scale 1-5); [3]Fc-car, fat colour score of carcass samples (scale 0-9).
† P<0.1,* P<0.05, ** P<0.01, ***P<0.001.

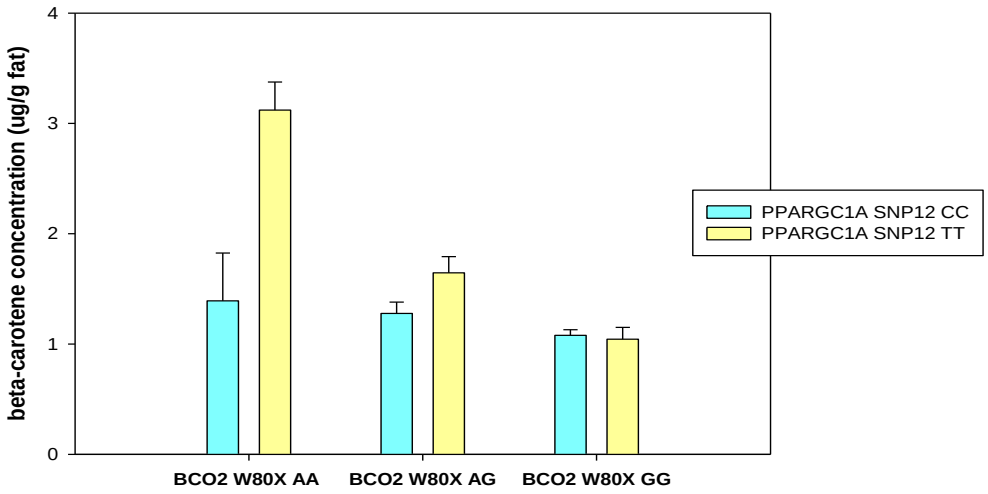


Figure 5-1 Interaction between *PPARGC1A* SNP 12 and *BCO2* W80X on β-carotene concentration

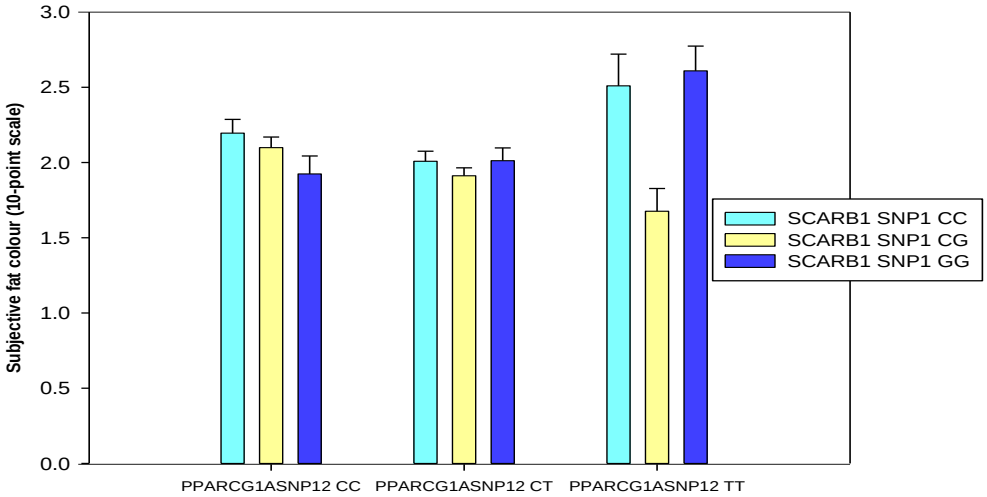


Figure 5-2 Interaction between *PPARGC1A* SNP 12 and SCARB SNP1 on biopsy fat colour

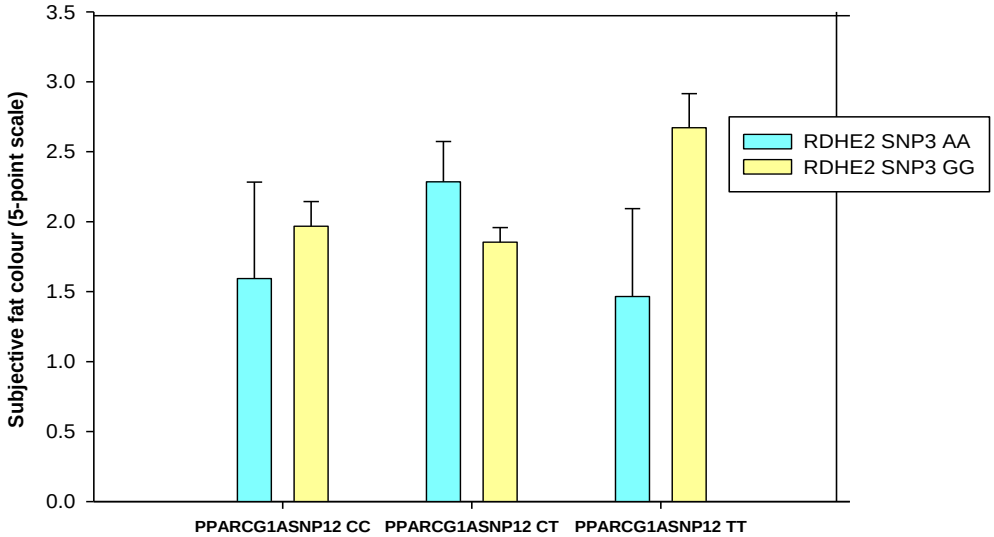


Figure 5-3 Interaction between *PPARGC1A* SNP 12 and RDHE2 SNP3 on carcass fat colour (10 point scale)

5.4 Discussion

5.4.1 Haplotype effects

Although the single SNP effects were not always significant, haplotypes formed from SNPs within genes or SNP interactions within genes could still have effects on the traits of interest. For example, none of the individual SNPs in *RDHE2* was significant for any fat colour traits, with the exception of *RDHE2* SNP2 effects on β-carotene concentration and carcass fat colour (Table 4-2). In contrast, the interaction between SNP3 and SNP4 and the *RDHE2* haplotype combination effect was highly significant for β-carotene concentration (Table 5-1 and Table 5-2). The individuals with diplotype combination 2/4 had the highest β-carotene concentration. This paradox indicates that the genotype effect of single SNP may be influenced by other SNPs within the gene and/or that the haplotype is in stronger linkage disequilibrium with the causative SNP.

Fallin *et al.* (2001) provided evidence that the inheritance of haplotype combinations is often more effective for detecting associations than that of a single SNP. The haplotype combination provides a more comprehensive approach of assessing the relationship between multiple-site variation and traits. Therefore, the analysis of haplotype combinations is more powerful than the analysis of single individual SNPs.

No SNP-SNP interaction effect on biopsy fat colour was observed, for instance, for either the *PPARGC1A* gene or the *SCARB1* gene. However, there were significant haplotype effects on biopsy fat colour for both *PPARGC1A* and *SCARB1* genes. This may be because the detection of within-gene SNP interaction effects was limited by the sample size and small numbers of homozygous animals with rare alleles. It is difficult to identify the effect when the interaction includes all SNPs within a gene in a model by

this approach. Therefore, the haplotype analysis provides a good alternative approach to associate genes with beef fat colour, especially if the haplotype is in stronger linkage disequilibrium with the causative SNP. On the other hand, there was evidence of SNP-SNP interactions that were not detected in the haplotype analysis (eg *ALDH8A1* SNP 15-SNP16, *APOM* SNP 6- SNP7) and these should not be disregarded.

5.4.2 Epistatic effects

The analysis of epistasis indicated that genes without significant main effects can interact with other genes and confer a yellower fat colour. For example, the *ALDH8A1* SNP10, *ALDH8A1* SNP16, *PPARGC1A* SNP9 and *SCARB1* SNP16 had no significant additive or dominance effects on β-carotene concentration, but the interactions between the *BCO2* W80X SNP and each of these SNPs showed a significant effect on β-carotene concentration ($P<0.05$). Similarly, the *BCMO1* SNP13 showed no individual effect on biopsy fat colour, but the interaction between the *ALDH8A1* SNP16 and the *BCMO1* SNP13 had a highly significant effect on biopsy fat colour ($P<0.001$), which accounted for 4.2% of the total sum of squares. Moreover, rare genotype combinations were associated with beef fat colour. For example, the combination of rare genotypes of *ALDH8A1* SNP15 (CC genotype) and *BCMO1* SNP7 (TT genotype) resulted in a relatively high biopsy fat colour score. Such findings would have been overlooked in the absence of an interaction analysis. These results emphasize the importance of epistatic interactions in understanding the mechanisms underlying beef fat colour.

It is worth noting that the *BCO2* W80X SNP interacted with the largest number of other genes to affect β-carotene concentration, beef carcass fat colour and biopsy fat colour. In general, for any of those genes, the combination of the AA *BCO2* homozygous

genotype with the other genotypes of these SNPs had a higher β-carotene concentration and yellower fat colour, compared to the *BCO2* GG and GA genotype cattle. This was expected as the A allele results in the truncated BCO2 protein.

In addition, some of the observed significant epistatic interactions suggested there is "cross talk" between the alleles of proteins of different biological pathways. For example, a highly significant effect was shown for the interaction between the *RDHE2* SNP3 and *SCARB1* SNP2. SCARB1 is reported to facilitate β-carotene transportation in both *in vitro* and *in vivo* (Kiefer *et al.*, 2002, During *et al.*, 2005). RDHE2 catalyzes the first and rate-limiting step that generates retinaldehyde from retinol (Duester, 2000). Single gene analysis showed no significant associations of the *RDHE2* SNP3 or the *SCARB1* SNP2 with biopsy fat colour. However, the interaction analysis showed that some genotypic combinations of these genes were associated with significant effects on biopsy fat colour. This suggests that the occurrence of higher β-carotene concentrations in adipose tissues, which is mediated by *SCARB1*, may affect the enzyme activity of RDHE2.

Chapter 6

BCO2 and *RDHE2* gene validation studies

6.1 Introduction

A number of SNPs in the candidate genes were significantly associated with fat colour related traits (Chapter 4). Some of the SNP genotypes explained a large proportion of the variance. For example, the *BCO2* W80X SNP explained up to 15% of the total SNP variation in beef fat colour (Table 4-3). And the RDHE2 SNP2 was also accounted for larger of the total SNP variation for two fat colour traits. Before SNP information can be used in breeding programmes, it is important that unbiased and independent validation studies in different populations are conducted to establish whether the observable effects are likely to be found in other breeds or populations under selection (Gill *et al.*, 2009). The aim of this study was to examine the association of *BCO2* and *RDHE2* genes with beef fat colour and milk fat colour in other populations in order to validate the effects of these two genes.

6.2 Materials and methods

6.2.1 Animal resources

There are 6 groups of animals used in the validation study, which were described in Chapter 2 in detail. Group 3 included the 119 Jersey dams from the Jersey-Limousin backcross mapping herd in Australia; Group 2 included 419 Jersey-Limousin backcross progeny from the AgResearch Gene Mapping Project; there are 590 Holstein-sired cows and 192 Jersey-sired cows in Group 4 and Group 6 separately; 309 cattle were slaughtered in 2006 (Group 5) and 119 cattle were slaughtered in 2009 (Group 7). Animals in Groups 5 and 7 were slaughtered at two local Hamilton abattoirs in New Zealand (as described in Chapter 2). For Group 5, the extremes in carcass fat colour were specifically chosen, whereas for Group 7, the samples were taken from a range of

fat colour scores.

6.2.2 Statistical analysis

The models used in this analysis were described in section 2.7. In brief, the model for Group 2 fitted with breed, cohort (sexes and 2 years of birth), sire and genotype. For the carcass fat colour score (scored "0" or "1"), slaughter group was also included as a fixed effect. For Group 3, year of sampling and genotype were included as fixed effects and age as a covariate in the model. Some cows had very high β-carotene values, so the data were log-transformed before analysis for this group. This transformation was not necessary for the fat colour score data. The model in Group 4 was sire (five levels), herd (21 levels), management group (42 levels-range of 1–4 management groups per herd), cow age (2–8 + years), breed (three levels pure-, ¾- and ½-Holstein- Friesian), genotype and the covariate "Days in Milk". For Group 5 and Group 7, genotype was the only fixed effect. For Group 6, age (3–6 + years) and genotype were fitted as fixed effects.

6.3 Results

6.3.1 *BCO2* validation

6.3.1.1 Australian Jersey dams

For the Australian Jersey dams (Group 3), year had a significant effect on both β-carotene concentration and fat colour score (Table 6-1). The SNP *BCO2* W80X genotype only had a significant effect on the fat colour score (Table 6-1). Animals with the SNP *BCO2* W80X AA genotype had the highest fat colour score (4.48±0.39), whereas animals with the heterozygous genotype were intermediate (4.19±0.17) and

animals with the SNP *BCO2* W80X GG genotype had the lowest fat colour score (3.61±0.13). The SNP *BCO2* W80X GG had no additive or dominance effects on β-carotene concentration. For the biopsy fat colour score of the Australian Jersey dams, only a significant additive effect was identified (Table 6-1).

Table 6-1 Means (SE) for year, BCO2 W80X genotype, additive and dominant effects in Australian Jersey dams

	No. of animals	log_eBc-biop[1] (µg/g fat)	Fc-biop[2]
Year[3]		***	***
1994	26	1.68 (0.16)	3.51 (0.23)
1995	13	1.36 (0.20)	2.80 (0.29)
1996	15	1.23 (0.18)	3.30 (0.26)
1998	87	2.36 (0.09)	4.40 (0.14)
Genotype		ns	**
AA	7	2.31 (0.27)	4.48 (0.39)
GA	41	2.00 (0.12)	4.19 (0.17)
GG	81	1.86 (0.09)	3.61 (0.13)
Additive		0.23 (0.14) ns	0.44 (0.21) **
Dominant		-0.08 (0.18) ns	0.15 (0.26) ns

[1] log_eBc-biop: β-carotene concentration of fat biopsy samples in log scale. [2]Fc-biop: fat colour of biopsy samples on a 5 point scale. [3]Some samples (12) were repeated across years. *** P<0.001; **P<0.01; *P<0.05; ns: not significant.

6.3.1.2 Validation data from New Zealand

In parallel studies within New Zealand, additional samples (described in section 2.1) were genotyped by AgResearch to confirm the *BCO2* effects. The effects of the SNP *BCO2* W80X allele substitutions on several measures of yellow fat were determined (Table 6-2). As expected, the SNP W80X had a highly significant effect on New Zealand Jersey-Limousin backcross mapping progeny (Group 2) for all adipose fat

colour traits. This SNP accounted for 19% and 41% of the phenotypic variance for β-carotene concentration in the fat and fat colour score, respectively. However, when cattle of unknown breed or origin (Group 5) with extreme adipose fat colour data were genotyped, a significant additive effect was only observed for the fat colour score. The effect of the W80X A allele on this trait was 0.29 out of a score of 5 and accounted for 11% of the phenotypic variance (Table 6-2). The SNP *BCO2* W80X had a significant additive effect on the milk fat colour in both the Holstein-Friesian-sired cows (Group 4) and Jersey cows (Group 6), and accounted for 18% and 25% of phenotypic variance, respectively.

Table 6-2 Substitution effects of *BCO2* W80X alleles for fat colour traits in the New Zealand cattle

Cattle resource[1]	Trait	Mean	Additive effect	Additive	Dominance	% Phen. Variance[6]
Group 2 (NZ J x L backcross)	Log_eBc-bio[2]	0.48	0.53 ± 0.13	P < 0.0001	P < 0.01	19
	Fc-bio[3]	1.69	1.04 ± 0.21	P < 0.0001	P < 0.001	23
	Fc-car[4]	0.037	0.46 ± 0.06	P < 0.0001	P < 0.0001	41
Group 4 (NZ Holstein)	Milk Absorbance[5]	0.31	0.05 ± 0.01	P < 0.0001	n.s.	18
Group 5 (NZ extreme cattle)	Fat Absorbance[5]	1.01	0.29 ± 0.08	P = 0.005	n.s.	11
Group 6 (NZ Jersey)	Milk Absorbance[5]	0.77	0.18 ± 0.09	P = 0.04	n.s.	25
Group 7 (NZ random cattle)	Bc-bio	10.14	3.35 ± 0.84	P < 0.001	P = 0.03	14

[1]Described in detail in chapter 2. [2]Log_eBc-bio: β-carotene concentration of fat biopsy samples on log scale. [3]Fc-bio: fat colour of biopsy samples on a 5 point scale. [4]Fc-car: fat colour of carcass (0 or 1) . [5]Absorbance: milk or fat colour was assessed by spectrophotometry (absorbance at 450nm). [6] calculation of percentage of Phen. Variance: *BCO2* W80X SNP sum of squares/ total sum of squares

6.3.2 *RDHE2* validation

The two amino acid substitution SNPs in *RDHE2* were genotyped in a series of additional experimental cattle populations with milk fat colour and adipose fat colour data. The variation explained by these 2 *RDHE2* SNPs was determined (Table 6-3). There was a small individual effect of *RDHE2* SNP2 on the carcass fat, but the *RDHE2* SNP3 explained no variation in the fat colour traits. However, both *RDHE2* SNPs accounted for some variation in the abattoir samples (Groups 5 and 7). The individual effect of the *RDHE2* SNP2 was quite large, explaining 8-17% of the variation in the fat colour traits (Groups 5 and 7) (Table 6-3). The *RDHE2* SNP3 has less of an effect, explaining 2% of the variation. Interestingly, the *RDHE2* SNPs were significant for adipose fat colour but not for milk fat colour. This is in contrast to the *BCO2* W80X SNP, which affected both adipose and milk fat colour (Table 6-2).

6.3.3 Interaction effects between *RDHE2* and *BCO2*

The SNPs in the *RDHE2* gene were also analysed for interactions with the *BCO2* nonsense mutation, W80X. Significant interactions were observed for some fat colour traits in the various populations. In the Australian and New Zealand Jersey-Limousin backcross progeny (Groups 1 and 2), the size of effect for the *BCO2* interaction with the *RDHE2* SNP2 was larger than for the *RDHE2* SNP2 alone. However, the interaction between *BCO2* and *RDHE2* was not significant in the other populations.

Table 6-3 Effects of RDHE2 SNPs on fat colour traits

Group	Source	Trait	RDHE SNP2	RDHE SNP3	BCO2 x RDHE2 SNP2
1	JxL backcross (AUS)	Fc-bio[1]	0.5% ns	0.1% ns	3.3 *
		Bc-bio[2]	1.8% *	0.6% ns	5.1% **
		Fc-car[3]	1.9% *	1.0% ns	3.7% **
2	JxL backcross (NZ)	Fc- bio	0.2% ns		1.4% ns
		Bc-bio	0.5% ns		1.3% ns
		lnBc- bio[4]	0.5% ns		0.4% ns
		Yfat@slaut[5]	1.8% *		4.0% **
3	Jersey cows (AUS)	Fc-bio	1.6% ns		0.7% ns
		Bc-bio	0.5% ns		0.1% ns
4	Holstein cows (NZ)	Milk Colour[6]	0.3% ns	0.3% ns	1.8% *
5	Extreme cattle (NZ)	Fat Absorbance[7]	16.8% ***	1.9% *	2.4%†
		Fc-car	15.0% ***	2.1% *	0.6% ns
6	Jersey cows (NZ)	Milk colour	0.7% ns	2.3% ns	0.5% ns
7	Random cattle (NZ)	Bc-bio	8.6%**		0.4 ns
		lnBc- bio	8.4%**		1.8 ns

[1]Fc-bio: biopsy fat colour score on 1-5 scale [2] Bc-bio: β-carotene concentration of fat biopsy samples [3]Fc-car: carcass fat colour score on a 0-9 point scale [4] lnBc- bio: β-carotene concentration of fat biopsy samples on log scale. [4]Fc-car: fat colour of carcass on a 0.1 scale. [5]Yfat@slaut: carcass fat colour score [6] Milk Colour: milk colour was assessed by spectrophotometry (absorbance at 450nm) [7] Fat Absorbance: fat colour was assessed by spectrophotometry (absorbance at 450nm). . *** P<0.001; **P<0.01; *P<0.05; † P<0.1; ns: not significant

6.4 Discussion

6.4.1 *BCO2* effects

The relationship between the *BCO2* W80X SNP and fat colour in cattle was confirmed in a series of experimental populations. The *BCO2* W80X mutation had significant effects on beef fat colour traits in all these populations consistently. Approximately 680 Jersey-Limousin backcross progeny in Australia and New Zealand were genotyped for the *BCO2* SNP W80X. Although the animals were raised in quite different environments and finished on different diets (grain versus green pasture), the size of the effect of the *BCO2* SNP W80X A allele on the fat colour score of the biopsy samples was the same in both countries (1.09 and 1.04, respectively). The additive and dominance effects indicated that the A allele homozygotes had the most yellow fat and that the trait is partially recessive.

Due to the low number of AA genotypes observed in the Jersey backcross progeny, 141 pure Jersey dams in Australia and 309 animals from abattoirs in NZ were genotyped to validate the association. As expected, these data also demonstrated that the AA genotype increases yellow fat scores. This association was also observed for milk fat colour in the NZ data set, with the *BCO2* SNP W80X A allele being related to increased milk colour scores.

The data from these different populations support the association of the *BCO2* gene with yellow fat colour and β-carotene concentration. However, because of the modest numbers of animals with the AA genotype, the true size of effect of the gene on fat colour and β-carotene concentration could not be estimated accurately even with inclusion of the additional data sets.

6.4.2 *RDHE2* effects

The effects of the *RDHE2* SNPs were also investigated in these other experimental populations. The individual effects of the *RDHE2* SNP2 and *RDHE2* SNP3 genotypes were greater in the random samples taken from carcasses at New Zealand abattoirs than the Jersey-Limousin backcross progeny from either Australia or New Zealand. The breed origin of the random samples could not be traced, but the animals were mature and fed on green pasture. The samples were taken from carcasses that ranged from obvious yellow to white fat colour. For one group (Group 5), the extremes were specifically chosen, whereas for the other group (Group 7), the samples were taken from a range of fat colour scores. The results for these groups were similar although the size effect of the *RDHE2* SNP2 was less in the latter group (15-17% for Group 5 versus 8% for Group 7). In addition, there were some differences in the effects of *RDHE2* observed in the Australian (Group 1) versus New Zealand (Group 2) Jersey-Limousin backcross progeny. These are most likely explained by the differences in feeding regimes (grain *vs.* green pasture) and age at sampling.

Given the kinetics of the β-carotene metabolic pathway, one can postulate that having the AA genotype of the *RDHE2* SNP2 results in increased RDHE2 enzyme activity such that more retinol is generated from retinal (Figure 1-2). Thus, less β-carotene would need to be cleaved into retinol by BCMO1. In combination with the *BCO2* W80X nonsense mutation, fat colour would further increase as even less β-carotene is cleaved by BCO2.

However, there did not appear to be any interaction between the *BCO2* W80X and the *RDHE2* SNP2 in the New Zealand random abattoir samples. Instead, in these samples,

there was a much larger effect of the individual *BCO2* and *RDHE2* SNPs. There are several possible explanations including breed, diet and age differences between the Jersey-Limousin backcross progeny and these random samples. Another possible explanation is that the allele frequency of the *RDHE2* SNP 2 and the *RDHE2* SNP 3 in these various populations is quite different (Table 6-4). Given the rarity of certain genotypes, some of the effects may have been unobserved because there were insufficient data.

Table 6-4 RDHE2 allele frequencies in experimental cattle populations

SNP	Group 1 Australia JxL backcross (fat)	Group 2 NZ JxL backcross (fat)	Group 3 Australia Jersey cows (fat)	Group 4 NZ Holstein cows (milk)	Group 5 NZ extreme cattle (yellow fat)	Group 6 NZ Jersey cows (milk)	Group 7 NZ Random cattle (fat)
RDHE2 SNP 2, C allele	0.50	0.54	0.78	0.58	0.70	0.96	0.67
RDHE2 SNP 3, T allele	0.23	NA	NA	0.08	0.23	0.37	NA

NA= not available

The 2 SNPs in *RDHE2* result in conservative amino acid substitutions (alanine to valine), and thus, the combination of the amino acids is not likely alter enzyme function. It is possible that the true functional variant within the *RDHE2* gene (or nearby gene) is merely in linkage disequilibrium with these *RDHE2* SNPs. However, the linkage disequilibrium must extend beyond the backcross families analysed herein, as the genotype effect was observed in the random populations (Group 5 and 7) as well.

The association of *RDHE2* SNPs with milk colour in the New Zealand animals showed that only the interaction between *BCO2* W80X and *RDHE2* SNP 3 had an effect in the Holstein cows (Group 4, Table 6-3). There was no individual SNP effect on milk colour for either of the *RDHE2* SNPs. In contrast, the effect of *BCO2* W80X on milk fat colour

was highly significant in both the Holstein and Jersey cows (Table 6-2). Unlike BCO2 which converts β-carotene directly to retinoic acid, RDHE2 only affects β-carotene concentration in the local tissues indirectly via the feedback of retinoic acid formed from retinol. The RDHE2 enzyme is expressed mainly in the epidermis (Markova *et al.*, 2003). If there is less expression in mammary tissue, then RDHE2 may have little or no effect on milk colour.

Chapter 7

Gene expression studies

7.1 Introduction

Gene-expression analysis has become increasingly important in biological research as it provides functional insight into the phenotype traits, thereby allowing the investigation of underlying cellular and molecular mechanisms. Traditionally, Northern blots were employed to detect mRNA levels (Alwine *et al.*, 1977). However, this method is time-consuming and does not allow precise quantification. Today, quantitative reverse transcription PCR (also known as RT-PCR, qPCR or qRT-PCR) is most commonly used for detection of mRNA levels. qRT-PCR is a sensitive, highly specific and rapid time-to-result technique that can quantify the amount of DNA (either genomic or cDNA derived from RNA) molecules in a polymerase chain reaction (Heid *et al.*, 1996). For detecting the amount of RNA, the process involves reverse transcription (RT) followed by real-time quantitative PCR (qPCR). Firstly, the total RNA is extracted from a biological sample (ribosomal RNA and mRNA). Secondly, a DNA template is generated (cDNA) from the mRNA by reverse transcription. Thirdly, real-time polymerase chain reactions are carried out in a thermocycler using the cDNA template. This process permits measurement of fluorescent detector molecules which increase as the DNA amplification progresses.

DNA variants in the *BCO2*, *BCMO1* and *RDHE2* genes have been shown to significantly affect beef fat colour herein (Chapters 4, 5 and 6). Genetic variants from differentially expressed genes may explain some of this phenotypic variation. The aim of this study was to determine if the expression of *BCO2*, *BCMO1* and *RDHE2* genes in hepatic tissues of animals with pigmented or non-pigmented carcass fat differs.

7.2 Materials and methods

7.2.1 Animal resources and methods

Samples from the 119 random cattle in Group 7 were used in the gene-expression study as described in Chapter 2. In brief, liver samples were preserved using RNAlater solution after the cows were slaughtered. The animals were scored as having either yellow or white fat. RNA was extracted following the manufacturer's protocol (TRI Reagent® RNA Isolation, *Applied Biosystems*). The reverse transcript reaction was conducted in a 20μl reaction system using Superscript III kit (*Invitrogen, Australia*). Real-time PCR was performed for the relative expression with the KAPA SYBR® FAST qPCR Kit (*Geneworks, Australia*) in the Rotor-Gene 6000 Real-Time PCR Detection System (*Corbett*). Beta-carotene was extracted as described in section 2.2 and genotyping was performed as described in section 2.5.5 for these animals.

7.2.2 Statistical analysis

Three models were used in the gene expression study (Group 7). Model 8 fitted fat colour group to compare the mean of the mRNA levels for *BCMO1, BCO2* and *RDHE2*. Model 9 fitted different gene genotypes (*BCO2* W80X or *RDHE* SNP2) to compare the means of mRNA levels for three genes (*BCMO1, BCO2 and RDHE2*) with the means of β-carotene concentration for each genotype. Model 10 fitted mRNA levels for *BCMO1, BCO2 and RDHE2* to determine the relationship between the mRNA levels (*BCMO1, BCO2 and RDHE2*) and the β-carotene concentration.

7.3 Results

7.3.1 RNA extraction

The quality and integrity of extracted RNA was tested by spectrophotometry and gel electrophoresis analysis (Appendix 9). The results demonstrated that the extracted RNA was of excellent quality and suitable for the qRT-PCR experiments.

7.3.2 Optimization of PCR

The SYBR green dye detection system was used for qRT-PCR. The SYBR green dye binds to all double stranded DNA and as a consequence, any non-specific amplified products, including primer-dimers, which can contribute to the overall fluorescent signal. Therefore, the amplification of a single specific PCR product with an optimized annealing temperature is critical for the success of quantification analysis. Generally, PCR was optimized for a range of annealing temperatures. The optimal annealing temperature of each primer pair was selected by the amplification of a specific PCR product without primer-dimers or other non-specific products. The optimal annealing temperature for each primer pair of the genes were used in the real-time PCR assays (Table 7-1).

Table 7-1 Optimal annealing temperature for primer pairs

Gene	Annealing temperature °C
Actin beta (*ACTB*)	60
60S acidic ribosomal protein P0 (*RPLP0*)	60
TATA box-binding protein (*TBP*)	60
Ubiquitin carboxyl-terminal hydrolase L5 (*UCHL5*)	60
Beta-carotene 15,15'-monooxygenase 1 (*BCMO1*)	58
Beta-carotene oxygenase 2 (*BCO2*)	55
Retinal short chain dehydrogenase reductase (*RDHE2*)	55

7.3.3 Evaluation of reference genes

Before measuring the expression of the target genes, all samples were compared using the same reference genes. The selection of the stable reference genes was based on the expression stability parameter (M), which was calculated using the geNorm software in the qbaseplus programme (version 2.0). The measure M reflects the expression stability of the gene compared to the other reference genes. A lower M-value means more stable gene expression and is the basis for the ranking of the genes in order of their expression stability (Vandesompele *et al.*, 2002). Of the four evaluated reference genes, *UCHL5* had the highest M value of 1.878 and *RPLP0* had the lowest M value of 1.291 (Table 7-2).

Table 7-2 M values of reference genes

Reference Genes	M
Actin beta (*ACTB*)	1.705
60S acidic ribosomal protein P0 (*RPLP0*)	1.291
TATA box-binding protein (*TBP*)	1.531
Ubiquitin carboxyl-terminal hydrolase L5 (*UCHL5*)	1.878

To determine the minimum number of reference genes required for accurate normalization, a pairwise variation analysis was used between the normalization factors Vn/n+1, which measures the effect of adding more reference genes to the normalisation factor (that is, the effect is calculated as the geometric mean of the expression values of the selected reference genes). Vandesompele (2002) recommended the use of at least three reference genes if the pairwise variation value is higher than the cut-off value of 0.15. Therefore, the three most stably-expressed genes *RPLPO, ACTB2* and *TBP* were used for normalization herein (Figure 7-1).

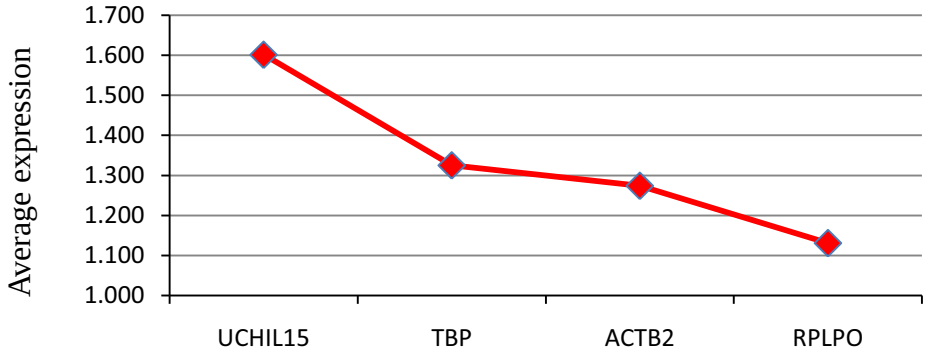


Figure 7-1 Average expression stability values (M) of the reference genes with stepwise exclusion of the least stable expressed reference genes

7.3.4 Gene expression analysis

7.3.4.1 Gene expression and fat colour

Analysis of the relative normalised *BCO2* and *BCMO1* mRNA levels revealed large variation between the samples. However, no significant difference in the mRNA levels of either gene was observed between the yellow fat and white fat samples (Figure 7-2, Figure 7-3, Figure 7-4). In contrast, the *RDHE2* mRNA level difference between the two groups was nearly significant (P=0.07). The mean value for the relative *RDHE2* mRNA levels in the white samples was 1.7 fold higher (in logarithm) than in the yellow samples (Table 7-3).

Table 7-3 Relative normalised gene mRNA expression[a]

fat colour group	logBCMO1	logBCO2	logRDHE2
white	0.10(0.17)	-0.03 (0.10)	0.19 (0.14)
yellow	-0.05 (0.11)	0.07 (0.07)	-0.11 (0.09)
P value	0.49	0.44	**0.07†**

[a] logarithm transformation

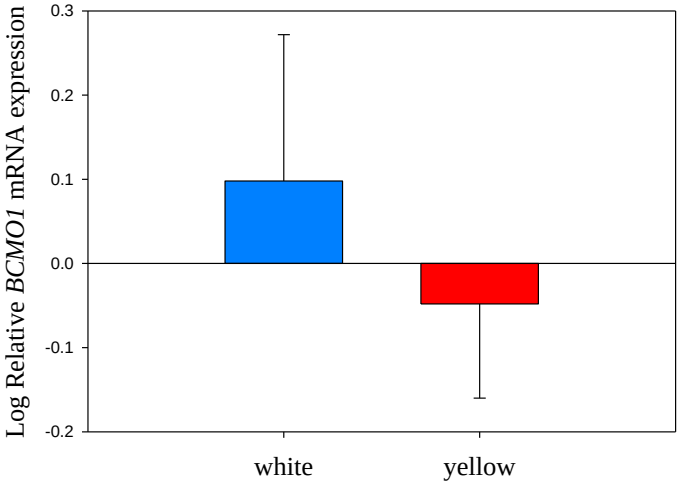


Figure 7-2 *BCMO1* mRNA expression in the two fat colour groups
(in logarithm) (P = 0.49)
White fat group n = 13
Yellow fat group n =31

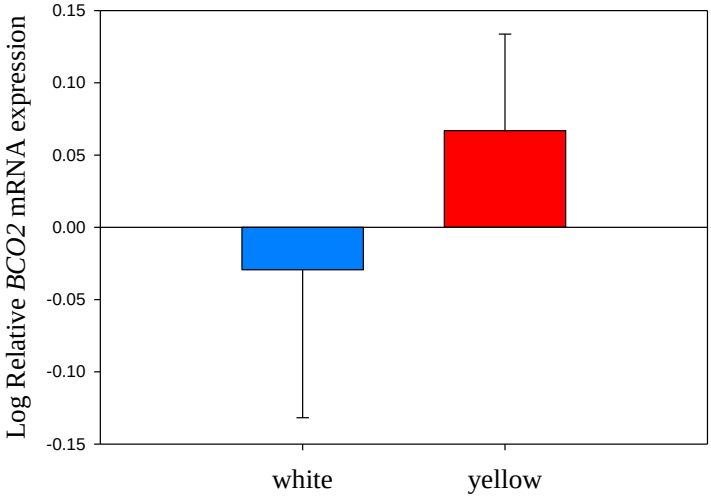


Figure 7-3 *BCO2* mRNA expression in the two fat colour groups
(in logarithm) (P = 0.44)
White fat group n = 15
Yellow fat group n =35

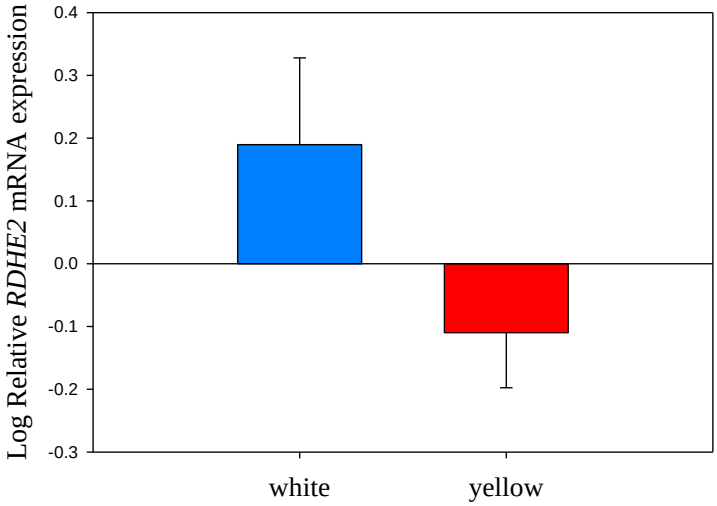


Figure 7-4 *RDHE2* mRNA expression in the two fat colour groups
(in logarithm) (P = 0.07)
White fat group n = 13
Yellow fat group n =32

7.3.4.2 Genotype effects on gene expression

The *BCO2* and *RDHE2* genotypes contribute to the colour difference in beef fat, and therefore, the effects of *BCO2* and *RDHE2* polymorphisms on the *BCO2*, *BCMO1* and *RDHE2* mRNA expressional levels were examined using least-square-effects from a fixed effect ANOVA model (Table 7-4). The *RDHE2* SNP2 genotype did not affect the mRNA levels for any of the 3 genes (Figure 7-5, Figure 7-6 Figure 7-7). The *BCO2* W80X SNP effect on *BCO2* mRNA expression level was near the significance threshold though (P=0.07) (Figure 7-5, Figure 7-6 Figure 7-7). Animals with the *BCO2* W80X SNP A/A genotype had the lowest *BCO2* mRNA expression level (-0.45 ±0.23 in logarithm) (Table 7-4), whereas the GG genotype had the highest *BCO2* mRNA expression level (0.10 ±0.09 in logarithm). However, no significant association was found between the *BCO2* W80X genotype and the *BCMO1* or *RDHE2* gene mRNA levels.

Table 7-4 Relative normalised gene mRNA levels among *RDHE2* SNP2 genotypes and SNP *BCO2* W80X genotypes

	logBCMO1	**logBCO2**	**logRDHE2**
RDHE2	P = 0.93	P = 0.81	P = 0.47
RDHE2 SNP2 AA	0.09 (0.33) n = 4	0.01 (0.20) n = 5	0.21 (0.23) n = 5
RDHE2 SNP2 GA	-0.04 (0.14) n = 21	0.12 (0.09) n = 23	-0.10 (0.11) n = 20
RDHE2 SNP2 GG	0.01 (0.15) n =19	-0.04 (0.10) n = 22	-0.01 (0.11) n = 20
BCO2	P = 0.19	**P = 0.07**	P = 0.25
BCO2 W80X AA	0.41 (0.31) n = 4	-0.45 (0.23) n = 4	-0.17 (0.29) n = 3
BCO2 W80X GA	0.09 (0.15) n = 18	0.06 (0.10) n = 20	0.13 (0.12) n = 18
BCO2 W80X GG	-0.16 (0.13) n = 22	0.10 (0.09) n =26	-0.12 (0.10) n =24

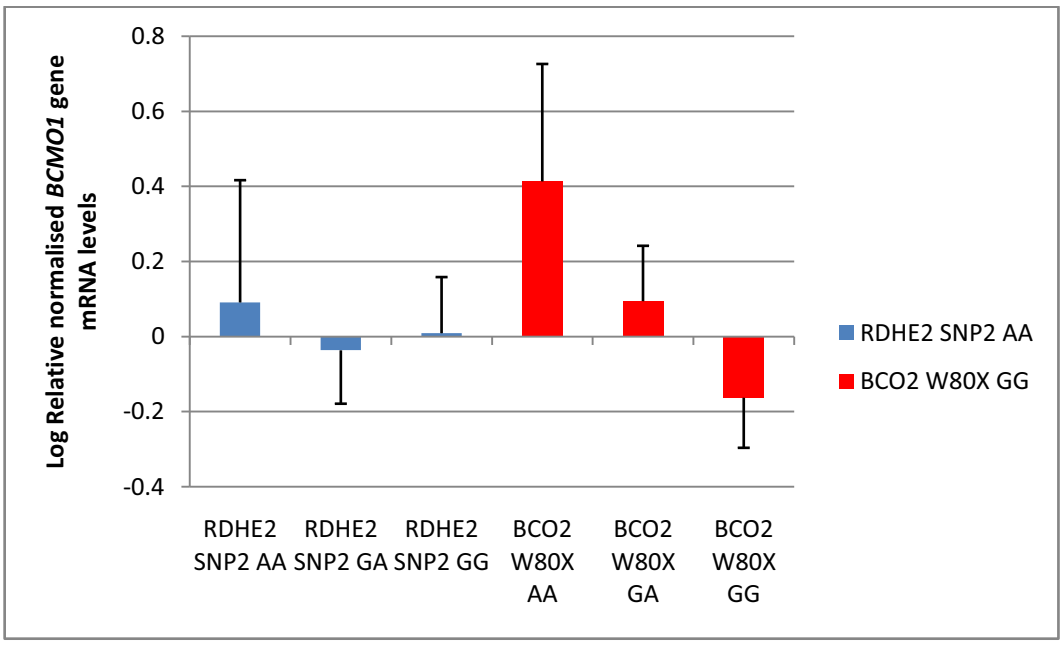


Figure 7-5 Relative normalised *BCMO1* gene mRNA levels for different gene genotypes [*RDHE2* SNP2 genotype (P = 0.93) and *BCO2* W80X genotype (P=0.19)]

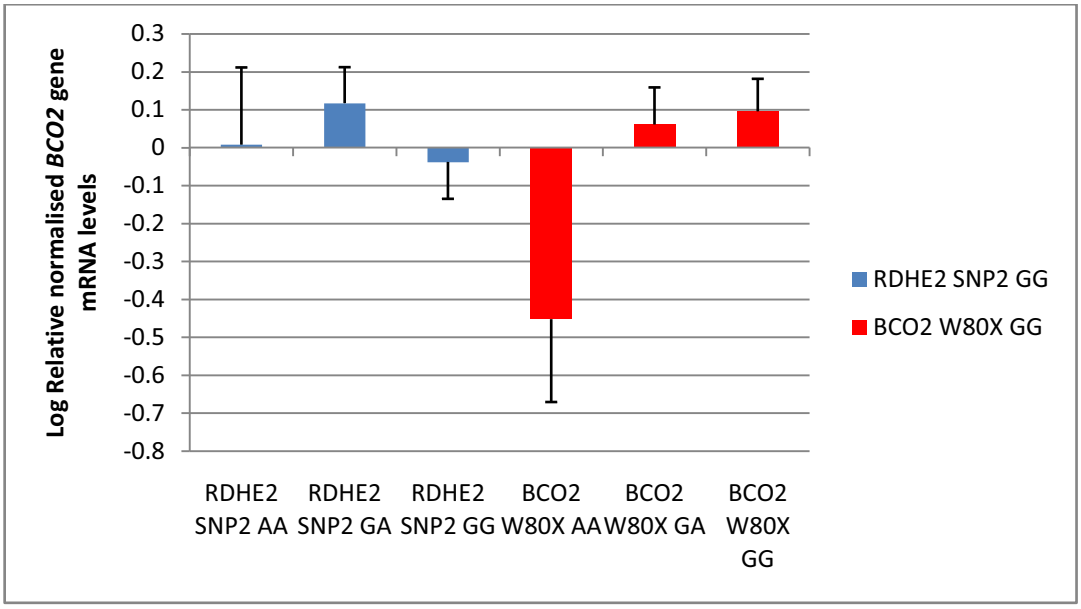


Figure 7-6 Relative normalised *BCO2* gene mRNA levels for different gene genotypes [*RDHE2* SNP2 genotype (P = 0.873) and *BCO2* W80X genotype (P=0.07)]

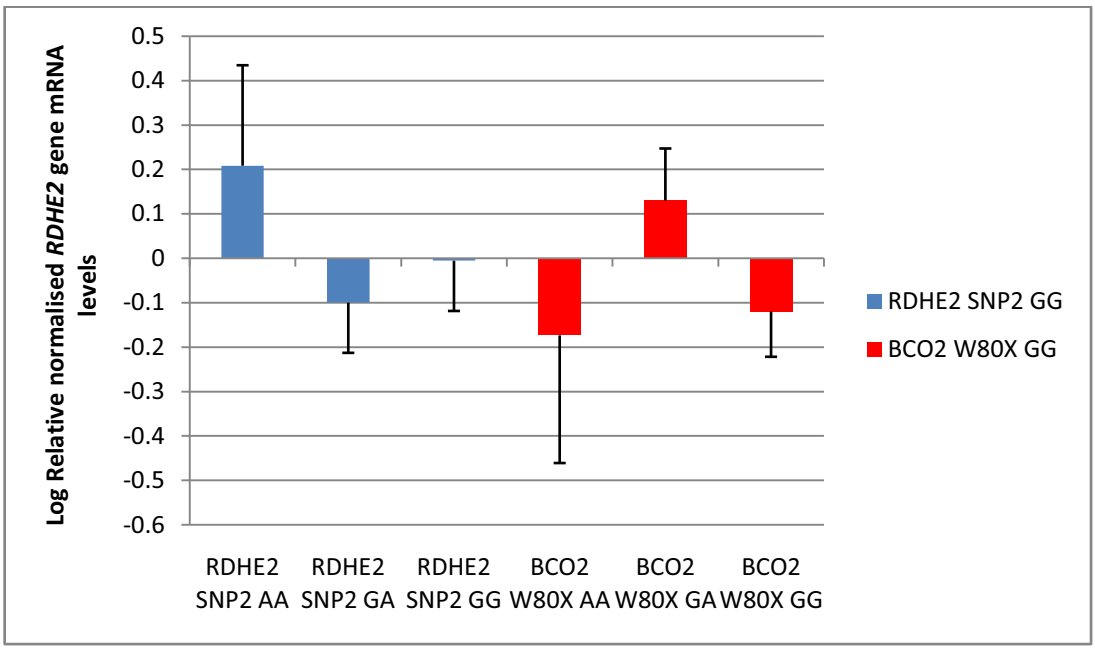


Figure 7-7 Relative normalised *RDHE2* gene mRNA levels for different gene genotypes [*RDHE2* SNP2 genotype (P = 0.47) and *BCO2* W80X genotype (P=0.25)]

7.3.4.3 Gene expression effects on β-carotene concentration

Regression analysis was also conducted to examine the relationships between β-carotene concentration and *BCO2, BCMO1* and *RDHE2* mRNA levels. The regression coefficients for each relationship were determined (Table 7-5). The β-carotene concentration was found to be negatively correlated to *BCO2* gene expression and was positively correlated to *BCMO1* and *RDHE2* mRNA levels. However, only the *BCO2* gene expression had a significant association with β-carotene concentration. In terms of gene interaction effects, only the interaction between *BCO2* and *BCMO1* expression levels had a nearly significant positively relationship with β-carotene concentration (P=0.07).

Table 7-5 Regression co-efficients (slope of linear regression analysis) for the relationship between β-carotene concentration and relative gene expression level

	logBCMO1	logBCO2	logRDHE2	logBCO2 *logBCMO1	logBCO2 *logRDHE2	logBCMO1 *logRDHE2
coefficients	0.01	-0.09	0.05	0.55	0.18	-0.3
P value	0.26	**0.05***	0.92	**0.07†**	0.87	0.62

7.3.4.4 Relationships between relative gene expression levels

The correlation between the relative mRNA levels between the genes was weak. The *BCO2* and *BCMO1* gene expression levels had a negatively moderate correlation (Figure 7-8, r=-0.49, P<0.001). The same negative trend was also observed between the *BCO2* and *RDHE2* gene expression levels, although the slope of relationship between *BCO2* and *RDHE2* gene expression levels was not significant (r= -0.21, P=0.15, Figure 7-10). In contrast, *BCMO1* gene expression level was positively correlated with *RDHE2* gene expression level (r=0.36, P=0.03) (Figure 7-9).

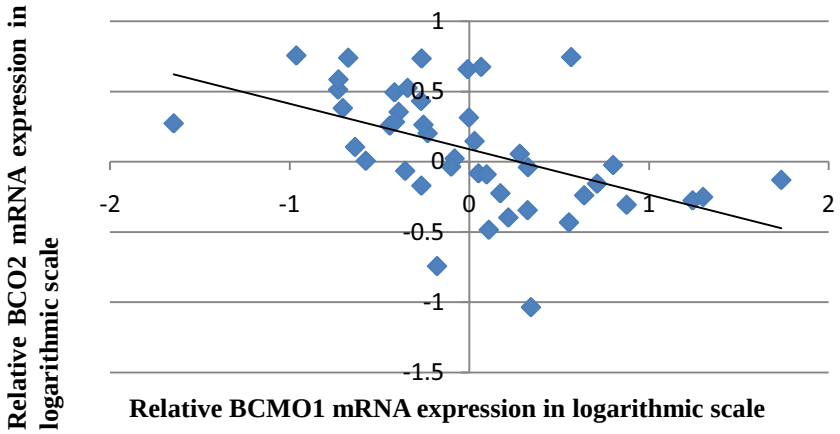


Figure 7-8 Correlation between *BCMO1* mRNA expression and *BCO2* mRNA expression
(r= -0.49, P<0.001) n=50, logarithmic scale (log 10), r = correlation

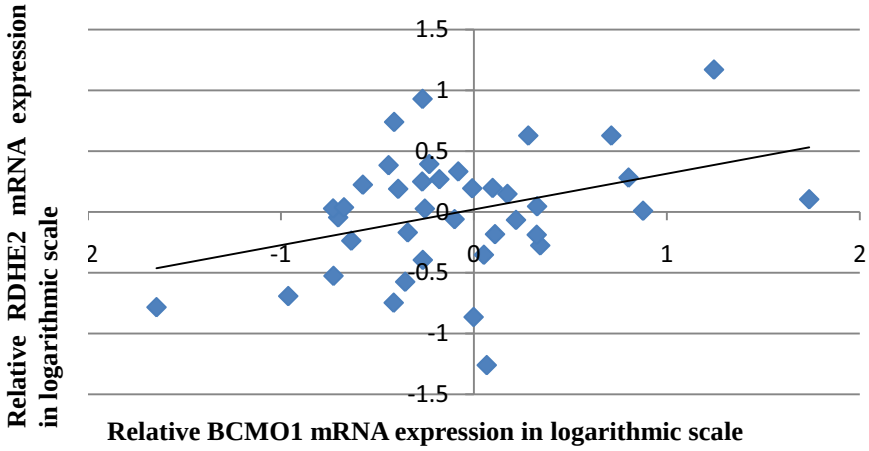


Figure 7-9 Correlation between *BCMO1* mRNA expression and *RDHE2* mRNA expression
(r= 0.36, P=0.03) n=50, logarithmic scale (log 10), r = correlation.

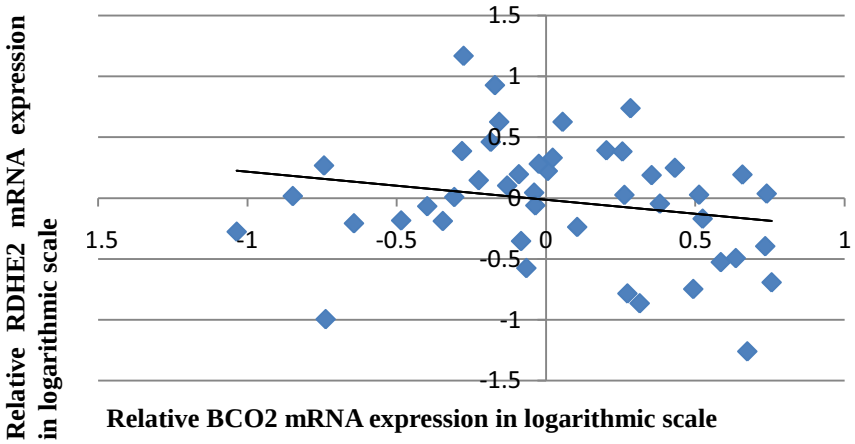


Figure 7-10 Correlation between *BCO2* mRNA expression and *RDHE2* mRNA expression
(r= -0.21, P=0.15) n=50, logarithmic scale (log 10), r = correlation.

7.4 Discussion

Given the significant effect of *BCMO1*, *BCO2* and *RDHE2* genes on beef fat colour, the expression of these genes was measured to investigate their role in fat colour. The main findings from the real-time PCR analyses were that 1) there was no difference in the expression of the *BCO2* and *BCMO1* genes between animals with yellow fat and white fat, 2) the *RDHE2* gene expression level was higher in white samples compared with yellow samples, 3) the expression of the *BCO2* gene was significantly associated with β-carotene concentration, and 4) the SNP *BCO2* W80X genotype was marginally associated with *BCO2* gene expression levels.

7.4.1 *BCMO1* gene expression analysis

The enzyme BCMO1 cleaves the central linkage of β-carotene producing two molecules of retinal or vitamin A (Glover, 1960). *BCMO1* mRNA levels in liver did not affect fat colour or β-carotene concentration herein. This result is in agreement with the Morales *et al.* (2007) finding that there is no difference in the expression levels of *BCMO1* in the duodenum and liver between cattle with yellow fat and white fat. However, mRNA expression level is not always necessarily correlated with the rate of synthesis of the corresponding protein. Differences in the level of enzymatic activity in the tissue can also affect the outcome. Morales *et al*. (2007) demonstrated that the BCMO1 enzyme activity in the liver is twice as high in pigmented cattle than non-pigmented cattle, even though their gene expression levels were not significantly different. Nevertheless, the higher enzyme activity in the liver of the pigmented animals was still not sufficient to cleave all the β-carotene and the excess must be being distributed and stored in different tissues of the body.

The effect of the *BCO2* polymorphism on the *BCMO1* gene mRNA levels was not statistically significant, perhaps due to the greater variation in the *BCMO1* mRNA levels among the samples. This result was not expected. The liver is the major storage organ for retinol and is involved in most aspects of vitamin A homeostasis (Mcclintick *et al.*, 2006). Vitamin A is formed by the cleavage of β-carotene both symmetrically and asymmetrically, as catalysed by the BCMO1 and BCO2 enzymes, respectively. Because of the truncated BCO2 protein encoded by the *BCO2* W80X A allele, a relatively low vitamin A concentration in the animals with *BCO2* W80X A allele would be expected without a compensatory mechanism. Thus, there should be increased *BCMO1* mRNA expression in order to maintain vitamin A homeostasis. However, the association between the *BCMO1* gene expression level and the SNP *BCO2* W80X was not significant. This could be a reflection of the lack of power due to the lower number of animals with the *BCO2* W80X AA genotype (n=4). Alternatively, the higher concentrations of β-carotene (due to the loss of BCO2 function) may have decreased the *BCMO1* gene expression. In a *BCO2* knockout mouse study, Ford *et al.* (2010) observed that the mRNA expression of *BCMO1* in liver did not differ between wild type *BCO2* and the *BCO2* knockout mice that were fed diets containing β-carotene for 4 or 30 days. Therefore, the regulation of the gene expression of *BCMO1* by vitamin A deficiency may be compensated by the effect of increasing β-carotene concentration in liver of animals with the *BCO2* W80X AA genotype.

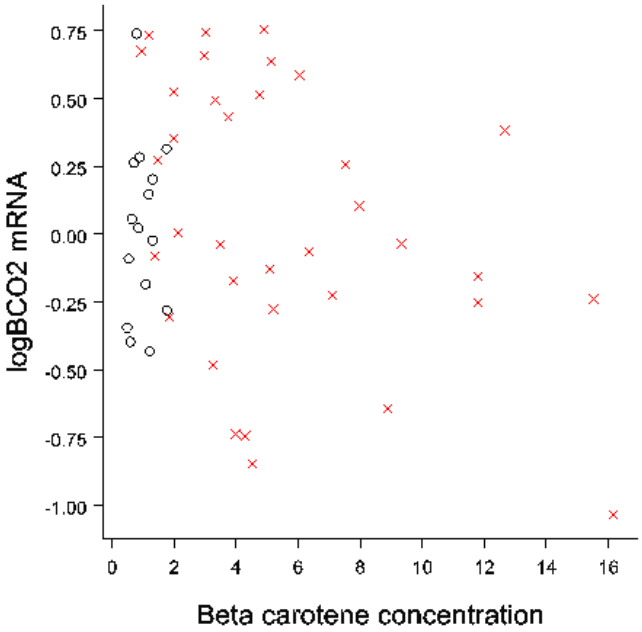
7.4.2 *BCO2* gene expression analysis

BCO2 cleaves β-carotene asymmetrically. It was hypothesised that the *BCO2* mRNA expression level would affect β-carotene concentration and consequently, there would be a difference in beef fat colour. Herein, *BCO2* mRNA expression was associated with

β-carotene concentration (P = 0.049, Table 7-5), but not with fat colour group (Table 7-3). Because the data for β-carotene concentration in yellow fat colour group were skewed (Figure 7-11), they were log-transformed and re-analysed (Figure 7-12). The relationship between β-carotene concentration (log-transformed) and *BCO2* gene expression was no longer significant (P = 0.17). Therefore, with this in mind, *BCO2* gene expression was not associated with either β-carotene concentration or fat colour group. The lack of relationship could result from the tissue-specificity of *BCO2* expression, i.e. adipose tissue versus liver, as the expression level of *BCO2* varies in different tissues. There is evidence of *BCO2* expression in several tissues in other species. The *BCO2* gene is fairly strongly expressed in the liver of chickens (Eriksson *et al.*, 2008), in the small intestine and liver of mice and humans (Kiefer *et al.*, 2001, Lindqvist *et al.*, 2005), and in the liver and testis of ferrets (Hu *et al.*, 2006). A relatively lower level was found in heart, spleen, lung, skeletal muscle intestine, colon, stomach, kidney, bladder, and prostate of ferrets, humans and mice (Kiefer *et al.*, 2001, Lindqvist *et al.*, 2005, Hu *et al.*, 2006). In a chicken study, qRT-PCR analysis demonstrated that *BCO2* mRNA in white skin is more than 90% higher than that in yellow skin. However, the expression level in liver was similar between yellow skin and white skin chickens (Eriksson *et al.*, 2008). Herein, the results are similar to the chicken study in that *BCO2* mRNA expression in the liver was not significantly different between the fat colour groups (Table 7-3).

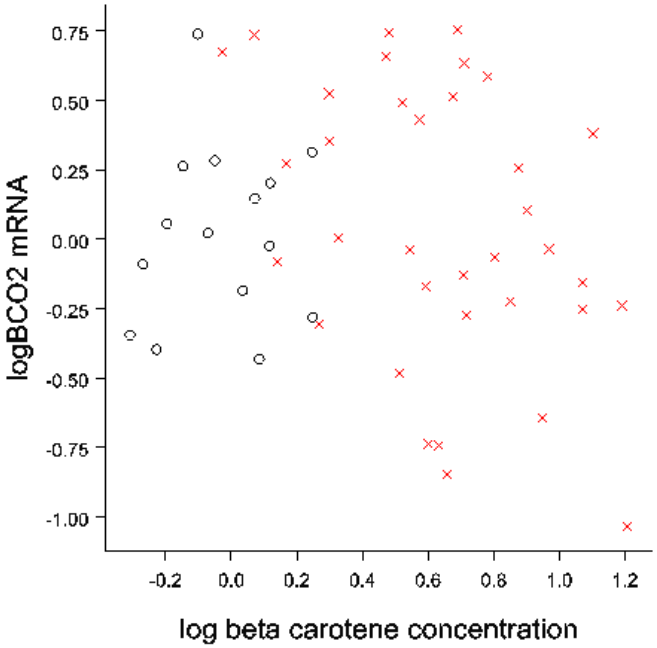
Although there is no significant relationship between β-carotene concentration (log-transformed) and *BCO2* mRNA expression, the negative relationship indicates a trend that the lower the *BCO2* mRNA expression, the more β-carotene was deposited in the subcutaneous fat (Table 7-5). This suggests that if less *BCO2* is present, the more β-

carotene would be available for distribution and storage in the other tissues of body, including subcutaneous fat.



logBCO2 vs. β-carotene concentration (White group) (n=15)
logBCO2 vs. β-carotene concentration (yellow group) (n=35)

Figure 7-11 Regression of *BCO2* mRNA level and beta carotene concentration



○ logBCO2 vs. log β-carotene concentration (White group) (n=15)
× logBCO2 vs. log β-carotene concentration (yellow group) (n=35)

Figure 7-12 Regression of *BCO2* mRNA level and log beta carotene concentration

Some animals had very high β-carotene values, so the data were also analysed with log-transformed beta carotene concentration.

There was a trend for the *BCO2* mRNA expression levels to differ between the *BCO2* genotype groups (P=0.07) ,which was in agreement with Berry *et al.* (2009). In their study, the same mutation in the *BCO2* gene resulted in four-fold lower levels of the *BCO2* mRNA in the liver of *BCO2*$^{-/-}$ cows with yellow milk. This indicates that one or more of the products resulting from cleavage of β-carotene by BCMO1 may influence the *BCO2* mRNA expression in a negative feedback mechanism. A similar mechanism has been observed for BCMO1, which is negatively regulated at transcriptional level by retinoic acid, one of the cleavage products of β-carotene by BCMO1 (Bachmann *et al.*, 2002). Furthermore, another two findings herein suggest that the *BCMO1* and *BCO2* genes are co-regulated by the same vitamin A derivative, retinoic acid. First, there was a negative relationship between *BCO2* and *BCMO1* mRNA expression (r=-0.49, P<0.001). Second, there was an interaction between *BCO2* and *BCMO1* mRNA expression on β-carotene concentration. Theoretically, more retinoic acid could be binding to the *BCMO1* promoter and less retinoic acid is binding to the *BCO2* promoter.

Another likely explanation for lower level of *BCO2* mRNA expression in animals with *BCO2* W80X AA genotype is that nonsense-mediated decay eliminates mRNA species containing premature termination codon. Nonsense-mediated decay is an important pathway to limit the synthesis of abnormal proteins because these truncated proteins have potentially deleterious gain-of-function or dominant negative activities (Chang and Waikan, 1979, Holbrook *et al.*, 2004, Chang *et al.*, 2007). Therefore, *BCO2* mRNA expression level may be lower in animals with *BCO2* W80X AA genotype compared to the wild type as the mRNA is targeted by the nonsense-mediated decay pathway.

7.4.3 *RDHE2* gene expression analysis

In addition to the formation of retinoic acid from retinal as the initial product of symmetric β-carotene cleavage, retinoic acid can be synthesised from retinol, which involves two enzymatic oxidation steps. Firstly, RDHE2, a member of the short-chain alcohol dehydrogenase/reductase (SDR) family, catalyses the rate-limiting reversible oxidation of retinol to retinaldehyde (Matsuzaka *et al.*, 2002); Secondly, retinaldehyde dehydrogenase catalyses the irreversible oxidation of retinaldehyde to retinoic acid (Bhat *et al.*, 1995, Penzes *et al.*, 1997).

The effects of *RDHE2* on the fat colour may be via this retinoic acid pathway. This is supported by two observations herein. Firstly, *RDHE2* mRNA expression was higher in the white fat colour group than that in yellow fat colour group, albeit not statistically significant (P=0.07). Secondly, there was a weak but positive relationship between *RDHE2* and *BCMO1* mRNA expression (r=0.36, P<0.05). Because of the oxidation of retinol to retinaldehyde by RDHE2, the higher *RDHE2* mRNA levels, the less retinol exists in the tissue. As a consequence, the *BCMO1* mRNA expression level will increase as *RDHE2* mRNA level increases in order to maintain vitamin A homeostasis in the local tissues. Furthermore, less β-carotene is circulated and deposited in the subcutaneous adipose tissue.

In addition to its cytoplasmic dehydrogenase activity, RDHE2 may be able to act as a transcriptional repressor in the nucleus. Recently, Markova *et al*. (2006) showed that RDHE2 is recruited to a protein complex on the profilaggrin promoter where it affects the function of the DNA-bound Sp1 transcription activator protein. This process involves protein–protein interactions rather than catalytic activity, since enzymes with

mutations in the active site are still able to repress profilaggrin promoter activity. Also, Markova *et al*. (2006) suggested that RDHE2 may affect transcription by modulating the amount of acetylation and deacetylation of histones near transcription initiation sites over the promoter. This suggests that RDHE2 could possibly have interactions with other proteins involved in fat colour besides the β-carotene cleavage pathway.

7.5 Conclusion

It should be noted that there are limitations to this study due to the small sample size and potential confounding factors. Firstly, the population was randomly selected from abattoir without background information other than the fat colour. Consequently, potential confounding factors (such as breed, age of animals feeding regimes and sex) may have affected the gene expression level comparisons. Secondly, because of the small sample size the power to detect associations between SNP genotypes and gene expression levels is not large.

The qRT-PCR experiments have demonstrated that the mRNA levels of 3 genes (*BCMO1*, *BCO2* and *RDHE2*) have no or only a weak relationship with the β-carotene concentration and beef fat colour. Neither of the *RDHE2* SNP2 and the *BCO2* W80X SNP genotype was not associated with the mRNA levels for any of the 3 genes. The observations indicate that the control of the retinol/retinoic acid pathway at the gene expression level is important for the β-carotene concentration in subcutaneous adipose tissue and consequently, for beef fat colour.

Chapter 8

General Discussion

8.1 Introduction

Consumer-focused research is seen as fundamentally important to underpin the future demand for red meat. Sixty two percent (62%) of beef produced in Australia is for export markets (Meat and Livestock Australia, 2010), and this has a large influence on the profitability of all sectors of the Australian beef industry. Overseas buyers, especially from Japan, place emphasis on consistent product quality, where premium meat has (i) a light bright red colour, (ii) a fine texture, (iii) a moderate amount of marbling, (iv) a high fat cover, and (v) white rather than yellow fat (Egan *et al.*, 2001). Therefore, understanding the biological mechanisms underlying beef fat colour is important in order to meet the consumer requirements of export markets.

Differences in carcass quality traits exist both between and within cattle breeds. Differences in beef fat colour between animals and breeds are primarily determined by the concentration of carotenoids in the adipose tissue. There is evidence that certain genes explain a significant proportion of the phenotypic variability in the beef fat colour. It was hypothesised herein that there are specific genetic variants specifically associated with the beef fat colour phenotype. This project was conducted with an aim to first evaluate DNA sequence variants that may be associated with this phenotype and then to clarify the metabolic pathways underlying fat colour.

8.2 SNP association studies

Single nucleotide polymorphisms (SNPs) are the simplest and most commonly occurring form of genetic variation in the general population (Lunetta, 2008). In the present study, candidate genes were selected based on their logical connection with

carotenoid metabolism and their location within an identified fat colour quantitative trait locus (QTL). SNPs in these genes were examined for association with fat colour and β-carotene concentration (Chapter 4 and 5). SNPs with significant associations may be mutations that affect beef fat colour directly or may be in linkage disequilibrium with the functional variant.

It is clear that even in the relatively small populations examined herein, there are significant associations between DNA variants and beef fat colour and/or β-carotene concentration. As expected, one polymorphism, or even one gene, did not explain all of the genetic variation in beef fat colour, and multiple polymorphisms within various genes seem to affect beef fat colour. Some of the SNPs with large effects, such those in the *BCO2* and *RDHE2* genes, were validated in other populations. An expansion of the sample size using independent populations will aid identification of other polymorphisms and may clarify or strengthen the associations found herein.

Additive effects of SNPs are estimated to predict the performance of the progeny in breeding programmes for marker assisted selection. The additive effect was estimated for Group1 using the model 7, which showed that most SNPs accounted for only a small amount of the variation of fat colour related traits (Table 8-1). Fat colour was expected to be a relatively simple trait in which to study gene effects as the results from breed studies herein and elsewhere suggested the presence of one or two major genes (Kruk, 2001). However, even for fat colour with a major effect from the *BCO2* W80X mutation, there were a large number of SNPs with small effects.

Table 8-1 Proportion of the sum of squares accounted for all SNP additive effects

SNP	Fc-bio[1]	Bc-bio[2]	Fc-car[3]
BCO2 W80X	**5.7*** **	**1.9*** **	**5.7*** **
ALDH8A1 SNP10	0.1	0.2	0.2
ALDH8A1 SNP15	0.1	0.1	0.1
ALDH8A1 SNP16	0.1	0.5	0.1
APOM SNP2	0.0	0.0	**1.5***
APOM SNP6	0.0	0.0	0.0
APOM SNP7	0.4	0.7	0.2
BCO2 SNP1	0.1	0.1	0.0
BCO2 SNP2	0.6	0.4	**1.4***
BCMO1 SNP4	0.0	0.7	0.0
BCMO1 SNP7	0.0	0.0	0.3
BCMO1 SNP8	0.0	0.0	0.0
BCMO1 SNP13	0.1	0.2	0.0
PPARCG1A SNP4	0.0	0.0	**1.6***
PPARCG1A SNP9	0.2	0.2	0.5
PPARCG1A SNP10	0.7	0.1	0.7
PPARCG1A SNP12	0.1	**1.1***	0.0
RARA SNP1	0.0	0.0	**1.3***
RARA SNP 3	0.4	0.0	0.0
RARA SNP7	**0.8†**	0.5	0.0
RDHE2 SNP2	0.0	**1.7***	**1.4***
RDHE2 SNP3	0.1	**1.2***	**0.8†**
RDHE2 SNP4	0.0	0.0	0.0
SCARB1 SNP1	0.1	0.2	0.0
SCARB1 SNP2	**0.8†**	**1.3***	0.1
SCARB1 SNP16	0.4	0.8	0.2
Total SNP additive	10.8	13.1	16.0

[1]Fc-bio, fat colour score of biopsy samples (scale 1-5); [2]Bc-bio, β-carotene concentration of biopsy sample (μg/g of fat); [3]Fc-car, fat colour score of carcass samples (scale 0-9). †P<0.1,* P<0.05, ** P<0.01, ***P<0.001. **Boldface**= effects with P values < 0.1.

Calculation: SNP additive%= (sum of squares of SNP additive/ total sum of squares excluding sum of squares of cohort, breed of dam, and sire)*100

8.3 Non-synonymous SNP effects

A non-synonymous SNP results in an amino acid substitution in the protein product due to a codon change. Much attention has been focused on the selection from this type of SNP, as amino acid changes can alter the function or stability of proteins and consequently, affect the trait of interest. Herein, there are several non-synonymous SNPs in the *ALDH8A1, BCO2*, *RDHE2* and *PPARGC1A* genes (Table 3-5).

The W80X SNP in the *BCO2* gene results in a truncated protein which obviously will affect function. To examine whether the other amino acid substitutions may affect the function or stability of the proteins, the tertiary and secondary structures of the ALDH8A1, PPARGC1A and RDHE2 proteins were predicted for the altered proteins using two computer software packages, the $(PS)^2$ program (http://ps2.life.nctu.edu.tw/) and SSpro8 program (http://scratch.proteomics.ics.uci.edu/). For ALDH8A1 (Figure 8-1, Figure 8-2), PPARGC1A (Figure 8-3, Figure 8-4) and RDHE2 (Figure 8-5, Figure 8-6), the non-conservative amino acid substitutions did result in potential changes to the conformation of these proteins. The number of α loops and the configuration of the β sheets are different between the structures of these proteins based on the predictions. For example, the substitution of threonine^{240} with methionine^{240} in the ALDH8A1 protein is located in the conserved "aldehyde/histidinol dehydrogenase superfamily domain". Threonine is one of two proteinogenic amino acids bearing an alcohol group and is classified as a polar amino acid. Methionine is one of two sulfur-containing proteinogenic amino acids and is also classified as a polar amino acid. Nevertheless, because of the alcohol and sulphur groups, the prediction of the secondary and tertiary structure with these 2 amino acids showed a difference in the number of loops and the structure of β-sheet (Figure 8-1, Figure 8-2). This could potentially result in an

enzymatic catalysis difference given the location of the substitution.

The conformation of the PPARGC1A protein with proline^{616} is also quite different from PPARGC1A protein with leucine^{616} (Figure 8-3, Figure 8-4). Leucine is a highly stabilizing residue within a α-helix, while in contrast, proline forms a tight turn between helices (Fersht, 1999). Therefore, this replacement would be expected to destabilize the loop conformation, resulting in reduced activity toward the substrate (Fersht, 1999).

For RDHE2, the alanine^{6} is a conservative substitution for valine^{6} as these non-polar amino acids are structurally very similar. Nevertheless, the α-helix structure around residue 6 is disrupted by the alanine substitution based on the predictions (Figure 8-6). The difference in side chain size between valine (isopropyl group) and alanine (methyl group) may be sufficient to interfere with the tight turn in the α-helix of the RDHE2 protein (Figure 8-5). Hence, this could potentially also affect the RDHE2 enzymatic catalysis.

The association study herein indicated that these non-synonymous SNPs had significant effects on beef fat colour traits (Table 4-3) and given the protein predictions, these SNPs may represent the QTN. However, only the *BCO2* and *ALDH8A1* SNPs eliminated their respective fat colour QTL when included in the linkage analysis model. The fat colour QTL on BTA 14 was not eliminated when either or both of the *RDHE2* SNP2 and SNP3 were included in the model. There was no evidence of an interaction between these 2 *RDHE2* SNPs either. Thus, the combination of the alleles at position 6 and position 33 does not appear to be important (eg alanine^{6} and alanine^{33} had the same effect as valine^{6} and valine^{33}). This would suggest that the *RDHE2* SNP2 and SNP3

may only be in linkage disequilibrium with the QTN, with the *RDHE2* SNP2 being in stronger linkage disequilibrium as it had the larger effect across populations.

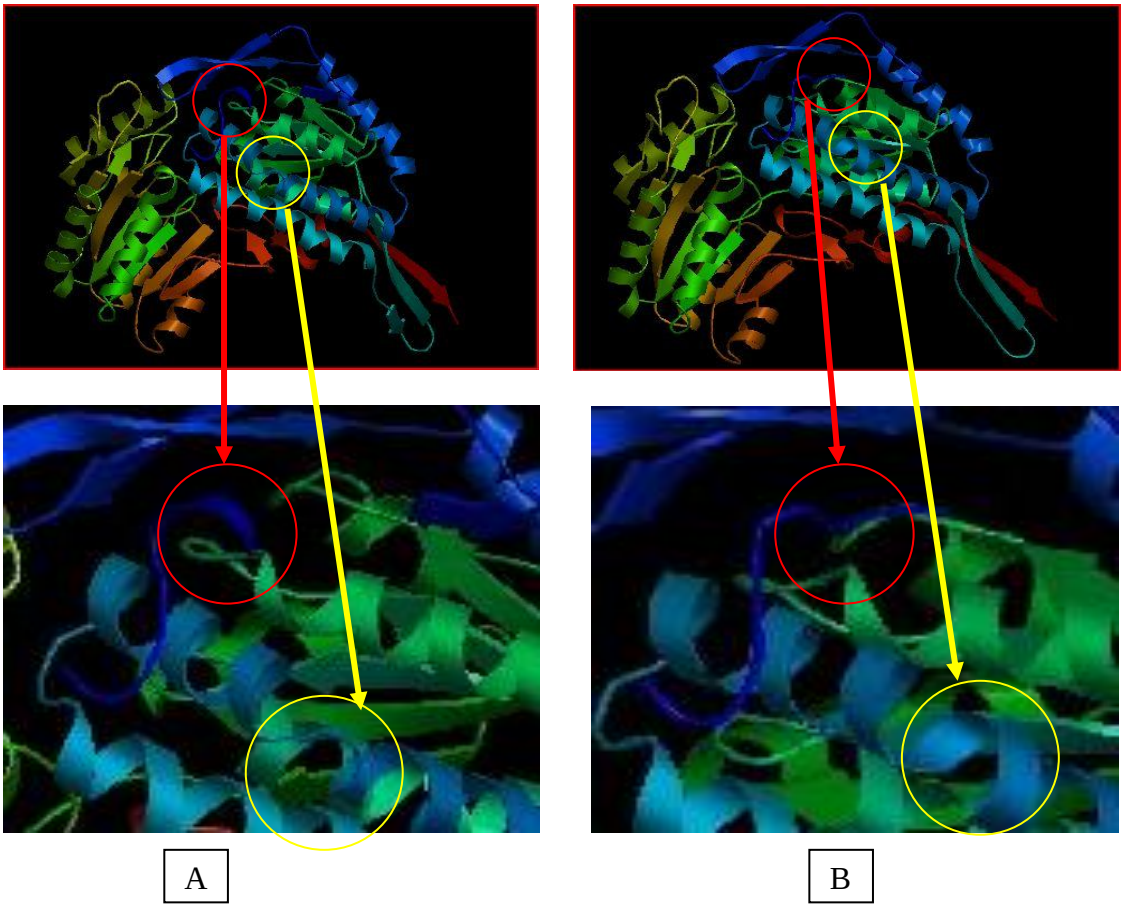


Figure 8-1 ALDH8A1 protein tertiary structure as predicted by the (PS)^2 program
(A) ALDH8A1 protein with threonine^{240}. (B) ALDH8A1 protein with methionine^{240}.
The structure of ALDH8A1 protein with threonine^{240} has one less loop than that of ALDH8A1 protein with methionine^{240} (yellow arrow). One β-sheet is also different between two structures (red arrow)

```
Amino acid No:    208. . . . . 240. . . . . 262
Amino acid:       FGTGPRVGEALVSHPEVPLISFTGSQPTAERITQLSAPHCKKLSLELGGKNPAVI
ALDH8A1(T):       ESCCHHHHHHHHHCTTCCEEEEESCHHHHHHHHHHHHHHCCCEEEETTCSCCEEE
Amino acid:       FGTGPRVGEALVSHPEVPLISFTGSQPTAERIMQLSAPHCKKLSLELGGKNPAVI
ALDH8A1(M):       ESCCHHHHHHHHHCTTCCEEEEESCHHHHHHHHHHHHHHCCEEEETTCSCCEEE
```

Figure 8-2 ALDH8A1 protein secondary structure as predicted by the (PS)^2 program

Only part of the ALDH8A1 protein secondary structure is shown (from amino acid sequence 208-262). ALDH8A1 protein (SNP 15) with threonine^{240} has one less amino acid in the helix than the ALDH8A1 protein (SNP15)with methionine^{240} (highlighted with yellow colour).
The first line is amino acid sequence number. The second line is ALDH8A1 amino acid sequence with threonine. The third line is secondary structure prediction of ALDH8A1 protein with threonine^{240}. The forth line is ALDH8A1 amino acid sequence with methionine^{240}. The fifth line is secondary structure prediction of ALDH8A1 protein with methionine^{240}.
SSpro8 adopts the full DSSP 8-class output classification for protein structure as follows:
H: = alpha-helix. E: = extended strand. T: = turn. S: = bend. C: = the rest (non-extended strand)

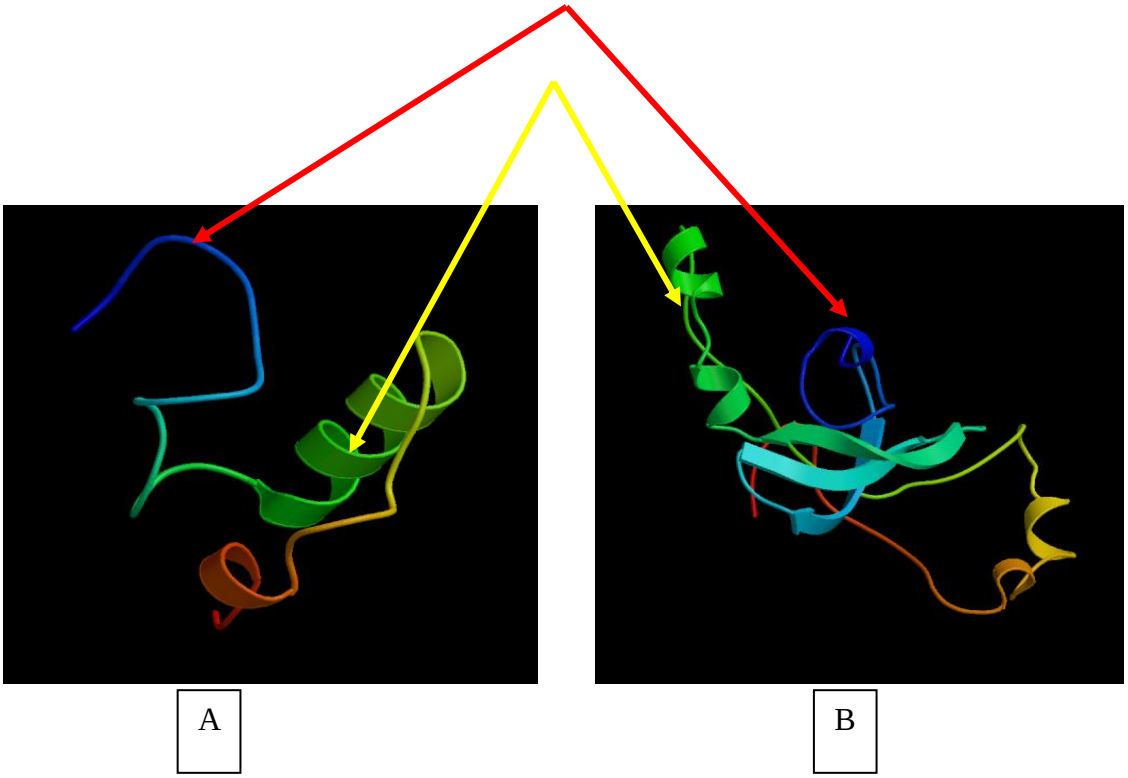


Figure 8-3 PPARGC1A protein tertiary structure as predicted by the $(PS)^2$ program
(A) PPARGC1A protein with proline^{616} (structure was only predicted for amino acid 604-656).
(B) PPARGC1A protein with leucine^{616} (structure was only predicted for amino acid 531-656).
The structure was only predicted for part of the protein due to limitations of the software. The structure of PPARGC1A protein with proline^{616} has less number of loops than that of PPARGC1A protein with leucine^{616} (yellow arrow). The extended strand is also different between two PPARGC1A proteins (red arrow).

Amino acid No:	567. . . . . 616. . 621
Amino acid:	RSRSFSRHRSCSRSPYSRSRSRSPGSRSSSRSCYYYESGHCRHRTHRNSPLCASR
PPARGC1A (P):	CCCCCCTCCCCCSCCCCCCCCCCCTTCCCCCEEEEEEETSCCEEECCCCCCEECCC
Amino acid:	RSRSFSRHRSCSRSPYSRSRSRSPGSRSSSRSCYYYESGHCRHRTHRNSLLCASR
PPARGC1A (L):	CCCCCCTCCCCCSCCCCCCCCCCCTTCCCCCEEEEEEETSCCCECCHHHEEHEECS

Figure 8-4 PPARGC1A protein secondary structure as predicted by the $(PS)^2$ program

Only part of the PPARGC1A protein secondary structure is shown (from amino acid sequence 567-621). The number of alpha-helix (H) for PPARGC1A protein (SNP12) with proline^{616} is less than that of PPARGC1A protein (SNP12) with leucine^{616} (highlighted with yellow colour).

The first line is amino acid sequence number. The second line is PPARGC1A amino acid sequence with proline^{616}. The third line is secondary structure prediction of PPARGC1A protein with proline^{616}. The forth line is PPARGC1A amino acid sequence with leucine^{616}. The fifth line is secondary structure prediction of PPARGC1A protein with leucine^{616}.

SSpro8 adopts the full DSSP 8-class output classification for protein structure as follows:
H: = alpha-helix. E: = extended strand. T: = turn. S: = bend. C: = the rest (non-extended strand)

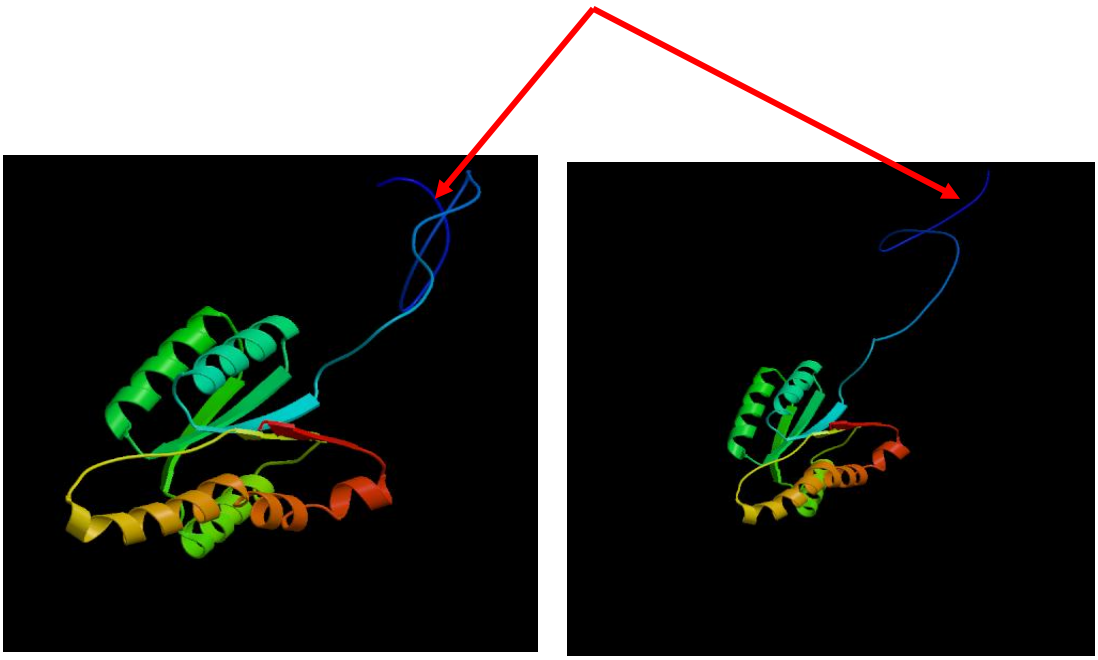


Figure 8-5 RDHE2 protein tertiary structure as predicted by the (PS)^2 program

(A) RDHE2 protein with valine[6]

(B) RDHE2 protein with alanine[6].

The structure was only predicted for amino acid 1-180 due to limitations of the software. The strand of tertiary structure of RDHE2 protein is different between protein with valine[6] and protein with alanine[6] (red arrow).

Amino acid No:	1. . . . 6. 50
Amino acid:	MSQNMVLKLKAAKKLLIFLGKSVLALVEAVVFVIIPKPRKNVAGEIVLIT
RDHE2(V):	CCHHCHCCHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCGGCCTTEEEEEE
Amino acid:	MSQNMALKLKAAKKLLIFLGKSVLALVEAVVFVIIPKPRKNVAGEIVLIT
RDHE2(A):	CCCTCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCGGCCTTEEEEEE

Figure 8-6 RDHE2 protein secondary structure as predicted by the (PS)^2 program

Only part of the RDHE2 protein secondary structure is shown (from amino acid sequence 1-50). The number of alpha-helix (H) and turn (T) for RDHE2 protein (SNP2) with valine[6] is different with that of RDHE2 protein (SNP2) with alanine[6] (highlighted with yellow colour).

The first line is amino acid sequence number. The second line is RDHE2 amino acid sequence with valine[6]. The third line is secondary structure prediction of RDHE2 protein with valine[6]. The forth line is RDHE2 amino acid sequence with alanine[6]. The fifth line is secondary structure prediction of RDHE2 protein with alanine[6].

SSpro8 adopts the full DSSP 8-class output classification for protein structure as follows:
H: = alpha-helix. E: = extended strand. T: = turn. S: = bend. C: = the rest (non-extended strand).
G: = 3-10-helix

8.4 Candidate pathways

Based on the identified fat colour QTL and known carotenoid metabolic pathways, four metabolic candidate mechanisms were considered as potentially regulating beef fat colour herein. These included 1) vitamin A synthesis from symmetric cleavage of β-carotene catalysed by BCMO1, 2) vitamin A synthesis from the asymmetric cleavage of β-carotene by BCO2, 3) absorption and transportation of β-carotene, and 4) β-carotene dilution in fat caused by increased fat deposition.

Vitamin A is a generic term that includes any fat-soluble compound that is similar in structure and possesses the biologic activity of retinol (International Union of Pure and Applied Chemistry—International Union of Biochemistry (Iupac-Iub), 1982). In mammals, there are three main forms of vitamin A (or vitamers) which differ in their oxidation state: the hydroxyl form (retinol), the aldehyde form (retinal) and the carboxylic form (retinoic acid) (Figure 8-7).

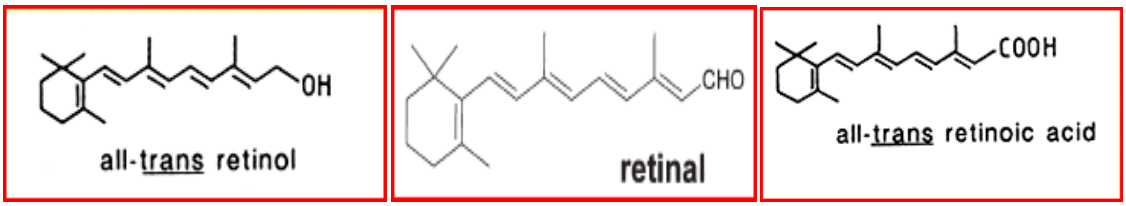


Figure 8-7 Three forms of vitamin A

Retinoic acid is a critical factor in many developmental processes, wherein it directly serves as an intracellular messenger that affects the transcription of more than 500 target genes (Balmer and Blomhoff, 2002). Retinoic acid (all-*trans* retinoic acid and 9-*cis* retinoic acid) regulates gene expression by functioning as a ligand for the peroxisome proliferator-activated receptor and two types of retinoid receptors in the

nuclear-hormone-receptor superfamily (the retinoic acid receptors and retinoid receptors) (Chambon, 1996).

Retinoic acid in animals is derived from food containing provitamin A carotenoids, of which β-carotene is the most active. The conversion of β-carotene to vitamin A in human and animal tissues is catalysed by BCMO1 and BCO2 (Figure 8-8). Two molecules of retinal are formed in the small intestine from cleavage of β-carotene by BCMO1, which appears to be rate-limiting for carotenoid utilization and hence, the supply of vitamin A is tightly controlled (Villard and Bates, 1986, Bachmann *et al.*, 2002). Retinal is also converted from retinol by both alcohol dehydrogenases and short-chain dehydrogenases/reductases (e.g. RDHE2) (Duester, 2000). Retinal is then irreversibly converted to retinoic acid by members of the ALDH family, of which ALDH8A1 is the only ALDH that oxidizes 9-*cis* retinal more efficiently than all-*trans* retinal (Lin and Napoli, 2000, Lin *et al.*, 2003). In addition to the biosynthesis of retinoic acid from retinal, retinoic acid can be formed from the eccentric cleavage of β-carotene by BCO2 (Kiefer *et al.*, 2001).

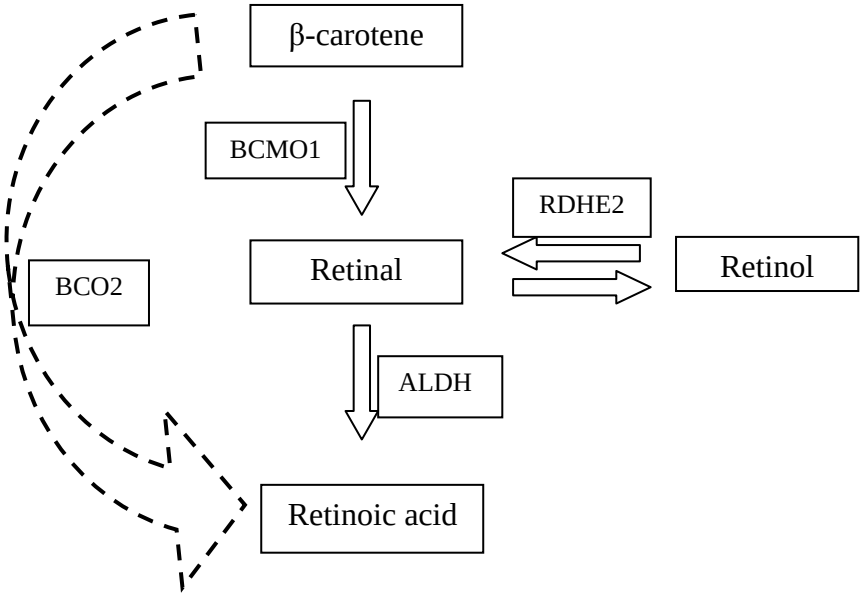


Figure 8-8 Retinoic acid formation from β-carotene and retinol

8.4.1 Vitamin A synthesis via BCMO1 pathway

As the major precursor of vitamin A in cattle, β-carotene must first be converted to retinoids by cleavage, and these primary cleavage products must then be further metabolized for storage and/or production of the biologically active retinoid derivatives. In the present study, *BCMO1* showed effects on β-carotene concentration and thereby, the beef fat colour.

8.4.1.1 BCMO1 direct effects

The association study (Chapter 4) showed that the *BCMO1* SNP4 had a strong association with β-carotene concentration ($p<0.01$, Table 4-2). Lindqvist *et al*. (2007) identified a T170M mutation in the *BCMO1* exon 5 in a human patient displaying hypercarotenemia and mild hypovitaminosis A. The replacement of a highly conserved threonine with methionine resulted in a 90% loss of function in the BCMO1 enzyme activity when analysed *in vitro* using purified recombinant enzymes. The patients with this mutation have orange-yellowish skin and variable degrees of mild vitamin A deficiency and high levels of circulating carotenoids. In a *BCMO1* knockout mouse study, the mice became vitamin A deficient on diets with β-carotene as the major source of vitamin A. Large amounts of the β-carotene accumulated, not only in the blood, but also in other tissues, such as the liver, lung, and adipose tissues. Furthermore, *in vivo* data established that intestinal *BCMO1* is a "gate keeper" that limits the amount of dietary β-carotene that is absorbed intact from the intestine (Fierce *et al.*, 2008). The findings herein strongly support the idea that a genetic variation exists in the bovine *BCMO1* gene and can affect the conversion efficiency of β-carotene to vitamin A. Thus, in order to supply vitamin A in animals, the *BCMO1* gene can directly affect β-carotene concentration and thereby, decrease yellow fat colour.

8.4.1.2 Regulation of BCMO1 by transcription factors

There were 17 SNPs found in the *BCMO1* gene, including 8 SNPs in the upstream sequence. The upstream sequence of genes contains protein-binding sites that determine whether these regions function in transcriptional regulation. If a SNP is located in one of transcriptional factor binding sites, the gene expression level may differ between individuals. Therefore, the upstream SNPs in the cattle *BCMO1* gene were examined to determine if they were potentially located in any known transcriptional factor binding sites.

PPARGC1A encodes peroxisome proliferator-activated receptor gamma coactivator 1-alpha protein, which can bind and co-activate one of the nuclear receptor family members, namely *PPAR*γ (Lin *et al.*, 2005). Peroxisome proliferator receptor responsive elements (PPRE) have been identified in the promoter region of mouse and human *BCMO1* genes (Boulanger *et al.*, 2003, Gong *et al.*, 2006). Site-directed mutagenesis and gel shift experiments demonstrated that this PPRE is essential for *BCMO1* promoter specificity and that PPARγ specifically binds to this element. Indeed, in the present study, interaction effects were found between the *BCMO1* SNPs and *PPARGC1A* SNPs (Table 5-3). Predictive analysis of the bovine *BCMO1* promoter identified two putative PPREs with the nucleotide sequences 5'-TGTACA A AGGCCA-3' and 5'-AGTGGA A AGGGAG-3' at 1201 and 255 base pairs upstream of the transcriptional start site, respectively, as determined by the PPRE search program (http://www.classicrus.com/PPRE/). Although the induction of *BCMO1* expression via the PPAR signalling pathway seems conclusive (Figure 8-9), the nearest *BCMO1* SNP7 was 74 nucleotides distant from the PPRE (5'-TGTACA A AGGCCA-3' -1201).

In addition to the regulation of the *BCMO1* gene by the PPARγ transcriptional factor, retinoic acid can also regulate *BCMO1* mRNA expression at the transcriptional level via the retinoic acid receptor (RAR) and retinoid X receptor (RXR) by binding the retinoic acid response element (RARE) in the promoter of *BCMO1* gene (Figure 8-9). All-*trans* retinoic acid and *9-cis* retinoic acid can bind with all three subtypes (α, β and γ) of the retinoic acid receptor and the retinoid X receptor, but the RXR subtypes only bind *9-cis* retinoic acid. A transcription factor binding site RARE search in the 5' flanking region of the human *BCMO1* gene (GenBank sequence database) has been performed and revealed a putative retinoic acid response element (GGGTCActtgAGGTCA), 600 bases upstream of the transcription start site (Chichili *et al.*, 2005). In this *in vitro* study, the authors found that *BCMO1* gene expression in the human retinal pigment epithelial cell line D407, which reacted in a time-dependent manner after β-carotene supplementation. *BCMO1* mRNA and protein levels increased after the first 2 hours, but decreased after longer incubations up to 24 hours. The results indicated that retinoic acid regulates *BCMO1* mRNA expression in a bidirectional manner by inducing expression at lower concentrations and inhibiting at higher concentrations. Furthermore, the authors suggested that the *RARA* is involved in the regulation of *BCMO1* expression as the treatment of D407 cells with the *RARA* antagonist Ro 41-5253 resulted in the up-regulation of *BCMO1* mRNA expression.

A significant interaction effect was observed for *BCMO1* with *RARA* (retinoic acid receptor alpha) and *BCMO1* with *ALDH8A1* (Table 5-3), which suggests retinoic acid signalling in the regulation of *BCMO1* gene expression in cattle. A search for RARE sites in the bovine *BCMO1* gene using the NHRscan program (http://www.cisreg.ca/cgi-bin/NHR-scan/nhr_scan.cgi) identified two potential RARE sequences

(TGACCCTGCTAGA and TGACCTTTGACCA) at 1223 and 186 base upstream of the transcriptional start site, respectively. However, the nearest SNP herein was again *BCMO1* SNP7, 96 nucleotides from the first RARE site (5'- TGACCCTGCTAGA-3' -1223). Thus, none of the 8 SNPs in the upstream region of the bovine *BCMO1* gene were in these putative transcription factor binding sites.

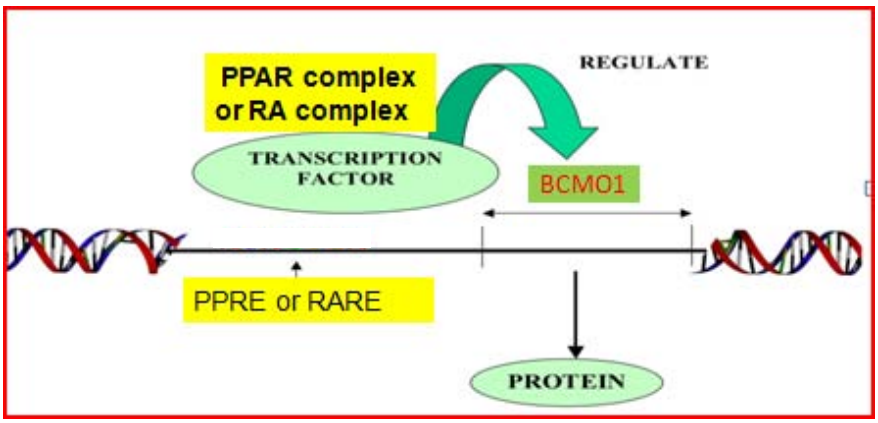


Figure 8-9 Putative transcription factor binding sites in the promoter region of *BCMO1*
PPRE: peroxisomal proliferator response element. RARE: retinoic acid response elements

8.4.1.3 Regulation of BCMO1 by negative feedback mechanisms

Upon absorption, provitamin A carotenoids are readily converted to vitamin A by the action of BCMO1 in the enterocytes of the intestinal mucosa. Animal studies have suggested that dietary β-carotene and its retinoid metabolites repress the intestinal BCMO1 enzymatic activity, and this regulation is exerted at transcriptional level by retinoic acid as described above. Moreover, Seino *et al.* (2008) and Lobo *et al.* (2010) provided further evidence that intestinal *BCMO1* gene expression is also regulated by a negative feedback mechanism in the intestines (Figure 8-10). The intestine specific homeobox (ISX), an intestine-specific transcription factor, plays a role in repressing the mouse *BCMO1* gene expression in the intestine (Seino *et al.*, 2008, Lobo *et al.*, 2010). A retinoic acid–responsive element was identified in the *ISX* gene promoter to which

RARs can bind. A study in which the *β-galactosidase gene* (*LacZ*) was knocked into the mouse *ISX* locus showed that vitamin A deficiency increased duodenum *BCMO1* gene expression in wild-type ($ISX^{+/+}$) mice but not in the duodenum of ISX-deficient mice ($ISX^{-/-}$) (Seino *et al.*, 2008). This suggests that the effect of retinoic acid on *BCMO1* gene expression is dependent on *ISX*. Moreover, recent studies in mouse and human colonic cell lines showed that *ISX* gene expression is induced by β-carotene derived retinoic acid. The carotene absorption and conversion to retinoids by BCMO1 was under negative feedback control (Lobo *et al.*, 2010). Based on their findings, the authors also predicted that high or a low vitamin A status would determine β-carotene utilization in humans by regulating *ISX* –mediated *BCMO1* gene expression. This implies that differences in β-carotene absorption in cattle depend on the vitamin A status. For example, a higher concentration of vitamin A will repress *BCMO1* expression level in the small intestine and result in a higher concentration of intact β-carotene in circulation, which thereby can result in more yellow colour in the subcutaneous fat.

Figure 8-10 Crosstalk between RAR and ISX signalling controls β-carotene absorption
(Lobo *et al.*, 2010). *A*) Vitamin A deficiency: *SR-BI* and *BCMO1* expression are increased significantly throughout the small intestine. Enhanced SR-BI activity facilitates the absorption of β-carotene. *B*) Vitamin A sufficiency: retinoids derived either from β-carotene conversion or preformed dietary retinoids promote the production of retinoic acid. Retinoic acid binds to RARs, inducing ISX expression. Induction of ISX then leads to the repression of intestinal expression of *SR-BI* and *BCMO1*.

8.4.2 Vitamin A synthesis via BCO2 pathway

8.4.2.1 Asymmetric cleavage of β-carotene

There is an eccentric cleavage pathway in the production of retinoic acid from β-carotene in mammals (Glover and Redfearn, 1954, Wang *et al.*, 1991). As the primary cleavage products in this pathway, long-chain β-apocarotenoids (>C20) are truncated to retinoic acid in a stepwise process that might be mechanistically related to the β-oxidation of fatty acids (Wang *et al.*, 1996). The isolation and biochemical characterization of a second carotenoid cleaving enzyme, BCO2, from mouse, human and ferret (Kiefer *et al.*, 2001, Lindqvist *et al.*, 2005, Hu *et al.*, 2006) proved the existence of the eccentric cleavage of β-carotene as an alternative pathway for retinoic acid formation. Herein, there is also evidence of *BCO2*-mediated conversion of β-carotene to vitamin A in cattle (Tian *et al.*, 2008, Tian *et al.*, 2010). The association studies and qPCR based expression analyses indicated that the *BCO2* gene had a significant effect on β-carotene concentration and fat colour traits. Since the *BCO2 W80X* mutation results in a premature termination codon, the aberrant mRNA is undoubtedly prone to nonsense-mediated RNA decay, which in turn, leads to complete loss of the corresponding protein (Chang and Waikan, 1979, Holbrook *et al.*, 2004, Chang *et al.*, 2007). Recently, Berry *et al.* (2009) also provided the evidence of eccentric cleavage of β-carotene by BCO2 in cattle. The authors described the effects of the same mutation in *BCO2* (*BCO2 W80X* described herein) on milk fat colour. They found *BCO2*$^{-/-}$ cows had higher levels of β-carotene in serum and milk, yellower milk fat colour and reduced levels of vitamin A in the liver compared to wild type cows. From the study herein and Berry's study (2009), Kiefer's supposition (2001) that eccentric cleavage of β-carotene is an alternative pathway is confirmed. The gene expression results herein also support the hypothesis that this eccentric cleavage by

BCO2 leads to retinoic acid formation because the mRNA levels of *BCMO1* and *BCO2* were correlated (Figure 7-8), and *BCMO1* gene expression is primarily controlled by the level of retinoic acid, which consequently regulates the cleavage of β-carotene.

8.4.2.2 Asymmetric cleavage of lutein

In addition to the cleavage of β-carotene, a critical role of BCO2 in non-provitamin carotenoid metabolism has been established in several studies. In domestic chickens (*Gallus gallus*), yellow skin colour is determined by a *cis*-acting and tissue-specific regulatory mutation(s) that inhibits the expression of *BCO2* in skin, allowing the deposition of yellow carotenoids (primarily, xanthophylls lutein and zeaxanthin) (Eriksson *et al.*, 2008). In sheep (*Ovis aries*), the main carotenoids in adipose tissues are lutein, flavoxanthin, and to a lesser extent, β-carotene. A nonsense mutation in the ovine *BCO2* gene (*BCO2* Q66X, NCBI_ss# BCO2-196 181341842), resulting in a premature stop codon, was strongly associated with the yellow fat phenotype in sheep (Vage and Boman, 2010). Furthermore, a study using a *BCO2* knockout mouse model provided evidence that BCO2 acts as a mitochondrial enzyme and converts both β-carotene and xanthophylls, such as lutein, to apocarotenoid breakdown products (Amengual *et al.*, 2011). In this study, when animals were fed a diet supplemented with lutein, both $BCO2^{-/-}$ and $BCO2^{+/-}$ mice accumulated lutein in the form of oxidized 3-dehydro metabolites in several tissues. In contrast, wild type mice expressed 7-fold higher *BCO2* mRNA levels and prevented this accumulation. All these results highlight the importance of *BCO2* in carotenoid homeostasis.

Despite the broad substrate specificity, BCO2 may have a preference for the certain substrates. This idea was supported in an *in vitro* study, where the baculovirus-

generated recombinant ferret BCO2 cleavage activities of various carotenoid substrates (zeaxanthin, lutein and β-cryptoxanthin) were investigated. Enzyme kinetic analysis indicated the xanthophylls zeaxanthin and lutein are preferentially cleaved by BCO2 over β-cryptoxanthin. The broad substrate specificity of BCO2 must be considered in terms of the understanding of the potential biological roles of BCO2 in carotenoid metabolism and function. Because the main pigment responsible for beef fat colour is β-carotene and only to a lesser extent lutein, the effect of BCO2 on beef fat colour is most likely related to eccentric cleavage of β-carotene. However, it may be also related to the cleavage of lutein.

8.4.3 Absorption and transportation of carotenoids

Absorption of carotenoids includes their release from the food matrix in the intestinal lumen, their solubilisation into mixed micelles, and their uptake by intestinal mucosal cells. Carotenoids are hydrophobic molecules that are transported in the plasma within the lipid core of lipoproteins to the various fat depots. In cattle, high-density lipoproteins constitute 80–85% of the total lipoprotein pool (Jonas, 1972, Kruk, 2001) and plasma carotenoids are primarily associated with high density lipoprotein (more than 800 g/kg) in pre-ruminant calves (Bierer *et al.*, 1995), steers (Yang *et al.*, 1992) and dairy cows (Schweigert *et al.*, 1987).

APOM is a novel apolipoprotein present mainly in high density lipoproteins in the blood plasma, and to a minor extent in chylomicrons, very low density lipoproteins, and low density lipoproteins (Xu and Dahlback, 1999, Christoffersen *et al.*, 2006). The expression of the *APOM* gene has high tissue specificity and is confined predominantly to the liver and kidney in humans and pigs (Xu and Dahlback, 1999, Pan *et al.*, 2010).

In the study herein, the *APOM* SNP2 had effect on carcass fat colour (Table 4-2). The involvement of APOM in the regulation of fat colour could be linked by one or more the following mechanisms. Firstly, APOM is mainly found within the high density lipoproteins and is involved in lipid and lipoprotein metabolism, which are associated with the fat deposition traits. In a porcine study, APOM was investigated as a positional candidate gene for a QTL related to fat deposition traits, including backfat thickness and intramuscular fat content (Pan *et al.*, 2010). The G2289C polymorphism in the porcine *APOM* gene was reported to significantly affect several fat deposition traits including backfat thickness at the shoulder, thorax-waist, and buttock. Considering the role of *APOM* in fat deposition traits and its association with carcass fat colour, β-carotene (and/or lutein) could be diluted with the increased fatness caused by *APOM* and consequently, the fat colour decreases. However, in the analysis herein, when fat depth was fitted as covariate, the variance of *APOM* was not altered (Appendix 6). This suggests that the effect of *APOM* on the beef fat colour is not via increased fatness.

APOM could affect β-carotene concentration by binding retinoic acid. APOM belongs to the lipocalin protein family. The lipocalins share a structurally conserved β-barrel as a central motif, which encloses a hydrophobic pocket serving as the ligand binding site, leading to the storage and transport of small lipophilic substances (Flower, 1996, Schlehuber and Skerra, 2005). APOM has the capacity to bind retinol and its two metabolites, all-*trans* retinoic acid and *9-cis* retinoic acid (Ahnstrom *et al.*, 2007). However, there is no evidence available to explain how APOM is involved in retinoid metabolism *in vivo*. Interestingly, there was a significant interaction on carcass fat colour between the *RDHE2* SNP2 and *APOM* SNP6, and a close to significant interaction on carcass fat colour between the *ALDH8A1* SNP16 and *APOM* SNP2.

Since *RDHE2* and *ALDH8A1* are involved in synthesis of all-*trans* retinoic acid and 9-*cis* retinoic acid *in vivo* respectively, *APOM* could affect β-carotene concentration through an interaction with those two genes.

In terms of β-carotene absorption, there was also an interaction effect between the *SCARB1*and *RDHE2* genes on biopsy fat colour. The scavenger receptor B1 (SCARB1) is an HDL-receptor identified in the plasma membrane of several cellular types in mammals. Both *in vivo* and *in vitro* studies have demonstrated that SCARB1 is a key mediator for uptake of β-carotene from the intestinal lumen into the enterocyte (Acton *et al.*, 1996, During *et al.*, 2005, Reboul *et al.*, 2005, Van Bennekum *et al.*, 2005). In a *SCARB1* knockout mouse study, the authors concluded that *SCARB1* is essential for β-carotene absorption, at least for mice consuming a high fat diet (Van Bennekum *et al.*, 2005). In transfected COS-7 cells (Van Bennekum *et al.*, 2005) and in intestinal Caco-2 cells (During and Harrison, 2007), it has been shown that inhibition of SCARB1 decreases uptake of β-carotene from mixed bile salt micelles and triacylglycerol emulsions. Thus, the evidence indicates that SCARB1 acts to facilitate β-carotene uptake into enterocytes. As an HDL-receptor, SCARB1 facilitates the uptake not only for β-carotene, but also other lipids, such as lutein and cholesterol. In fact, SCARB1 was first characterized by probing its involvement in the bidirectional cholesterol flux between circulating high-density lipoproteins and target cells in mice (Acton *et al.*, 1996).

There is competition between cholesterol and carotenoids for the central lipid core of the lipoprotein. This was suggested from the observation that increased plasma cholesterol concentration is associated with increasing plasma concentrations of

lipoproteins and reduced yellowness and carotenoid concentration in fat (Knight *et al.*, 2001). Moreover, lutein, as a nonprovitamin A carotenoid, is also absorbed by a facilitative transporter in Caco-2 TC-7 mono-layers, a model for human intestinal epithelium (Reboul *et al.*, 2005). The absorption rate was decreased up to 20% in the monolayer when lutein was co-incubated with β-carotene. Therefore, competition between the uptake of these lipids by SCARB1 could result in a change in β-carotene absorption rate, leading to altered deposition in adipose tissue.

In addition to its competition with other lipids, β-carotene absorption is also dependent on *SCARB1* gene expression level. In ISX-deficient mice, *SCARB1* intestinal mRNA expression is significantly enhanced (Choi *et al.*, 2006). Furthermore, it was demonstrated that *SCARB1* gene expression is subject to control by retinoid signalling, especially retinoic acid derived from the cleavage of β-carotene (Lobo *et al.*, 2010). With the identification of a retinoic acid responsive element in *ISX*, it was suggested that a complex of a retinoic acid and retinoic acid receptors regulates the intestinal gene expression of *ISX*. ISX then represses the expression of *SCARB1*, leading to reduced β-carotene and/or lutein levels in the adipocytes.

In adipocytes, *SCARB1* gene expression is regulated by hormones, such as insulin, epinephrine and angiotensin II. In an *in vitro* study, mobilization of SCARB1 from cytoplasmic compartments to the cell membrane of adipocytes was induced when 3T3-L1 cells were exposed to either insulin or angiotensin II for 1-2 hours (Tondu *et al.*, 2005). This translocation of SCARB1 is concomitant with an increase in HDL-delivered cholesterol uptake and cell cholesterol content. In another mouse study, it was demonstrated that insulin and angiotensin II are also involved in plasma HDL

homeostasis through the regulation of SCARB1 membrane translocation (Yvan-Charvet *et al.*, 2007). Furthermore, the effects of insulin and epinephrine on β-carotene content in fat was examined in a study using explants from bovine adipose tissue cultured *in vitro* (Arias *et al.*, 2009). Beta-carotene content was affected positively by insulin and negatively by epinephrine. Thus, for β-carotene, it is proposed that these hormones could have a stimulatory effect on mobilization of SCARB1, allowing increased transport of β-carotene into the adipocytes.

In the study herein, both the *SCARB1* SNP1 genotype (Table 4-2) and the *SCARB1* haplotype (Table 5-2) showed significant effects on biopsy fat colour. However, it remains unclear whether fat colour results from transportation differences due to competition between β-carotene and other lipids or the regulation of *SCARB1* gene expression by hormones.

In summary, both APOM and SCARB1 can affect high density lipoproteins transport of β-carotene. As a consequence, the concentration of β-carotene in bovine adipose tissues and beef fat colour may be affected.

8.4.4 Dilution of carotenoids in the fat

Reduction of fat colour score is accompanied with the increased fat thickness when cattle are finished with a short period grain feeding (Forrest, 1981, Pitchford *et al.*, 2002), which suggests that increased adipose tissue accretion dilutes the previously accumulated carotenoids. The study herein showed that there is weak correlation of fat colour related traits with fat depth (Figure 8-11, Figure 8-12 and Figure 8-13). Fat depth accounted for only a small proportion of fat colour variation (0-3%). Therefore, genes

affecting the fatness are likely to play just minor roles in fat colour.

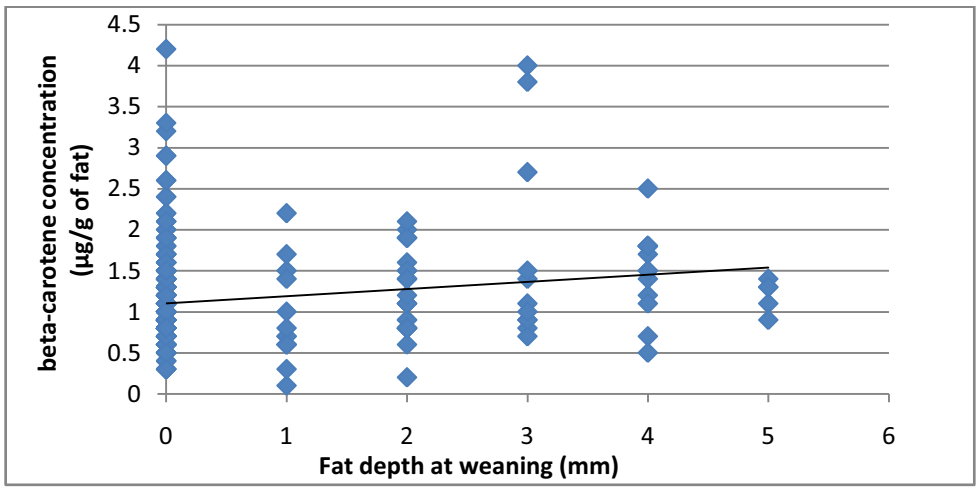


Figure 8-11 Correlation between beta-carotene concentration and fat depth (r=0.18, P<0.001)

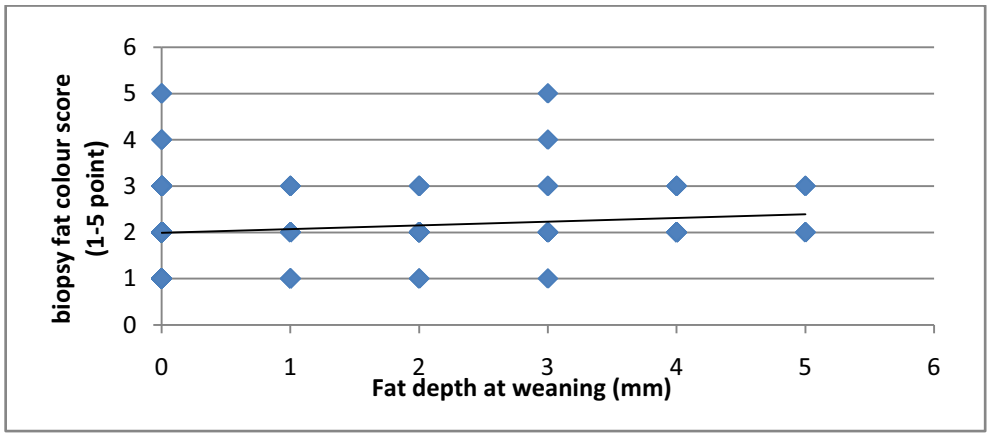


Figure 8-12 Correlation between biopsy fat colour and fat depth (r=0.15, P<0.01)

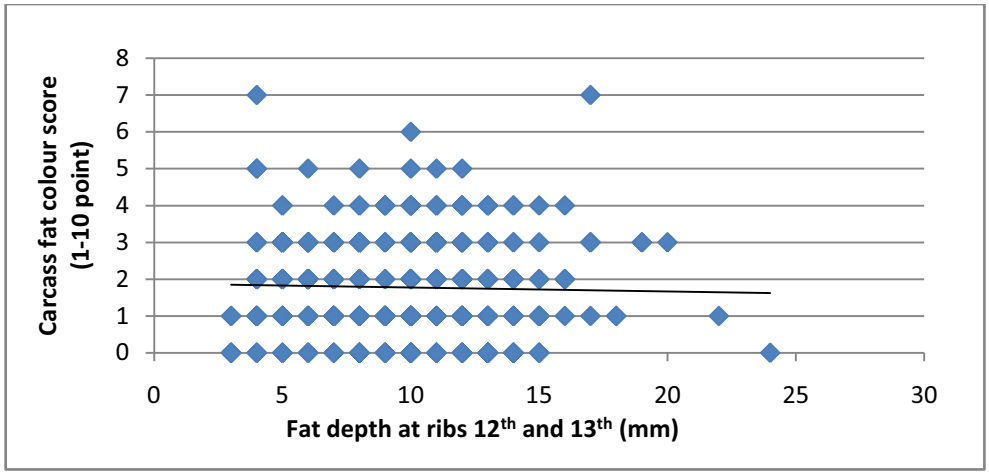


Figure 8-13 Correlation between carcass fat colour and fat depth (r = -0.15, P<0.01)

Growth hormone stimulates important physiological processes in cattle, particularly post-natal growth and milk production (White *et al.*, 2007). By binding to extracellular domain of transmembrane GH receptor (GHR), growth hormone activates an intracellular signalling pathway that induces the transcription of many genes including the insulin-like growth factor 1 (*IGF1*) gene (Zhou and Jiang, 2005). An association study conducted in 848 Holstein–Friesians indicated that variants in the *growth hormone receptor* gene are associated with performance traits in cattle, such as fat yield, protein yield, milk yield, fat percentage and protein percentage (Zhou and Jiang, 2005). Japanese Black steers are known to have a greater proportion of carcass fat and smaller proportions of carcass lean and bone than Holstein steers (Matsuzaki *et al.*, 1997). A study comparing the expression of the *GHR* gene in the adipose tissue between these two breeds indicated that the expression of the *GHR* gene was significantly greater in Japanese Black steers compared with Holstein steers (Komatsu *et al.*, 2005). When fat depth was fitted as a co-variate herein, *GHR* still affected fat colour suggesting the effect of *GHR* on fat colour is not likely to involve fat deposition *per se*. That is, while GHR affects fatness, dilution of β-carotene in the fat is not likely to explain the role of GHR in beef fat colour.

The *PPARGC1A* gene has a vital role in energy and fat metabolism, and is involved in adipogenesis, muscle fibre type formation, mitochondrial biogenesis/respiration, fat distribution and adipocyte differentiation (Wu *et al.*, 1999, Dulloo and Samec, 2001, Schennink *et al.*, 2009, Benton *et al.*, 2010). Due to its function, *PPARGC1A* has a strong impact on animal meat and carcass quality traits. *PPARGC1A* has been selected as a potential candidate gene for QTL related to fat metabolism traits in several species. In cattle, *PPARGC1A* was a potential candidate gene located a QTL for milk fat yield on

BTA6 (Weikard *et al.*, 2005). In pigs, *PPARGC1A* is the only gene so far that has been mapped to the QTL-region for backfat on chromosome 8 (Jacobs *et al.*, 2006). Association studies in two independent populations indicated that a polymorphism in the porcine *PPARGC1A* gene was significantly associated with multiple carcass fat traits, including both average backfat depth and belly weight (Jacobs *et al.*, 2006, Erkens *et al.*, 2010).

Adipocyte differentiation results from sequential changes in gene expression. PPARγ is expressed predominantly in adipose tissue and is a master regulator of adipogenesis (Macdougald and Lane, 1995). The PPARγ dimerises with the retinoid X receptor to bind DNA and activate transcription (Ziouzenkova and Plutzky, 2008). PPARGC1A is the PPARγ main coactivator, a ligand of PPARγ that binds and activates transcription regulating adipocyte differentiation and lipid filling (Lin *et al.*, 2005). In an *in vitro* study, the combination of *9-cis* and all-*trans* retinoic acid was the best activator of *PPARGC1A* expression in bovine adipocytes over various treatments, including rosiglitazone, unsaturated fatty acids, *9-cis* and all-*trans* retinoic acid, β-carotene and lutein (Garcia-Rojas *et al.*, 2010). However, when fat depth was added as a co-variate for the fat colour traits herein, this did not alter the results and the *PPARGC1A* gene still affected fat colour (Appendix 6). Therefore, fat dilution caused by the *PPARGC1A* gene effects on fat deposition is not a likely explanation for the effect of *PPARGC1A* on beef fat colour.

RARA is known to mediate retinoic acid effects in adipose tissue. It has been established that RARA is required for the inhibition of adipocyte differentiation (Kamei *et al.*, 1994, Xue *et al.*, 1996). In a human study, the *RARA* mRNA levels in the

subcutaneous abdominal adipose tissue in lean women were compared to obese women to investigate the role of *RARA* in adipose tissue. The results showed that the *RARA* mRNA levels in the subcutaneous abdominal adipose tissue of the obese subjects were significantly lower than in control subjects (56%, $P<0.01$) (Redonnet *et al.*, 2002). The authors suggested that the effect of retinoic acid on adipocyte differentiation is mediated by RARA. In the study herein, when fat depth was fitted as covariate, biopsy fat colour was no longer significantly affected by the *RARA* gene (Appendix 6). Thus, it is conceivable that the bovine *RARA* gene may affect adipocyte differentiation through retinoic acid mediated actions and results in bovine fattening.

To summarise, although *GHR* and *PPARGC1A* have effects on fatness, it is not likely that these two proteins play roles in affecting fat colour via dilution of the β-carotene content in the fat. On the other hand, the *RARA* effect on β-carotene concentration was related to fatness. Thus, in this instance, *RARA* may increase bovine fat deposition and dilute the β-carotene concentration in the fat and thereby, reduce carcass fat yellowness.

8.5 Epistasis

One of the aims of this study was to understand the contribution of relevant SNP-SNP interaction between genes (epistasis) to the fat colour related traits. The SNP *BCO2* W80X genotype accounted for much of the variation in single-marker association analysis. An epistatic network constructed by a locus interacting with a large number of other loci would be of interest because many significant epistatic effects involving one locus at a lower significance levels are less likely to be random than a single epistasis effect at the same significance level (Ma *et al.*, 2007). The results indicate that both polymorphisms in single genes and combinations of genes are important in determining

the phenotypic variation in fat colour traits. The SNP *BCO2* W80X had interaction effects with SNPs in the *ALDH8A1*, *BCMO1*, *PPARGC1A*, *RARA*, *RDHE2* and *SCARB1* genes on the fat colour related traits (Table 5-3). Therefore, *BCO2* was the single most important gene contributing to the phenotypic variation in the fat colour herein both individually and epistatically.

When one of genes in a metabolic pathway affects a trait, other genes in the same pathway are likely to do so as well. The results indicate that there are many interaction effects between genes were involved in the retinoic acid pathway. Genes that interacted with *BCO2* included *ALDH8A1*, *PPARGC1A*, *SCARB1*, *BCMO1* and *RARA* (Figure 8-14). Genes that interacted with *RDHE2* included *ALDH8A1*, *PPARGC1A*, *BCO2*, *APOM* and *RARA*. Genes involved in interactions with *BCMO1* included *ALDH8A1*, *PPARGC1A* and *SCARB1*. Of these, *ALDH8A1* and *PPARGC1A* interacted with all 3 of the biosynthetic enzyme genes, the *BCMO1*, *BCO2* and *RDHE2* genes. Therefore, it is concluded that the retinoic acid pathway is the key for fat colour in beef.

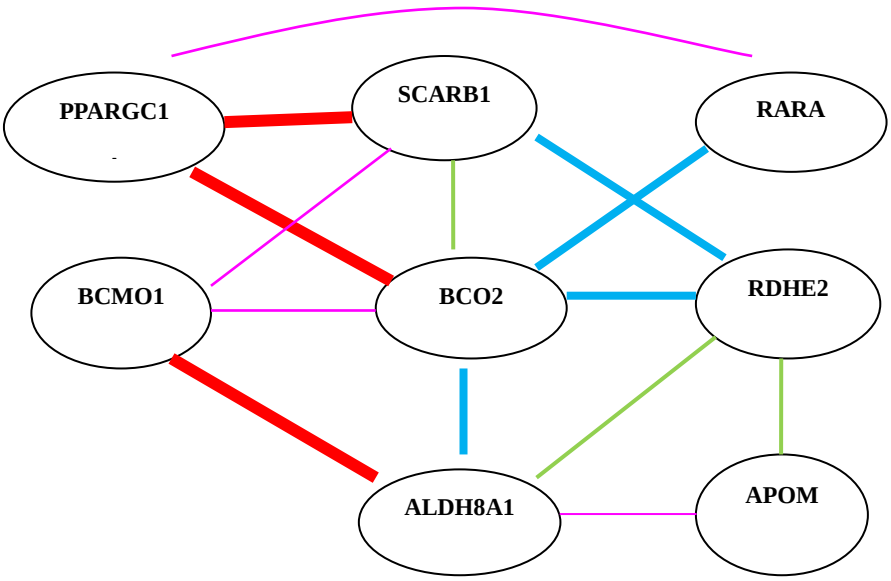


Figure 8-14 Interactions between genes

SNP interactions 0.1<P<0.05 — SNP interactions P<0.05

SNP interactions P<0.01 — SNP interactions P <0.001

Moreover, the gene expression studies (Chapter 7) supported the theory that the *BCMO1, BCO2* and *RDHE2* genes are tightly regulated together. Firstly, the *BCMO1* gene mRNA expression was correlated to the *BCO2* gene mRNA expression with a negative relationship (Figure 7-8). Secondly, even though there were only 4 animals with the *BCO2* W80X AA genotype, there was a significant difference between the *BCMO1* gene mRNA expression and the *BCO2* gene mRNA expression for these animals (Table 7-4). This mutation in the *BCO2* gene results in a premature stop codon and abolishes a functional BCO2 protein; thus, the biosynthesis of retinoic acid from β-carotene in the eccentric cleavage pathway is blocked. Consequently, there were elevated *BCMO1* mRNA levels in order to maintain of retinoic acid homeostasis. Likewise, in animals with the wild type *BCO2* W80X GG genotype, the *BCMO1* mRNA levels decreased with an increase in *BCO2* mRNA expression levels. Thirdly, the *RDHE2* mRNA expression was significantly different between white and yellow fat colour groups. There were higher *RDHE2* mRNA levels in the animals with white subcutaneous fat than those with yellow subcutaneous fat (Chapter 7). A positive correlation between the *RDHE2* mRNA expression and the *BCMO1* mRNA expression also was observed. This indicates that the higher the *RDHE2* mRNA expression, the lower the retinol concentration in the local tissues (e.g. liver). The *BCMO1* mRNA levels increases, therefore, in order to maintain retinol homeostasis. As a consequence, less β-carotene is present and white subcutaneous fat is the consequence. These interaction effects between genes and gene expression showed that the biosynthesis of retinoic acid in the tissue is tightly regulated through the synthesizing enzymes.

8.6 Future studies

There were 7 candidate genes representing 7 QTL selected for the association studies

herein. The remaining QTL were not investigated in this study and should be also examined. In addition, inclusion of the SNPs in *ALDH8A1*, *BCO2*, *RDHE2* and *PPARGC1A* genes in the statistical model for any newly identified QTL is worth investigation. There was less knowledge of the candidate gene functions in carotenoid metabolism and the bovine Ensembl genome database was not as well annotated (http://www.ensembl.org) at the beginning of the project. Therefore, identification of DNA variants and association studies of other candidate genes within the original QTL may contribute to additional markers for the selection of beef fat colour.

The *BCO2* gene mRNA expression in liver was not significantly different between cattle with yellow fat and white fat. However, the SNP *BCO2* W80X explained 12-16% genetic variation for fat colour and β-carotene concentration was correlated *BCO2* mRNA levels. A greater number of animals with a range of β-carotene concentrations and more *BCO2* W80X AA genotypes represented are needed to verify potential correlations with *BCO2* mRNA levels. In addition to *BCO2* mRNA expression in the liver, the *BCO2* mRNA level should also be investigated in other tissues, such as the small intestine (Zaripheh *et al.*, 2006) and subcutaneous fat. Moreover, the enzymatic rate of β-carotene cleavage in adipose tissues could be different. Therefore, the BCO2 enzyme activity in cattle with different fat colours should be measured. Similarly, because of the important role of *BCMO1*, *BCMO1* mRNA levels and enzyme activities need to be examined in these tissues. Because the important role of intestine specific homeobox (ISX) in repressing the mouse *BCMO1* gene expression in the intestine (Seino *et al.*, 2008, Lobo *et al.*, 2010), it would be interesting to investigate whether the bovine ISX regulates *BCMO1* gene expression and controls the absorption of β-carotene.

Beef fat colour can be reduced by grain feeding cattle before slaughter. The current evidence suggests that once carotenoids accumulate in adipose tissue, they persist or are at least turned over very slowly. The activity of enzymes, such as BCMO1, BCO2 and RDHE2, could be measured in subcutaneous fat tissues between animals with different fat colour at various time points of grain feeding to determine if the grain effects are due to fattening or whether β-carotene is being metabolised. In addition, since grain-fed cattle generally accrue more adipose tissue than cattle of a similar age fed grass or forage, the genes that play a role in fatness should be further investigated to examine whether the increased adipose tissue accretion dilutes the carotenoids.

The DNA variants in the non-coding regions in genes could potentially alter the enzymatic activity of the protein (Thomas *et al.*, 2007). None of the DNA variants herein were located in known microRNA sites using the cattle microRNA database (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=bta). However, it could be worthwhile to re-examine whether the variants which have effects on fat colour herein are located in microRNA sites once the cattle microRNA database is further developed.

Lastly, given that a limited number of cattle were genotyped in this study, all DNA variants associated with beef fat colour herein should be verified by genotyping in other breed populations with a larger number of animals. These verified DNA variants can be then used in animal breeding selection programs to eliminate the yellow fat.

8.7 Conclusion

The aim of this thesis was to obtain a better understanding of the genetic control of beef

fat colour and to investigate opportunities to improve selection for beef carcass traits. The first objective of this study was to select candidate genes for beef fat colour. The major problem for selection of candidate genes from the fat colour QTL is that the regions contain many genes, and in some cases, current knowledge is insufficient to make precise predictions of their function. Only those genes known to involve in the metabolism of carotenoids were examined for association herein (Chapter 3).

The second objective was to obtain insight into the role of these candidate genes in beef fat colour and to determine whether these genes might be useful for selection. A total of 111 polymorphisms were detected, of which, 27 SNPs were selected for association studies. However, the genotype frequency for some SNPs was extremely unbalanced in this study. Therefore, rare genotypes could not be evaluated with sufficient statistical power with regards to beef fat colour. Most of the genotyped SNPs were associated with fat colour related traits although the size of the effects was relatively low for most SNPs (Chapter 4-5). The variants within the *BCMO1*, *PPARGC1A*, *RDHE2* and *SCARB1* genes had haplotype effects on beef fat colour, and many interactions between the genes excluding *BCO2* (Chapter 5) had small to intermediate effects on fat colour related traits. However, *BCO2* was one gene that accounted for a large proportion of the phenotypic variance in fat colour related traits. A nonsense mutation in the *BCO2* gene (*BCO2* W80X) explained 12-16% of total SNP variation (Table 4-3). Furthermore, validation of this SNP in a sister cattle mapping herd and other independent herds in New Zealand confirmed the *BCO2* W80X genotype has large effect on fat colour related traits (Table 6-2).

The third objective was to investigate the differences in the expression of genes

involved in beef fat colour (Chapter 7). The *RDHE2* gene mRNA transcript level was higher in the animals with white adipose tissue. In addition, the expression pattern of *BCO2* was highly correlated with β-carotene concentration. This further supports the role of *BCO2* in cleaving β-carotene eccentrically and the association of *RDHE2* with β-carotene concentration.

The fourth objective was to clarify the most likely pathways involved in the beef fat colour. The association studies showed that the *BCMO1*, *BCO2*, *RARA*, *RDHE2*, *PPARGC1A* and *ALDH8A1* genes and their interactions account for a large proportion of the variation (78-89%) in beef fat colour. These genes are involved in retinol and retinoic acid synthesis. Therefore, it is concluded that the retinol/retinoic acid synthesis pathway is most important in terms of contribution to the β-carotene concentration in bovine adipose tissue. In this pathway, BCMO1 and BCO2 are primary enzymes to control the retinol and retinoic acid synthesis and thereby, to determine the beef fat colour. In addition to retinol and retinoic acid synthesis pathway, effects of APOM and SCARB1 indicate that transportation of β-carotene is also important in the regulation of regulate the β-carotene concentration in fat. Lastly, the minor effects of genes on fat colour via dilution of β-carotene suggest that this mechanism is less likely to affect beef fat colour significantly.

The results in this thesis lend support to the hypothesis that candidate genes involved in conversion of β-carotene to vitamin A and in carotenoid transportation are regulate beef fat colour. However, the hypothesis that fat deposition affects beef fat colour is not supported because the effects of the candidate genes involved in fatness were marginal herein. More candidate genes involved in fatness should be investigated to test this

hypothesis.

The study conducted herein contributes to the understanding for metabolism of carotenoids and their numerous derivatives. This may advance the knowledge of human health problems, such as the hypervitaminosis A syndrome. Furthermore, the use of variants in major key genes for marker assisted selection is economically expedient and desirable to accelerate breeding. Variants that have a large influence on fat colour, such as *BCO2* W80X and *RDHE2* SNP2, can be used in marker selection systems to reduce the incidence of yellow fat colour rapidly. However, most other gene variants are likely to have relatively small effects.

Appendices

Appendix 1 Protocols

Appendix 1.1 Extraction and quantification of carotenoids

1. Accurately measure the weight of a partially frozen sample. If the fat is very white, use 0.3-0.5g samples. If the fat is yellow, measure 0.1-0.2g samples.
2. Hydrolyse solid samples at 65°C by adding KOH in ethanol (20% w/v).
3. Dilute sample after hydrolyses with 3ml of water, and extract carotene twice with organic solvents, shaking sample vigorously by hand for 20sec.
4. Wash combined extracts with water, and transfers the organic fraction to another bottle.
5. Evaporate extracts to dryness under the heated nitrogen gas (-40°C).
6. Resuspend the residue in ethanol and centrifuge at 8400xg for 10min.
7. Determine total carotenoids on a spectrophotometer at 45nm using pure β-carotene for plotting a standard curve or separate and quantify individual carotenoids on HPLC or spectrophotometry.

Appendix 1.2 DNA purification (Ultra Clean PCR Clean-up Kit, MoBio)

1. Shake to mix the SpinBind before use. Add 250 µl of SpinBind to PCR reaction 50µl (combination of 2 reactions).
2. Transfer PCR/SpinBind mixture to a Spin Filter unit.
3. Centrifuge 30 seconds at 10,000 x *g* (13,000 rpm) in a tabletop microcentrifuge.
4. Remove the Spin Filter basket and discard the liquid flow-through from the tube by decanting.
5. Replace the Spin Filter basket in the same tube.
6. Add 300 µl SpinClean Buffer to the Spin Filter.
7. Centrifuge 30 seconds at a minimum 10,000 x *g*.
8. Remove Spin Filter basket and discard liquid flow through by decanting then replace basket back into the same tube.
9. Centrifuge 60 seconds at minimum 10,000 x *g*.
10. Transfer Spin Filter to a clean 2 ml Collection Tube (provided).
11. Add 30µl of Elution Buffer (10 mM Tris) solution provided directly onto the centre of the white Spin Filter membrane.
12. Centrifuge 60 seconds at a minimum 10,000 x *g*.
13. Discard Spin Filter basket from the inside of the Spin Filter unit. Purified DNA is now in the 2 ml Collection Tube.

Appendix 1.3 Purification of PCR products for sequencing

1. Transfer the sequencing PCR products to new tubes.
2. Add 80 µl of 75% isopropanol into each tube.
3. Vortex them briefly and leave them at room temperature for 15 min.
4. Spin the tubes in 10,000 x *g* (13,000 rpm) for 20 minutes.
5. Remove the supernatant from the tubes.
6. Add 250 µl of 75% isopropanol into each tube.
7. Spin the tubes in 10,000 x *g* (13,000 rpm) for 20 minutes.
8. Remove the supernatant from the tubes.
9. Dry the sample in the incubator at 37 ºC.

Appendix 1.4 Ambion TURBO DNA-*free*™ DNase Treatment

1. Add 4 µl TURBO DNase Buffer (10X) and 1 µL (2U) TURBO DNase to the RNA, and mix gently.
2. Incubate at 37 ºC for 30 minutes.
3. Add 4 µl resuspended DNase Inactivation Reagent and mix well.
4. Incubate 5 minutes at room temperature, mixing occasionally.
5. Centrifuge at 10,000 *g* for 1.5 minutes and transfer the RNA to a fresh tube.

Appendix 1.5 TRI Reagent® DNA Isolation

Generally, DNA was isolated from the interphase and organic/phenol phase of the lysate from the TRI Reagent® RNA Isolation Protocol (*Applied Biosystems*).

Step 1: DNA Precipitation: add 0.3 ml ethanol into phenol phase and interphase and incubate at room temperature for 3minutes. Centrifuge at 2000 x g for 5 min at 4°C to sediment the DNA, then discard the supernatant.

Step 2: DNA Wash: Add 1 ml DNA Wash Solution and incubate for 30 minutes at room temperature with periodic mixing, Centrifuge at 2000 x g for 5 minutes at 4°C. Carefully remove the supernatant. And repeat step 2.

Step 3: Add 1.5 ml 75% ethanol and incubate for 20 minutes at room temperature with periodic mixing. Centrifuge at 2000 x g for 5 minutes at 4°C and remove the ethanol.

Step 4: DNA Solubilisation: Briefly air dry the DNA pellet by keeping the tube open for 3–5 min at room temperature and add 300 µl 8 mM NaOH. Centrifuge at 12000 x g for 10 minutes to pellet any insoluble material and transfer the DNA-containing supernatant to a new tube, then adjust pH to 8.0.

Appendix 2 Chemicals and solutions

2.1 TE Buffer pH=8 PBS (1X) 100 mL

1 M Tris-HCl / 1 ml

0.5 M EDTA / 0.2 ml

Milli Q water: up to 100 ml

Adjust to pH 8.0

2.2 TAE buffer (50X) 1L

TRIZMA: 242 gm

EDTA (disodium salt): 18.62 gm

Glacial acetic acid: 57.1 ml

Milli Q water: up to 1 litre

Adjust pH to 8.0

2.3 Formamide Loading Buffer (2X) 1ml

80% formamide, deionised

10mM EDTA

1mg/ml xylene cyanole

1mg/ml bromophenol blue

2.4 1M Tris-HCl (pH 8.0) 1L

Tris base 121.1g

HCL (1M) 42ml

Dissolve in 500mls of Milli Q H_2O and adjust to pH 8.0 with HCl. Adjust volume to 1L with MQ H_2O and autoclave.

Appendix 3 PCR conditions

Touchdown program:

TD1

Step 1: 95ºC for 10 minutes (1 repeat)

Step 2: 95ºC for 1 minute, (68 – 58°C drop 1°C/ cycle for 10 cycles) until 58°C for 1 minute, 72ºC for 1 minute (24 replicates)

Step 3: 72ºC for 10 minutes (1 repeat)

Step 4: 4 ºC for forever

TD2

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 1 minute, (60 – 50°C decrease 1°C/ cycle, x 10 cycles) until 50°C for 1 minute, 72ºC for 1 minute (24 replicates)

Step 3: 72ºC for 10 minutes

Step 4: 4 ºC

TD3

Step 1: 95ºC for 10 minutes

Step 2: 95ºC for 1 minute, (65 – 55°C drop 1°C/ cycle, x 10 cycles) until 55°C for 1 minute, 72ºC for 1 minute (24 replicates)

Step 3: 72ºC for 10 minutes

Step 4: 4 ºC

Appendix 3.1 PCR conditions for the candidate gene primers

Gene	Region	Primers sequence		PCR Condition				
		Forward primer	Reverse primer	Size (bp)	Program	Taq	Primer conc.	$MgCl_2$ conc.
ALDH8A1	5'flanking 1	GACCCAGCAACCACAAATC	ACCACCCACCAACTTCTACC	675	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	5'flanking 2	TGTCATCATCTTCATAGCACCTC	ACCTCCTCTTCCAGCCATAGT	692	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 1	TGGGAACTCTTCATTCAAAGC	AGTGGCAAATTCAGGCAAAC	658	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 2	TGATTTCCTTTTGACTGGTTTG	TTTATCGCTGCCAAGAGAAG	605	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 3	TCAATTCTAACCACTTCTACTTCA	TCAGTGTCATTCCTTTCCCT	592	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 4 part1	GGGAGAGGTGACAGCATTTTAG	CAGCAGATAGAGGGGCAGAT	390	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 4 part2	CTGGAATCTGCCCCTCTATCT	GCTCTACTCAATGCTCTGTGGT	385	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 5	GGCAAAGCACCCAGAGAA	AGGGGGAGAAAAACAGAGG	565	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 6	TTGCGTTATTGCCTGTCTT	AGGGTTTTACTTACTTTCTCCA	363	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 7 part1	GGCAAGAGAGCAGGAGAAAG	TATCAAAGGGGACGACACAC	746	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	Exon 7 part2	TTCTGTGTGGTGAGGGAGTG	AGGAGGAGCCCAATGTTTC	644	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	3'UTR	GCAAAGGAAGCCACAGAAAC	AATATCCAAGGCAAGCATGAA	875	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	3'flanking 1	CTTTGCCTCTCCCTCCTCTC	GCCAACATACGCTAAATCCTACA	361	TD2	AmpliTaq	0.1µM	2.5mM
ALDH8A1	3'flanking 2	GGCCCTAGTGTATTGGTTG	CTGGTCTTGTTTTTGCTGA	675	TD2	AmpliTaq	0.1µM	2.5mM
APOM	5'flanking 1	AGCAATAGGGGGTAGTGGTG	CAGGGTCTTGTGTTTCATTCC	512	TD1	AmpliTaq	0.1µM	2.5mM
APOM	5'flanking 2	CGTTGTTGCCGGTGTATCT	GTATGTGGATGGGAGGCTTT	872	TD1	AmpliTaq	0.1µM	2.5mM
APOM	5 UTR, Exon 1	TTCTTCAAGGCCAGGGACTA	CACACCCTTTCTTCCTTCCA	684	TD1	AmpliTaq	0.1µM	2.5mM
APOM	Exon 2	TGGAAGGAAGAAAGGGTGTG	CAGAAGTGGTGGTGGCAAG	725	TD1	AmpliTaq	0.1µM	2.5mM
APOM	Exon 3& Exon 4	GCGGTCTCTGCCTAAAGTGT	CCCACCTCCCCTCTTTTTC	570	TD1	AmpliTaq	0.1µM	2.5mM
APOM	Exon 5& Exon 6	GAAAAAGAGGGGAGGTGG	GGGGAGGACTGAAATGAGAG	857	TD1	AmpliTaq	0.1µM	2.5mM
APOM	3'flanking	CTCGTCTCATTTAGGGGTTTGT	TGGGAGCAAGGATGAAGATAG	829	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	5'flanking 1	CGTTTGAACCAGACAGCAGA	ACCTCCCAGCAAGAAAAAGA	880	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	5'flanking 2	CTGGAGAAAGAAATGGCAACA	AGCTCTCCCTTTCCACTTCC	432	TD2	AmpliTaq	0.1µM	2.5mM

Appendix 3.1 continued

Gene	Region	Primers sequence		PCR Condition				
		Forward primer	Reverse primer	Size (bp)	Program	Taq	Primer conc.	$MgCl_2$ conc.
Bcmo1	5 UTR, Exon 1	TTCAGCCAAATGTGGAAGTG	CTGACTCCCCTTGAAAGCTG	544	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 2	AGTGGTAGAGGGGCTGTGTG	CAACACTGGCAACCTGACTG	617	TD1	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 3	AGCCTCAGTTCCGCCATAC	CTTTGCCTTGCCTGCTATCT	554	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 4	GCTGATTGGTTGTGGTGGT	GCAGGAAGAGGCAAAGAAG	669	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 5	GGGACAACCGTCAGAGAACA	GCCATCAACCAGAACCAGAA	472	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 6	GAAAGGCTGGGCTGTAGGA	GGCTGGATGGGGAGAAGTA	586	TD1	AmpliTaq	0.1µM	1.5mM
Bcmo1	Exon 7	ATGGCTTCTGGTGAGGTTTG	TCTTCCTGTCTATTTGCCTGTCT	706	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 8	CGGTTCTCTCTGCTGCTCTT	ACATCCACCCCTTCCTCTG	589	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 9& Exon10	CTTTGTCAGCCATGTCGTCTC	TTGTAAATCAGCCCCGTTGT	739	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 11	CTGCTTTCCTGTTTTTGTGCT	ATCCCACTCGCCTTTTCTCT	597	TD2	AmpliTaq	0.1µM	2.5mM
Bcmo1	Exon 12, Exon 13 & Exon 14	ACAAAGAACAGGGAGACAGAG	GGAATGACCACAGAGCACAC	803	TD1	AmpliTaq	0.1µM	1.5mM
BCO2	5'flanking	TCTCAAATCATCCCACCGTC	CTTTGTCCTTCTGCCCTTCT	1022	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	5 UTR, Exon 1	GAAGGACAAAGGATGAAAGGTAG	CTGAATCCTGCCCCTGTATG	763	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 2	ACCAGGGATCAAACCCATAC	CAGGTCCCACACGAAGTAGA	628	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 3	GGACATTCATCTTATTTGCTTACTT	AGCAGTTGAAACATCCTCTCAT	813	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 4	AGCCTGGTAAGCACCCTTTAT	CCAATACCACAGGCCATTCT	507	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 5	GGCAAAGGAGACACAGCATA	ATGGCACTCTGGGAAGACA	735	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 6	TTCCTGGCTGCTATCCTATCTT	AATGTGCTGGGAAGGAACTG	763	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 7	TCTCGCATACAGGGTTATTGTT	GCTAACATGCCAAGAGGATTT	804	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 8	CACCAAAGTCCAAAGCGTATT	CAAGCCCAAAGCCAAAACT	761	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 9	TTCTTATCTATTACCATTCTTCCTCC	TCCATGATCCTACCCTGCTC	549	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 10	ATAAAAGCCCATGTCCACCTC	GGAAGGGATAGTTAGGGTGTTTG	780	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	Exon 11	ATGCTGGGGCTGACAAAA	ACAGAGAGAAACAAAAGGGAGAA	320	TD2	AmpliTaq	0.1µM	2.5mM

Appendix 3.1 continued

Gene	Region	Primers sequence		PCR Condition				
		Forward primer	Forward primer	Size (bp)	Program	Taq	Primer conc.	$MgCl_2$ conc.
BCO2	Exon 12	GCCCAGTGATGAGAATGGA	GGCAGGAGAGTGAGCAAGA	835	TD2	AmpliTaq	0.1µM	2.5mM
BCO2	3'flanking	GGATTGTCTCCTGACCTTGTG	TGAGAACCTCTGTTACCCATCTAT	622	TD2	AmpliTaq	0.1µM	2.5mM
RARA	5'flanking 1	CGACCCTGCTTGTCCTACCT	CCCCCTCCTCTTCCCTAAAC	705	TD1	AmpliTaq	0.1µM	2.5mM
RARA	5'flanking 2	AGACTCGCCCGTTCCCTG	GTCCGTTGGGCATCCGAT	721	TD1	AmpliTaq	0.3µM	1.5mM
RARA	5 UTR, Exon 1	GCCCACTCACCTGTCTTTTC	CTCCCCGACCCCACTTAC	577	TD1	AmpliTaq	0.1µM	2.5mM
RARA	Exon 2	AGCCACATCCACATACCAGTC	GATGCTGGGGGAAAGAGTC	673	TD1	AmpliTaq	0.1µM	2.5mM
RARA	Exon 3	CTGACCTGACTCCCTGCTCT	CCTCCTTCTTTCCCTCCATC	721	TD1	AmpliTaq	0.1µM	2.5mM
RARA	Exon 4	CGGCTGTGTGTGGATGTC	TGTTGTTCTGGGAGGATGG	653	TD2	AmpliTaq	0.2µM	1.5mM
RARA	Exon 5 & 3UTR	CAGGAAGTGAAGGCCAGTAGA	GCAGCCAGCACTTATCACAG	739	TD1	AmpliTaq	0.1µM	2.5mM
RDHE2	5'flanking 1	TCTCAGGTGCTTGTTAGGAAAG	CCCTTGCAGAAGTGAAAGAAA	916	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	5 UTR & Exon 1	GAACACATAACAGGCATTCAGG	GGCACATAGCAAGAACTCAAAA	712	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	Exon 2	GCACCAGGAAACCAAAACA	CAAGAGGAAGCCTAAGTCGC	524	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	Exon 3	TTACTGGTTTGTGGACTGTGG	CCTCCTTATCCCACCCATC	708	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	Exon 4	ATGTGTCCTTCTGCCCATTT	GTGGTGCTCATGCTTGTTTG	816	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	Exon 5	CCCAAGCTGTCCTTCTCTCT	TCCTCTCTGTTGGCTGTGG	206	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	Exon 6	AGTGGGAGGAACGGAGTGT	CAAAAGGTCGGATGTGATTTAG	743	TD2	AmpliTaq	0.1µM	2.5mM
RDHE2	3'flanking	TTGCGCTCTGTTTTTCTGTT	CTTCCCAGATGGTGCTAGTG	750	TD2	AmpliTaq	0.1µM	2.5mM
PPARGC1A	5 UTR, Exon 1	GGAGGCTGGGTGAGTGACA	TTCCAACCAGGCCACTAAAC	601	TD1	KapaTaq	0.2µM	-
PPARGC1A	Exon 2	GGAGGTAGAGTTTGGGATGG	GGTGACAGCAAAGCAAGAAC	513	TD2	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 3	GGACGTGATTTTTGTGTTATGG	CTGAGTGTGCTTGGGGATTA	481	TD1	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 4	TCTCGCTTTCCCTCCTTC	TAGACTACTGCTTTTGGAGACC	315	TD1	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 5	ATACTGGGCTCTGGCAATC	CAGAGATTCCTACAAACCACAC	837	TD2	KapaTaq	0.2µM	-
PPARGC1A	Exon 6 & EXON 7	TGGTTATTGAGGGAGTTTGG	TGGGAGGAGGTTTATTTGTG	945	TD1	KapaTaq	0.2µM	-

Appendix 3.1 continued

Gene	Region	Primers sequence		PCR Condition				
		Forward primer	Forward primer	Size (bp)	Program	Taq	Primer conc.	$MgCl_2$ conc.
PPARGC1A	Exon 8 part1	CCTAACTCCACCCACAACTC	GAACAAGAAGGCGACACATC	747	TD2	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 8 part2	CCGTGCTACCTGAGAGAGA	CTTTGGGTGGGTTTTGAC	530	TD2	KapaTaq	0.2µM	-
PPARGC1A	Exon 9& Exon10	CCTTTTTGTTTTTCCTTCTCTC	CACCTCCTCATTTTCCACTC	775	TD2	KapaTaq	0.2µM	-
PPARGC1A	Exon 11	CTTCTCACTTCCCCCTCCA	GAACCACTGCATCCATTTATCA	372	TD1	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 12	ACCAGCGAAGTCTCCAAAGT	GGATTCAGGGTAGGCACAAG	578	TD1	AmpliTaq	0.2µM	2.5mM
PPARGC1A	Exon 13	TGTTTTTGAATCGGGTTGTC	CACACGCACACTCCATCAC	472	TD1	AmpliTaq	0.2µM	2.5mM
SCARB1	5'flanking 1	GCTGTGCCTGTTTGCTTATGT	GCTCCCTGCTTATCCCTTTC	757	TD1	AmpliTaq	0.2µM	3.5mM
SCARB1	5'flanking 2	GGCGAAATGAGAGGAGGAG	GAAGAGGAGCCCGATGAAA	602	TD1	AmpliTaq	0.1µM	2.5mM
SCARB1	5 UTR & Exon 1	CCTCTATCACCTCCGTCCC	TCACAGGAAGCAAGCAAAGT	691	TD1	KapaTaq	0.1µM	-
SCARB1	Exon 2	GCCACCTCCCAGTCTCTTG	TCCTCATAACCACTCCACCA	730	TD1	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 3	ACAGGGAGTTCAGGCACAAG	AACACCACAGACAAGCACACA	475	TD1	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 4	TAACCAGGGCTAAGGCACAC	TTTCTTCCAGCACCAACCTC	757	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 5	CCCTCAAGTCCTCCTCCTCT	GCAATCCCCTCCTCTGTTT	615	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 6	TGTCTGCCCACTTCCTGTCT	CCATCTTGCCCATTTTCTGT	732	TD1	KapaTaq	0.1µM	-
SCARB1	Exon 7	GGCAGGGAGACACATAAGGA	GCAACAGATACACACGGCAC	505	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 8	CTGTGTTTTCGTCGCCTGT	TATGCTCCTGGTCCCTGTTT	659	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 9	CCAGGGAGAGGGGGATTAG	AGGGCAGAAACAAAGCAACA	547	TD1	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 10	AGCAGGGGATCAGCATAAAC	ATCTCAGGCAGCTTCGTCTT	604	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 11	TGGCAAAGAGATGGAGAGAA	TCCCAGAACAGGCAGAGAA	668	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	Exon 12	TGCCACTTGCTCACCTCTCT	CCTCCATCCCCTACCACATC	653	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	3 UTR part1	TGCTCTGCCCTTTTCTCTGT	GCCCTTGTTGACCAGTCTTT	602	TD2	KapaTaq	0.1µM	-
SCARB1	3 UTR part2	CAACTTCCCAGCCCCTACAC	CAACCCAGCCTCCCATAAAC	941	TD2	AmpliTaq	0.1µM	2.5mM
SCARB1	3'flanking	GTTTATGGGAGGCTGGGTTG	CAAAAGGTGGGTCAAAAGATG	493	TD1	AmpliTaq	0.1µM	2.5mM

Appendix 3.2 PCR conditions for the genotyped SNPs

Gene	SNP ID	SNP sequence context	primers	PCR product size	TA	Reagents or Taq
ALDH8A1	SNP 10	GGACCACCTGG[G/A]CTGTCTGCACT	F: CTTCGCCTCCTCCATCCT R: CCGCACTGTGTAGTGCAGAC	79	57ºC	Sensimix
ALDH8A1	SNP 15	GAGCGCATCA[C/T]GCAGCTGAGCG	F: GCCCCTCATCTCCTTCACTG R: GACATACCTGGTTGGCAAAGC	185	57ºC	Sensimix
ALDH8A1	SNP 16	GCGCTCTG[A/G]TCAGTAAAGCT	F: TCTGATCCATCGGCTGAC R: CTGAAACTTACACAGAGGGTT	83	TD3	AmplitaqGold
APOM	SNP 2	ACTTTTCAGG[A/G]CTGTTGTCTT	F: GGAGAAGCCAAGGATTTAGGA R: CCACTCTGACCCCATTTGTT	96	TD1	KapaTaq
APOM	SNP 6	TCTTACTTTT[A/G]TGTTCCCAAG	F: CAAGGCCAGGGACTATGTCT R: GCAGCACTGAAGAGAGATGGA	142	TD1	Sensimix
APOM	SNP 7	CTGAGCAGATG[G/T]GAAGCTGCT	F: AGCACAGCCAACTGACAACT R: CACACCCTTTCTTCCTTCC	153	TD3	Sensimix
Bcmo1	SNP 4	AAAGGGAGGA[A/C]CATGAATCTA	F: TTACAGATAAGGAAACGGAG R: CCAAGGAAAGACGCTACA	112	TD2	Sensimix
Bcmo1	SNP 7	CTTGAAGCAA[C/T]GTAAGTAAA	F: TCCACCCAGAGAGGCAAC R: TCACCAGAAATCCACACTCAC	105	TD3	Sensimix
Bcmo1	SNP 8	TATTGAGTA[G/C]AGTTTCCTGTG	F: TGGACACAACACAGCGACTAA R: TTGGGATTGATACATACACACT	243	TD2	KapaTaq
Bcmo1	SNP 13	GCAGAGAAAA[C/T]AGAGCTGAAA	F: CCTATGCTCGCTGGTGTG R: CGAAATGAATGGTTGCAGAG	109	TD3	Sensimix
BCO2	SNP W80X	ACAATCACTG[G/A]TTTGATGGAA	F: AACCCATCCCACTTCCTTATC R: GCTGAAATCAAACCCCAAAG	525	TD2	AmplitaqGold
BCO2	SNP 1	GCCTGGTAAG[C/T]ACCCTTTATT	NA	NA	NA	NA
BCO2	SNP 2	TATGGATCTT[G/T]ATATTTATTT	NA	NA	NA	NA
BCO2	SNP 3	TAGTTATTAT[C/G]AGAAGAAAAA	NA	NA	NA	NA
RARA	SNP 1	AGGTGTTCAC[G/T]TACTGATTCC	F: GAGTAGGCACTTCCCCTTACA R: GCGGACAGAAAGAGGAATCA	115	TD1	Sensimix
RARA	SNP 3	CCCTCCCTCC[C/T]GCC[G/A]TAGACGG	F: GAGTAGGCACTTCCCCTTACA R: ACGAGCACACACCCTTCAC	717	64 ºC	AmplitaqGold
RARA	SNP 7	GCATCATCAA[C/T]AAGGTGACCC	F: CGCAGCATCCAGAAGAACA R: GTCGGTGGCACTCACACTC	145	TD1	Sensimix

Appendix 3.2 continued

Gene	SNP ID	SNP sequence context	primers	PCR product size	TA	Reagents or Taq
RDHE2	SNP 2	CAGAACATGG[C/T]GCTCAAGCTG	F: TTTATAGGTATGAGCCAGAA R: TCCACAAGAGCCAACAC	92	TD2	Sensimix
RDHE2	SNP 3	GTAGTTTTTG[C/T]TATAATCCCC	NA	NA	NA	NA
RDHE2	SNP 4	CTGGAGCCAC(G/T)AGGGTCTATGCCT	NA	NA	NA	NA
PPARGC1A	SNP 4	ATGTATCATGT[G/A]CTCATTTGGT	F: TTGAGAAGGACGTGATTTTTG R: TGAGTTGACACTGGGAGATGA	126	TD3	Sensimix
PPARGC1A	SNP 9	AGGAGCTCCA[C/T]GACTCCAGAC	F: GGACACGAGGAAAGGAAGG R: GAGGAGGGGGCATCTTTAT	155	TD3	Sensimix
PPARGC1A	SNP 10	GAGAGAGACC[G/A]CGGAGGTGAG	F: GGGCAAATACACTCTTCCACA R: GCTCGGATTTCCTGGTCTT	119	TD3	Sensimix
PPARGC1A	SNP 12	CGAAATTCGC[C/T]CCTGTGCGCG	F: TGCAGACACCGCACACAC R: GGGCAGGGTTTTGGACTA	151	TD3	AmplitaqGold
SCARB1	SNP 1	GAAGCTCGGG[G/C]GCCGGATTCC	F: CAGCCAGAAACGGAAGCTC R: CGGGGCCTAAGTTAGCAAG	110	TD3	Sensimix
SCARB1	SNP 2	CGCAGGCTGA[G/A]TTTGGGGGCCA	F: GAGAGACCAGAGGGCATGAG R: GGCTTTCCCAACCCCAG	141	TD1	Sensimix
SCARB1	SNP 16	TTGCTTGCCT[G/C]CTTGGGGGAA	F2: GGGCTGCGTCCTACTGCT R2: GAAGGGACAGTTGGGGAATC	146	TD3	Sensimix

Appendix 3.3 Real-time PCR conditions

Gene	Forward primer
Beta-carotene oxygenase 2 (BCO2)	Forward: TCTTCTATGGCTGTGGCTTC Reverse: CAAACCCTCCGTGTCTTGT
Beta-carotene 15,15'-monooxygenase 1 (BCMO1)	Forward: TACATCAGGAGGATCAACCCC Reverse: TTGCCAGATTTACAGCCACATA
Retinal short chain dehydrogenase reductase (RDHE2)	Forward: AACAATGCCGGAATCGTAAC Reverse: AAATGCACACCAAATGTCCA
Actin beta (ACTB)	Forward: GAGCGGGAAATCGTCCGTGAC Reverse: GTGTTGGCGTAGAGGTCCTTGC
60S acidic ribosomal protein P0 (RPLP0)	Forward: TTCCAGGCTTTAGGCATCAC Reverse: GCTGGCGCCTACTTTGTCT
TATA box-binding protein (TBP)	Forward: GTGCTTACCCACCAACAGTTC Reverse: TCTCGGTTTGATCATTCTGTAGAT
Ubiquitin carboxyl-terminal hydrolase L5 (UCHL5)	Forward: CAAGTGGCAACCAGGAGAA Reverse: GGCTTGAGTAGCACAAGCATT

Appendix 4 Identified DNA variants

Gene	Variant ID	variant	SNP sequence context	Variant location
RARA	1	GT	AGGTGTTCAC[G/T]TACTGATTCC	5UP flanking
RARA	2	CT	CCCTCCCTCC[C/T]GCCGTAGACGG	5UP flanking
RARA	3	GA	CCCTCCCTCCCGCC[G/A]TAGACGG	5UP flanking
RARA	4	GA	CGGGACGGAAG[G/A]GGGGGTGGGC	5UTR
RARA	5	CT	CGGCCTGAAGCC[C/T]GCCAGACTGT	5UTR
RARA	6	GA	GGACTGTACC[G/A]GCAGTGGGG	INTRON2-3
RARA	7	CT	GCATCATCAA[C/T]AAGGTGACCC	EXON3
RARA	8	GA	GTCCATGGGC[G/A]GACAGCCCTT	INTRON 3-4
RARA	9	GA	TCTGAGGACA[G/A]GGGTGCTGCT	INTRON 3-4
RARA	10	CT	GCTCCAGGCA[C/T]CGGGATCTAA	INTRON 3-4
RARA	11	CA	TGTGGATGTCC[C/A]CGCCTGCCTGT	INTRON3-4
RARA	12	GA	GGAACAAGAA[G/A]AAGAAGGAGGT	EXON4
RARA	13	CT	TCACCCTCCT[T/C]AAGGCTGCCT	EXON5
BCMO1	1	CT	TTGCTGGACT[C/T]TTGACTCCGA	5UP flanking
BCMO1	2	CT	TTGACTC[C/T]GAATAACCTT	5UP flanking
BCMO1	3	CT	TTTTGGTAT[C/T]ACTATACCTCC	5UP flanking
BCMO1	4	CA	AAAGGGAGGA[C/A]CATGAATCTA	5UP flanking
BCMO1	5	CG	GGCCCAGCAC[C/G]GGCACTGCTA	5UP flanking
BCMO1	6	GA	GGCACTGCT[G/A]AAGCTCCCAA	5UP flanking
BCMO1	7	CT	CTTGAAGCAA[C/T]GTAAGTAAA	5UP flanking
BCMO1	8	GC	TATTGAGTA[G/C]AGTTTCCTGTG	5UP flanking
BCMO1	9	indel T	CTTTTTTTTT[INDEL T]CCTGCTTAT	INTRON 1-2
BCMO1	10	CT	GGGCTTCCTG[C/T]CTGGCCTTCC	EXON 6
BCMO1	11	CT	AACCCACCAG[C/T]TTTGCCTTCT	INTRON 7-8
BCMO1	12	GC	AGAGAGTGAG[G/C]TCAGTTTGGG	INTRON 9-10
BCMO1	13	CT	GCAGAGAAAA[C/T]AGAGCTGAAA	INTRON 9-10
BCMO1	14	CT	CCTTCCTATT[C/T]GCCGGGAAT	INTRON 10-11
BCMO1	15	GA	TTCATTTCGC[G/A]TTTCTCCCCT	INTRON 10-11
BCMO1	16	GA	TTTGCATGC[G/A]CAGATAATAA	INTRON 10-11
BCMO1	17	GA	TCTCTCATCA[G/A]GCAAGATTCC	INTRON 1-2 SPLICE SITE
SCARB1	1	GC	GAAGCTCGGG[G/C]GCCGGATTCC	5UP flanking
SCARB1	2	GA	CGCAGGCTGA[G/A]TTTGGGGGCCA	INTRON 1-2
SCARB1	3	indel GG	GAGGTCCAGGGAGGG[INDEL GG]CAGACAAGCT	INTRON 3-4
SCARB1	4	GT	AAAACCTAAC[G/T]GAAACTGGCT	INTRON 4-5
SCARB1	5	GA	CAGGGACCAG[G/A]TGGGGGTGGC	INTRON 5-6
SCARB1	6	GA	GTGACAGGTCC[G/A]CTTTACAGAT	INTRON 5-6
SCARB1	7	GT	ACAGATGAAG[G/T]TACTGAGTCC	INTRON 5-6
SCARB1	8	CT	GTGTGTGAAG[C/T]TGGAGCTCCA	INTRON 5-6
SCARB1	9	CT	TGCTCAGAGC[C/T]GAACCAAGTG	INTRON 6-7
SCARB1	10	CT	AGGCTGCTGG[C/T]GGTTGTTGGG	INTRON 6-7
SCARB1	11	indel A	AGGCGGGGGA[INDEL A]CGGGGGTGG	INTRON 6-7
SCARB1	12	indel A	AAAAAAAAAAA[DEL A] TGTGCCGTGTGTA	INTRON 7-8
SCARB1	13	CT	TATAAGGGAA[C/T]TGTCTCTATAA	INTRON 8-9
SCARB1	14	GA	ATTAATTAGG[G/A]GTTATCAACCT	INTRON 8-9

Appendix 4 continued

Gene	Variant ID	variant	SNP sequence context	Variant location
SCARB1	15	CT	AGGGCTAG[C/T]CCCTCACTGT	INTRON 8-9
SCARB1	16	GC	TTGCTTGCCT[G/C]CTTGGGGGAA	INTRON 11-12
SCARB1	17	GC	GGTGGCCGGC[G/C]TTAGTGATGC	INTRON 12
APOM	1	indel G/CT	TTGTGCCTCG[INDELG/CT]AATCTATGCT	5UP flanking
APOM	2	GA	ACTTTTCAGG[G/A]CTGTTGTCTT	5UP flanking
APOM	3	indel C	ATAGTGAGCCC[INDEL C]TAGTTGGTGC	5UP flanking
APOM	4	indel T	TTTTTTTTTT[DEL T]AAATATAATG	5UP flanking
APOM	5	GC	TGCCTACCTC[G/C]TATTACTTGA	5UP flanking
APOM	6	GA	TCTTACTTTT[G/A]TGTTCCCAAG	5UP flanking
APOM	7	GT	CTGAGCAGATG[G/T]GAAGCTGCT	INTRON 1-2
APOM	8	CT	GCTCTGCCCC[C/T]ATGCAGCTCC	EXON2
APOM	9	GA	GCTTCCAGAA[G/A][G/C]AGTAAGATGG	INTRON 2-3
APOM	10	GC	GCTTCCAGAA[G/A][G/C]AGTAAGATGG	INTRON 2-3
APOM	11	CT	CTGTTTCTCCT[C/T]CTTGCCACCA	INTRON 2-3
APOM	12	TA	AAGTGATGTC[T/A]CCAGGGGGCA	INTRON 5-6
APOM	13	GA	GACACTGAAC[G/A]GTGCTAACTC	3DN flanking
APOM	14	GC	CCCTAATCCA[G/C]CCAACTAGGA	3DN flanking
PPARGC1A	1	CT	TGGTGGTGT[C/T]GTTGAATTAA	INTRON 1-2
PPARGC1A	2	indel T	TTTTTTTTTTTT[INDEL T]CTCCCTGC	INTRON 1-3
PPARGC1A	3	CT	TCTCCCTGC[T/C]TCCTGCAGTGT	INRON 1-2
PPARGC1A	4	GA	ATGTATCATGT[G/A]CTCATTTGGT	INTRON 2-3
PPARGC1A	5	TA	CAGAGAGTTC[T/A]ACACCTTATG	INTRON 5-6
PPARGC1A	6	GT	CTGCAGGTAA[G/T]CCTTACATTT	INTRON 7-8
PPARGC1A	7	GA	TGAACAAGAA[G/A]TTATCTATAA	INTRON 7-8
PPARGC1A	8	GA	TCAGTTGTTT[G/A]TTTTTTTTAA	INTRON 7-8
PPARGC1A	9	CT	AGGAGCTCCA[C/T]GACTCCAGAC	EXON 8
PPARGC1A	10	GA	GAGAGAGACC[G/A]CGGAGGTGAG	EXON 8
PPARGC1A	11	CT	TTGATGACAG[C/T]GAAGATGAAA	EXON 8
PPARGC1A	12	CT	CGAAATTCGC[C/T]CCTGTGCGCG	EXON 9
PPARGC1A	13	CT	TAGATTTTAT[C/T]CTATTTTTTT	INTRON 12-13
PPARGC1A	14	indel T	TTTTTTTTTTT[INDEL T]ACTTTTTAAC	INTRON 12-13
RDHE2	1	GA	ATACAAGCAG(G/A)ATTTCACATT	5UP flanking
RDHE2	2	GA	CAGAACATGG(T/C)GCTCAAGCTG	EXON1
RDHE2	3	GA	GTAGTTTTTG(T/C)TATAATCCCC	EXON1
RDHE2	4	GA	CTGGAGCCAC(G/T)AGGGTCTATGCCT	EXON1
RDHE2	5	GA	TATTTGCCTTCCTC(G/A)GTGCTAATTTGCCTCT	INTRON 1
RDHE2	6	GA	TCAACAATGC(C/T)GGAATCGTAA	EXON2
RDHE2	7	GA	TGACTTGGTG(C/T)ATTAACAATA	INTRON 3-4
RDHE2	8	GA	CTGGGTTTAA(C/T)AGTGATGGTT	INTRON 4-5
RDHE2	9	GA	TATACTTCAT(C/T)GTGTTTTTAA	EXON 5
ALDH8A1	1	GC	TGGTGGAAAGA[G/C]AGAGTCTGCCC	5UP flanking
ALDH8A1	2	CT	CCTCAACCAC[C/T]TCTGATGGTGA	5UP flanking
ALDH8A1	3	GC	AGGGCCTGGCA[G/C]TATCCGTCTG	5UP flanking
ALDH8A1	4	GT	GCAAAGCCAAG[G/T]GGAAGGGCAGGC	5UP flanking

Appendix 4 continued

Gene	Variant ID	variant	SNP sequence context	Variant location
ALDH8A1	5	GA	TCATATGCAGAA[G/A]TGACGAATAAG	INTRON 1-2
ALDH8A1	6	GT	TGCAGAA[G/A]TGAC[G/T]AATAAGCTAGAT	INTRON 1-2
ALDH8A1	7	CT	TTTCTGTTTG[C/T]TCCTGCAAGGTC	INTRON 1-2
ALDH8A1	8	CT	GTATGGAAATG[C/T]GCTTACTCAG	INTRON 2-3
ALDH8A1	9	GA	TTCTGCTGTT[G/A]ACTTCTTACAGGG	INTRON 2-3
ALDH8A1	10	GA	GGACCACCTGG[G/A]CTGTCTGCACT	EXON3
ALDH8A1	11	GA	GCCGTGCAATG[G/A]CAGCTGGGCCA	INTRON 3-4
ALDH8A1	12	CA	CAGACTTTAAA[C/A]AAAGAAACTT	INTRON 3-4
ALDH8A1	13	GA	GTCAAATTCCCT[G/A]CTTATCCCTACA	INTRON 3-4
ALDH8A1	14	GA	CTTTGGAGAT[G/A]TATGTTTGTAGG	INTRON 4-5
ALDH8A1	15	CT	GAGCGCATCA[T/C]GCAGCTGAGCG	EXON5
ALDH8A1	16	GA	GCGCTCTG[A/G]TCAGTAAAGCT	EXON6
ALDH8A1	17	GT	TATTAGTGATA[G/T]TCTTAGCTCTT	3DN flanking
BCO2	1*	CT	GCCTGGTAAG[C/T]ACCCTTTATT	INTRON 3-4
BCO2	2*	GT	TATGGATCTT[G/T]ATATTTATTT	INTRON 3-4
BCO2	3*	CG	TAGTTATTAT[C/G]AGAAGAAAAA	INTRON 4-5
BCO2	W80X*	GA	ACAATCACTG[G/A]TTTGATGGAA	Exon 3
BCO2	5	GT	CCAGAAATAA(G/T)ACAAAATTTAACA	INTRON 6-7
BCO2	6	C/G	CTTTTGTGCC(C/G)TCTTTTAGAT	INTRON 9-10
BCO2	7	AG	TGCTCGCTAC(A/G)TAAGAGAGAG	3UTR
BCO2	8	AG	CTCACAGAAAA(A/G)TCATTAAAGC	3UTR
BCO2	9	GA	TCCTTGGAGC(G/A)CAGAAACCCA	3DN flanking
BCO2	10	GA	CAGAAACCC(G/A)TCTTGCTCAC	3DN flanking

* SNP identified earlier as part of Coursework Masters research project.

Appendix 5 Candidate gene haplotypes

Gene	SNP ID	Haplotype							
		HAP 1	HAP 2	HAP 3	HAP 4	HAP 5			
ALDH8A1	SNP 10	G	A	G	A	A			
	SNP 15	C	T	T	T	C			
	SNP 16	A	G	A	A	G			
Hap Frequency (%)		19.2	29.8	48.1	2.3	0.6			
		HAP 1	HAP 2	HAP 3	HAP 4				
APOM	SNP 2	A	A	A	G				
	SNP 6	A	G	A	A				
	SNP 7	T	T	G	G				
Hap Frequency (%)		29.9	10.6	45.1	14.4				
		HAP 1	HAP 2	HAP 3	HAP 4	HAP 5			
BCMO1	SNP 4	A	A	C	A	A			
	SNP 7	C	C	C	C	T			
	SNP 8	G	C	C	G	G			
	SNP 13	T	C	C	C	C			
Hap Frequency (%)		37.4	7.2	11.8	20.7	22.9			
		HAP 1	HAP 2	HAP 3	HAP 4	HAP 5	HAP 6	HAP 7	HAP 8
PPARGC1A	SNP 4	G	A	G	G	G	G	A	G
	SNP 9	C	T	T	C	T	T	C	T
	SNP 10	A	G	G	G	G	G	G	A
	SNP 12	C	C	T	C	C	C	C	C
Hap Frequency (%)		11.0	24.1	39.1	13.7	0.1	10.5	0.8	0.7
		HAP 1	HAP 2	HAP 3	HAP 4	HAP 5	HAP 6		
RARA	SNP 1	G	G	T	T	T	G		
	SNP 3	G	A	A	A	G	A		
	SNP 7	C	C	C	T	C	T		
Hap Frequency (%)		10.6	27.0	34.8	26.3	0.1	1.3		
		HAP 1	HAP 2	HAP 3	HAP 4				
RDHE2	SNP 2	T	C	C	C				
	SNP 3	C	C	C	T				
	SNP 4	G	G	T	G				
Hap Frequency (%)		49.8	18.3	9.8	22.1				
		HAP 1	HAP 2	HAP 3	HAP 4				
SCARB1	SNP 1	C	G	G	G				
	SNP 2	G	A	A	G				
	SNP 16	G	C	G	G				
Hap Frequency (%)		56.7	18.6	13.7	11.0				
		HAP 1	HAP 2	HAP 3	HAP 4				
BCO2	W80X	G	G	G	A				
	SNP 1	C	C	T	C				
	SNP 2	G	T	G	G				
	SNP 3	C	G	C	C				
Hap Frequency (%)		63.3	11.7	13.9	11.1				

Appendix 6 Effects of candidate gene SNPs on fat colour traits with covariates

	Bc-bio	Bc-bio/weanfat[1]	Fc-bio	Fc-bio/weanfat	Fc-car	Fc-car/rftms[2]
cohort	P<0.001	P<0.001	P<0.05	ns	P<0.001	P<0.001
breed	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
sire	P<0.01	P<0.01	ns	P<0.05	P<0.05	P<0.05
ALDH8A1 SNP10	ns	ns	ns	ns	ns	ns
ALDH8A1 SNP15	ns	ns	ns	ns	P<0.05	P<0.05
ALDH8A1 SNP16	ns	ns	P=0.05	P=0.05	ns	ns
APOM SNP2	ns	ns	ns	ns	0.05	0.05
APOM SNP6	ns	ns	ns	ns	ns	ns
APOM SNP7	ns	ns	ns	ns	ns	ns
BCMO1 SNP4	P<0.01	P<0.05	ns	ns	ns	ns
BCMO1 SNP7	ns	ns	ns	ns	ns	ns
BCMO1 SNP8	ns	ns	ns	ns	ns	ns
BCMO1 SNP13	ns	ns	ns	ns	ns	ns
BCO2 SNP1	ns	ns	P<0.01	P<0.01	ns	ns
BCO2 SNP2	ns	ns	ns	ns	ns	ns
BCO2 SNP3	ns	ns	ns	ns	ns	ns
BCO2 W80X	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
PPARCG1A SNP4	ns	ns	ns	ns	ns	ns
PPARCG1A SNP9	ns	ns	ns	ns	ns	ns
PPARCG1A SNP10	ns	ns	ns	ns	ns	ns
PPARCG1A SNP12	P<0.01	P<0.01	P=0.005	P=0.005	P<0.05	P<0.05
RARA SNP1	ns	ns	ns	ns	ns	ns
RARA SNP 3	ns	ns	ns	ns	ns	ns
RARA SNP7	ns	ns	P=0.05	ns	ns	ns
RDHE2 SNP2	ns	ns	ns	ns	ns	ns
RDHE2 SNP3	ns	ns	ns	ns	ns	ns
RDHE2 SNP4	ns	ns	ns	ns	ns	ns
SCRB1 SNP1	ns	ns	P<0.05	P<0.05	ns	ns
SCRB1 SNP2	ns	ns	ns	ns	ns	ns
SCRB1 SNP16	ns	ns	ns	ns	ns	ns

Model 2 (Chapter 2) was employed to analyse the effect of SNP (weanfat and rftms were fitted as covariate). [1]weanfat: Fat depth at weaning (mm), [2]rftms: Fat depth at ribs 12^{th} and 13^{th}(mm)

Appendix 7 QTL

Appendix 7.1 Most likely position and F-statistic values of putative QTL detected by individual family analysis (Sire 361 family)

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
1	Fc-bio	9cM	4.58	9cM	4.84	9cM	4.57	9cM	4.55	36cM	4.85
1	Bc-bio	70cM	6.41	70cM	5.72	70cM	6.48	70cM	6.38	67cM	6.12
1	Fc-car	4cM	6.80	3cM	6.62	4cM	6.34	4cM	6.74	3cM	7.51
2	Fc-bio	50cM	1.66	52cM	1.72	50cM	1.68	49cM	1.76	51cM	1.62
2	Bc-bio	64cM	7.61	64cM	7.77	64cM	7.11	64cM	7.54	64cM	7.25
2	Fc-car	39cM	4.18	37cM	4.20	37cM	3.71	36cM	4.79	38cM	4.00
3	Fc-bio	106cM	5.09	106cM	4.34	106cM	5.10	105cM	4.83	106cM	4.58
3	Bc-bio	99cM	1.62	99cM	1.40	100cM	1.73	99cM	1.62	101cM	1.79
3	Fc-car	109cM	0.93	109cM	0.92	109cM	1.02	51cM	0.86	109cM	1.03
4	Fc-bio	59cM	0.44	61cM	0.60	59cM	0.45	0cM	0.50	0cM	0.50
4	Bc-bio	76cM	3.87	76cM	3.96	76cM	4.24	76cM	3.83	73cM	5.12
4	Fc-car	59cM	2.30	21cM	2.10	21cM	2.14	59cM	2.17	59cM	1.96
5	Fc-bio	12cM	0.91	81cM	1.90	12cM	0.91	12cM	0.83	12cM	0.88
5	Bc-bio	12cM	4.43	12cM	4.13	12cM	4.03	12cM	4.41	12cM	5.11
5	Fc-car	0cM	1.37	91cM	1.53	91cM	1.22	0cM	1.30	0cM	1.35
6	Fc-bio	46cM	9.03	46cM	9.15	46cM	8.96	46cM	8.74	45cM	8.84
6	Bc-bio	0cM	2.58	0cM	2.45	0cM	2.39	0cM	2.57	0cM	3.58
6	Fc-car	54cM	7.35	53cM	7.03	54cM	7.03	54cM	7.14	53cM	7.01
7	Fc-bio	25cM	4.38	25cM	3.78	25cM	4.34	25cM	4.25	25cM	4.55
7	Bc-bio	4cM	4.04	4cM	3.72	3cM	4.17	4cM	4.02	11cM	4.74
7	Fc-car	0cM	2.73	0cM	2.87	0cM	2.94	0cM	2.63	0cM	2.65
8	Fc-bio	41cM	6.52	42cM	6.34	40cM	6.64	41cM	5.98	41cM	6.00
8	Bc-bio	33cM	1.96	33cM	1.83	33cM	2.29	33cM	2.02	31cM	2.79
8	Fc-car	52cM	14.91	53cM	15.9	51cM	15.49	53cM	14.16	52cM	14.73
9	Fc-bio	46cM	3.29	68cM	1.70	46cM	3.26	46cM	3.08	46cM	3.09
9	Bc-bio	89cM	2.95	88cM	2.18	89cM	2.65	89cM	3.01	87cM	2.72
9	Fc-car	0cM	2.13	0cM	1.59	0cM	2.03	0cM	2.50	0cM	2.52
10	Fc-bio	89cM	5.81	87cM	4.41	88cM	5.86	89cM	5.95	88cM	5.40
10	Bc-bio	32cM	2.86	32cM	2.85	32cM	2.84	32cM	2.98	32cM	2.60
10	Fc-car	10cM	4.22	9cM	3.70	10cM	4.31	10cM	5.06	10cM	4.29
11	Fc-bio	111cM	0.66	110cM	1.21	111cM	0.66	0cM	0.95	0cM	0.72
11	Bc-bio	2cM	2.63	2cM	2.44	2cM	2.42	2cM	2.67	2cM	2.09
11	Fc-car	10cM	2.61	10cM	2.81	10cM	2.26	10cM	2.78	10cM	2.39
12	Fc-bio	77cM	4.11	79cM	5.71	77cM	4.07	80cM	4.49	76cM	4.06
12	Bc-bio	98cM	8.46	98cM	9.37	98cM	8.38	98cM	8.46	98cM	8.01
12	Fc-car	97cM	0.71	97cM	0.73	97cM	0.76	97cM	0.53	97cM	0.77
13	Fc-bio	68cM	0.57	58cM	1.09	68cM	0.58	69cM	0.65	68cM	0.52
13	Bc-bio	43cM	5.13	43cM	5.75	44cM	5.26	43cM	5.11	43cM	4.35
13	Fc-car	0cM	5.84	0cM	5.39	0cM	5.67	0cM	5.15	0cM	7.04
14	Fc-bio	0cM	15.45	0cM	14.47	0cM	15.75	0cM	16.64	0cM	22.16
14	Bc-bio	46cM	3.05	47cM	2.81	47cM	2.76	46cM	3.03	47cM	2.77
14	Fc-car	54cM	2.70	53cM	2.60	54cM	2.36	0cM	2.80	45cM	5.63

Appendix 7.1 continued

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
15	Fc-bio	5cM	2.51	3cM	2.40	5cM	2.49	96cM	2.33	5cM	2.28
15	Bc-bio	5cM	2.82	5cM	2.70	5cM	2.81	5cM	2.84	5cM	4.06
15	Fc-car	29cM	0.71	29cM	0.73	29cM	0.78	29cM	0.67	29cM	0.71
16	Fc-bio	52cM	2.72	51cM	4.20	52cM	2.70	52cM	2.84	51cM	2.62
16	Bc-bio	51cM	7.64	51cM	8.21	51cM	7.66	51cM	7.57	51cM	8.87
16	Fc-car	86cM	0.51	40cM	0.51	86cM	0.50	86cM	0.50	86cM	0.52
17	Fc-bio	40cM	1.36	15cM	1.08	40cM	1.40	90cM	1.34	40cM	0.95
17	Bc-bio	73cM	1.39	71cM	1.24	71cM	1.62	73cM	1.39	75cM	1.39
17	Fc-car	64cM	1.02	64cM	1.21	90cM	0.86	64cM	0.76	64cM	1.09
18	Fc-bio	81cM	1.36	81cM	0.87	81cM	1.35	81cM	1.81	81cM	1.30
18	Bc-bio	44cM	1.52	44cM	1.42	44cM	1.58	44cM	1.57	45cM	2.06
18	Fc-car	44cM	2.78	44cM	3.40	44cM	2.71	44cM	3.49	44cM	2.79
19	Fc-bio	0cM	0.90	25cM	0.71	0cM	0.91	0cM	0.80	0cM	1.05
19	Bc-bio	59cM	2.99	58cM	3.12	55cM	3.13	59cM	3.02	59cM	3.60
19	Fc-car	108cM	3.35	108cM	4.02	108cM	3.47	108cM	3.24	108cM	3.18
20	Fc-bio	2cM	1.38	2cM	1.81	2cM	1.36	2cM	1.64	2cM	1.24
20	Bc-bio	54cM	1.19	54cM	0.97	54cM	1.33	54cM	1.19	54cM	1.30
20	Fc-car	56cM	0.67	29cM	0.52	56cM	0.57	56cM	0.84	56cM	0.63
21	Fc-bio	13cM	1.08	12cM	0.75	13cM	1.07	65cM	1.20	65cM	0.98
21	Bc-bio	7cM	1.25	6cM	1.07	9cM	1.17	8cM	1.31	5cM	2.33
21	Fc-car	0cM	0.67	0cM	0.68	0cM	0.61	0cM	0.44	0cM	0.86
22	Fc-bio	0cM	2.07	1cM	2.41	0cM	2.13	0cM	1.96	0cM	1.74
22	Bc-bio	28cM	0.55	28cM	0.63	28cM	0.52	28cM	0.55	1cM	0.55
22	Fc-car	16cM	5.07	16cM	4.88	15cM	5.20	16cM	4.95	14cM	5.48
23	Fc-bio	26cM	3.57	20cM	2.99	27cM	3.58	26cM	3.21	25cM	3.22
23	Bc-bio	0cM	6.09	0cM	5.93	0cM	5.94	0cM	6.11	0cM	6.63
23	Fc-car	59cM	3.37	59cM	3.20	59cM	3.41	59cM	2.81	59cM	3.32
24	Fc-bio	46cM	6.90	46cM	6.27	46cM	6.84	46cM	7.45	46cM	6.47
24	Bc-bio	18cM	0.68	18cM	0.80	18cM	0.62	18cM	0.69	18cM	0.84
24	Fc-car	31cM	1.49	35cM	1.71	35cM	1.61	31cM	1.21	33cM	1.66
25	Fc-bio	0cM	1.42	0cM	1.52	0cM	1.41	0cM	1.31	0cM	1.53
25	Bc-bio	0cM	0.49	2cM	0.49	1cM	0.50	0cM	0.49	10cM	0.64
25	Fc-car	0cM	1.44	0cM	1.40	0cM	1.41	0cM	1.59	0cM	1.49
26	Fc-bio	0cM	0.25	0cM	0.21	0cM	0.25	0cM	0.30	0cM	0.18
26	Bc-bio	0cM	2.14	0cM	2.05	0cM	2.06	0cM	2.12	0cM	3.57
26	Fc-car	69cM	1.14	69cM	1.12	69cM	1.21	69cM	1.23	69cM	1.20
27	Fc-bio	38cM	0.11	18cM	0.17	37cM	0.11	38cM	0.08	48cM	0.07
27	Bc-bio	8cM	3.53	8cM	3.24	8cM	3.46	8cM	3.49	0cM	2.29
27	Fc-car	1cM	1.84	5cM	1.56	0cM	1.78	2cM	1.94	0cM	1.56
28	Fc-bio	52cM	2.4	52cM	1.91	52cM	2.37	52cM	2.89	52cM	2.56
28	Bc-bio	41cM	0.93	41cM	1.10	41cM	1.11	41cM	0.94	41cM	1.02
28	Fc-car	50cM	0.57	48cM	0.78	47cM	0.61	48cM	0.36	51cM	0.66
29	Fc-bio	58cM	0.57	44cM	1.07	58cM	0.58	44cM	0.60	44cM	0.85
29	Bc-bio	9cM	2.12	9cM	2.11	9cM	1.99	9cM	2.11	9cM	1.70
29	Fc-car	64cM	0.42	64cM	0.40	64cM	0.34	64cM	0.39	64cM	0.46

Appendix 7.2 Most likely position and F-statistic values of putative QTL detected by individual family analysis (Sire 368 family)

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
1	Fc-bio	19cM	2.00	112cM	2.06	116cM	1.90	19cM	2.47	19cM	2.23
1	Bc-bio	95cM	1.25	23cM	1.29	95cM	1.31	23cM	1.49	100cM	1.40
1	Fc-car	43cM	1.77	43cM	1.49	43cM	2.17	43cM	2.59	43cM	2.12
2	Fc-bio	46cM	0.34	46cM	0.47	46cM	0.25	0cM	0.23	46cM	0.40
2	Bc-bio	64cM	1.03	98cM	1.35	2cM	0.83	98cM	1.27	99cM	1.73
2	Fc-car	54cM	1.84	104cM	2.34	54cM	2.44	56cM	2.39	55cM	2.64
3	Fc-bio	109cM	4.84	109cM	4.66	109cM	5.26	109cM	5.05	109cM	4.91
3	Bc-bio	35cM	1.00	31cM	1.29	33cM	1.97	33cM	0.71	26cM	1.26
3	Fc-car	109cM	4.37	109cM	4.26	109cM	3.77	109cM	4.05	108cM	4.09
4	Fc-bio	50cM	1.50	50cM	1.39	59cM	1.45	50cM	1.82	50cM	1.51
4	Bc-bio	88cM	2.90	87cM	2.91	88cM	3.40	86cM	2.49	86cM	1.67
4	Fc-car	0cM	1.72	0cM	1.95	0cM	1.52	0cM	1.80	0cM	2.16
5	Fc-bio	27cM	1.00	82cM	0.69	26cM	1.07	23cM	1.06	27cM	1.01
5	Bc-bio	91cM	3.33	91cM	3.20	91cM	2.75	91cM	2.89	91cM	2.42
5	Fc-car	65cM	6.59	65cM	5.63	65cM	6.79	65cM	7.09	65cM	6.27
6	Fc-bio	68cM	7.96	67cM	8.77	67cM	7.62	60cM	10.45	68cM	8.69
6	Bc-bio	0cM	2.06	0cM	1.65	0cM	2.37	3cM	2.25	0cM	2.94
6	Fc-car	47cM	3.55	46cM	3.06	50cM	3.62	47cM	2.56	51cM	4.41
7	Fc-bio	0cM	1.72	0cM	1.86	126cM	1.75	0cM	2.10	126cM	1.97
7	Bc-bio	46cM	5.58	46cM	6.49	43cM	7.21	47cM	4.27	47cM	3.27
7	Fc-car	132cM	3.85	132cM	3.76	132cM	4.52	132cM	4.07	132cM	2.48
8	Fc-bio	29cM	1.03	29cM	1.01	29cM	1.17	29cM	1.40	29cM	1.10
8	Bc-bio	7cM	0.31	29cM	0.45	6cM	0.18	2cM	0.23	35cM	0.60
8	Fc-car	112cM	1.97	112cM	2.40	112cM	1.98	112cM	2.48	112cM	2.19
9	Fc-bio	104cM	3.66	104cM	3.49	104cM	3.76	104cM	3.20	104cM	3.36
9	Bc-bio	4cM	1.70	86cM	3.19	5cM	2.04	4cM	1.90	4cM	2.14
9	Fc-car	73cM	6.41	72cM	2.86	72cM	6.36	75cM	6.63	76cM	8.83
10	Fc-bio	69cM	2.66	71cM	2.61	69cM	2.44	69cM	2.79	69cM	3.13
10	Bc-bio	73cM	0.75	69cM	1.05	13cM	0.74	73cM	0.87	42cM	0.84
10	Fc-car	49cM	2.45	49cM	2.96	49cM	2.57	49cM	2.60	49cM	2.72
11	Fc-bio	98cM	15.29	98cM	15.17	104cM	16.89	97cM	14.29	104cM	16.29
11	Bc-bio	53cM	0.51	51cM	0.39	52cM	0.70	51cM	0.58	54cM	0.78
11	Fc-car	51cM	1.33	51cM	1.53	51cM	1.67	51cM	1.74	51cM	1.56
12	Fc-bio	0cM	2.85	0cM	3.30	0cM	2.59	0cM	3.13	0cM	3.17
12	Bc-bio	37cM	1.88	102cM	1.64	37cM	1.72	37cM	1.89	102cM	2.32
12	Fc-car	74cM	17.87	75cM	17.74	74cM	18.23	75cM	16.94	73cM	19.91
13	Fc-bio	82cM	3.45	82cM	3.08	82cM	3.58	82cM	3.43	82cM	3.18
13	Bc-bio	7cM	3.14	3cM	3.44	9cM	2.49	4cM	3.83	11cM	4.53
13	Fc-car	65cM	2.28	65cM	1.62	65cM	2.28	65cM	2.55	88cM	2.64
14	Fc-bio	11cM	0.75	12cM	0.92	10cM	0.67	8cM	0.93	22cM	5.50
14	Bc-bio	30cM	1.76	30cM	1.35	30cM	1.32	30cM	1.90	21cM	5.44
14	Fc-car	54cM	3.20	54cM	2.81	54cM	2.86	54cM	3.21	30cM	1.67
15	Fc-bio	80cM	6.18	80cM	6.15	80cM	6.08	80cM	6.78	80cM	6.55
15	Bc-bio	21cM	5.97	21cM	5.43	20cM	5.72	19cM	5.15	26cM	5.90

Appendix 7.2 continued

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
15	Fc-car	48cM	0.38	50cM	0.71	73cM	0.41	45cM	0.42	75cM	0.80
16	Fc-bio	81cM	0.79	81cM	0.78	81cM	0.95	81cM	0.89	81cM	0.86
16	Bc-bio	85cM	6.94	82cM	6.51	85cM	6.91	85cM	6.18	78cM	7.81
16	Fc-car	0cM	3.92	0cM	3.28	0cM	3.78	0cM	3.55	0cM	4.61
17	Fc-bio	81cM	5.69	80cM	5.78	79cM	6.36	81cM	5.56	81cM	5.11
17	Bc-bio	70cM	7.53	72cM	7.72	70cM	6.83	67cM	8.27	70cM	4.95
17	Fc-car	23cM	2.8	83cM	2.54	26cM	2.39	23cM	2.74	19cM	2.52
18	Fc-bio	81cM	0.79	81cM	0.72	81cM	0.77	45cM	0.69	81cM	0.9
18	Bc-bio	2cM	2.57	3cM	3.86	4cM	2.30	4cM	2.17	2cM	1.62
18	Fc-car	44cM	0.98	44cM	0.94	44cM	1.01	44cM	1.40	44cM	1.00
19	Fc-bio	89cM	1.28	90cM	1.41	84cM	1.53	90cM	1.23	87cM	1.27
19	Bc-bio	90cM	1.44	90cM	1.67	90cM	1.00	90cM	1.49	90cM	1.74
19	Fc-car	90cM	1.62	90cM	2.00	90cM	2.22	90cM	1.96	90cM	1.85
20	Fc-bio	18cM	5.56	18cM	5.49	17cM	5.95	19cM	5.51	17cM	5.36
20	Bc-bio	28cM	5.43	28cM	7.09	28cM	6.53	28cM	5.04	28cM	5.43
20	Fc-car	17cM	7.82	17cM	7.64	17cM	9.04	17cM	7.66	17cM	8.63
21	Fc-bio	63cM	1.33	63cM	1.32	63cM	1.37	63cM	1.7	63cM	1.24
21	Bc-bio	75cM	0.44	75cM	0.56	75cM	0.41	75cM	0.64	75cM	0.90
21	Fc-car	0cM	4.93	0cM	4.70	0cM	4.59	0cM	4.99	0cM	5.61
22	Fc-bio	28cM	0.70	28cM	0.53	28cM	0.65	28cM	0.71	28cM	0.71
22	Bc-bio	26cM	0.77	26cM	1.28	26cM	0.67	26cM	0.88	26cM	1.08
22	Fc-car	45cM	1.54	48cM	1.42	48cM	1.72	47cM	1.44	43cM	1.22
23	Fc-bio	60cM	2.34	61cM	2.16	59cM	2.60	62cM	2.28	59cM	2.46
23	Bc-bio	63cM	2.13	65cM	2.61	64cM	1.68	59cM	2.18	57cM	1.76
23	Fc-car	68cM	5.27	69cM	6.88	68cM	4.68	67cM	4.75	67cM	6.13
24	Fc-bio	44cM	3.89	42cM	4.13	44cM	3.61	45cM	4.79	45cM	4.04
24	Bc-bio	45cM	0.78	0cM	1.22	48cM	1.23	0cM	0.91	48cM	1.36
24	Fc-car	48cM	6.82	48cM	8.27	48cM	5.95	48cM	6.61	48cM	7.32
25	Fc-bio	32cM	0.65	32cM	0.68	32cM	0.60	32cM	0.62	32cM	0.56
25	Bc-bio	0cM	1.32	0cM	1.70	0cM	1.11	0cM	0.83	0cM	1.57
25	Fc-car	32cM	2.18	32cM	1.82	32cM	2.45	32cM	2.35	32cM	1.84
26	Fc-bio	24cM	0.68	24cM	0.65	24cM	0.56	24cM	0.72	24cM	0.88
26	Bc-bio	35cM	0.66	43cM	0.6	32cM	0.97	30cM	0.53	30cM	1.62
26	Fc-car	31cM	3.89	32cM	4.42	32cM	3.37	31cM	3.93	34cM	2.85
27	Fc-bio	30cM	0.67	28cM	0.52	28cM	0.74	31cM	0.91	27cM	0.58
27	Bc-bio	0cM	6.29	0cM	6.11	0cM	5.94	0cM	5.46	0cM	5.36
27	Fc-car	7cM	2.87	8cM	2.70	7cM	3.30	5cM	2.61	3cM	3.56
28	Fc-bio	35cM	2.78	36cM	2.41	35cM	2.66	36cM	3.24	35cM	2.89
28	Bc-bio	51cM	0.29	51cM	0.35	51cM	0.18	51cM	0.63	51cM	0.15
28	Fc-car	35cM	0.43	35cM	0.81	35cM	0.54	17cM	0.25	35cM	0.54
29	Fc-bio	40cM	2.18	40cM	2.36	40cM	2.29	40cM	2.37	40cM	2.17
29	Bc-bio	42cM	4.93	40cM	5.48	41cM	4.56	45cM	4.82	44cM	5.93
29	Fc-car	34cM	3.21	36cM	2.78	34cM	2.99	37cM	3.58	35cM	2.99

Appendix 7.3 Most likely position and F-statistic values of putative QTL detected by individual family analysis (Sire 398 family)

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	Traits	position	F-value	position	F-value	position	F-value	position	F-value	position	F-value
1	Fc-bio	23cM	2.47	23cM	2.01	0cM	6.72	23cM	3.36	23cM	3.36
1	Bc-bio	133cM	2.06	133cM	2.44	3cM	2.21	133cM	1.55	133cM	1.55
1	Fc-car	133cM	0.55	95cM	1.15	9cM	2.49	107cM	0.44	107cM	0.44
2	Fc-bio	87cM	1.09	93cM	0.99	99cM	3.35	93cM	1.87	93cM	1.87
2	Bc-bio	5cM	0.42	5cM	0.38	104cM	0.66	104cM	0.65	104cM	0.65
2	Fc-car	28cM	2.95	27cM	3.60	28cM	3.94	28cM	3.00	28cM	3.00
3	Fc-bio	82cM	3.74	82cM	3.93	90cM	3.38	83cM	2.96	83cM	2.96
3	Bc-bio	78cM	3.65	78cM	3.70	26cM	2.99	82cM	2.89	82cM	2.89
3	Fc-car	57cM	2.14	57cM	2.28	51cM	3.28	48cM	2.13	48cM	2.13
4	Fc-bio	0cM	3.96	0cM	3.31	0cM	1.99	0cM	2.32	0cM	2.32
4	Bc-bio	0cM	2.38	1cM	2.14	3cM	0.81	10cM	1.12	10cM	1.12
4	Fc-car	0cM	4.55	2cM	3.94	2cM	2.38	2cM	3.16	2cM	3.16
5	Fc-bio	12cM	1.90	15cM	2.55	28cM	1.29	109cM	0.64	109cM	0.64
5	Bc-bio	91cM	0.57	8cM	0.60	95cM	0.64	91cM	1.91	91cM	1.91
5	Fc-car	115cM	4.22	114cM	3.30	116cM	4.23	117cM	5.22	117cM	5.22
6	Fc-bio	74cM	0.85	74cM	0.41	46cM	0.36	74cM	0.78	74cM	0.78
6	Bc-bio	61cM	1.45	58cM	1.19	62cM	0.52	0cM	0.82	0cM	0.82
6	Fc-car	0cM	1.01	118cM	0.67	46cM	1.00	92cM	1.01	92cM	1.01
7	Fc-bio	130cM	0.89	67cM	1.67	39cM	0.40	65cM	0.61	65cM	0.61
7	Bc-bio	25cM	1.39	25cM	0.95	25cM	0.79	25cM	1.52	25cM	1.52
7	Fc-car	131cM	2.45	131cM	2.42	134cM	0.99	130cM	1.44	130cM	1.44
8	Fc-bio	8cM	1.29	31cM	1.15	113cM	1.74	8cM	1.57	8cM	1.57
8	Bc-bio	0cM	3.65	0cM	3.94	0cM	7.33	0cM	3.56	0cM	3.56
8	Fc-car	53cM	2.17	53cM	1.42	41cM	2.89	53cM	1.66	53cM	1.66
9	Fc-bio	93cM	1.07	92cM	0.95	91cM	3.10	46cM	1.52	46cM	1.52
9	Bc-bio	15cM	2.56	13cM	2.29	27cM	2.35	40cM	4.02	40cM	4.02
9	Fc-car	12cM	0.98	6cM	0.85	51cM	1.10	46cM	1.15	46cM	1.15
10	Fc-bio	49cM	3.73	49cM	3.35	50cM	5.55	49cM	2.95	49cM	2.95
10	Bc-bio	43cM	1.92	43cM	1.78	43cM	2.91	43cM	1.66	43cM	1.66
10	Fc-car	61cM	2.60	64cM	2.77	65cM	4.27	62cM	2.07	62cM	2.07
11	Fc-bio	3cM	4.46	87cM	4.51	44cM	5.42	5cM	5.50	5cM	5.50
11	Bc-bio	19cM	2.24	42cM	2.04	42cM	3.19	61cM	3.64	61cM	3.64
11	Fc-car	48cM	2.88	47cM	2.53	50cM	5.33	63cM	3.84	63cM	3.84
12	Fc-bio	37cM	2.76	37cM	1.78	81cM	2.65	37cM	2.54	37cM	2.54
12	Bc-bio	36cM	0.58	35cM	0.41	94cM	1.60	64cM	0.34	64cM	0.34
12	Fc-car	0cM	0.45	102cM	0.76	101cM	2.47	0cM	0.67	0cM	0.67
13	Fc-bio	33cM	0.88	33cM	0.60	88cM	1.19	0cM	1.39	0cM	1.39
13	Bc-bio	88cM	1.12	88cM	1.03	81cM	2.11	86cM	0.92	86cM	0.92
13	Fc-car	30cM	4.02	30cM	3.39	32cM	2.83	35cM	3.89	35cM	3.89
14	Fc-bio	34cM	2.22	33cM	1.81	0cM	0.13	38cM	1.91	38cM	1.91
14	Bc-bio	82cM	1.16	82cM	1.40	0cM	2.99	0cM	2.11	0cM	2.11
14	Fc-car	0cM	2.34	0cM	2.63	21cM	10.39	0cM	3.89	0cM	3.89
15	Fc-bio	45cM	7.23	46cM	8.75	89cM	4.25	22cM	5.04	22cM	5.04
15	Bc-bio	98cM	2.21	98cM	2.64	96cM	1.73	95cM	1.18	95cM	1.18

Appendix 7.3 continued

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
15	Fc-car	53cM	3.46	53cM	3.90	94cM	2.39	94cM	1.92	94cM	1.92
16	Fc-bio	17cM	0.42	14cM	0.40	13cM	2.51	20cM	0.42	20cM	0.42
16	Bc-bio	20cM	0.70	20cM	0.73	25cM	0.14	86cM	1.06	86cM	1.06
16	Fc-car	75cM	0.39	70cM	0.54	11cM	0.75	72cM	1.10	72cM	1.10
17	Fc-bio	40cM	1.90	40cM	1.46	64cM	2.89	0cM	0.89	0cM	0.89
17	Bc-bio	0cM	2.64	0cM	2.36	0cM	2.72	10cM	3.27	10cM	3.27
17	Fc-car	0cM	2.01	0cM	1.66	0cM	2.20	0cM	2.41	0cM	2.41
18	Fc-bio	60cM	1.13	62cM	2.43	0cM	0.82	58cM	2.41	58cM	2.41
18	Bc-bio	52cM	0.26	52cM	0.54	27cM	0.05	52cM	1.36	52cM	1.36
18	Fc-car	18cM	1.94	18cM	2.14	18cM	1.21	18cM	2.08	18cM	2.08
19	Fc-bio	78cM	3.83	83cM	3.80	53cM	1.79	78cM	2.71	78cM	2.71
19	Bc-bio	52cM	3.45	52cM	3.19	49cM	3.83	54cM	2.09	54cM	2.09
19	Fc-car	16cM	1.55	101cM	0.86	16cM	0.52	14cM	1.14	14cM	1.14
20	Fc-bio	17cM	1.34	21cM	1.26	17cM	2.21	1cM	0.57	1cM	0.57
20	Bc-bio	31cM	1.45	31cM	1.66	17cM	1.21	69cM	0.46	69cM	0.46
20	Fc-car	49cM	1.47	49cM	1.39	17cM	1.48	1cM	1.56	1cM	1.56
21	Fc-bio	21cM	0.90	63cM	0.88	21cM	1.95	0cM	0.59	0cM	0.59
21	Bc-bio	21cM	1.03	21cM	0.81	21cM	1.52	0cM	0.95	0cM	0.95
21	Fc-car	0cM	0.68	0cM	1.14	64cM	1.72	0cM	1.30	0cM	1.30
22	Fc-bio	68cM	2.44	68cM	2.01	71cM	4.76	16cM	2.49	16cM	2.49
22	Bc-bio	0cM	1.62	0cM	1.42	0cM	2.51	0cM	2.59	0cM	2.59
22	Fc-car	80cM	0.88	80cM	1.23	0cM	1.14	80cM	2.72	80cM	2.72
23	Fc-bio	69cM	0.55	69cM	0.82	53cM	0.96	69cM	0.70	69cM	0.70
23	Bc-bio	67cM	0.68	67cM	0.56	67cM	1.66	67cM	0.54	67cM	0.54
23	Fc-car	4cM	3.01	4cM	2.71	6cM	0.60	6cM	2.30	6cM	2.30
24	Fc-bio	44cM	2.55	44cM	2.67	44cM	3.31	44cM	1.89	44cM	1.89
24	Bc-bio	40cM	0.76	40cM	0.71	46cM	0.95	46cM	0.24	46cM	0.24
24	Fc-car	47cM	0.54	47cM	0.52	46cM	0.83	47cM	0.99	47cM	0.99
25	Fc-bio	32cM	0.34	32cM	0.50	9cM	0.99	9cM	0.31	9cM	0.31
25	Bc-bio	32cM	0.21	32cM	0.29	11cM	0.41	32cM	0.21	32cM	0.21
25	Fc-car	32cM	1.96	32cM	2.18	32cM	1.22	32cM	1.70	32cM	1.70
26	Fc-bio	64cM	0.37	64cM	0.17	13cM	0.95	64cM	0.34	64cM	0.34
26	Bc-bio	62cM	1.14	62cM	0.95	62cM	0.91	62cM	1.17	62cM	1.17
26	Fc-car	48cM	2.81	57cM	2.23	48cM	3.47	48cM	2.84	48cM	2.84
27	Fc-bio	0cM	1.99	0cM	1.66	45cM	1.72	0cM	1.65	0cM	1.65
27	Bc-bio	10cM	0.45	64cM	0.51	45cM	0.78	64cM	1.72	64cM	1.72
27	Fc-car	0cM	0.91	64cM	1.66	64cM	0.29	63cM	2.14	63cM	2.14
28	Fc-bio	0cM	1.56	0cM	1.64	0cM	3.64	0cM	1.35	0cM	1.35
28	Bc-bio	18cM	1.58	20cM	1.42	17cM	1.19	25cM	1.13	25cM	1.13
28	Fc-car	52cM	3.23	52cM	2.15	52cM	4.11	52cM	3.36	52cM	3.36
29	Fc-bio	40cM	2.32	40cM	2.07	64cM	2.98	40cM	3.10	40cM	3.10
29	Bc-bio	61cM	0.67	61cM	0.60	14cM	1.58	61cM	1.34	61cM	1.34
29	Fc-car	64cM	1.49	64cM	1.66	40cM	1.62	64cM	1.05	64cM	1.05

Appendix 7.4 Position and F-statistic values of putative QTL detected by across family analysis

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
1	Fc-bio	25	2.07	25	2.06	0	4.36	25	2.54	27	2.04
1	Bc-bio	107	1.61	107	1.40	1	2.13	76	1.48	107	1.63
1	Fc-car	38	1.44	38	1.61	9	1.96	39	1.86	37	1.53
2	Fc-bio	96	0.70	98	0.54	46	1.25	97	0.80	95	0.65
2	Bc-bio	6	1.82	5	1.55	64	1.86	4	2.02	5	1.93
2	Fc-car	47	2.52	46	2.28	54	3.23	49	2.62	47	2.69
3	Fc-bio	82	2.79	79	3.25	106	1.66	82	2.70	82	2.76
3	Bc-bio	77	3.49	77	3.63	77	1.85	77	3.30	77	3.77
3	Fc-car	80	3.90	71	4.00	105	2.75	87	3.76	80	4.22
4	Fc-bio	0	3.36	0	2.86	0	2.09	0	2.81	0	3.60
4	Bc-bio	2	4.64	2	4.89	5	3.37	4	4.02	2	4.77
4	Fc-car	0	2.99	0	2.64	0	2.23	0	2.72	0	3.04
5	Fc-bio	22	1.14	24	2.03	21	1.26	105	0.86	100	1.22
5	Bc-bio	21	1.06	23	1.26	8	1.24	8	0.90	21	1.09
5	Fc-car	109	2.70	109	2.64	109	2.09	113	2.55	109	2.79
6	Fc-bio	74	1.38	74	1.24	47	1.84	50	1.37	52	1.38
6	Bc-bio	71	1.27	70	1.14	0	1.06	74	1.02	70	1.38
6	Fc-car	60	2.19	60	2.09	60	3.21	60	1.90	60	2.11
7	Fc-bio	130	1.07	131	1.16	0	1.03	0	1.20	0	1.13
7	Bc-bio	49	0.82	132	0.66	49	1.11	51	1.15	25	0.74
7	Fc-car	134	1.07	134	1.11	82	1.00	134	0.89	134	0.99
8	Fc-bio	91	0.89	8	1.02	19	0.90	8	0.66	91	0.92
8	Bc-bio	31	0.82	36	0.88	33	1.38	32	1.30	37	0.86
8	Fc-car	50	1.75	50	1.52	47	2.76	50	1.94	50	1.68
9	Fc-bio	46	1.98	102	1.41	90	2.68	46	2.03	46	1.85
9	Bc-bio	47	4.26	32	3.93	52	3.81	32	4.48	48	4.39
9	Fc-car	16	3.19	0	3.31	0	3.12	16	2.93	16	3.24
10	Fc-bio	50	2.73	51	2.29	54	3.83	52	2.69	49	2.65
10	Bc-bio	60	2.97	43	2.55	43	4.13	43	2.99	60	3.01
10	Fc-car	79	2.20	80	1.95	80	3.00	78	2.05	79	2.31
11	Fc-bio	87	5.31	87	5.27	110	3.56	87	3.81	87	5.62
11	Bc-bio	13	2.99	42	3.14	42	2.73	59	3.05	43	3.05
11	Fc-car	10	1.43	10	1.37	109	1.33	60	1.34	10	1.54
12	Fc-bio	39	1.09	47	1.11	92	1.73	0	1.35	41	1.05
12	Bc-bio	102	2.19	102	1.85	87	3.39	81	1.89	102	2.23
12	Fc-car	37	2.29	81	2.35	38	3.19	37	2.30	39	2.48
13	Fc-bio	88	1.04	88	1.03	43	1.02	43	1.30	88	1.15
13	Bc-bio	66	3.34	66	3.19	61	3.85	60	3.45	62	3.47
13	Fc-car	43	1.64	33	1.47	33	1.5	43	2.12	43	1.58
14	Fc-bio	0	3.86	0	3.15	0	4.00	0	4.10	20	5.38
14	Bc-bio	66	1.15	66	1.13	66	2.47	66	0.67	23	2.58
14	Fc-car	60	0.97	58	1.15	24	2.48	60	0.83	62	0.60
15	Fc-bio	16	6.49	15	7.68	83	3.71	19	6.16	17	6.10
15	Bc-bio	5	4.60	7	4.74	11	2.19	9	3.87	5	4.85

Appendix 7.4 continued

		no additional SNP		with *ALDH8A1* SNP16		with *BCO2* W80X		with *PPARGC1A* SNP12		with *RDHE2* SNP2	
chromosome number	**Traits**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**	**position**	**F-value**
15	Fc-car	48	2.41	48	2.80	66	1.76	50	2.55	47	2.41
16	Fc-bio	81	0.75	24	0.78	14	1.25	81	0.68	15	0.64
16	Bc-bio	51	2.46	51	2.35	51	2.3	53	2.74	51	2.41
16	Fc-car	0	1.97	1	1.81	0	2.35	0	1.98	0	2.06
17	Fc-bio	78	1.04	0	0.85	40	1.38	81	1.08	79	1.10
17	Bc-bio	11	2.47	10	2.22	45	2.35	13	2.43	10	2.48
17	Fc-car	0	1.99	0	1.91	0	1.43	0	1.89	0	2.08
18	Fc-bio	81	0.89	0	0.97	27	0.98	52	0.86	81	0.85
18	Bc-bio	40	0.95	40	0.91	40	1.07	40	1.50	40	0.99
18	Fc-car	27	1.03	37	1.04	27	1.21	37	1.34	37	1.02
19	Fc-bio	16	1.50	77	1.31	21	1.33	72	1.10	16	1.76
19	Bc-bio	72	2.68	72	2.92	53	2.73	70	2.26	72	2.75
19	Fc-car	93	2.08	93	2.25	94	2.51	95	2.14	93	2.09
20	Fc-bio	20	1.93	21	2.02	21	1.59	22	1.53	19	1.85
20	Bc-bio	26	1.93	25	2.18	26	1.49	25	1.39	26	2.01
20	Fc-car	17	3.45	17	3.53	17	4.00	17	3.56	17	3.69
21	Fc-bio	21	0.42	75	0.58	21	0.36	21	0.44	21	0.41
21	Bc-bio	75	1.30	75	1.51	75	0.91	75	1.14	75	1.33
21	Fc-car	0	1.70	0	1.63	0	1.89	0	2.01	0	1.96
22	Fc-bio	66	2.53	64	2.54	67	3.72	62	1.77	66	2.75
22	Bc-bio	61	0.93	61	0.87	61	1.10	80	1.08	61	0.95
22	Fc-car	28	0.65	1	0.70	0	0.89	80	0.61	6	0.61
23	Fc-bio	61	1.10	60	1.40	27	1.23	61	1.12	61	1.19
23	Bc-bio	69	0.66	69	0.58	0	0.99	69	0.78	69	0.62
23	Fc-car	0	1.81	0	1.71	59	1.55	0	1.51	0	1.82
24	Fc-bio	41	3.27	43	3.15	40	4.22	44	4.15	40	2.97
24	Bc-bio	0	0.61	0	0.69	0	0.84	48	0.85	48	0.59
24	Fc-car	48	2.34	48	2.33	48	2.43	48	2.00	48	2.28
25	Fc-bio	9	0.40	9	0.39	9	0.62	9	0.42	32	0.40
25	Bc-bio	9	0.60	9	0.49	9	1.02	9	0.69	9	0.78
25	Fc-car	32	1.25	32	1.41	32	1.23	32	1.26	32	1.00
26	Fc-bio	46	0.47	49	0.49	35	0.89	20	0.39	46	0.54
26	Bc-bio	69	1.02	69	0.95	69	1.47	35	1.01	69	0.97
26	Fc-car	24	1.70	24	1.72	24	2.07	24	1.55	24	1.53
27	Fc-bio	10	0.68	10	0.74	45	0.86	10	0.51	10	0.72
27	Bc-bio	16	2.15	16	2.05	12	2.46	19	3.14	15	2.14
27	Fc-car	0	2.32	0	2.55	0	2.35	0	1.98	0	2.32
28	Fc-bio	52	1.84	52	1.77	52	1.67	52	1.80	52	1.89
28	Bc-bio	41	1.78	41	1.84	41	1.64	41	1.83	41	1.81
28	Fc-car	52	1.36	52	1.22	52	0.87	52	1.20	52	1.30
29	Fc-bio	40	2.07	40	1.84	49	1.53	40	2.43	40	1.90
29	Bc-bio	40	0.49	4	0.55	7	0.58	40	0.79	40	0.53
29	Fc-car	40	1.49	40	1.51	39	1.83	40	1.87	40	1.48

Appendix 8 Sequencing alignment

Appendix 8.1 RDHE2 and RDHE2 similar genes from bovine and human

Seq No	Species	Gene name	Length (aa)	Description	Accession No	Database
1	BOVINE	RDHE2 (SDRC6)	316	Bovine epidermal retinal dehydrogenase 2	ENSBTAP00000048107	ENSEMBL
2	BOVINE	SDR16C5 (RDHE2 SIMILAR)	313	short chain dehydrogenase/reductase family 16C, member 5 similar to epidermal retinal dehydrogenase 2	ENSBTAP00000024714	ENSEMBL
3	HUMO	RDHE2 (SDR16C5-001)	309	short chain dehydrogenase/reductase family 16C, member 5	ENSP00000307607	ENSEMBL
4	HUMO	RDHE2 (SDR16C5-002)	318	short chain dehydrogenase/reductase family 16C, member 5	ENSP00000431010	ENSEMBL
5	HUMO	RDHE2 (SDR16C5-003)	265	short chain dehydrogenase/reductase family 16C, member 5	ENSP00000379947	ENSEMBL
6	HUMO	RDHE2 (SDR16C5)	309	Human epidermal retinal dehydrogenase 2 Homologs of the BOVINE SDR16C5 gene	NP_620419	NCBI

Appendix 8.2 Pairwise alignments score for bovine, human *RDHE2* and *RDHE2 similar* genes

Sequence aligned*	Score (%)
Sequences (1:2)	54
Sequences (1:3)	55
Sequences (1:4)	52
Sequences (1:5)	54
Sequences (1:6)	55
Sequences (2:3)	78
Sequences (2:4)	71
Sequences (2:5)	77
Sequences (2:6)	78
Sequences (3:4)	90
Sequences (3:5)	100
Sequences (3:6)	100
Sequences (4:5)	88
Sequences (4:6)	90
Sequences (5:6)	100

*** Sequence No: described to in Appendix 8.1**

Appendix 8.3 Bovine, human *RDHE2* and *RDHE2 similar* genes amino acid sequence alignments

```
5       ----MSFNLQSSKKLFIFLGKSLFSLLEAMIFALLPKPRKNVAGEIVLITGAGSGLGRLL 56
6       ----MSFNLQSSKKLFIFLGKSLFSLLEAMIFALLPKPRKNVAGEIVLITGAGSGLGRLL 56
3       ----MSFNLQSSKKLFIFLGKSLFSLLEAMIFALLPKPRKNVAGEIVLITGAGSGLGRLL 56
4       ----MSFNLQSSKKLFIFLGKSLFSLLEAMIFALLPKPRKNVAGEIVLITGAGSGLGRLL 56
2       MSQNMVLKLKAAKKLLIFLGKSVLALVEAVVFVIIPKPRKNVAGEIVLITGAGSGLGRLL 60
1       ----MNVLLDTS----IFLGKFLYYFLESLYYKIIPKKKKDVTGEIVLITGAASGLGRLL 52
            * . *.::    ***** :  ::*:: : ::** :*:*:*********.*******

5       ALQFARLGSVLVLWDINKEGNEETCKMAREAGATRVHAYTCDCSQKEGVYRVADQ----- 111
6       ALQFARLGSVLVLWDINKEGNEETCKMAREAGATRVHAYTCDCSQKEGVYRVADQVKKEV 116
3       ALQFARLGSVLVLWDINKEGNEETCKMAREAGATRVHAYTCDCSQKEGVYRVADQVKKEV 116
4       ALQFARLGSVLVLWDINKEGNEETCKMAREAGATRVHAYTCDCSQKEGVYRVADQVKKEV 116
2       ALKFAQLGSVLVLWDISQESNEETCKMAVEAGATRVYAYTCDCSRKEEIYRVANQVKKEV 120
1       AIKFASLGAILVLWDINEEGNMETCRIIKEERDAKVFAYTCDCSNRQDVYRVADQVKKEV 112
          *:** **::******.:*.* ***::  *   ::*.*******.:: :****:*

5       ------------------------------------TYKAFLPAMIANDHGHLVCIS 132
6       GDVSILINNAGIVTGKKFLDCPDELMEKSFDVNFKAHLWTYKAFLPAMIANDHGHLVCIS 176
3       GDVSILINNAGIVTGKKFLDCPDELMEKSFDVNFKAHLWTYKAFLPAMIANDHGHLVCIS 176
4       GDVSILINNAGIVTGKKFLDCPDELMEKSFDVNFKAHLWTYKAFLPAMIANDHGHLVCIS 176
2       GDASILINNAGIVTGRKFMDCPDELIEKSLDVNFKSHIWTYKAFLPAMIANNRGHLVCIS 180
1       GNVTILINNAGVVTGREFLKTPDHMVERSFLVNVMSHFWTYKAFLPAMLEANHGHLVCIS 172
                                             *********:  ::*******

5       SSAGLSGVNGLADYCASKFAAFGFAESVFVETFVQKQKGIKTTIVCPFFIKTGMFEGCTT 192
6       SSAGLSGVNGLADYCASKFAAFGFAESVFVETFVQKQKGIKTTIVCPFFIKTGMFEGCTT 236
3       SSAGLSGVNGLADYCASKFAAFGFAESVFVETFVQKQKGIKTTIVCPFFIKTGMFEGCTT 236
4       SSAGLSGVNGLADYCASKFAAFGFAESVFVETFVQKQKGIKTTIVCPFFIKTGMFEGCTT 236
2       SSAGLIGMNGLADYCASKFAAYGFAESIFLESFTKGQNGIKTTIVCPFFIKTGMFDGCTT 240
1       SFAGIVGINGLSDYCASKFAAYGFAESLHFELKLLQKSKINTTIVCPYFIKTGMFEGCST 232
        * **: *:***:*********:*****:..*     :. *:******:*******:**:*

5       GCPSLLPILEPKYAVEKIVEAILQEKMYLYMPKLLYFMMFLKSFLPLKTG-LLIADYLGI 251
6       GCPSLLPILEPKYAVEKIVEAILQEKMYLYMPKLLYFMMFLKSFLPLKTG-LLIADYLGI 295
3       GCPSLLPILEPKYAVEKIVEAILQEKMYLYMPKLLYFMMFLKSFLPLKTG-LLIADYLGI 295
4       GCPSLLPILEPKYAVEKIVEAILQEKMYLYMPKLLYFMMFLKRLHLAQDGSECLTEAMWD 296
2       GCPSLLPILEPEYAVRKIVDAILQENMYLYMPKFIYFIVFLKSFLPLKLG-LLLGEYLGA 299
1       KYPLLLPMLTQEYAAQSILNAILEEQLYLIMPRFSHVALFLKQIISTNMM-MTMAEYLGM 291
          * ***:*  :**...*::***:*::** **:: :. :*** :   :     : : :

5       LHAMDGFVDQKKKL----------- 265
6       LHAMDGFVDQKKKL----------- 309
3       LHAMDGFVDQKKKL----------- 309
4       RAKDPGQAIRNSGLRSHYSLSL--- 318
2       FNLMDGFTGAKKKN----------- 313
1       DISLASFIEREKSGEVQTKTERKQQ 316
```

Appendix 9 RNA quality

Sample ID	RNA concentration ng/ul	liver Homogenised (mg)	260/280 ratio	Total RNA extracted (ug)
1	224.09	71	1.85	67.23
2	303.98	87	1.96	91.19
3	450.68	77	1.97	135.20
7	391.28	67	1.97	117.38
8	397.4	57	1.96	119.22
11	575.69	77	1.99	172.71
13	457.56	79	1.96	137.27
15	381.82	61	1.95	114.55
18	433.76	82	1.98	130.13
21	563.27	80	2.00	168.98
22	248.85	56	1.94	74.66
23	400.51	67	1.94	120.15
24	217.99	58	1.93	65.40
26	316.23	73	1.96	94.87
27	262.51	69	1.95	78.75
30	238.93	68	1.94	71.68
31	533.27	67	1.96	159.98
32	271.59	44	1.94	81.48
36	352.33	67	1.96	105.70
37	386.7	65	1.97	116.01
44	531.98	75	1.97	159.59
47	673.17	66	2.03	201.95
49	361.02	64	1.94	108.31
53	334.92	50	1.94	100.48
54	440.49	69	1.95	132.15
55	468.17	77	1.94	140.45
61	405.59	71	1.95	121.68
62	368.26	67	1.95	110.48
65	304.24	57	1.93	91.27
69	352.86	67	1.92	105.86
70	318.03	66	1.92	95.41
72	302.18	58	1.93	90.65
73	217.7	68	1.92	65.31
74	344.76	59	1.96	103.43
78	413.41	67	1.96	124.02
79	418.9	61	1.95	125.67
82	415.38	56	1.95	124.61
83	393.41	69	1.94	118.02
85	332.84	73	1.94	99.85
90	518.97	77	1.97	155.69
91	706.63	78	1.96	211.99
95	314.11	56	1.95	94.23
98	446.06	69	1.96	133.82
99	379.69	55	1.95	113.91
100	423.49	66	1.94	127.05
102	288.5	75	1.91	86.55
106	262.77	69	1.86	78.83
107	335.97	73	1.88	100.79
109	344.19	81	1.92	103.26
115	403.86	74	1.94	121.16

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