Histology and genetic control of intramuscular fat deposition and desaturation

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Jose L Valenzuela 04 April 2020

Abstract

There is an increasing world demand for food production. Contemporaneously the market seeks assurance in terms of provenance, quality, and healthiness. While consumers demand tasty, highly and fine marbled meat, its healthiness has become extremely important.

An important component of the organoleptic characteristics and level of healthiness is the quantity of intramuscular fat of the meat and its fatty acid composition. Higher intramuscular fat content, measured as marbling, results in better tenderness, juiciness and flavour, and is often healthier due to a higher content of UFA and MUFA, i.e. oleic acid. The intramuscular fat quantity, measured as marbling, is part of the quality grading and pricing of the carcase.

This thesis examines the deposition of intramuscular fat, contributing to the understanding, and consequently the improvement, of marbling and fat desaturation. The different areas examined are (a) genetics of marbling and fat desaturation, (b) nature of marbling and possibilities of measuring in live animals and (c) similarities between marbling and muscular dystrophy in human, and opportunities for translation of scientific findings between the two species.

One of the simplest ways of assessing the level of desaturation of the fat is measuring its melting temperature (Tm). Fats with higher proportions of unsaturated fatty acids melt at lower temperatures. Current techniques are practical and repeatable and can be done in conjunction with some DNA tests. Here I validate the measurement of Tm of fat obtained in the process of DNA extraction. Using this method, we proved the effects of the breed on the Tm for Red Wagyu (Akaushi) and Black Wagyu, as well as individual differences among Black Wagyu bulls.

I evaluated the effect of breed-specific haplotypes in Bota19 over Tm, establishing the importance of these haplotypes in the inheritance of the trait. I show synteny between the region that contains these haplotypes in cattle and an important region in Hosa 17 involved in some muscular dystrophies. Additionally, these ancestral haplotypes also proved to be practical and successful in the confirmation of the post mortem identity.

The lack of knowledge and deep understanding of intramuscular fat deposition is a big limitation. The inability to reliably measure the trait in live animals limits its measurement and analysis to the carcass stage, and even then, the traditional measurements of marbling are still not objective, reliable and repeatable.

I analysed and described marbling at a microscopic level, designing a microscopic scoring system for marbling. We defined areas suitable for fat and muscle biopsies and measured their Tm and marbling. Later, I compared and validated the results against the muscle commonly used for marble score (*Longissimus dorsi*), showing similarities on histology, marble score and Tm of the surrounding fat. This gives the opportunity for the development of a test to monitor marbling and fat desaturation throughout the animal's life.

Following the previously established genetic relation between marbling and muscular dystrophy, a histological comparison of the two processes was made, revealing common features. Due to the histological commonalities between marbling and human muscular dystrophy, and the relation between the areas of the genome involved in fat and muscle metabolism in both species, I proposed the translation of scientific findings from one species to the other. For example, I observed fat acting as an invasive tissue, which could also be a factor in muscular dystrophy.

An example of a practical way of performing farm trials was presented, as an alternative to achieve statistical significance with small sample size and minimal interference in production

systems, allowing beef farmers to team up with scientists to generate practical knowledge, and to minimise the number of animals needed for research with live animals.

This thesis provides new information that could lead to the development of practical techniques to establish more effective and efficient production systems, favouring grass-feeding and shorter feeding times, with the consequent improvement on animal welfare and reduction of the environmental impact.

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Publications from this thesis and contribution

Lloyd, S.S., **Valenzuela, J.L.**, Steele, E.J. and Dawkins R.L. (2017). Genetics of marbling in Wagyu revealed by the melting temperature of intramuscular and subcutaneous lipids. *International Journal of Food Science*, Volume 2017, Article ID 3948408, 6 pages. (Chapter 3).

Lloyd, S.S., Steele, E.J., **Valenzuela, J.L.** and Dawkins R.L. (2017). Haplotypes for type, degree, and rate of marbling in cattle are syntenic with human muscular dystrophy. *International Journal of Genomics*, Volume 2017, Article ID 6532837, 14 pages. (Chapter 4).

Valenzuela, J.L., Lloyd, S.S., Mastaglia F.L. and Dawkins R.L. (2020). Adipose invasion of muscle in Wagyu cattle: monitoring by histology and melting temperature. *Meat Science* 163, Volume 163, Article ID 108063, 10 pages. (Chapter 6).

Valenzuela, J.L., Lloyd, S.S., Steele, E.J., Mastaglia F.L. and Dawkins R.L. (2019). Interspecies translation: bovine marbling to human muscular dystrophy. *Sakuma, K., Muscular Dystrophies. Intech Open.* (Chapter 7).

Valenzuela, J.L., Lloyd, S.S., Sweeney, J. and Dawkins R.L. (2020). Comparison of feeds for marbling may be facilitated by haplotype pairing. Submitted to *Translational Animal Science.* (Chapter 8).

Confirmation of contribution

Chapters 3, 4, 6, 7 and 8 have been prepared as co-authored manuscripts. The publication status for each manuscript is noted at the beginning of each chapter, as well as my personal contribution in them, summarised as follows:

Chapter 3: maintaining herd records, sampling, DNA testing of animals and carcasses, Tm measuring, and revising and writing part of the manuscript.

Chapter 4: sampling, haplotype testing and parental verification of calves, Tm testing, and revising and correcting the final version of the paper.

Chapter 6: designing the experiment and research plan, developing the microscopic marbling scoring technique, identifying areas for future muscle and fat biopsy, sampling muscle and fat, Tm measuring, evaluating the histology of muscle samples, analysing data, writing of the manuscript.

Chapter 7: identifying histological similarities between marbling and muscular dystrophies, sampling, processing of frozen samples, describing and analysing muscle histology, writing the manuscript.

Chapter 8: designing the experiment and research plan, executing and evaluating feed trials, measuring the different variables and generating the data. Laboratory testing for Tm, data analysis and writing of the manuscript.

I confirm that the contribution attributed to Jose L Valenzuela is correct.

Professor Roger L Dawkins

Dr Sally S Lloyd

Acronyms and terms

AAT	Area of adipose tissue
ACACA	Acetyl-CoA carboxylase alpha, gene or protein
ACC	Acetyl-CoA carboxylase, protein
ADG	Average daily gain
AH	Ancestral haplotype
AK	Akaushi (Red Wagyu)
AWA	Australian Wagyu Association
BMS	Beef marbling score
Bota	Bovine Chromosome
BOTA 19	Bos Taurus Chromosome 19
COP II	Coat protein 2
CVD	Cardiovascular disease
CYO	CY O'Connor Foundation
CYOEVF	CY O'Connor ERADE Village Foundation
DEXA	Dual-energy X-ray absorptiometry
DFAT	Dedifferentiated fat cells
DNA	Deoxyribonucleic acid

DOF	Days on feed
EBV	Estimated breeding value
ELOVL6	Elongase protein 6
ER	Endoplasmic reticulum
EU	European breed
F1	First cross 50% Wagyu
F2	Second cross 75% Wagyu
F3	Third cross 87.5% Wagyu
F4	Forth cross 93.75% Wagyu
FA	Fatty acid
FABP4	Fatty acid binding protein 4, gene or protein
FASN	Fatty acid synthase, Gene on BOTA 19
FASN	Fatty acid synthase, gene or protein
FFA	Free fatty acids
GH	Growth hormone
GLUT 4	Glucose transporter type 4, protein
H&E	Hematoxylin and Eosin
HDL	High-density lipoprotein
Hosa	Human Chromosome

IgA	Immunoglobulin A
IMF	Intramuscular fat
INSIG-2	Insulin induced gene 2
IT	Ischiatic tuberosity
LCFA	Long-chain fatty acids
LD	Longissimus dorsi muscle
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MLA	Meat and Livestock Australia
MLA	Meat and Livestock Australia
MPRIP	Myosin phosphatase rho-interacting protein, gene or protein
MRIP	Genetic marker near MPRIP
MS	Marble score
MSA	Meat Standards Australia
MSA MB	Marble score as defined by Meat Standards Australia
MSC	Mesenchymal stem cells
MUFA	Monounsaturated fatty acids
NFI	New fineness index of marbling
NLIS	National livestock identification system

PCR	Polymerase chain reaction
PFB	Polymorphic frozen block
PPARG	PPARγ, Peroxisome proliferator-activated receptor gamma
PPARγ	Peroxisome proliferator-activated receptor gamma, gene or protein
PUFA	Polyunsaturated fatty acids
QTL	Quantitative trait locus
RFID	Radio-frequency identification
SCAP	SREBP cleavage-activating protein
SCD	Stearoyl-CoA Desaturase, gene or enzyme
SDM	Sacrocaudalis dorsalis medialis muscle
SFA	Saturated fatty acids
SNP	Single-nucleotide polymorphism
SREB	Genetic marker near SREBF1
SREBF1	Sterol regulatory element binding transcription factor 1, gene
SREBP	Sterol regulatory element-binding protein
SREBP1	sterol regulatory element-binding protein 1, protein encoded by SREBF1
STAT5A	Signal transducer and activator of transcription 5A, gene
TCA	Tricarboxylic acid
TCAP	Titin-cap, Telethonin, gene

TGF	Transforming growth factor
THRSP	Thyroid hormone responsive, gene or protein
Tm	Melting temperature of the fat
UFA	Unsaturated fatty acids
UTS2R	Urotensin 2 Receptor, gene
VFA	Volatile fatty acids
WY	Wagyu

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1 General introduction

1.1 *Preamble*

There is an increasing world demand for food production. Contemporaneously the market seeks assurance in terms of provenance, quality, and healthiness. While consumers demand tasty, highly and fine marbled meat, its healthiness has become extremely important.

Marbled beef tastes better. The Meat Standards Australia (MSA) grading system of Meat and Livestock Australia (MLA) relates increased intramuscular fat to higher eating quality scores. Such a system estimates the amount of intramuscular fat, which determines in high proportion the value of Wagyu carcasses used in premium steak restaurants. However, every country uses a different approach to score marbling, and some have been shown to be subjective and unreliable. As a benchmark, this thesis has adopted the MSA approach developed by Pethick and colleagues at Murdoch University.

The fatty acid composition of the diet directly affects human health. Reducing consumption of saturated fatty acids (SFA) by replacement with unsaturated fatty acids (UFA) is highly beneficial (Mensink et al., 2003), although Booker and Mann, 2008 concluded that more evidence was needed. Meat with high levels of monounsaturated fatty acid (MUFA), predominantly oleic acid, lowers LDL cholesterol and therefore improves cardiovascular health (Adams et al., 2010).

The improvement in taste and healthiness of the meat can be attributed to increased oleic acid (MUFA) itself (Smith et al., 2009; Wood et al., 2004). It follows that objective and accurate measurements, such as the melting temperature of fat (Tm), may be used to promote and develop healthy beef (Lloyd et al., 2014b).

The research program at CY O'Connor ERADE Village Foundation (CYOEVF) has developed innovations that enable the efficient production of healthy and tasty beef. For example, CYOEVF has identified Wagyu haplotypes responsible for softer, healthier fat and superior flavour of marbled steak. Selective breeding could allow these characteristics to be merged with the growth and robustness of other breeds (Williamson et al., 2011).

Traditionally such Wagyu are fed proprietary, essentially secret, diets for at least 300 and often 600 days. Increasingly, there is evidence of market resistance to such regimes. The cost of feeding at approximately $5 per day is one issue. Lack of transparency is another. Most important, however, is the consumer demand for more natural grass feeding to replace grain and to avoid additives such as antimicrobials and growth promotants. The ultimate goal is to produce fine marbled beef with healthy fat using only sustainable pasture systems.

The variability in marbling and Tm between breeds, but also within breeds, is a reality that meat producers have to live with and pay for. It would, therefore, be of great benefit to beef producers and to the health of the consumers if simple genetic and physical testing could be shown to be useful in a commercial setting for managing that issue.

An understanding of the multiple factors involved in marbling is an essential prerequisite for the analysis and improvement of this trait. This review focuses on particular aspects of bovine intramuscular fat (IMF), which has provided the bases for the processes analysed in this thesis.

1.2 *Thesis research questions*

As a consequence, at the beginning of this project, the following questions were posed:

a) Do standardised and automated measurement of melting temperature of the fat reflect known differences between breeds and sires?

b) Is it possible to use the information pertaining to syntenic regions in man so as to improve translation of findings in cattle?

c) Is haplotyping valuable for maintaining Australia's enviable reputation as a producer of healthy food?

d) Does histological examination of marbling reflect the AUS meat marble score?

e) Can haplotypes be used to obtain clearer results from feed trials intended to improve animal welfare?

1.3 *Thesis chapters outline*

This thesis addresses these questions and gives answers in the following chapters:

Chapter 3: Genetics of marbling in Wagyu revealed by the melting temperature of intramuscular and subcutaneous lipids.

This chapter demonstrates the presence of high concentrations of oleic acid in the adipose tissue of Wagyu cattle, highly recognised and valued for its ability of marbling. It explains the complexity of fat melting temperature dependence on genetic and environmental components and gives important considerations to be taken to optimise it and as a result, benefit the health of the consumer.

This chapter shows:

- Low Tm is heritable and affected by sire.
- Tm decreases with Days on Feed and with the increment of Wagyu composition.
- Samples used for DNA tracing can be used for Tm measurements without any interference with the results.

Chapter 4: Haplotypes for type, degree, and rate of marbling in cattle are syntenic with human muscular dystrophy.

This chapter presents the ancestral haplotypes and their importance in the degree of expression of multigene traits, using a principle developed in the major histocompatibility complex which can be applied throughout the genome. It explains how the identification of ancestral haplotypes gives answers to questions that SNP approaches have failed to do, in production traits like muscle development and intramuscular fat deposition, responsible for organoleptic characteristics and degree of healthiness of beef.

This chapter shows:

- The importance of synteny as a guide for exploring and understanding different regions of the genome in different species. It presents several genes within a region of Hosa Chr 17 that are implicated in Limb-girdle muscular dystrophy and the homologous region of Bota Chr 19, responsible for muscle and fat metabolism. Individual genes cannot explain significant differences, as these traits depend on the presence and interaction of multiple genes, and therefore, haplotypes must be involved.

- C19 Ancestral Haplotypes of the marbling region of the genome and polymorphic markers that define them.

- Segregation of the mentioned haplotypes through generations.

- Breed-specific haplotypes associated with marbling, showing the differences between Wagyu and Simmental, using W plots.

- The effect of different ancestral haplotypes over Tm within Wagyu, and the effect of the infusion of Wagyu haplotypes in other breeds.

- A novel mathematical fat desaturation model that integrates multiple parameters to explain the desaturation of fat and decrease of Tm over time.

Chapter 5: Carcass identification reliability: importance, impact and DNA contribution.

This chapter presents the importance of the use of accurate and reliable information for traceability and for the assessment of parental worth. It talks about the advantages of DNA tracing as the only reliable method for traceability after slaughter.

With the same DNA markers used for the determination of ancestral haplotypes relevant for meat quality traits, it was possible to investigate the meat supply chain from Melaleuka Stud, discovering more than 8% mismatches between meat cuts and animals.

As a result, C19 haplotyping is proposed as a single multipurpose test for paddock to plate traceability, parentage, ancestry and breed composition.

Chapter 6: Adipose invasion of muscle in Wagyu cattle: monitoring by histology and melting temperature.

This chapter investigates some aspects of the histology of the muscle at different levels of marbling, showing the invasion of the muscle by fat, in an arborisation pattern along the connective tissue, distinguishing differences in connective tissue disruption and fineness of marbling.

It introduces the microscore as an excellent measure of marbling, especially for lesser and greater degrees which are not quantified reliably by other methods

By comparing anatomical regions, it’s concluded that the tailhead is a suitable site for sequential monitoring, showing useful correlations with marbling and Tm of the loin area.

The findings are exciting. A high correlation between the histological characteristics of those two muscles is shown, allowing us to infer the level and type of marbling in the sweet cuts of an animal from a muscle biopsy at the base of its tail. This opens the possibility of using sequential biopsies for monitoring the progression of the intramuscular deposition.

Chapter 7: Interspecies translation: Bovine marbling to human muscular dystrophy.

This chapter delves into histology for the visualisation and understanding of the intramuscular fat deposition process, showing similarities between marbling in bovines and the changes described in human muscular dystrophy. It shows the aggressive invasion of the muscle by fat, in an arborisation pattern, resulting in disruption of the connective tissue, small fascicles and finally fine marbling.

Two muscles are examined: *Longissimus dorsi*, used for routine marbling assessment post mortem, *Sacrocaudalis dorsalis medialis*, identified as a candidate region for sequential *in vivo* muscle biopsies.

Chapter 8: Feed trials for marbling may be facilitated by haplotype pairing.

This chapter introduces the use of Bota Chr 19 ancestral haplotypes as a valuable and simple technology to reduce the variation from genetic factors in studies aiming to measure the effect of feed over marbling and fat melting temperature. It demonstrates how, by identifying those haplotypes, it is possible to reduce the genetic heterogeneity and obtain statistically significant results with considerably smaller groups of animals, improving the use of resources and the potential compromise of the welfare of the animals.

Table 1.1 *Research questions, associated research projects and thesis chapters.*

Question	Thesis chapter
Do standardised and automated measurement of melting temperature of the fat reflect known differences between breeds and sires?	Chapter 3: Genetics of marbling in Wagyu revealed by the melting temperature of intramuscular and subcutaneous lipids
Is it possible to use the information pertaining to syntenic regions in man so as to improve translation of findings in cattle?	Chapter 4. Haplotypes for type, degree, and rate of marbling in cattle are syntenic with human muscular dystrophy Chapter 7. Interspecies translation: bovine marbling to human muscular dystrophy
Is haplotyping valuable for maintaining Australia's enviable reputation as a producer of healthy food?	Chapter 5. Carcass identification reliability: importance, impact and DNA contribution
Does histological examination of marbling reflect the AUS meat marble score?	Chapter 6. Adipose invasion of muscle in Wagyu cattle: monitoring by histology and melting temperature
Can haplotypes be used to obtain clearer results from feed trials intended to improve animal welfare?	Chapter 8. Feed trials for marbling may be facilitated by haplotype pairing

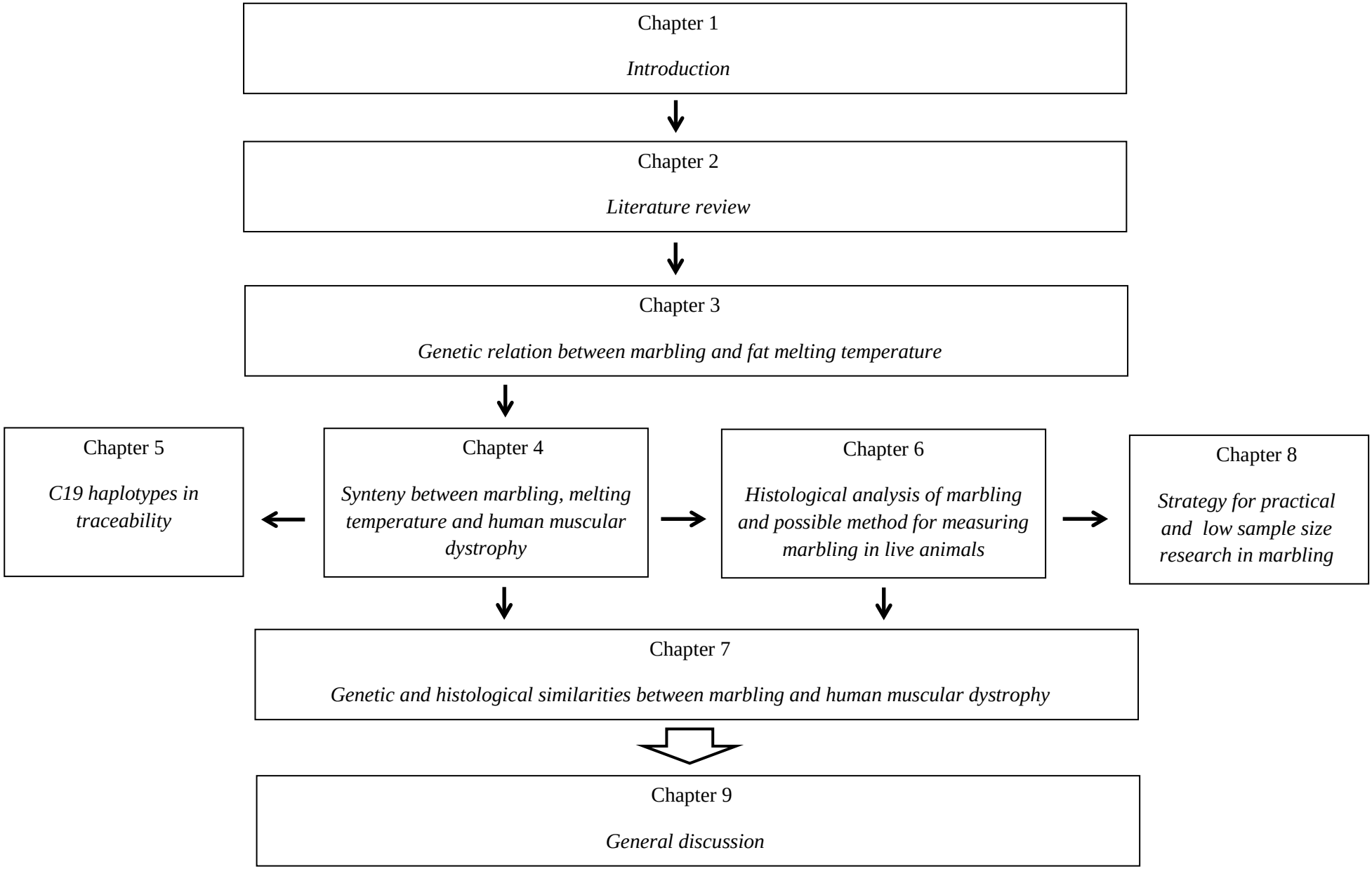

Figure 1.1 *Roadmap of thesis chapters and interrelationship of thesis topics.*

2 Literature review

2.1 *Introduction*

Intramuscular fat and the associated monounsaturated fatty acids are thought to be beneficial to human health. Numerous factors and genes are involved, but it is not clear how they interact. There is an obvious need for a) analysis by histopathology with the potential for translation between species and b) multigenic analysis, for example by defining relevant ancestral haplotypes.

As described in the General Introduction, quantity and quality (level of desaturation, fat composition) of IMF are key to meat quality. Understanding the multiple factors involved in marbling is crucial for the improvement of this trait.

This literature review will examine the different factors known to play a role of importance in the generation, development and desaturation of intramuscular fat. In particular, it will examine:

- Importance of marbling to meat quality.
- Impact of the human consumption of fats of different nature on health.
- Changes that occur to fats in ruminants, from their consumption, digestion, break down to fatty acids, saturation in the rumen, absorption, distribution, deposition in muscle and intramuscular desaturation.
- Synthesis of fat from starch, glucose and fermentation of cellulose.
- Genetics involved in fat desaturation and adipogenesis.
- Possible methods of monitoring marbling in life animals.

2.2 *Marbling and palatability*

Marbled beef tastes better (Smith et al., 1982; Lorenzen et al., 1999; Lorenzen et al., 2003; Emerson et al., 2013; Pannier et al., 2014; Corbin et al., 2015). IMF, referred to in the industry as marbling, improves tenderness, juiciness, and flavour (Corbin et al., 2015), and therefore is one of the main factors that improve organoleptic quality and palatability in beef (Jackman et al., 2009, 2010 a, b; Platter et al., 2003) (Figure 2.1).

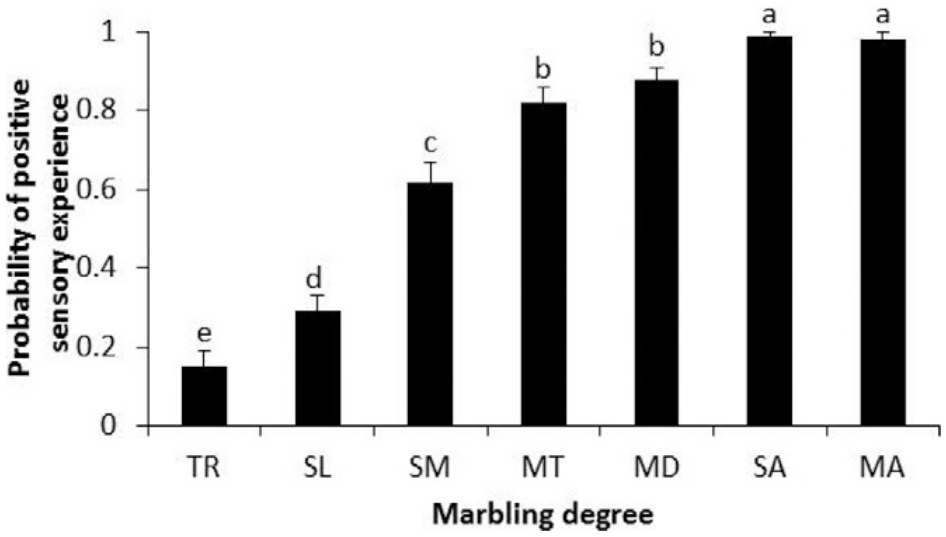

Figure 2.1 *Marbling and sensory experience*. Reproduced with permission from Emerson et al., 2013. Effect of marbling degree (TR = traces, SL = slight, SM =small, MT = modest, MD = moderate, SA = slightly abundant, MA = moderately abundant (USDA, 1997)) on the probability of a steak receiving a positive rating (>7.5 on a 15-cm scale) for overall sensory experience (probabilities that do not share a common superscript letter differ; $p < 0.05$).

Tenderness is considered one of the main attributes in meat quality. As animals mature, the mechanical strength of intramuscular connective tissue increases, with a negative effect on meat tenderness. In the opposite way, while the intramuscular adipose tissue develops, the perimysium suffers changes that affect its conformation. As a result, the honeycomb structure of the endomysium breaks down, with the consequent decrease on its mechanical strength and increase on tenderness (Tian et al., 2005; Jackman et al., 2009; Gotoh et al., 2014).

The balance between toughness due to aging and tenderness due to IMF accumulation has been shown to favour tenderness in muscles with IMF of 8% or more (Nishimura et al., 1999).

2.3 *Effects of dietary fatty acids on human health*

The relation between Cardiovascular Disease (CVD) and dietary fats has been of great medical interest since the Soviet professor Nikolaj Anitschkow, in 1913, discovered the correlation between the amount of cholesterol intake and the severity of atherosclerosis formation and cholesterol-induced alterations (Finking and Hanke, 1997).

The correlation between fat consumption and cholesterol levels led to the recommendations of reducing fat intake for patients considered high-risk of heart disease in the 1940s. This recommendation started to expand to the entire population in the 1960s, transitioning to a low-fat diet ideology in the 1980s, promoted by physicians, governments, food industry and the media. Paradoxically, in America, what accompanied this ideology was an obesity epidemic (La Berge, 2008).

Since then, the medical recommendations for decreasing the incidence of CVD have evolved from a reduction of fat consumption to specifically SFA. However, just reducing the consumption of SFA is not sufficient to guarantee a positive effect on cardiovascular health as it depends on the nature of the nutrient selected for replacement.

Multiple studies comparing diets with different components of saturated and unsaturated fats have been published with contradictory results. However, in 2016 Mensink released a regression analysis, aiming to assess the effect of SFA intake modifications on serum lipid and lipoprotein levels when replacing SFA with MUFA, PUFA or carbohydrates (Mensink, 2016).

The analysis selected studies done just with healthy people, with designs that eliminate the effect of non-specific drifts to the outcome variables, and with thorough control of diets. The analysis excluded sequential and longitudinal studies and considered *trans*-fatty acid intakes of 2% or less of the total energy intake.

84 studies published between 1970 to 2013 were selected. To put it in perspective, for the 2009-2013 period, only 8 publications out of 629 qualified for the analysis.

Within Mensink's conclusions, the isocaloric replacement of SFA intake with MUFA and PUFA produces a significant reduction in the concentration of total cholesterol and triglycerides, and decreases the LDL:HDL cholesterol ratio. A similar but smaller effect was found in the replacement with carbohydrates.

There is a lack of consensus regarding the replacement of SFA with proteins. Even though there are some indications of a positive effect on the reduction of CVD risk, further research seems necessary (Briggs et al., 2017).

Based on the previous evidence, a direct benefit in human health for improving the fatty acid ratio of beef would be expected.

2.4 *Fatty acid consumption, absorption and deposition*

Ruminants consume lipids in two forms, galactolipids, predominant in forage-based diets, and triglycerides, predominant on grain-based diets. Triglycerides are formed of glycerol and fatty acids, whereas galactolipids are formed of galactose, glycerol and fatty acids, generally PUFA.

Lipids reach duodenum mostly adsorbed on feed particles and bacteria, where bile salts and lysolecithins are needed for desorption to then be available for absorption (Doreau and Ferlay, 1994).

To be absorbed, lipids need to be available as free fatty acids (FFA). Triglycerides and galactolipids are broken down by pancreatic enzymes, resulting in FFA, galactose, and glycerol. Then, these products can be absorbed by the intestinal cells (enterocytes) (Doreau and Chilliard, 1997; Burns, 2011).

Once in the enterocytes, FFA are assembled or re-esterified to glycerol, forming triglycerides, and transported by lipoproteins to the liver and peripheral tissues through blood and lymph, where they can be oxidised for energy production, deposited in cellular membranes, or stored into adipocytes (Doreau and Chilliard, 1997; Millen et al., 2016).

2.4.1 *Ruminal biohydrogenation*

In monogastrics, the nature of the fatty acids present in blood and tissues reflect the fatty acid composition of their diet (Doreau and Chilliard, 1997; Wood et al., 2004; Nuernberg et al., 2005). Ruminants' digestive system is more complex. Their rumen has evolved to digest cellulose through microorganisms, and dietary UFA (Table 2.1) have a toxic effect on them.

Table 2.1 *Type, structure, common name and formula of fatty acids present in ruminant's diets* (Arrigoni et al., 2016).

Type	Structure	Common name	Form
SFA	$C_{16}H_{32}O_2$	Palmitic acid	C16:0
	$C_{18}H_{36}O_2$	Stearic acid	C18:0
MUFA	$C_{18}H_{35}O_2$	Oleic acid	C18:1
PUFA	$C_{18}H_{34}O_2$	Linoleic acid	C18:2
	$C_{18}H_{33}O_2$	Linolenic acid	C18:3

To avoid this toxic effect, fatty acids undergo saturation in the rumen. This process, called Ruminal Biohydrogenation, produces mainly stearic and palmitic acid as final products (Noble, 1981). The process occurs primarily due to the effect of ruminal bacteria, but protozoa also contribute. For that reason, beef SFA/UFA ratio cannot be reduced by simply adding UFA to the diet (Harfoot, 1981). Interestingly, some of the UFA not affected by biohydrogenation in the rumen can be exposed to a similar process later, in caecum and colon (Ward et al., 1961).

Rumen bacteria are divided into two groups, A and B. Group A hydrogenate linoleic acid into C18:1 trans-11, and group B generates stearic acid (C18:0) as the final product. Figure 2.2 (Arrigoni et al., 2016) shows the steps for ruminal conversion of linoleic acid into stearic acid.

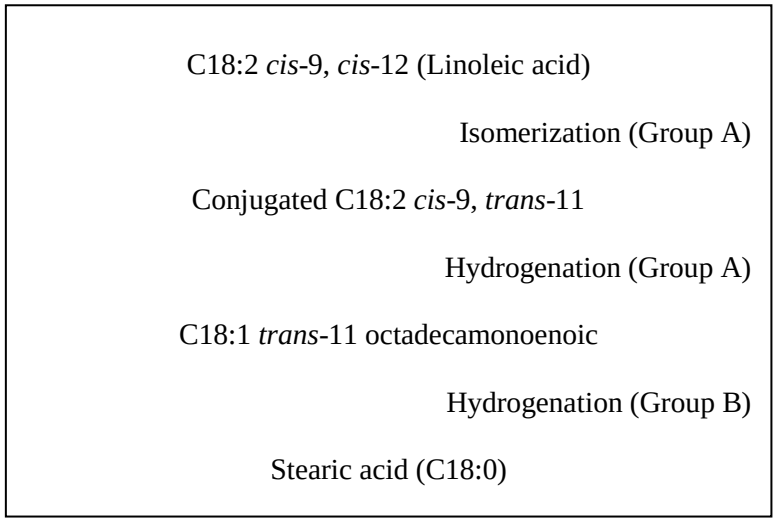

Figure 2.2 *Scheme of linoleic acid biohydrogenation* (Arrigoni et al., 2016).

2.4.2 *Volatile fatty acids*

In ruminants, the absorption of lipids starts in the rumen, absorbing volatile fatty acids (VFA) of up to 12 carbon atoms (Doreau and Ferlay, 1994).

Volatile fatty acids are one of the major products of ruminal fermentation, supplying 60% to 70% of the total dietary energy in cattle (Dijkstra, 1994; Seymour et al., 2005). The three main VFA are acetic, propionic and butyric, with different effect over the body. The effect of acetate/propionate ratio over milk production has been largely studied, as the acetate/propionate ratio is directly proportional to milk fat content (Seymour et al., 2005).

While acetate and butyrate are precursors of lipids, propionate is only glucogenic, via gluconeogenesis (Seymour et al., 2005; Nafikov and Beitz, 2007).

In ruminants, acetate is the most important source of carbon for fatty acid synthesis, followed by glucose (Nafikov and Beitz, 2007; Smith et al., 2018). This may be a consequence of the high activity of the enzyme acetyl-CoA synthetase in ruminants (Hanson and Ballard, 1967).

However, the different fat depots of the body differ in their pathways for fatty acid synthesis. While subcutaneous adipose tissue uses acetate as a primary source for the synthesis of fatty acids, intramuscular adipose tissue uses glucose, (Gilbert et al., 2003; Chung et al., 2006; Rhoades et al., 2007; Smith and Johnson, 2014). For that reason, propiogenic diets (e.g., high-grain diets) promote higher marbling than acetogenic diets (roughage-based diets) and is expected that glucogenic diets (e.g., dry-rolled corn) promote even greater marbling development than propiogenic diets (Rhoades et al., 2007; Smith et al., 2018).

Grain-based rations and starch offer free glucose for absorption, and also increase propionate production, which will synthesise glucose, available for intramuscular fat development, and therefore may favour marbling (Smith and Johnson, 2014; Park et al., 2018).

In addition to the acetogenic nature of hay, the results of Rhoades et al. in 2007 suggest that hay limits glucose supply and its utilisation in response to insulin, reducing the development of intramuscular fat.

These facts explain part of the success of Japanese production system of highly marbled Wagyu beef, with large amounts of concentrate fed during the fattening period, and consumptions of 4,000 to 5,000 kg per animal between 11 to 30 months of age (Gotoh et al., 2014).

2.4.3 *Fatty acid synthesis*

There are two possible origins for the fat stored in adipocytes: Diet and synthesis (lipogenesis).

The definition of lipogenesis varies in the literature. Some define it as the metabolic pathways undertaken to synthesise fatty acids, specifically from carbohydrates (Martin, 2015; Martin and McFerran, 2016), also known as *de novo* lipogenesis (Ameer et al., 2014). Others use the term as the synthesis of fatty acids and later triglycerides, regardless of the source of origin (Kersten, 2001; Fuller, 2004; Bender, 2014). This review will use the last definition.

The storage and accumulation of fat are determined by the balance between lipogenesis and lipolysis, and it is regulated by nutrition, hormones and the expression of genes connected with cholesterol and fatty acid metabolism, which are controlled by transcription factors (including SREBP1 and PPARγ) (Kersten, 2001).

For the synthesis of triglycerides, there are two options. They can be formed after dietary fatty acids have been absorbed or after new fatty acids have been synthesised during lipogenesis (Ladeira et al., 2016).

Lipogenesis occurs mainly in liver and adipose tissue, and their relative importance varies among species. In chicken, liver is more important than adipose tissue, in rats they are similar and in ruminants, adipose tissue dominates over the liver (Vernon et al., 1999). The process

occurs in the cytoplasm of the cells with acetate as the primary precursor for the synthesis of fatty acids in ruminants (Shingfield et al., 2010).

The different steps for the synthesis of fatty acids in the adipocyte are shown in Figure 2.3.

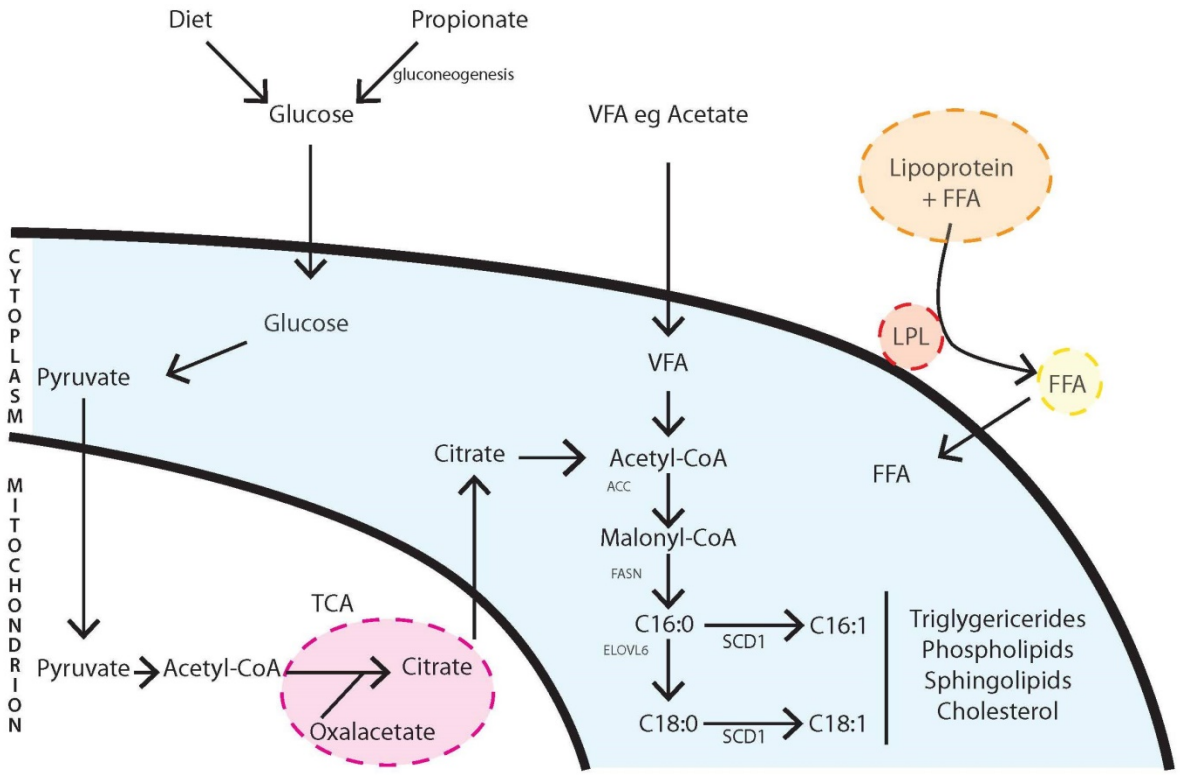

Figure 2.3 *An overview of fatty acid synthesis in the ruminant adipocyte* (Burns, 2011; Ladeira et al., 2016; Park et al., 2018).

- Lipoproteins
  - Lipids packaged in lipoproteins are transported to adipocytes.
  - Lipoprotein lipase (LPL) releases free fatty acids.
  - Fatty acids diffuse through the cell membrane.
  - Free fatty acids can undergo fatty acid synthesis reactions such as desaturation by stearoyl-CoA desaturase 1 (SCD1).
- Volatile fatty acids (VFA)
  - Freely diffuse across the cell membrane and undergo de novo fatty acid synthesis.
  - Acetyl-CoA carboxlyase (ACC) : acetyl-CoA, forming malonyl-CoA.

- Fatty acid synthase (FASN): generates SFA 2C longer than malonyl-CoA or another fatty acyl-CoA, to reach a final 16C in length.
- Bound to the endoplasmic reticulum membrane, Elongase protein 6 (ELOVL6) can further elongate fatty acyl-CoA to more than 16 C.
- SCD1 can add a double bond at the 9 position.

- Glucose
  - Derived from propionate through gluconeogenesis in liver, and from diet, glucose is transported into the cytoplasm, undertakes glycolysis and generating Pyruvate, for the production of citrate in the TCA cycle in the mitochondrion. Citrate is then transported to the cytoplasm, degraded into oxaloacetate and acetyl-CoA, which can be used for FA synthesis.
- Fatty acids can be packaged into
  - Triglycerides for storage.
  - Phospholipids for membrane components.
  - Sphingolipids for membrane components.
  - Cholesterols and eicosanoids for hormone production.

2.4.3.1 *Vitamin A*

Another factor strategically managed to improve marbling is Vitamin A (retinol). Despite the mechanism for which Vitamin A affects marbling not being completely understood, studies suggest that restrictions of vitamin A increase IMF, as it acts as an inhibitor of adipocyte differentiation (Sato et al., 1980) and conditions the activity of PPARγ, nuclear receptor and master regulator in fat metabolism (Frey and Vogel, 2011).

By restricting Vitamin A on the rations, intramuscular fat increases. It affects body composition and growth, as the nutrients consumed favour fat production instead of muscle (Nade et al., 2003). Interestingly, that increase in fat production happens only in muscle, not affecting subcutaneous fat (Kruk et al., 2018).

2.4.4 *Adipogenesis*

Adipose tissue is categorised as connective tissue. It originates in the mesenchyme during the embryological development and contains a variety of cells, including mesenchymal stem cells (MSC), preadipocytes, fibroblasts, and adipocytes.

Adipogenesis is the term used to describe the process for which new adipocytes are formed, involving (Hausman et al., 2008):

- Determination, where MSC commit to pre-adipocytes.
- Proliferation of pre-adipocytes.
- Differentiation of pre-adipocytes into adipocytes with the ability to store lipids in the form of triacylglycerol.

Knowing that fat depots are formed by adipocytes that store lipids into their cytoplasm, the question is; Does the adipose tissue expand due to an increase of the number of adipocytes or only due to the increasing size of the pre-existent adipocytes, by storing more lipids in their cytoplasm? In other words, is the increase in fat tissue due to hypertrophy or also hyperplasia?

At least four decades ago, there was already information relevant to the answer to those questions. In 1985 the results of Cianzio et al. suggested that in bovines the main cause of the increment of adipose tissue during growth is hypertrophy particularly from 11 to 17 months of age for most fat depots, whereas in skeletal muscle, hypertrophy and also hyperplasia can occur, the findings of Hood and Allen in 1973.

Later, other researchers confirmed that marbling is a result of both processes; hypertrophy, with the increase of the size of the adipocytes and hyperplasia, with the appearance of new

adipocytes and the consequent increase of intramuscular fat deposition (Cianzio et al., 1985; Du et al., 2011; Ladeira et al., 2016).

That also explains the selective effect of Vitamin A over intramuscular fat, as Vitamin A affects adipocyte hyperplasia, and not hypertrophy (Kruk et al., 2018).

Research has shown that the development of muscle and adipose tissue is predetermined or at least partly conditioned at early stages of life. Nutrition of the dam during gestation may impact fibrogenesis, myogenesis and also adipogenesis from MSC (Du et al., 2011; Ladeira et al., 2016), including intramuscular adipogenesis, which begins at mid-gestation (Du et al., 2011).

Du et al., 2010 explain that inhibition of the differentiation of stem cells into myocytes can happen in utero and can promote their differentiation into adipocytes. In the same way, Zhu et al. in 2006 proved a reduction in muscle mass together with an increase in intramuscular triglycerides in lambs from dams exposed to nutritional restrictions.

Another factor that affects the accumulation of fat, the number of adipocytes and possibly marbling is cell plasticity. Mature adipocytes, despite having already undergone differentiation and having already stored lipids in the cytoplasm, have the ability to dedifferentiate to preadipocytes, known as DFAT (Dedifferentiated fat cells). In vitro, DFAT can proliferate, replicate, and finally redifferentiate into adipocytes or transdifferentiate into osteoblasts, chondrocytes, and also skeletal muscle cells (Kazama et al., 2008; Wei et al., 2013).

2.4.5 *Bypass fats*

Due to the extremely high energy requirements of dairy cows to express their production potential, in the last 35 years, supplementation of fats and oils has become standard practice.

Due to its energy density, extra lipids in the diet improve energy balance and can favour body condition, milk production and reproductive indexes (Drackley, 2004; DeFrain et al., 2005).

The toxicity of UFA over many of the ruminal microorganisms involved in fibre digestion limits the amount that can be safely added to the diet without harmful effects. The dairy industry has found in by-pass fats a solution to that issue. In addition to protecting ruminal microorganisms from the toxic effect of unsaturated fats, bypass fats also protect UFA from saturation, which allows the absorption of the fatty acids in their unsaturated form.

The use of by-pass fats allows reaching high levels of energy in the diet without having to increase the amount of grain in the ration (Chalupa et al., 1986), avoiding the risk of ruminal acidosis and with no adverse effect on rumen fermentation of fibre, feed intake or digestibility of nutrients (Naik, 2013).

There are four main types of by-pass fat products tested in ruminants.

a) Crystalline or prilled fatty acids, made by spraying liquefied SFA into a cooled atmosphere (Naik, 2013). The melting temperatures of these fatty acids are higher than the ruminal temperature. Therefore, although not completely inert in the rumen, they are not exposed to ruminal microorganisms (Chalupa et al., 1986).

b) Free or esterified fatty acids encapsulated in formaldehyde-treated protein (de Veth et al., 2005).

c) Fatty acylamide, formed from a fatty acid and an amine (Jenkins, 1998).

d) Calcium salts of long-chain fatty acids (Ca-LCFA). Due to the acidic pH of the abomasum, these insoluble soaps dissociate the fatty acid from Ca-LCFA, which are then efficiently absorbed in the small intestine (Naik et al., 2009; Warner et al., 2015).

Fatty acids encapsulated on formaldehyde-treated protein and fatty acyl amides have been shown to efficiently increase UFA in milk. The first group has also shown the same results in IMF of lambs (Hogan and Hogan, 1976; Jenkins, 1993; Jenkins, 1998; Warner et al., 2015).

It seems clear that by-pass fats are a valid alternative for milk production. Thus, to consider the use of that technology in beef production, further research is required to measure their effect on bovine IMF composition and the economic factors.

However, as the tendency is the production of good quality low Tm meat at a low cost, and favouring extended periods of grass and shorter periods of grain feeding, improvements in the use of favourable genetics seems to be a better approach.

2.5 *Fatty acid composition of IMF*

Multiple factors influence the final fatty acid composition of IMF in bovines. The more we understand them, the better we can manage them to achieve final desirable results.

Breed, environment, age, fatness, and dietary energy are important factors to consider for the quantity of IMF but also for the fatty acid composition of the fat. The level of desaturation of fatty acids increases with age, fatness and high energy diets, partly by up-regulation of mRNA expression of several genes (ACACA, SCD, FASN, SREBP-1c, PPARγ, and FABP4) (Leat, 1975; Perry et al., 1998; Li et al., 2011; Yokota et al., 2012; Kaplanova et al., 2013; Yang et al., 2017).

Even though the mechanisms whereby some polymorphisms in the genome are associated with differences in fatty acid composition are not fully understood, correlations have been found, i.e. SREB and FASN (Bhuiyan et al., 2009).

As in traits like milk production, growth, feed conversion, etc., genome-wide association studies have been done for fatty acid composition in beef, showing multiple significant genomic regions (Ishii et al., 2013; Cesar et al., 2014), including Chromosome 19 with several SNPs close to the FASN gene that correlate with oleic acid concentration (Uemoto et al., 2010).

Effects of polymorphisms in individual genes, as well as several gene-gene interactions (Lee et al., 2014), have been identified, for instance in SREBP1 and FASN (Lee et al., 2004; Shogo Hoashi et al., 2007; Bhuiyan et al., 2009; Oh et al., 2012; Lee et al., 2013), SCD1 (Taniguchi et al., 2004; Milanesi et al., 2008; Ohsaki et al., 2009; Barton et al., 2010) and UTS2R (Sasazaki et al., 2014).

2.5.1 *The process of desaturation of fat within the muscle*

In ruminants, as intramuscular fat deposition occurs and adipocytes increase in size, there is a concomitant increase in intracellular MUFA. This is due primarily to the conversion of the intramuscular SFA into oleic acid, which results from the Δ9 desaturation of fatty acids. This process is mediated by the activity of the enzyme SCD. Further, as desaturation of the fatty acids increases, fat melting temperature (Tm) declines (Smith et al., 1998; Smith et al., 2009). This dynamic and complex process has to be genetically mediated and therefore heritable.

The genetics and environmental factors affecting the de novo production of saturated fatty acids and their desaturation dominate the fatty acid composition of beef. Dietary fatty acid composition has little influence due to ruminal biohydrogenation, and therefore, there is a low conversion rate of dietary UFA into beef UFA content (Harfoot, 1981). This fact, plus the high heritability of the trait, has prompted scientists to investigate the genetics behind the fatty acid composition in cattle.

Nakahashi et al. (2008) found that within individuals, the percentage of MUFA over total fatty acid differs among subcutaneous, intramuscular and intermuscular fat, and is significantly higher in subcutaneous fat than intramuscular fat. In 2012, Smith et al. showed differences between 8 subcutaneous fat depots along the body, with the brisket showing higher palmitoleic:stearic acid ratio than all other depots. They show that time on feed increases MUFA:SFA ratio and the activity of the enzyme SCD, responsible for fat desaturation.

Williamson et al., (2011) discussed the influence of several genes on Chromosome 19 relevant to lipid metabolism and muscle growth, which may have an important role in intramuscular fat Tm (Table 2.2).

Table 2.2 *Genes in C19 important in the metabolism of lipids.* (Lloyd et al. 2013, NCBI).

Gene	Influence
SREBF1	Cholesterol and FA biosynthesis regulation of intracellular lipids.
MPRIP	Myosin phosphatase Rho-interacting protein. Regulation of muscle cell contraction.
TCAP	Muscle Structural function. Mutations in this gene are associated with Limb-girdle muscular dystrophy.
STAT5A	Mammary tissue development. Milk fat percentage. Fat cell formation and function.
GH	Breakdown of lipids.
Urotensin 2 receptor	Glucose metabolism. Insulin resistance. Skeletal muscle fat deposition. Fatty acid metabolism.
FASN	Fatty acid synthesis.

The complex system of lipid synthesis and desaturation, determined by multiple proteins and enzymes is represented in Figure 2.4 and listed in Table 2.3. The transcription of the relevant genes is regulated by the nuclear receptor PPRG, as a result of the influence of insulin and thyroid hormones over cellular regulatory proteins (Graugnard et al., 2009).

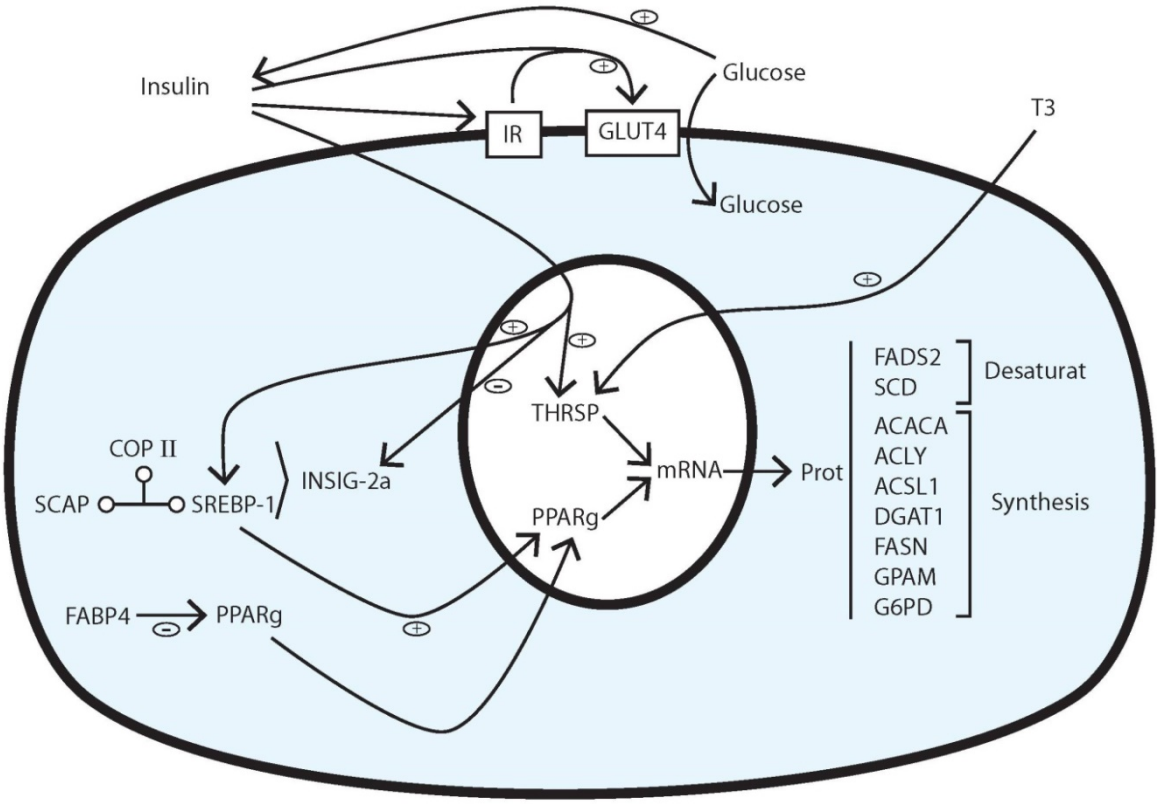

Figure 2.4 *Major trends in mRNA expression for lipid synthesis and desaturation.*

- Insulin enhances the transcription of the gene SREBP-1 (Yellaturu et al., 2009) and the affinity of the SCAP/SREBP-1 complex for COP II vesicles by reducing the level of INSIG-2a protein via degradation of its cognate mRNA, and subsequently promoting the migration from ER to Golgi (Yellaturu et al., 2009).
- SREBP-1 directly enhances the expression of the PPARg gene at a transcriptional level (Kim et al., 1998; Fajas et al., 1999).
- FABP4 downregulates PPARg triggering its degradation in the cytoplasm (Garin-Shkolnik et al., 2014).
- Insulin stimulates the transport protein GLUT4, which regulates glucose homeostasis (Sonksen and Sonksen, 2000; Huang and Czech, 2007).
- Insulin interacts with insulin receptors (IR) and stimulates the activity of the transport protein GLUT4, resulting in glucose uptake (Lee and Pilch, 1994; Sonksen and Sonksen, 2000; Huang and Czech, 2007).
- The transcription of genes involved in the synthesis and desaturation of fatty acids is mediated by the nuclear receptor PPARg, acting as a transcription factor (Garin-Shkolnik et al., 2014) and the lipogenic transcription regulator THRSP, activated by thyroid hormone triiodothyronine (T3), glucose and insulin (Graugnard et al., 2009; Thering et al., 2009).

Table 2.3 *Major proteins involved in lipid production and desaturation.*

Protein	Long name	Function	Gene	Bota	Position	Reference
ACACA	Acetyl-CoA carboxylase alpha	Carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis.	ACACA	19	13752492-13996058	https://www.ncbi.nlm.nih.gov/gene/31
ACLY	ATP citrate lyase	Synthesis of cytosolic acetyl-CoA in many tissues. Lipogenesis and cholesterogenesis.	ACLY	19	42691368-42735634	https://www.ncbi.nlm.nih.gov/gene/47
ACSL1	Acyl-CoA synthetase long-chain family member 1	Converts free long-chain fatty acids into fatty acyl-CoA esters. Lipid biosynthesis and fatty acid degradation.	ACSL1	27	14223447-14288333	https://www.ncbi.nlm.nih.gov/gene/2180
AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1	Enzyme that converts LPA into PA, part of the membrane lipid bilayer. Contributes to the membrane's physical properties.	AGPAT1	23	27017073-27024546	https://www.ncbi.nlm.nih.gov/gene/10554 https://www.sciencedirect.com/topics/neuroscience/phosphatidic-acid
DGAT1	Diacylglycerol O-acyltransferase 1	Transmembrane protein. Catalyzes the conversion of diacylglycerol and fatty acyl CoA to triacylglycerol. Transfer acyl CoA to retinol. Associated with obesity Esterifying exogenous fatty acids to glycerol.	DGAT1	14	1795425-1804838	https://ghr.nlm.nih.gov/gene/DGAT1
DGAT2	diacylglycerol O-acyltransferase 2	Catalyses the final reaction in the synthesis of triglycerides in which diacylglycerol bound to long-chain fatty acyl-CoA	DGAT2	15	55940757-55973229	https://www.ncbi.nlm.nih.gov/gene/84649

FABP4	Fatty acid binding protein 4	Protein found in adipocytes. Fatty acid uptake, transport, and metabolism. FABP4 regulates adipogenesis by downregulating PPARγ. Suppression of PPARγ by FABP4 in visceral fat may explain the reported role of FABP4 in the development of obesity-related morbidities.	FABP4	14	46833665-46838053	https://www.ncbi.nlm.nih.gov/pubmed/24319114 Diabetes. 2014 Mar;63(3):900-11. doi: 10.2337/db13-0436. Epub 2013 Dec 6 https://www.ncbi.nlm.nih.gov/gene/2167
FADS2	Fatty acid desaturase 2	Introduction of double bonds between defined carbons of the fatty acyl chain.	FADS2	29	41045232-41082249	https://www.ncbi.nlm.nih.gov/gene/9415
FASN	Fatty acid synthase	Synthesis of palmitate (salts and esters of palmitic acid, saturated fatty acid found in animals, plants and microorganisms) from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids.	FASN	19	51384922-51403614	https://www.ncbi.nlm.nih.gov/gene/2194
GLUT4	Solute carrier family 2 member 4	Transports glucose across the cell membrane.	SLC2A4	19	27616613-27622206	https://www.ncbi.nlm.nih.gov/gene/6517
GPAM	Glycerol-3-phosphate acyltransferase, mitochondrial	Enzyme which prefers saturated fatty acids as its substrate for the synthesis of glycerolipids.	GPAM	26	32963400-33003349	https://www.ncbi.nlm.nih.gov/gene/57678
G6PD	Glucose-6-phosphate dehydrogenase	Production of NADPH for tissues involved in biosynthesis of fatty acids or isoprenoids, such as the liver, mammary glands, adipose tissue, and the adrenal glands.	G6PD	X	40484210-40500959	https://www.ncbi.nlm.nih.gov/gene/2539 https://en.wikipedia.org/wiki/Glucose-6-phosphate_dehydrogenase
INSIG1	Insulin induced gene 1	Endoplasmic reticulum membrane protein that regulates cholesterol metabolism, lipogenesis, and glucose homeostasis. It binds SREBP activating SCAP and HMG-CoA reductase.	INSIG1	4	117906798-117920023	https://www.ncbi.nlm.nih.gov/gene/3638

IR	Insuline receptor	Activates the insulin signaling pathway. Synthesis and storage of carbohydrates, lipids and protein.	INSR	7		https://www.ncbi.nlm.nih.gov/gene/3643
MDH2	Malate dehydrogenase 2	Oxidation of malate to oxaloacetate, utilizing the NAD/NADH cofactor system in the citric acid cycle (Krebs cycle)	MDH2	25	34766932-34779637	https://www.ncbi.nlm.nih.gov/gene/4191
PPARG	Peroxisome proliferator activated receptor gamma	Type II nuclear receptor. Sensing steroid and thyroid hormones. Regulates the expression of specific genes. Development, homeostasis and metabolism. Regulator of adipocyte differentiation.	PPARG	22	57367106-57432321	https://www.ncbi.nlm.nih.gov/gene/5468
SCAP	SREBF chaperone	Binds to SREBPs and mediates their transport from the ER to the Golgi.	SCAP	22	52649470-52818676	https://www.ncbi.nlm.nih.gov/gene/22937
SCD	Stearoyl-CoA desaturase	Biosynthesis of monounsaturated fatty acids. Catalyzes the D^9-cis desaturation of a range of fatty acyl-CoA substrates.	SCD	26	21137945-21148318	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2711665/
SREBP1	Sterol regulatory element binding transcription factor 1	Transcription factor that binds to the sterol regulatory element-1. Promoter of the low density lipoprotein receptor gene and other genes involved in sterol biosynthesis.	SREBF1	19	35234637-35250672	https://www.ncbi.nlm.nih.gov/gene/6720
THRSP	Thyroid hormone responsive	Gene expression controlled by nutritional and hormonal factors. Expressed in liver and adipocytes. Role in controlling tumor lipid metabolism. Increased triacylglycerol levels and enhanced the expression of FAS, PPARγ, and SREBP1. Local activation of thyroxine (T_4), key mechanism of TH regulation of metabolism.	THRSP	29	18084680-18090586	https://www.ncbi.nlm.nih.gov/gene/7069 https://www.ncbi.nlm.nih.gov/pubmed/25608868

Having identified the genes of relevance for marbling and desaturation, it is crucial to understand that “the relevant unit of inheritance is not the allele but the ancestral haplotype” (Lloyd et al., 2013).

These sequences, containing multiple genes, are inherited untouched over many generations. Therefore the key to producing healthy beef is the identification of ancestral haplotypes, as undoubtedly many of these characteristics are under complex polygenic control and reflect the mix of ancestral haplotypes (Williamson et al., 2011).

2.6 *Genetics of marbling*

2.6.1 *Ancestral haplotypes for marbling and desaturation*

Through the genome, recombination is suppressed over large stretches of DNA, leaving the internal sequences stable and unexposed to variation, in other words, “frozen” (Yunis et al., 2003). These blocks are polymorphic, and they are referred to as “polymorphic frozen blocks” (PFBs) (McLure et al., 2013).

Ancestral haplotypes are defined as alternative sequences of a PFB inherited over generations without recombination (Dawkins, 2015). There are multiple ancestral haplotypes at each PFB, and they can be regarded as the principal unit of inheritance (Lloyd et al., 2015).

The same version of a PFB can be found in unrelated individuals, implying conservation from ancestors over multiple generations, leading to their designation of Ancestral Haplotypes (AH).

Numerous ancestral haplotypes with effect on intramuscular fat content and distribution, and therefore marbling, have been identified in Chromosome 19 of bovine, in a 14-Mb region, by Williamson et al. 2011. Williamson explains the different steps for the identification of the haplotypes, including the validation of the results examining segregation in multigenerational

families (Figure 2.5), after which the biological significance needs to be confirmed by demonstrating associations with the trait in study (Lloyd et al., 2015) (Figure 2.6).

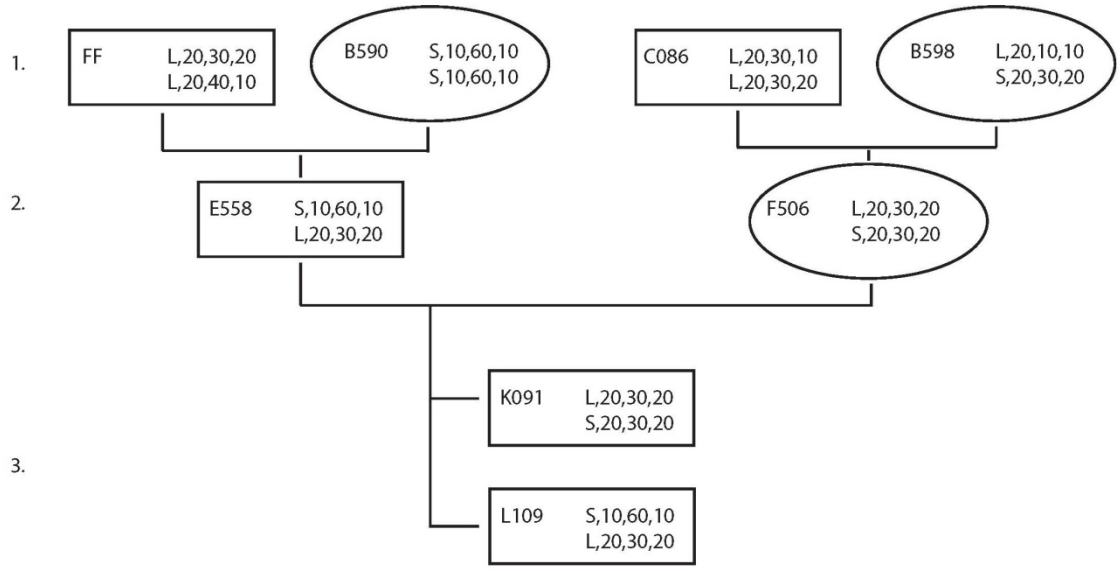

Figure 2.5 *Tracing segregation through a three-generation family of cattle from Melaleuka Stud.* The haplotypes are defined by alleles in 4 different loci; SREB:L and S; NT5M: 22, 20 and 10; MRIP: 60, 40, 30, 20 and 10; TCAP: 20 and 10. Some combinations of alleles are inherited untouched from the first to the third generation (L,20,30,20 and S,20,30,20). Dams are represented as ovals and sires as rectangles.

One example of segregation showed in Figure 2.5 is the haplotype S,10,60,10 from the dam B590, inherited unchanged to E558 and then L109. The haplotype is not present in K091. This is one example of the hundreds of progeny from E558, where there is no recombination in the PFB between SREB and TCAP.

	Allele								
Haplotype	A	Cw	B	Bf	C2	C4A	C4B	DR	Disease
8.1	1	7	8	S	C	Q0	1	3	MG, SLE, IDDM
18.2	-	-	18	F1	C	3	Q0	3	IDDM
18.1	25	-	18	S	Q0	4	2	2	C2 deficiency

Figure 2.6 *MHC haplotypes and disease associations.* MG = myasthenia gravis, SLE = systemic lupus erythematosus, IDDM = insulin-dependent (type 1) diabetes mellitus. Adapted with permission from (Lloyd et al., 2015).

2.6.2 *Syntenic regions*

Comparative genomics is the comparison of the genome of different organisms with the purpose of understanding the structure and function of the genomes, allowing the translation of findings from one species to another.

One of the main tools used in comparative genomics is the identification of syntenic blocks (Ghiurcuta and Moret, 2014), which can be defined as blocks of DNA with evolutionarily conserved features (Sinha and Meller, 2007).

The main advantages of the identification and analysis of synteny for genome comparison are the viability of the analysis despite individual variability, the identification of new candidate regions of interest for studies, and the possibility of the use of visualisation tools for whole-genome comparisons (Ghiurcuta and Moret, 2014).

Figure 2.7 shows an example of a whole-genome analysis in comparative genomics, using the Cinteny® software (BMC Bioinformatics) to visualise synteny blocks for human and bovine. The genome shown in the right panel (Human) is, in this case, the source genome, and the genome on the left (Cow) is the target genome.

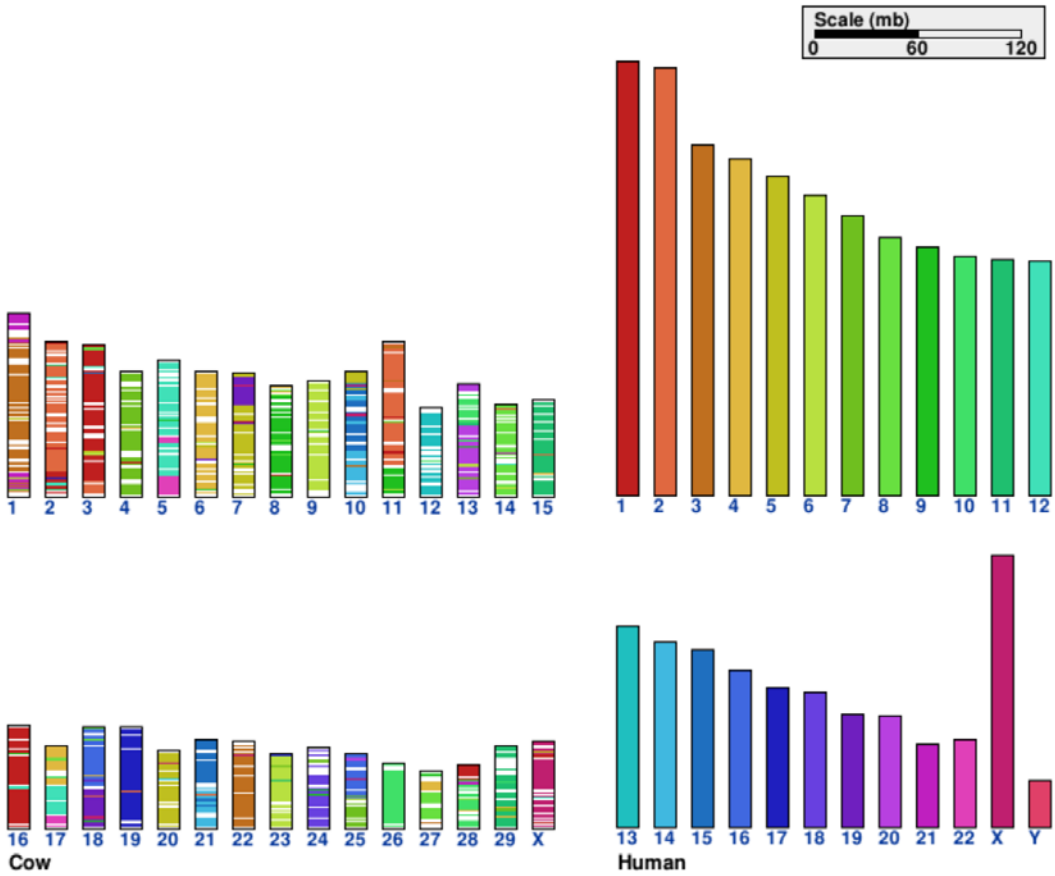

Figure 2.7 *Visualisation of synteny between bovine and human genomes.* Chromosomes of the human genome are shown in solid colours, and each Chromosome in the bovine genome is composed of segments of Chromosomes from the human genome (Cinteny®, BMC Bioinformatics).

The figure shows that most of bovine Chromosome 19 (Bota 19) is composed of, and syntenic to, human Chromosome 17 (Hosa 17). A similar situation occurs in Bota 6 and Hosa 4, Bota 15 and Hosa 11, Bota 16 and Hosa 1, and X Chromosomes.

Figure 2.8 shows a simple synteny analysis at a Chromosome level using Cinteny® software (Sinha and Meller, 2007). In this example, I chose Bota 19 and Hosa 17, where each synteny block is shown in a unique colour and connected to the corresponding synteny block on the other Chromosome.

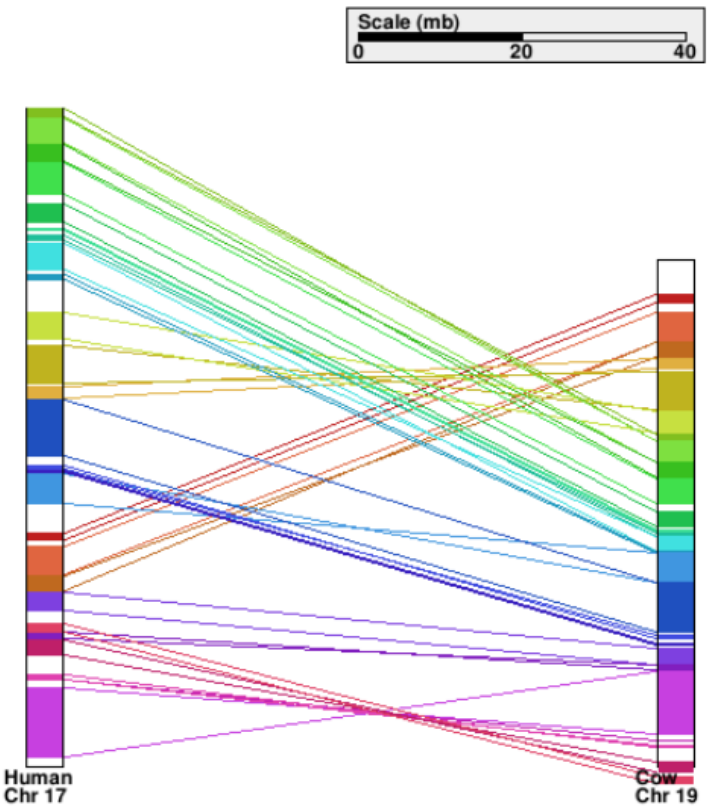

Figure 2.8 *Visualisation of synteny between bovine Chromosome 19 (Bota 19) and human Chromosome 17 (Hosa 17)* (Cinteny®, BMC Bioinformatics).

Once identified the synteny of some areas of interest on a different species, then it is possible to translate the findings from one species to the other, in this case, from human to cattle and from cattle to human.

2.7 *Measuring marbling*

As a response to a declining beef consumption, MLA (Meat and Livestock Australia) created MSA (Meat Standards Australia) in 1998. The large variability of eating quality was identified as the main contributor to the problem, and the key to address the issue was to design a system able to predict eating quality prior to consumption.

The system was designed using almost 800,000 consumer taste tests by more than 114,000 consumers from 11 countries, leading to the implementation of the MSA grading system,

which identifies marbling as a key factor for eating quality (Meat and Livestock Australia, 2018).

Multiple systems have been tried in an attempt to measure marbling. Cheng et al., 2015, described the different methods for measuring marbling, summarised in Table 2.4.

T**able 2.4** Marbling measurement methods.

Method			Characteristics	Advantages	Drawback
Conventional Methods	Visual appraisal		Uses human graders. Used in the USA, Japan and Australia. United States: Marbling Scores to the LD muscle between the 12th and 13th rib into 7 grades. In Japan, 12 categories in the LD muscle between the 6th and 7th rib. 5th to 13th rib Australia.	Traditional and easily accepted. Widely used for many years.	Laborious and tedious, subjective, and inconsistency.
	Chemical analysis		Uses solvents as ether or chloroform/methanol to calculate IMF content.	Accuracy.	Destructive, expensive and time-consuming, It cannot reflect the visual appearance of samples.
Instrumental techniques	Spectroscopic techniques	Near-infrared spectroscopy. NIR	Measures wavelength of light when sample irradiated with near-infrared light.	Low cost of instruments, high sensitivity to fat and fast.	Cannot provide spatial information of the samples.
		Bioelectrical impedance spectroscopy. BI	Measures the electronic conductivity of meat.	Fast and objective.	Good for ground meat. The system needs improvement for intact meat
		Nuclear magnetic resonance spectroscopy. NMR	Gives IMF content based on the fat–water chemical shift in the NMR spectrum. The different methods tried are Fast inversion-recovery (FIR), Carr-Purcell-Meiboo-Gill pulse sequence (CPMG), continuous wave-free precession (CWFP).	Non-destructive and potentially fast and accurate depending on the method used.	High cost of instruments and strict testing environment.
	Imaging techniques	Computer image analysis. CIA	Uses a camera and an algorithm. Requires the segmentation of marbling from the muscle and marbling feature extraction.	Non-destructive and objective. Accurate and repeatable. It is the most promising technique. It is the best match to human eyes.	Image segmentation is an unsolved problem. Up until now: not robust enough, not adaptable real-time inspection.

		Ultrasonic imaging. UI	Measures the echo reflected from the fat/muscle interface.	Allows measurements on live animals, allowing genetic studies of breeding and sorting of feedlot cattle.	Low prediction accuracy.
		X-ray computed tomography. X-ray CT	Measures the difference of the attenuation of an X-ray when it passes through tissues of different densities.	Nondestructive. Promising for carcass and live animals.	More investigation is necessary to improve robustness.
	Spectral imaging technique	Hyperspectral imaging technique. HIS	Integrates conventional imaging and spectroscopic techniques.	Accurate and fast assessment of marbling levels.	Slow. The dimensionality of the hyperspectral data needs to be reduced for real-time inspection.

Out of all the methods described, the most successful and widely used is visual appraisal, used in the United States, Japan and Australia. However, it requires human graders, becoming laborious, tedious, subjective, and inconsistent. The beef industry urgently needs an objective, practical, efficient and repeatable system to reach the standards of quality required.

2.7.1 *Fine marbling*

Intramuscular fat accumulation is a complex trait that cannot be grouped under one single concept. The lack of detail in its description is a reflection of limited understanding.

In addition to the quantity of intramuscular fat, there is another factor to consider, which is the way intramuscular fat is distributed within the muscle. Kato et al. (2014) calculated the heritability of the New Fineness Index of Marbling (NFI) and compared it with the traditional Japanese Beef Marbling Score (BMS). Later, in 2017, Asa et al. established that NFI is a better indicator of palatability than BMS. Meanwhile, the Australian Wagyu Association

(AWA) has included the Camera Fineness Index as a trait of importance for Wagyu beef, with estimations of its heritability and EBV already available.

2.8 *Histology*

Histology has been widely used in human research to visualize and describe the morphological changes that the muscle undergoes during the process of intramuscular fat deposition at a cellular level. The same techniques can be applied to animal muscle, with the use of different stains that target different tissues and their components.

Microscopy allows us to observe the muscle structure and the real location of fat depots in the muscle. It is possible to differentiate connective tissues, like epimysium, perimysium and endomysium, and categorise the adipocytes based on the connective tissue in which they are located. With the use of microscopy, it is also possible to analyse cellular changes that muscle and adipose cells present during the process of accumulation of intramuscular fat.

As previously mentioned, some researchers (Tian et al., 2005; Jackman et al., 2009; Gotoh et al., 2014) have histologically described some changes that the honeycomb structure of the endomysium suffers during marbling, which explains in part the effect of the process on tenderness.

A good example is the description of changes published by Nishimura et al. in 1999, using electron microscopy. They describe the changes in the structure and the effect on mechanical properties of the connective tissue of the *Longissimus* muscle of Wagyu during fattening. With an initial toughening of the beef due to an increase of the mechanical strength of the connective tissue at initial stages, with a later increase of intramuscular fat and disruption and disorganisation of the honeycomb structure of the connective tissue and a resultant tenderisation of the beef.

In Limb-girdle type 2B, using the histological dye Oil Red O, is possible to see intramyocellular lipid droplets, sometimes with considerable levels of muscle fibres replaced by fat (Grounds et al., 2014). In 2016 Listrat et al. stated that in normal healthy muscles, intramyocellular lipid droplets can reach up to 20% of the intramuscular fat. However, the origin of that data could not be confirmed in this review.

Intramyocellular fat is not visible to the naked eye and consequently is not considered in the traditional intramuscular fat estimation. Fortunately, once again, histology gives us alternatives. As mentioned above, Oil Red O dye can be used to stain, visualise and analyse the presence of fat into the muscle fibre.

2.9 *Sequential monitoring*

There is no doubt that IMF deposition and fat desaturation are conditioned by genetics and environment, and mediated by complex enzymatic pathways.

Many studies have measured different variables at slaughtering, including the activity of SCD in fat and muscle (Taniguchi et al., 2004; Smith et al., 2012), but to achieve a greater and practical level of understanding, it would be essential to analyse and observe the processes of intramuscular fat accumulation while occurring, analysing the process at a molecular level but also the anatomical changes.

2.10 *The problem/need*

The lack of understanding of the process of IMF deposition has led to huge inefficiencies and waste of resources in meat production.

Every Wagyu beef producer runs a different system based on his knowledge, experience, individual environment and market condition. However, the common denominator is the

period of grain feeding to induce the changes that give the meat its intramuscular fat responsible for the flavour, juiciness, and tenderness.

With periods that vary from 300 to 600 days and feeding costs around $5 per day, grain feeding becomes one of the main costs of producing well-marbled beef.

There is no doubt that different breeds have different marbling potential. Wagyu beef is at the top of the list, making it very appreciated in some markets. The issue is that even within breeds, the differences in performance are still significant.

Unfortunately, as science have not yet generated the knowledge nor provided the tools to determine the production potential of individual animals, Wagyu producers have no other option than blindly put all their animals through a common feeding regime, where just a fraction of them are expected to perform well enough to cover the costs of the total number of animals and generate the profits for the business, while the rest are hoped to break even at best.

In general terms, animals on a traditional Wagyu farm in Australia would break even when reaching a Marble Score 6 or 7 (0 to 9 scale). 20% to 40% of the carcasses are at or below that point. That means that the profits are made out of just 60% to 80% of the animals. This is an evident and painful waste of resources and effort, which generate a huge level of frustration in high-quality meat producers, but also a great opportunity for improvement.

Animals that under the current system do not generate profits after long feeding periods have always had the potential of performing perfectly well under a different system with shorter times on feed, being able to become a product that satisfies some markets that demand less marbled good quality for a lower cost.

A practical and reliable tool to predict performance is not existent at the moment. By creating it, there is an opportunity to generate a great contribution to agriculture and human health.

2.11 *Conclusions*

From the literature review, it is clear that the improvement of the quantity and quality of the intramuscular fat in beef is a great challenge, and the considerable implications for the beef industry and also for human health justify the significant efforts that researchers and beef producers have been making in the last decades.

We know that environment plays an important role in intramuscular fat deposition in conjunction with genetics, and that successful strategies used in monogastrics to affect the fatty acid composition of the meat cannot be applied to cattle. However, the issue remains poorly understood.

Fortunately, superior genetics are available. In Japan, some breeds have developed over the years showing an outstanding performance in quantity and quality of fat. Now the aim is to investigate and fully understand what's behind that superiority to then generate technological outcomes that allow us to contribute to the improvement of these traits.

3 Genetics of marbling in Wagyu revealed by the melting temperature of intramuscular and subcutaneous lipids[1]

The importance of fat desaturation in the human diet has been well established, and the advantages of the replacement of UFA for MUFA and MUFA are clear.

At the same time, advances in measuring the melting temperature of animal fat (Tm) on a reliable, practical and repeatable way have been made and are currently published and available.

The variability of the Tm of bovine fats depends on many factors, like age, climate, nutrition, genetic and possibly many others. However, the way those factors contribute to the variability of that important trait is not completely understood.

In this paper, we have considered multiple traits that are expected to be related/correlated to fat desaturation in bovines. We have maintained the animals under the same environmental conditions; we have compared genetically similar animals and their performance over time and the effect of the use of different sires and breeds over Tm of the offspring.

Furthermore, we have validated and tested the practicality of CYO Tm measurement technique for bovine fat Tm analysis. We have confirmed the correlation between the measurement of Tm of rendered fat and the Tm of the fat obtained during the process of DNA extraction.

The results are promising and provide evidence for the effect of environmental factors but also the heritability of Tm, reinforcing the consideration of DNA analysis and selective breeding to make sustainable improvements.

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[1] Published as: Lloyd, S., Valenzuela, J., Steele, E., and Dawkins, R. (2017). Genetics of marbling in Wagyu revealed by the melting temperature of intramuscular and subcutaneous lipids. *International Journal of Food Science*. Volume 2017, Article ID 3948408, 6 pages. https://doi.org/10.1155/2017/3948408

There is no doubt that the process of fat desaturation is complex and multifactorial. However, the use of the right genetic testing combined with the appropriate technique for measuring Tm will be the key to achieving substantial results.

Being part of the CY O'Connor Foundation and Melaleuka Stud allowed me to actively participate in all areas of this research, from the activities with the cattle in the field, the processing of the biological samples, to the analysis of the data.

As a co-author of this paper, my contribution was:

i. Maintaining records of Melaleuka Stud herd, including feeding and joining for further parental verification.

ii. Tissue sampling of Melaleuka Stud's dams, sires and calves.

iii. DNA processing for parental verification of the calves and for DNA matching of the carcasses.

iv. Monitoring nutritional management of animals.

v. Sampling of the carcasses.

vi. Tm measuring.

vii. Revision and corrections of the paper.

viii. Writing part of the Material and Methods section.

During the analysis of the data for this study, I came to the realisation that on multiple occasions, the DNA extracted from the carcasses did not match the DNA of the animals delivered to the abattoir. The impact of that issue can be of huge proportions in the Australian beef industry, and the need for analysis, quantification and impact of the problem became

evident. Therefore, further quantification and analysis of the problem became necessary. This issue is addressed in Chapter 5 of this thesis.

3.1 *Abstract*

Extreme marbling or intramuscular deposition of lipid is associated with Wagyu breeds and is therefore assumed to be largely inherited. However, even within 100% full blood Wagyu prepared under standard conditions, there is unpredictable scatter of the degree of marbling. Here, we evaluate melting temperature (Tm) of intramuscular fat as an alternative to visual scores of marbling. We show that “long fed” Wagyu generally has Tm below body temperature but with a considerable range under standardised conditions. Individual sires have a major impact indicating that the variation is genetic rather than environmental or random error. In order to measure differences of lower marbling breeds and at shorter feeding periods, we have compared Tm in subcutaneous fat samples from over the striploin. Supplementary feeding for 100 to 150 days leads to a rapid decrease in Tm of 50% Red Wagyu (Akaushi): 50% European crosses, when compared to 100% European. This improvement indicates that the genetic effect of Wagyu is useful, predictable, and highly penetrant. Contemporaneous DNA extraction does not affect the measurement of Tm. Thus, provenance can be traced and substitution can be eliminated in a simple and cost-effective manner.

3.2 *Introduction*

Marbling (or the accumulation of intramuscular fat) is the holy grail for beef producers, chefs, and their customers, but there is still no agreed definition and therefore no universal standard of measurement (Johnson et al., 1986; Cheng et al., 2015). So as to increase commercial returns based on superior taste and health benefits, there have been countless

attempts to improve the reproducibility of visual and scanning scores but with limited success (Kuchida et al., 2000; Cheng et al., 2015).

Lipid profiles of highly marbled samples have revealed a high content of oleic acid and therefore a reduction in melting temperature (Tm) (De Smet et al., 2004; Chung et al., 2006; Smith et al., 2009). A precise and high throughput method for the measurement of Tm exists(Lloyd et al., 2014b) and is used here to interrogate the complex interplay between the genetic and environmental factors which can be optimised by the producer to the benefit of the health-conscious consumer.

Because of the association of Wagyu breeds with high marbling and high oleic acid content, these traits can be assumed to be genetically determined and faithfully inherited (Zembayashi et al., 1995; Malau-Aduli et al., 2000). However, in spite of numerous studies (Shibata et al., 2006; Cheong et al., 2007; Zhang et al., 2008; Matsuhashi et al., 2011; Han et al., 2013; Saatchi et al., 2013; Xu et al., 2013; Hayakawa et al., 2015; Bartoň et al., 2016; Papaleo Mazzucco et al., 2016), it has not been possible, hitherto, to identify markers which allow a breeder to quantify superior genetics in individual sires and dams. Some of the explanations for the slow progress include the following:

i. Complexity due to interactions of several metabolic processes and their regulatory mechanisms (Smith et al., 2009; McGilchrist et al., 2011a; McGilchrist et al., 2011b).

ii. Contribution of many genes with small effects (Hill, 2014).

iii. Uncontrolled environment factors associated with supplementary feeding (Durmic et al., 2008; Durmic et al., 2014; Myer et al., 2015).

iv. Difficulty in quantifying marbling reproducibly (Johnson et al., 1986).

v. Unreliable tracing of meat from paddock to plate.

vi. Perception that fat is dangerous.

Recently it has been demonstrated that low fat diets have not improved health (Teicholz, 2014). In fact, higher oleic acid and therefore low Tm are preferable in terms of lipid profiles (Mensink et al., 2003; Adams et al., 2010; Gilmore et al., 2011). This has led to the increasing popularity of the Wagyu brand world wide.worldwide. Not surprisingly, mislabeling is now rife, resulting in the need to be able to confirm the provenance of retail samples.

Here we show that low Tm is heritable and that the same fat sample can be used for the DNA tracing without affecting the measurement of Tm.

3.3 *Materials and methods*

Postmortem samples of meat and fat were taken from carcasses of animals harvested for routine food production. Therefore, ethics approval was not required.

3.3.1 *Dataset 1 Full Blood Wagyu with identified sires*

Two cohorts of Wagyu steers (n=126) were fed for 300±20 days with a proprietary ration within the same commercial feedlot. One-gram samples of meat from the *Longissimus dorsi* were taken from between the 10th and 11th rib. AUS-MEAT marbling score (MS) was scored between the 10^{th} and 11^{th} rib, with an average of 7.5 and a range from 2 to 11. Steers for the comparison of sires had their paternity confirmed by DNA testing (Williamson et al., 2011). Only one progeny of each dam was included so as to focus on the effect of the sire.

3.3.2 *Dataset 2 European and Wagyu cross breeds with varied feed time*

Melaleuka stud, located in the Peel region of Western Australian, 100 km south of Perth, runs a variety of European breeds including Simmental, Gelbvieh and Angus. This herd was selected to produce high quality beef on pasture, finished with 2 to 4 months of supplemental feeding. Black and Red Wagyu (full blood or purebred) have been mated with these European breeds.

Calves stay on milk until 4 months of age when they are weaned and male calves are castrated. After weaning, they continue grazing Kikuyu and Ryegrass pasture until they reach 300 kg. Their feed is then supplemented with 9mm EasyBeef pellets (Milne Feeds, Perth, Australia) *ad libitum*. The main ingredients of the EasyBeef pellets are lupins, barley, oats, wheat, and triticale.The nutritional composition, based on dry matter, is crude protein (min) 14.5%, metabolizable energy (est.) 11.0 MJ/kg., crude fiber (max) 20.0%, urea (max) 1.5%, and monensin 26.6 ppm. The feeders are considered ready for slaughter when they reach a weight of 400 kg and are slaughtered to match demand. Some animals were kept on feed longer to test the effect of increased feeding on Tm and meat quality. The average live weight at slaughter for animals in this study was 461 kg, average age at slaughter was 15.4 months (range 8 to 23), and the average days on feed was 104 days (range 17 to 288). Body numbers from abattoirs were matched to farm records and pedigrees via their RFID tags, where possible identity was confirmed by in-house proprietary DNA testing (Williamson et al., 2011).

Subcutaneous fat overlying the striploin (HAM number 2140) of these cattle was collected after boning and wet aging for 1 to 3 weeks.

3.3.3 *Fat extraction and Tm measurement*

Intramuscular fat was extracted from dataset 1 samples by digestion with proteinase K. This method allows for simultaneous extraction of intramuscular fat and DNA from 0.5 gram samples of meat if the fat content is above 20%. The samples were incubated at 56°C, digested in a proteinase K mixture for 4 hours and centrifuged at 10,000 ×g for 2 minutes to separate the fat from the dissolved DNA and protein solution. Fat was removed for Tm measurement by pipette. DNA was extracted from the remaining mixture using a standard salting out method.

Intramuscular fat content for many of the carcasses of dataset 2 was too low to allow extraction by the above method. Instead, fat was extracted from 1-gram samples of subcutaneous fat by rendering at 90°C for at least eight hours.

Samples from 17 sirloin steaks with intramuscular fat higher than 20% were used to determine whether fat separated during a DNA extraction process could be used for Tm measurement. Fat was extracted by both digestion and rendering from the same samples and the Tm measurements compared.

Tm of all fat samples was determined in triplicate according to the thermocycler method (Lloyd et al., 2014b), which is closely correlated to slip points, although the values are higher by 2°C for animal fat with a Tm of 40°C.

3.4 *Results*

3.4.1 *Tm is affected by sire*

Samples were taken from long fed Wagyu steers differing only by sire and dam (dataset 1). The steers were fed, harvested, and tested in two cohorts two months apart. The cohorts did not differ significantly in feeding, genetics, or initial Tm (as shown in Supplemental Table 1

in Supplementary Material available online at https://doi.org/10.1155/2017/3948408) and have therefore been combined for further analysis. Tm and marble score were analysed by sire for the three sires with more than ten progenies. As shown in Figure 1, the Tm of the progeny of Sire 2 fell consistently, whereas Sire 1 had little impact. In fact, 14 progenies of Sire 1 were above 37°C, compared to only 3 of Sire 2. The cross-product ratio is 104/6 or 17, as shown in Figure 1. This difference is highly significant (p value < 0.01 by $\chi 2$). It is noteworthy that there is more scatter with Sire 3 and all remaining sires.

By contrast with Tm, visual scores of marbling gave greater scatter, did not demonstrate a sire effect, and must be misleading in their present form.

3.4.2 *In Wagyu, Tm falls with days on feed and proportion of Wagyu*

Notwithstanding the genetic effects, there is also a major environmental effect on Tm and marbling. Tm results of dataset 2, grouped by proportion of black Wagyu, are shown in Figure 2. Tm falls with increase in Wagyu and days on feed. Separating these two variables is not yet possible but, in the meanwhile, the results suggest that increasing the content of Wagyu genes allows the benefit of long feeding. The European cattle included in this study do not show the same benefit as the Wagyu.

Importantly, the benefits are seen with only 25% Wagyu, again emphasizing the high penetrance of the Wagyu genetics.

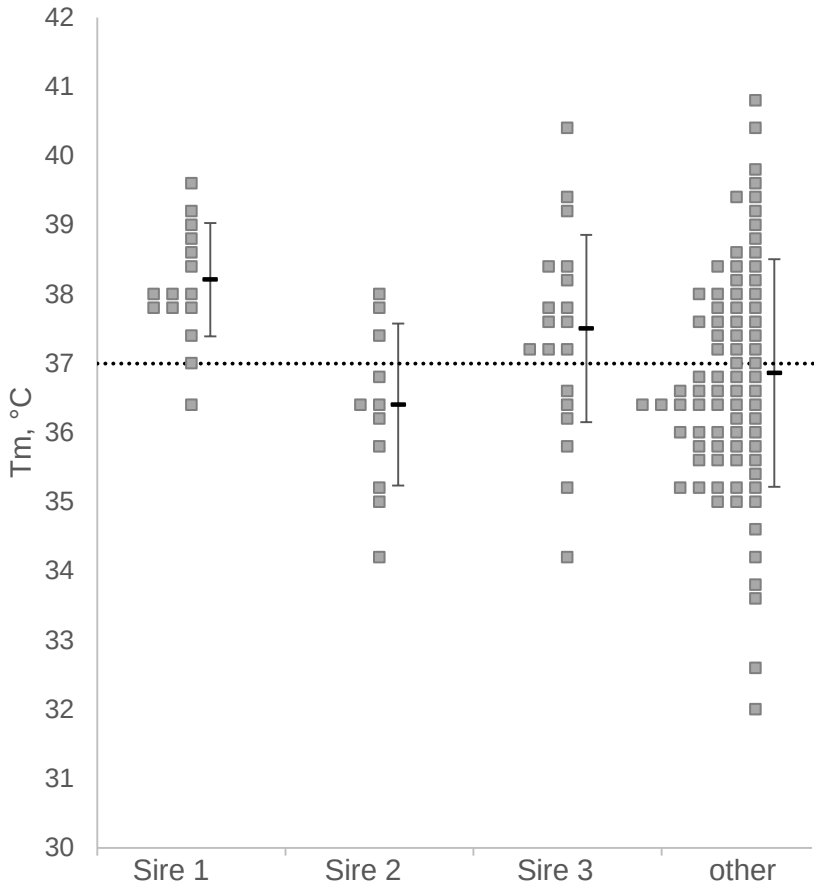
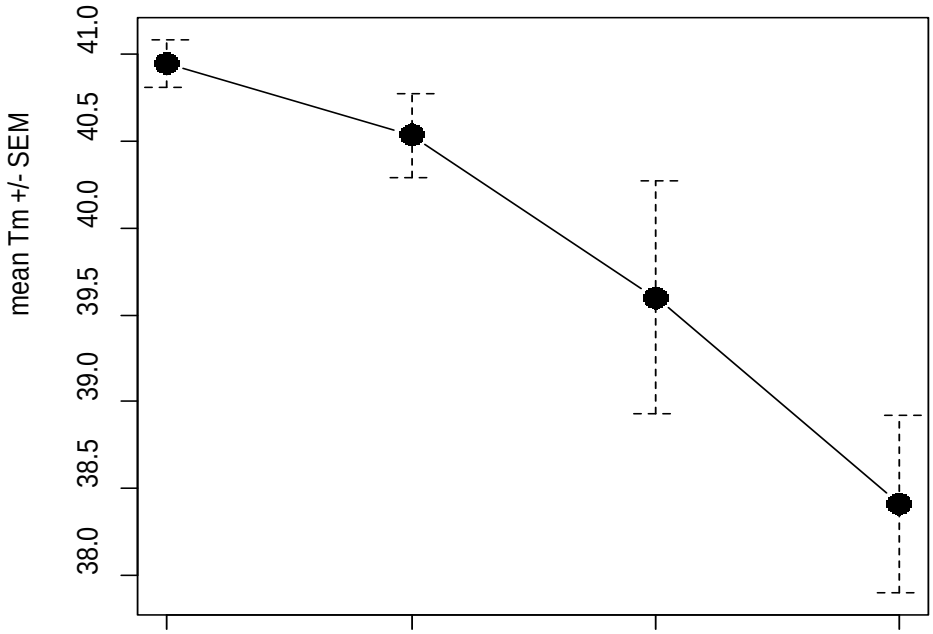


Figure 3.1: *Tm distributions of Wagyu carcasses differ by sire*. The melting temperature of intramuscular fat samples taken from between the 10thand 11th rib of 126 carcasses of full blood Wagyu steers. All animals were fed the same ration for 300±20 days. Individual Tm measurements of carcasses are grouped by sire (mean and standard deviation). Animals with either an uncertain sire or a sire with less than 10 progeny are grouped under “other” sires. Progeny of Sire 3 shows considerable scatter, whereas 8/11 of those of Sire 2 are below 37 degrees compared with 1/15 in the case of Sire 1. The difference between Sire 1 and Sire 2 is statistically significant with a chi-square statistic of 12.2 and thus a p value < 0.01.



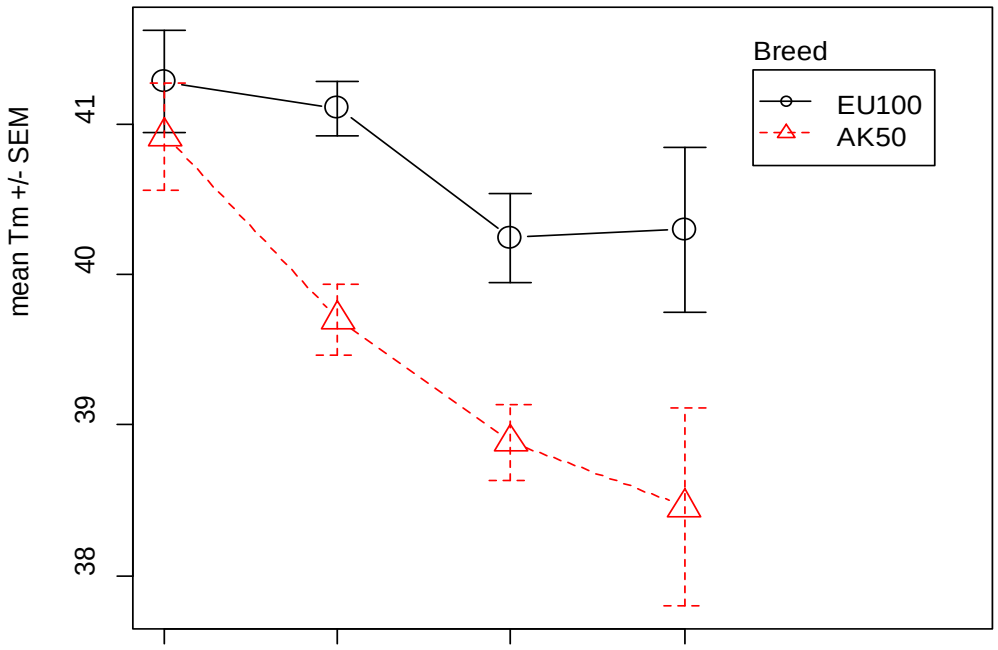
Breed	EU100	WY25	WY50	WY75+
N	176	29	11	15
DOF. Mean ± SD	84±41	103±52	167±103	225±79

Figure 3.2: *Tm decreases with feeding and increasing proportion of Wagyu ancestry*. Tm of subcutaneous fat samples over the loin of a mix of breeds and crossbreeds including Simmental, Gelbvieh, Angus, Dexter, and Wagyu. 176 samples (EU100) came from 100% European breeds fed for an average of 81 days. WY25, WY50, and WY75+ samples had 25%, 50%, and 75–100% Wagyu ancestry, respectively. There were 29 samples of WY25, 14 samples of WY50, and 11 samples of WY75+ with average days on feed of 103, 167, and 225, respectively.

3.4.3 *Quantitative effect of feeding*

So as to address the complex interaction between genetics and environment, we compared two breed groups from dataset 2: a control group of purely European cattle (EU100) and the F1 Red Wagyu, also known as Akaushi and recorded as AK50. The dams have a similar breed composition and history to the EU100 control group. So as to avoid the complexity of

sampling intramuscular fat before it is visible, we have relied on Tm measurements of overlying subcutaneous fat. The effect of feeding is clear, as shown in Figure 3. Tm falls progressively even with only a 50% infusion of Akaushi.



Days on Feed	1 to 50	51 to100	101 to150	Over 150
Number of EU100	26	84	34	9
Number of AK50	5	33	31	7

Figure 3.3: *Red Wagyu sired carcasses have lower Tm for equivalent DOF*.Tm was measured for subcutaneous fat samples taken from the loins of 229 carcasses. The cattle were backgrounded on pasture and then fed on pellets until they reached a satisfactory weight and fatness. The results are grouped by days on feed and by breed of sire (European or Akaushi). The dams of all carcasses were European breeds. Breed and days on feed were both statistically significant influences on Tm, with $p < 0.01$ calculated by multiway ANOVA. The difference between the two groups was significant after only 51–100 days on feed.

3.4.4 *DNA extraction does not invalidate measurement of Tm*

In Figure 4 we show that extracting DNA with the proteinase K does not affect the measurement of Tm on the same extract. Oxidation of the polyunsaturated fatty acids in the sample that may have occurred during rendering at 90°C did not have a measurable effect on the melting point, as expected (Wood et al., 2004; Chung et al., 2006).

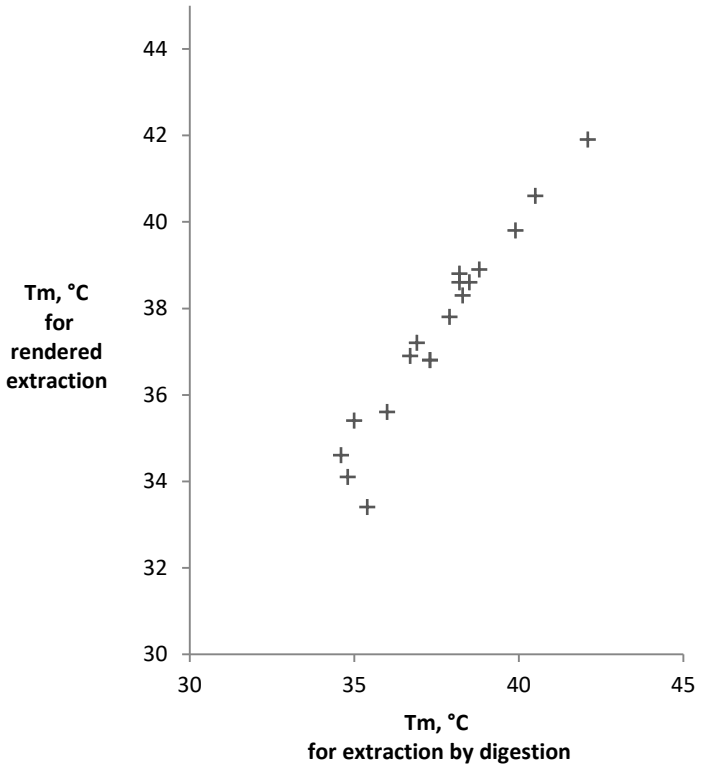


Figure 3.4: *Simultaneous extraction of fat and DNA does not change Tm.* There is excellent correlation between Tm measurements of fat harvested during DNA and extracted by rendering (Pearson's R = 0.97). There was no measurable bias (mean difference 0.13, SEM = 0.14). Either extraction method can be used for direct comparison without adjustment.

3.5 *Discussion*

The intention of these studies is to resolve, in part, the manifest confusion facing producers and consumers of healthy beef.

It is clear that Wagyu beef is superior, as reflected by the commercial returns for highly marbled beef, but increasingly the brand is amenable to misuse.

A major issue is the lack of a reproducible measurement of the degree of marbling. Multiple and incompatible systems of scoring may have been retained perhaps to the advantage of some sectors. The measurement of Tm is possible at successive stages of the production line so that quality can be confirmed. At the same time, DNA can be extracted so that provenance can be confirmed.

The difficulty faced by the breeder is even more important. Nonreproducible measurement obfuscates attempt to identify breeding values and therefore confound selection of superior sires. This issue becomes particularly important in an attempt to upgrade first crosses.

The present results show that even WY25 can have reduced Tm but the scatter is substantial leading to lack of consistency. Future studies may identify those sires which are well suited to crossbreeding.

So as to reduce the number of variables we sampled AK50 at differing days on feed. All had European dams. The initial results are promising in that there was a progressive decline in Tm. Further work may define the preferred type and length of supplementary feeding. Importantly, there is also the potential to examine the controversies surrounding the use of grass versus grain. Whilst there is growing consumer demand for less intensive feeding and especially for “grass-fed”, there is also the perception that corn and perhaps other grains are necessary for extreme marbling. Given reproducible measurements, it should be possible to define acceptable compromises between supplementation, on the one hand, and tastiness and healthiness, on the other hand.

A major finding of this study is the difference in Tm between the progeny of two full blood Wagyu sires. Sire 1 and Sire 2 share a paternal and a maternal grand sire and were imported from the same prefecture in Japan. Pedigree analysis alone would not predict large differences in lipid composition. It is noteworthy however, that sire 1 and sire 2 are quite different in their C19 haplotypes, as described elsewhere (Lloyd et al., 2017a). A major issue remains unresolved. The degree of marbling and the lipid profile differ depending upon the site of sampling; as an approximation the intramuscular accumulation of lipid progress from the brisket backwards with the more caudal fat deposits having somewhat lower proportions of oleic acid and higher Tm (Smith and Johnson, 2014). Therefore, comparable samples need to be from a fixed location. Even within the same muscle group there is variation depending upon sampling (Nakahashi et al., 2008). We recommend further experience using subcutaneous fat so that its utility can be extended. Ultimately, it should be possible to take *in vivo* samples so as to monitor changes with time, genetics, and feed.

4 Haplotypes for type, degree, and rate of marbling in cattle are syntenic with human muscular dystrophy[2]

In the previous chapter, we confirmed some of the environmental factors that play an important role in the level of marbling and desaturation of the fat accumulated in the muscle. We also established clear differences between breeds and between sires within breeds. The evidence for a significant genetic contribution provoked us to search for the genes responsible for marbling and low Tm.

Many others have tried to identify genetic markers for these traits. Their lack of success is evident, as well as the reasons. As with many other traits, marbling and Tm are controlled by multiple genes, in many cases conserved in stable multigene sections of DNA, where recombination does not occur. These sequences are inherited together faithfully for many generations. Therefore, the gene combination and many of their interactions are preserved. These sequences, called ancestral haplotypes, are polymorphic and the genes included in them will condition the potential of each individual for marbling and production of healthy fat.

This paper addresses how, due to the relatively recent evolutionary divergence between human and bovines, blocks of genes of importance in Limb-girdle muscular dystrophy in human are also found in bovine. That concept, known as synteny, occurs in many positions of the genome. In this paper, the syntenic region of interest is located in chromosome 17 of humans (Hosa 17) and 19 of bovines (Bota 19).

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[2]Published as: Lloyd, S.S., Steele, E.J., Valenzuela, J.L. and Dawkins R.L. (2017). Haplotypes for type, degree, and rate of marbling in cattle are syntenic with human muscular dystrophy. *International Journal of Genomics.* Volume 2017, Article ID 653283714 pages. https://doi.org/10.1155/2017/6532837

In Bota 19, the genes associated with fatty acid composition, which define the haplotypes analysed in this document, are MPRIP, TCAP, SREBF1 and NT5M.

Using W plots, we compare the frequencies of haplotypes in two different breeds, Simmental and Wagyu, showing that while some haplotypes are present in both breeds, others are breed-specific, which could explain the differences in phenotype related to muscle and fat metabolism.

We review the effects of different Bota 19 haplotypes in animals within the same Wagyu breed. Also, we show how the infusion of Wagyu specific haplotypes in different breeds can affect Tm. Of particular interest, this work shows that the rate and degree of marbling are genetically determined and will be different between animals entering the feedlot.

We present the multiparameter mathematical model for fat melting temperature, (developed by Dr Lloyd, CY O'Connor Foundation), to assist the understanding of the process. We also show how differences in TCAP allele result in curves of desaturation similar to the prediction of the model.

As a co-author of this paper, my contributions were:

i. Collection of tissue samples for DNA testing.

ii. Parental verification of calves for breed confirmation.

iii. Laboratory testing for haplotype testing and Tm.

iv. Revision, correction and analysis of the content of the paper.

The similarities between the genome of human and bovine, shown in this paper, allows one species to benefit from the research and discoveries made in the other.

There are commercial and welfare issues which limit the duration of lot feeding, indicating that there is a need for a method to quantify marbling during life and preferably at several stages during feeding. Other measures, such as DEXA, may prove helpful, but I have chosen to focus on melting temperature and histology.

With further development of the technique, it is possible that Tm can serve as a useful and minimally invasive screening test (see Chapter 6).

It was my responsibility to examine the cattle for evidence of muscle weakness which might suggest dystrophy. There were no clinical signs of dystrophy.

4.1 *Abstract*

Traditional analyses of a QTL on Bota 19 implicate a surfeit of candidates, but each is of marginal significance in explaining the deposition of healthy, low melting temperature fat within marbled muscle of Wagyu cattle.

As an alternative approach, we have used genomic, multigenerational segregation to identify 14 conserved, ancestral 20Mb haplotypes. These determine the degree and rate of marbling in Wagyu and other breeds of cattle.

The melting temperature of intramuscular fat is highly heritable and traceable by haplotyping. Fortunately, for the production of healthy beef, some of these haplotypes are sufficiently penetrant to be expressed in heterozygous crossbreds, thereby allowing selection of sires which will improve the healthiness of beef produced under even harsh climatic conditions.

The region of Bota 19 is syntenic to a region of Hosa 17 known to be important in muscle metabolism and in determining susceptibility to a form of human muscular dystrophy.

4.2 *Introduction*

Conserved ancestral haplotypes have been highly informative in revealing the functional significance of polymorphic sequences especially when extensive enough to encode regulatory regions as well as structural elements (Dawkins et al., 1999; Dawkins, 2015; Lloyd et al., 2015).

In *Homo sapiens* (Hosa), there are now many examples of conserved haplotypes regulating the degree of expression of a trait or disease such as an enzyme deficiency, immune competence, drug reactions, and disease severity. The best known examples are the extended haplotypes of the major histocompatibility complex (Awdeh et al., 1983; Dawkins et al., 1999; Alper et al., 2000; Yunis et al., 2003; Lloyd et al., 2015; Raj et al., 2016) but the principle applies throughout the genome, (Steinberg et al., 2012) with the important caveat that the haplotypes are identified and tagged correctly (Johnson et al., 2001; Alper et al., 2006). Many of these haplotypes were discovered through the finding of genetic associations with quantitative traits, such as diseases. It became obvious that there are tens or even hundreds of alternative haplotypes encoding functionally important regions of the genome and that these interact with each other positively or negatively. A potent example is IgA deficiency (Cobain et al., 1983; Wilton et al., 1985; French and Dawkins, 1990; Dawkins et al., 1999; Alper et al., 2000) which is associated with the 8.1 haplotype *inter alia*. The precise mechanisms underlying this regulatory defect are not clear from analysis of SNPs and coding sequences (Sekine et al., 2007).

For the breeding of livestock, it is traditional (Simm, 1998; Khatib, 2015) to follow the precepts of the infinitesimal model which assumes there are independent genes separated by free recombination (Hill, 2014). However, it is quite clear that this assumption is not correct (Hill, 2014; Dawkins, 2015). The genomic architecture of cattle (Fadista et al., 2010; Hou et

al., 2011; Bickhart et al., 2012; Hou et al., 2012), sheep (Fontanesi et al., 2011; Liu et al., 2013), goats (Cameron et al., 1990), dogs (McLure et al., 2005; Alvarez and Akey, 2012), mice (Locke et al., 2015), and pigs (Fadista et al., 2008; Wang et al., 2012) is remarkably similar to that present in Hosa, where there are kilobase and even megabase blocks of polymorphism at which there are retroviral and other indels, segmental duplications, and differences in copy number in addition to nucleotide substitutions (Dawkins et al., 1999; Shiina et al., 2004; McLure et al., 2013; Lloyd et al., 2015).

In cattle, there are many traits of proven importance to the production of healthy food. There have been numerous attempts to find useful genetic markers if only for selection of superior breeding pairs for commercial gain. Results have been disappointing for several reasons, generally reflecting an oversimplified understanding of the genomic structure. Thus, conserved ancestral haplotypes can be confounding if not identified. For example, desirable traits such as increased milk production can be associated with disadvantages such as infertility (Dawkins, 2015). In other instances, the genetic marker may appear useful in one breed but not another, as illustrated by polling (Dawkins, 2015).

Here, we address these disappoinTments by identifying megabase haplotypes of critical interest to healthy food and public health (Teicholz, 2014; Teicholz, 2015) and potential relevance to the metabolic changes underlying Limb-girdle muscular dystrophy.

True or fine marbling describes the deposition of lipid between and within muscle fibres after supplementary feeding for a year or more occurs. The genesis is very complex, including differentiation of satellite or progenitor cells, and is obviously tightly regulated, (Smith and Johnson, 2014), but the ultimate effect is to improve taste and juiciness thereby increasing the value of beef several folds. More importantly, marbling increases the content of the healthy, monounsaturated fatty acid (MUFA) and is therefore “cholesterol lowering” and statin-

sparing (Adams et al., 2010; Gilmore et al., 2011). These features are characteristic of Japanese cattle, known as Wagyu, black and red, but may be found to lesser degrees in other breeds and crossbreeds. It is important to distinguish true or fine marbling from the deposition of subcutaneous and interfascicular fat as a characteristic of European breeds.

For more than 20 years, initially in Japan, there have been attempts to find predictors of

i. how much a given animal will marble;

ii. which matings are preferable;

iii. whether the very costly feeding can be reduced.

At face value, the results are contradictory but also tantalising since the inconsistencies may be due to the complexity of the marbling pathway and the sheer number of genes involved.

A simple polymorphism of the SCD (delta-9 desaturase on Bota 26) appears to affect the amount of marbling presumably by encouraging desaturation and therefore the production of MUFA and particularly oleic acid. Polymorphisms of growth hormone (GH) and fatty acid synthase (FASN) amongst many others may also have an effect. The evidence for some of these individual genes is reviewed and presented in Table 4.1 (Cheong et al., 2007; Hoashi et al., 2007; Zhang et al., 2008; Abe et al., 2009; Ardiyanti et al., 2009; Bhuiyan et al., 2009; Uemoto et al., 2010; Matsuhashi et al., 2011; Oh et al., 2012; Han et al., 2013; Ishii et al., 2013; Saatchi et al., 2013; Xu et al., 2013; Sasazaki et al., 2014; Chen et al., 2015; Hayakawa et al., 2015; Bartoň et al., 2016; Papaleo Mazzucco et al., 2016). From these 14 studies, the only fair conclusion is that, although the genomic region controls fat deposition, multiple genes and therefore haplotypes must be involved.

Table 4.1 *Candidate genes at c19 35Mb to 55Mb with reported associations with fatty acid composition.*

Gene	Reference	Effect on Intramuscular Fatty Acid Composition in Beef Cattle Muscle Tissues
SREBF1	(Hoashi et al., 2007)	In Japanese Black cattle: S allele associated higher MUFA and 1.6^{o}C lower Tm of IMF.
	(Bhuiyan et al., 2009)	In Korean Hanwoo cattle: SS alleles IMF stearic acid (C18:0) lower than LL ($P<0.05$) but linoleic and PUFA contents higher in SS compared to LL ($p<0.05$).
	(Matsuhashi et al., 2011)	In Japanese Black cattle: No associations with fatty acid composition or meat yield traits.
	(Han et al., 2013)	Canadian crossbred steers S/L polymorphism associated with 9c C17:1 ($P=0.013$).
	(Xu et al., 2013)	Simmental Bulls, Snow Dragon Black: LL higher palmitic acid (C16:1), triglycerides, and C16 index but lower stearic acid (C18:0) and SFA compared with the LS genotype ($P < 0.05$).
TCAP	(Cheong et al., 2007)	In Korean Hanwoo cattle: a 6 bp Leu-Gln deletion as well as SNP in intron 1 associates with Marbling Score ($P=0.02$, $P=0.003$ respectively).
GH	(Ardiyanti et al., 2009)	In Japanese Black cattle: A allele associated low Tm fat; allele B gave higher % C18:1n=9 IMF ($P<0.05$); allele C gave higher C18:1 MUFA, higher USFA & ($P<0.05$). Allele C also gave lower % Saturated fatty Acid (SFA), a higher MUFA/SFA ratio and lower Tm of fat ($P<0.01$).
	(Matsuhashi et al., 2011)	GH L127V polymorphism (A/B Ardiyanti et al., 2009) claimed association with IMF fatty acid composition, but not as strong as FASN, SCD or SREBF1.
UTS2R	(Sasazaki et al., 2014)	Japanese Black x Holstein cattle: Reported differential association between breeds with marbling and non-synonymous SNP in coding region.
FASN	(Zhang et al., 2008)	Angus bulls: SNP coding region g.17924A>G (as GG genotype) associated FA composition – lower myristic acid (C14:0 $p < 0.00001$), palmitic acid (C16:0 $p < 0.05$) and total saturated FA ($P<0.01$) in total lipids and TAG than g.17924AA genotype.
	(Abe et al., 2009)	Japanese Black x Limousin F2 exon 34 SNPs (g.16024A>G (T1950A), g.16039T>C (W1955R). TW changes together increase C18:0, C18:1 content, increase MUFA:SFA ratio.
	(Bhuiyan et al., 2009)	Korean Hanwoo cattle: g17924G>A SNP as GG genotype with higher oleic acid (C18:1) palmitic acid (C16:0).
	(Uemoto et al., 2010)	GWAS Oleic Acid signal g.16024A>G.
	(Ishii et al., 2013)	GWAS reveal significant SNP signals associated FA composition near FASN gene. Confirms Uemoto et al. 2011.
	(Matsuhashi et al., 2011)	Japanese Black cattle: significant effects on C14:0, C14:1, C18:0, oleic acid C18:1 and MUFA (all $p < 0.001$).
	(Oh et al., 2012)	Hanwoo Korean cattle: All 5 known exonic SNPs associated with FA composition IMF and Marbling Score.
	(Saatchi et al., 2013)	Angus cattle: GWAS 51st Mb window on c19 harboring FASN associated with FA composition of IMF.
	(Hayakawa et al., 2015)	Japanese Black cattle: Seven known SNPs. Promoter g.841G>C- improved FA composition IMF.
	(Bartoň et al., 2016)	Fleckvieh bulls: Known FASN SNPs significant associations C14:0, C16:0, C18:1n-9 in IMF.
	(Chen et al., 2015)	Canadian study: GWAS, same bead chip as Uemoto et al. 2011 "markers have large effects near FASN and SCD".
	(Papaleo Mazzucco et al., 2016)	Angus,Hereford, Limousin crossbreds: g.16024A>G SNP. AG genotype with higher IMF than GG genotype

SREBF1—transcription factor that regulates gene expression levels of stearoyl-CoA desaturase (SCD) leading predominantly to monounsaturated fatty acid (MUFA) oleic acid C18:1n = 9 in intramuscular fat (IMF); TCAP—titin-cap or Telethonin, interacts with titin-cap structure and regulates, by inhibition, myostatin hormone secretion; GH1—growth hormone; UTS2R—urotensin 2 receptor; FASN—fatty acid synthetase.

Our interest increased when it became apparent that a regulator of SCD, SREBF1, maps to the Bota 19 Chromosome, together with GH and FASN. Taken with the pleiotropic regulatory role of SREBF1, in humans at least (Lecomte et al., 2010), and with the evidence for the influence of Bota 19 on fatty acid composition in Japanese black cattle (Uemoto et al., 2010; Ishii et al., 2013), we asked whether extensive, multimegabase haplotypes could be identified. Previous investigations (Cheong et al., 2007; Hoashi et al., 2007; Zhang et al., 2008; Abe et al., 2009; Ardiyanti et al., 2009; Bhuiyan et al., 2009; Uemoto et al., 2010; Matsuhashi et al., 2011; Oh et al., 2012; Han et al., 2013; Ishii et al., 2013; Saatchi et al., 2013; Xu et al., 2013; Sasazaki et al., 2014; Chen et al., 2015; Hayakawa et al., 2015; Bartoň et al., 2016; Papaleo Mazzucco et al., 2016) have suggested that some intervening and adjacent genes could be relevant. Such apparent clustering of plausible candidates is often illusory, but in this case, we were encouraged by the fact that Japanese breeders are sufficiently convinced to be prepared to pay handsomely for SCD and GH typing even though it seems clear that these genes are unlikely to be involved directly.

Accordingly, we have been defining conserved ancestral haplotypes within the region. The many conserved megabase haplotypes have very different frequencies when breeds are compared (Williamson et al., 2011). The need for a simple summary of breed differences led to the development of the W plots which separate haplotypes common to all cattle from those which are found in, and potentially defining for, one breed but not in the other (Lloyd et al., 2013; Lloyd et al., 2014a). Of course, since Wagyu cattle marble, it follows that all Wagyu-specific haplotypes must be associated with marbling irrespective of their function; all too often, such passive associations have been misinterpreted as causal. Common haplotypes may

be implicated unless non-marbling breeds are compared to determine which are specific for marbling breeds. The challenge is to assess the Wagyu haplotypes in different backgrounds such as can be obtained by crossing Wagyu with other breeds which are relatively resistant to marbling.

As a strategy, we asked whether Wagyu-specific haplotypes, once defined, are penetrant when heterozygous in F1 crossbreds. If so, it would be fair to conclude that these haplotypes have direct effects on the marbling process. We also asked whether non-Wagyu haplotypes affect the rate of marbling alone or in combination. Therefore, we started the investigation by determining whether fine marbling of progeny is a function of parentage.

In order to undertake such studies, it is critical to have a reliable measurement of marbling. In spite of many attempts, including the use of photographic and ultrasound images, there is still no agreed international benchmark, largely because the deposition of the preferred fat is very fine and barely visible. The most objective approach is the cumbersome slip test for the measurement of melting temperature (Tm) of the lipid fraction and effectively the predominance of oleic acid (Smith et al., 2009; Smith and Johnson, 2014). In Wagyu, Tms below body temperature (37°C) correlate with fine marbling thereby explaining the cholesterol lowering and statin-sparing effects (Smith and Johnson, 2014).

We have introduced a more convenient and highly reproducible variation using the same thermocycler as used for the polymerase chain reaction (Lloyd et al., 2014b).

With this method, elsewhere we will show that low Tm is highly heritable as expected given its extreme expression in only the Wagyu breed. Furthermore, we show that the effect is determined by multiple, conserved, megabase haplotypes. These can be used to predict marbling in crossbreds. There is evidence for *cis*-interactions within, and *trans*-interactions

between, haplotypes. We also describe a strategy for improving the quality and healthiness of beef by judicious crossing.

The step-wise approach used here can be applied to other challenges in livestock genetics, especially where regulatory ancestral haplotypes are suspected. Systematically, we address

i. synteny as a guide to functions and interactions already defined in Hosa;

ii. breed effects as an initial screen for heritability;

iii. heritability as the only variable;

iv. genomic markers of inheritance;

v. haplotypes in crossbreds;

vi. mechanisms of haplotype effects.

The conclusions of all these complementary studies lead to a model of the genetic control of fatty acid composition.

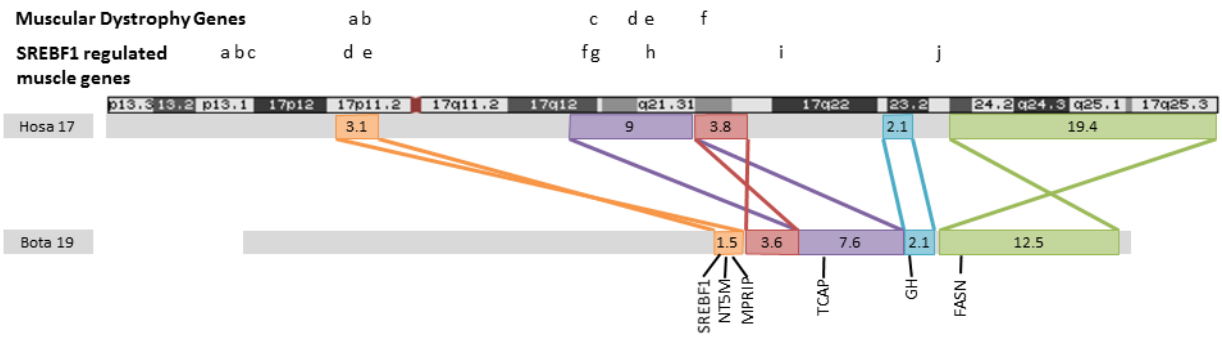
4.3 *Results*

4.3.1 *Synteny as a guide*

In Figure 4.1, we show the regions of Hosa Chr 17 which correspond to SREBF1 to FASN on Bota Chr 19. Note also the regulatory effects of SREBF1 on remote sites many of which map closely with locations implicated in Limb-girdle muscular dystrophy.

In the dot plot of GH to FASN, it is apparent that there are two separate components in Hosa; the blue and green sections are distinct but note how they remain contiguous even though the green has been inverted. Similar phenomena are present elsewhere in the synteny plot.

Together, these suggest that there are multiple interacting haplotypes from SREBF1 to FASN.



Muscular Dystrophy Genes

a	TRPV2	Muscular dystrophy is ameliorated in dystrophin-deficient mdx mice by dominant-negative inhibition of TRPV2(Iwata et al., 2009)
b	SREBF1	Mutations of LMNA that cause Emery-Dreifuss muscular dystrophy (EDMD2-AD) and familial partial lipodystrophy (FPLD2) result in less binding of lamin A to SREBP1.(Lloyd et al., 2002)
c	TCAP	Limb-girdle muscular dystrophy type 2G (LGMD2G) is caused by mutations in the TCAP gene(Moreira et al., 2000)
d	PTRF	Congenital generalised lipodystrophy, type 4; (CGL4) is caused by mutations in PTRF that result in CAV 3 deficiency.(Hayashi et al., 2009)
e	BECN1	Expression of BECN1 was reduced in patients with muscular dystrophies BTHLM1 and UMCD1 which were caused by COL6A1 mutations.(Grumati et al., 2010)
f	SGCA	Sarcoglycans form part of the dystrophin-glycoprotein complex. Mutations in SGCA cause Limb-girdle muscular dystrophy type 2D (LGMD2D). SGCB, SGCD and SGCG are associated with LGMD types 2E, 2F and 2C respectively. (Trabelsi et al., 2008)

SREBF1-BHBLB2 regulated muscle genes

a	MYH13
b	MYH2
c	MYH3
d	MPRIP
e	SREBF1
f	CACNB1
g	TCAP
h	BECN1
i	ENO3
j	CACNG1

Figure 4.1 *Marbling and muscular dystrophy are syntenic on Bota 19 and Chromosome 17.* Coloured boxes represent segments with the same gene content. Crossed joining lines indicate inverted translocations. Numbers represent Mb. Synteny was determined by the positions of homologous genes in the human assembly Hg 38 and Bovine assembly BosTau8 located using the UCSC Genome browser. Inverted sections and the exact location of boundaries between blocks were determined by dotplots (Krumsiek et al., 2007) comparing the two sequences. The annotated dotplots used are shown in Supplementary Figure 4.1 available online at https://doi.org/10.1155/2017/6532837. The positions of genes associated with muscular dystrophy are shown in the first row of letters above Hosa 17. The association to muscular dystrophy is shown in the table below (Trabelsi et al., 2008; Iwata et al., 2009). The positions of

genes involved in the regulation of muscle development by SREBF1, either directly or through BHLHE40 and BHLHE41 (previously known as BHLHB2 and BHLHB3 resp.), are shown in the second row of letters above Hosa 17. Adapted from (Dawkins, 2015) with permission. We thank Dr. Joe Williamson for assistance with this figure.

4.3.2 *Breed effects and heritability*

Most Wagyu have Tm of intramuscular fat below human body temperature of 37°C and distinctly lower than fat from Simmentals which is mostly subcutaneous and generally above 40°C (Lloyd et al., 2014b). The length of feeding was much greater for Wagyu due to the fact that the Simmentals grow rapidly and fatten quickly through deposition of largely subcutaneous fat. Wagyu are much slower to both grow and fatten as they deposit intramuscular rather than subcutaneous fat.

We compared Wagyu cattle with various European breeds and crossbreeds which were fed the same ration under the same environmental conditions, but with days on feed varied to achieve the desired finish. The results showed that the Tm of the subcutaneous fat was lower in animals with higher proportions of Wagyu ancestry (data not shown here).

Differences were noticeable in animals with only 25% Wagyu ancestry indicating that the breed effect can be highly penetrant.

Wagyu steers were fed together for 300 days and assessed identically. The only significant variable was parentage which was confirmed by DNA haplotyping. All sires are considered to be elite in terms of their breeding, reputation, and estimated breeding value (EBV) when available. The progeny groups differ significantly, with mean Tm for the progeny of two sires differing by nearly 2 degrees. Importantly, this effect was not seen with the visual marbling score. There is an unequivocal genetic effect detectable within one generation. Thus, in spite

of the undoubted complexity of the inheritance, sires can be ranked according to heritability of low Tm.

Thus, breed differences, individual sires, and presumably identifiable genetic factors, determine the type, rate, and location of lipid deposition. However, it is also clear that there is a major effect of environment in that Tm falls with increasing DOF irrespective of breed, and this fact compounded by imprecision may have contributed to past confusion.

In the analysis of genetic differences presented here, we define alleles and haplotypes common in Wagyu but rare in other breeds that may explain some of the differences in the type, rate, and location of lipid deposition.

4.3.3 *Genomic markers of inheritance*

The genomic structure of the relevant region of Bota 19 contains the previously implicated SREBF1 and GH, as shown in Figures 1 and 2. Note the extensive duplication as found in other genomic regions of polymorphism including differences in copy number. The degree of polymorphism (at the insensitive gel level) ranges from at least 7 alleles at the most polymorphic locus (MPRIP) to only 2 at the least (TCAP). In the SREBF1 to GH (S-G) segment, there are $3 \times 5 \times 7 \times 2 \times 3 = 630$ possible haplotypes on each paternal or maternal Chromosome. Therefore, there should be some thousands of genotypes if the alleles at each locus are segregating randomly due to free recombination as specified in the infinitesimal model of population genetics.

Here, we report the actual frequencies in Wagyu and in some of the breeds with which they have been crossed. Only 14 haplotypes occur commonly, say greater than 5%, in the breeds studied here. Importantly, as shown in Figures 3 and 4, these are the conserved ancestral haplotypes. Four of the fourteen are present in the three-generation family which also shows

the segregation of the (a) haplotype in three genotypes. None of the haplotypes could be identified through linkage disequilibrium; they must be observed empirically. They are *not* recent recombinants or mutants. They are *not* those expected from the allele frequencies and random assortment. Random segregation and free recombination can be excluded at least in this 20-megabase region.

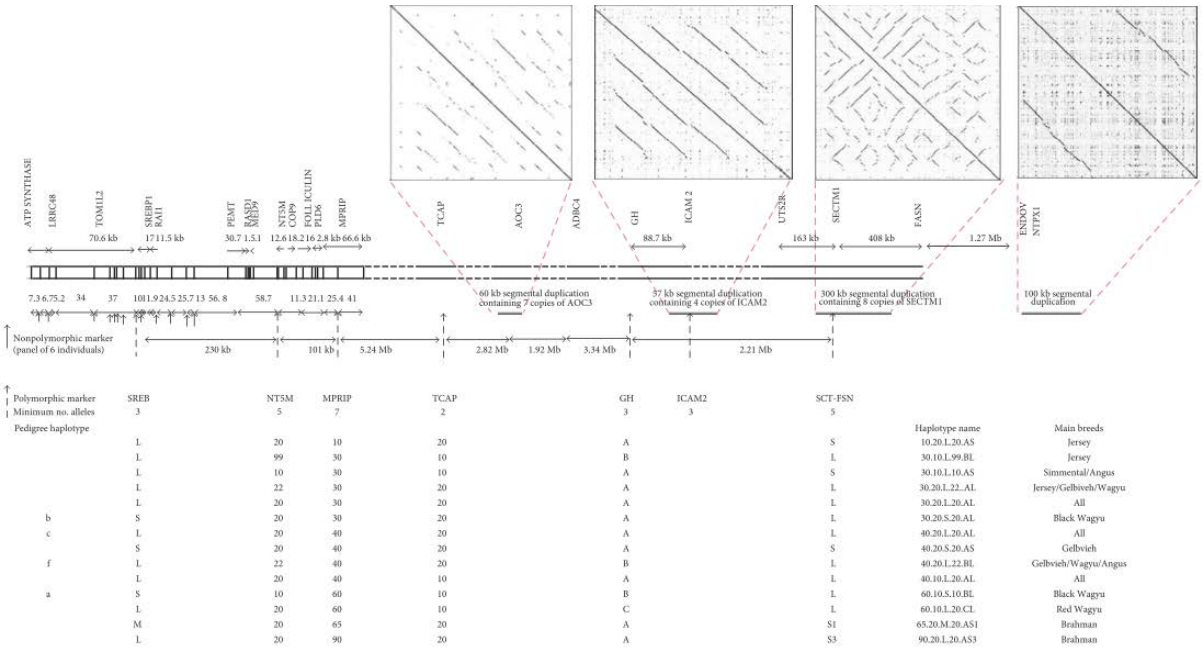
As shown in Figure 4.2, haplotypes are designated by the alleles at MPRIP, TCAP, SREBF1, and NT5M in that order so as to emphasize the conservation over the 5 megabases between MPRIP and TCAP (M-T segment). Note in Figure 4.1 that MPRIP and TCAP are adjacent in Bota but not in Hosa.

The 60.10.S.10 is the most common Wagyu-specific haplotype. Other haplotypes are found in all breeds (e.g., 30.20.L.20 and 40.20.L.20) and yet others are characteristic of a breed such as Simmental (e.g., 30.10.L.10). These breed differences are shown as a W plot in Figure 4.4.

It can be seen that the conserved ancestral haplotypes extend from SREBF1 to FASN. The breed differences are especially striking at GH and FASN. Note that Wagyu-specific haplotypes have L at FASN whereas Simmental-specific haplotypes have S (Figure 4.4(a)). This remarkable difference might suggest that FASN is critical, but similar breed specificity is apparent at GH. Further, an association with 60.10.S.10.B.L or 30.20.S.20.C.L in Wagyu could be misinterpreted as implicating the shared alleles SREBF1 S and FASN L which are megabases apart. Note also that the remarkable but unpredictable associations between alleles at SREBF1, GH, and FASN have the potential to explain why there have been 20 years of confusion and argument as to which particular gene is important. As also shown in Figure 4.4(a), the five haplotypes in the family are also present in the W plot. Three are essentially

Wagyu specific whereas two are common to cattle generally implying conservation over at least thousands of generations.

By comparing the haplotypes in the Red and Black Wagyu, it can be seen that the 60.10 segment at M-T is shared but the SREBF1 to NTM5 (S-N) segments differ (see Figure 4.2). Both types of Wagyu are known for their fine marbling, suggesting that M-T is crucial.



Polymorphic marker	SREB	NT5M	MPRIP	TCAP	GH	ICAM2	SCT-FSN		
Minimum no. alleles	3	5	7	2	3	3	5		
Pedigree haplotype								Haplotype name	Main breeds
	L	20	10	20	A		S	10.20.L.20.AS	Jersey
	L	99	30	10	B		L	30.10.L.99.BL	Jersey
	L	10	30	10	A		S	30.10.L.10.AS	Simmental/Angus
	L	22	30	20	A		L	30.20.L.22..AL	Jersey/Gelbvieh/Wagyu
	L	20	30	20	A		L	30.20.L.20.AL	All
b	S	20	30	20	A		L	30.20.S.20.AL	Black Wagyu
c	L	20	40	20	A		L	40.20.L.20.AL	All
	S	20	40	20	A		S	40.20.S.20.AS	Gelbvieh
f	L	22	40	20	B		L	40.20.L.22.BL	Gelbvieh/Wagyu/Angus
	L	20	40	10	A		L	40.10.L.20.AL	All
a	S	10	60	10	B		L	60.10.S.10.BL	Black Wagyu
	L	20	60	10	C		L	60.10.L.20.CL	Red Wagyu
	M	20	65	20	A		S1	65.20.M.20.AS1	Brahman
	L	20	90	20	A		S3	90.20.L.20.AS3	Brahman

Figure 4.2 *Haplotypes of the Marbling Region.* Polymorphic markers define conserved extended C19 ancestral haplotypes: This region on BTAChr 19 is bounded by markers SREBF1 and FASN (See Williamson et al., 2011 for details. The FASN marker is more correctly known as SCT-FSN since the present marker is adjacent to the coding region for FASN, within the segmental duplication containing SECTM1. The map positions of PCR markers and the number and type of alleles at each locus are indicated, including the main high frequency and largely breed-specific haplotypes extending from SREBF1 to TCAP. We have extended the haplotyping through the region and plan to develop more markers based on the potential polymorphism revealed by the structural duplications extending from 43Mb to 52Mb (figure and below). Note the regions where PCR product polymorphism was not detected. (Williamson et al., 2011). In structural duplications in C19 35-55Mb region, we used the current Cow genome assembly (Bos_tauros_UMD_3.1.1/bostau8 Assembly) on the UCSC Web Browser and searched the Chr 19 region from 35Mb to 55Mb in 500Kb sectors for large structural segmental duplications using standard dot-plotting methods aligning each 500Kb sector against itself (Gepard 1.30)

(Krumsiek et al., 2007). We found clusters of rolling, sometimes clustered-inverted, segmental duplications in the reference genome on Chr 19 at 43.51 Mb (~60Kb in length), 43.86 Mb (~90Kb), 48.86 (~57Kb) and 50.846Mb (~300Kb). We also found a long single imperfect duplication of 103-112Kb at 52.73Mb and 52.88Mb. Some of these dot plots are shown as cutaways in the figure. This region has a relatively low density of protein coding genes.

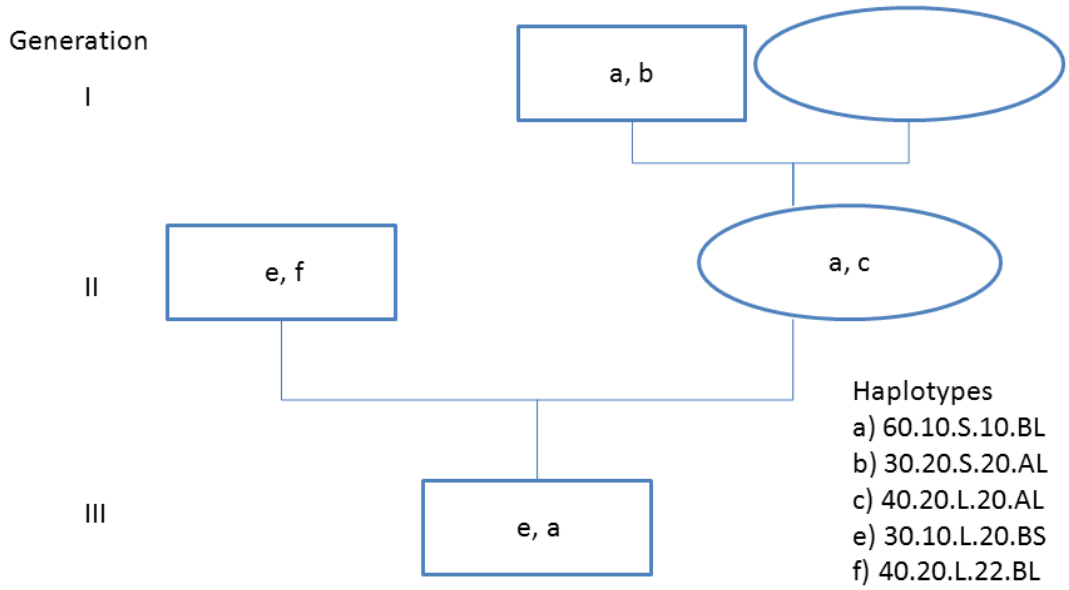


Figure 4.3 *Segregation of 20MB haplotype through three generations of Wagyu pedigree*. Haplotype (a) is inherited intact from the maternal grandsire. The calf, dam and maternal grandsire are all heterozygous at the SREBF1 or NT5M and GH or FASN markers, which allows the segregation of haplotype (a) to be seen clearly**.**

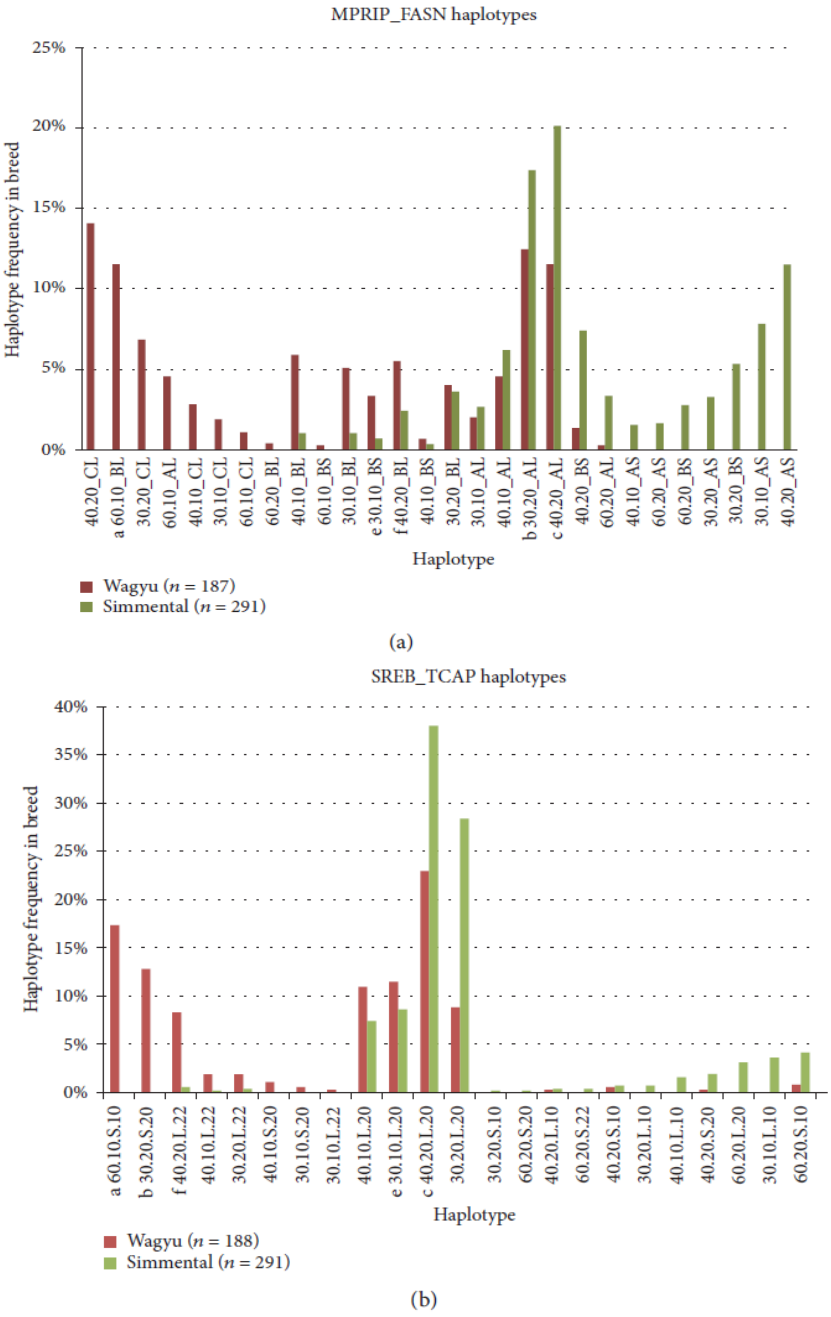


Figure 4.4 *W plot comparing haplotype frequencies in Wagyu and Simmental*. Adapted from (Lloyd et al., 2013). (a) The haplotypes are from MPRIP to FASN (see Figure 4.2). Note SCT-FSN L in Wagyu and S in Simmental, also GH C in Wagyu and GH A in Simmental with GH B largely in the haplotypes common to both breeds. Haplotype designation is MPRIP.TCAP_GH SCT-FSN. (b) Haplotypes are from SREBF1 to TCAP. Haplotype designation is MPRIP.TCAP.SREB.NT5M. The four most common haplotypes of Simmental are also found at high frequency in Wagyu (and many other breeds not shown here), while two of the three most common haplotypes of Wagyu are not found in Simmental (and are not common in any other breed tested).

4.3.4 *Haplotypes in crossbreds*

A key question is which Wagyu haplotypes can have an effect in crossbreds when the genetic background is foreign. Samples were taken from exhibits submitted to a steak competition intended to compare production systems rather than genetics. The results of Tm measurements are shown in Figure 4.5. The full bloods are below 36°C in 7/9 as expected. Although the crossbreds, taken as a group, have higher Tm, remarkably, 4/16 are below 36°C. Of the 11/16 crossbreds with Tm below 38°C, 9/11 have one or the other of two *Wagyu-specific* haplotypes. Thus, these two haplotypes (60.10.S.10 and 30.20.S.20) are associated with, if not directly responsible for, the dramatic fall in Tm obtained by crossing a high Tm breed with a Black Wagyu which transmits one of these haplotypes. Note that these two haplotypes differ in the M-T segment but share S at SREBF1.

So as to examine the effect of haplotypes in another setting, we compared marbling scores in crossbreds differing by whether they inherited haplotypes classified as specific for either Wagyu or *Bos indicus*. As shown in Table 4.2, the former group had greater marbling.

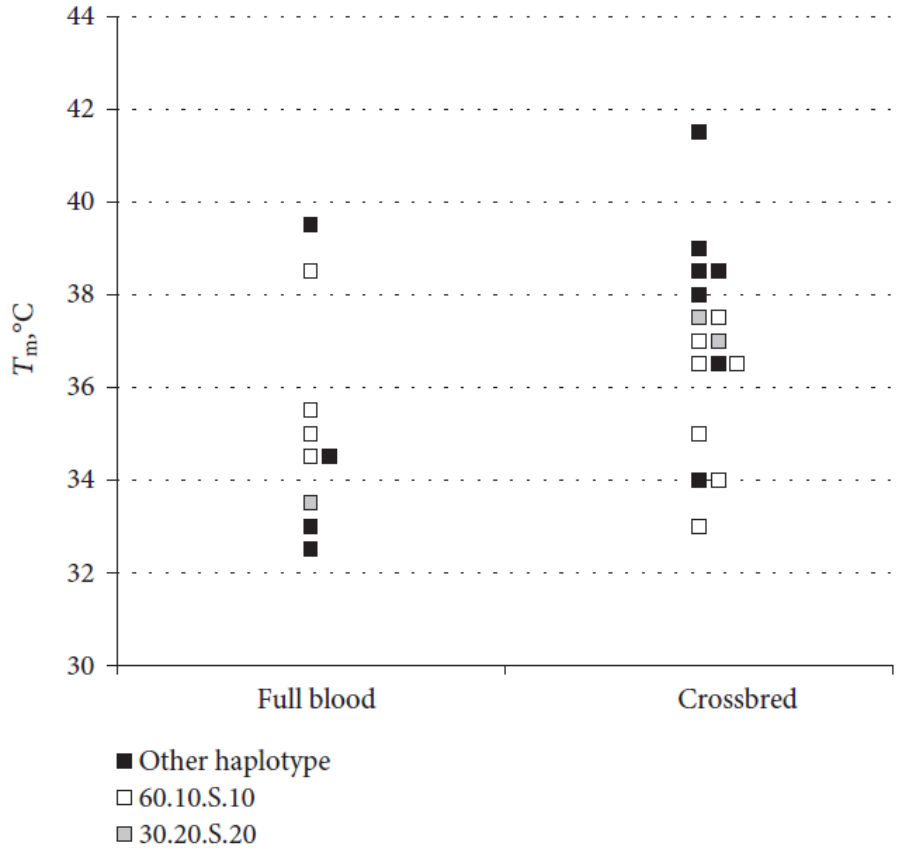


Figure 4.5 *Crossbreds perform as Wagyu if they possess a Wagyu haplotype.* Tm measurements from 9 full blood and 16 crossbred entries to AWA branded beef competition from 2014 and 2015. There are crossbred Wagyu with Tm as low as full bloods. Mean Tm with error bars ±1 SEM. SREB-TCAP haplotypes. White squares indicate animals with at least one 60.10.S.10 haplotype, pale grey squares indicate animals with at least one 30.20.S.20 haplotype, and dark grey squares indicate animals with neither common Wagyu haplotype.

Table 4.2 *Higher marble score in F1 and F2 Wagyu with Wagyu-specific haplotypes compared to Bos indicus-specific haplotypes.*

	Average MS	N	SEM
Bos indicus Haplotypes	4.2	6	0.54
Wagyu Haplotypes	5.3	22	0.22

Crossbred and purebred Wagyu fed for 450 days marbling assessed visually between the 10th and 11th rib, using the AUS-Meat marble scale. Breeds of dams include Brahman, Shorthorn, and Angus. Haplotypes were determined for SREB to TCAP markers by a combination of

pedigree analysis, homozygosity, and breed-based haplotype frequencies. Haplotypes classified as Wagyu specific were 60.10.S.10, 30.20.S.20, and 30.10.S.20 with frequencies in Wagyu shown in Figure 4.4(b). Haplotypes classified as *Bos indicus* specific were those with MRIP > 60 or NT5M > 22. For simplicity, animals with neither a *Bos indicus*-specific nor a Wagyu-specific haplotype have been excluded from the table.

4.3.5 *Mapping mechanisms to haplotypes*

It is unrealistic to expect to map single functions to ancestral haplotypes as if these were just a string of biallelic coding genes. The sequence conservation is so extensive, so complex, and yet, so poorly understood! However, it is reasonable to expect that the differences will be quantitative and that penetrance will be dependent on multiple variables. At minimum, there will be multiple cis- and trans-interactions between the various polymorphisms. It follows that we expect to find associations at multiple markers. The results given above appear to implicate sequences around S and within the M-T segment of Wagyu haplotypes irrespective of effects marked by GH and FASN (see Figure 4.2).

In order to evaluate such conclusions and so as to assess other markers and haplotypes, we have taken advantage of the availability of two very different data sets:

Mayura—fixed environment including 300 DOF, 100% black Wagyu, permitting examination of genetic differences within Black Wagyu.

Melaleuka—fixed environment but variable DOF and breed, permitting examination of all haplotypes, Wagyu haplotypes within crossbreds and effects on the *rate* of marbling.

Table 4.3 gives some of the results of the Mayura set. As already shown, see Figure 4.4, this is the set which revealed the heritability of Tm confirming its value in dissecting complex genetics. For convenience, we show a cut-off of 37°C, giving approximately 50% in the

above and below groups. By comparing alleles at four of the loci shown in Figure 4.2, it is clear that the TCAP 20 allele is associated with lower Tm. The MPRIP 60 is associated with higher Tm. Although SREBF1 S is a marker for low Tm in some earlier studies and patents, here we find the opposite. At the NT5M locus, the 22 allele is associated with low Tm.

Table 4.3 *Mayura data 300±20 DOF - 100% Wagyu. Associations of alleles with low or high Tm.*

(a)

SREBF1 Alleles			
Tm	S	L	Total
<37°C	41	89	130
≥37°C	52	82	134
Total	93	171	264

Fisher's exact test statistic 0.25. Not significant at $p < 0.05$.

(b)

NT5M Alleles				
Tm	10	20	22	Total
<37°C	19	84	27	130
≥37°C	32	82	20	134
Total	51	166	47	264

Chi-squared statistic is 4.32. The p-value is 0.1. Not significant at $p < 0.05$.

(c)

MPRIP Alleles				
Tm	30	40	60	Total
<37°C	48	60	18	126
≥37°C	34	66	32	132
Total	82	126	50	258

Chi-squared statistic is 6.5. The p-value is 0.04. Result is significant at $p < 0.05$. The total is lower as some animals with Tm were not typed for MPRIP allele.

(d)

TCAP Alleles			
Tm	10	20	Total
<37°C	55	75	130
≥37°C	74	60	134
Total	129	135	264

Fisher's exact test statistic 0.04. Result is significant at $p < 0.05$.

The inconsistencies between past and present results and between locus and haplotype analyses can be illustrated as follows. The prototypic Wagyu ancestral sequence, 60.10.S10, can be recognised by the 60.10.S.10 combination, but these alleles, taken individually, are *not* haplospecific. As shown in Figure 4.2, each is found on more than one haplotype.

When the alleles are examined without regard to whether they are carried by the ancestral haplotype, Tm would be higher at MPRIP, TCAP, SREB-P, and NT5M (see Table 4.3). However, as shown in Figure 4.5, the 60.10.S.10 ancestral haplotype actually lowers the Tm of crossbreds. Thus, there may be multiple cis- and trans-interactions contributing to the effect of a particular haplotype.

As a further approach to address the complexity, we asked whether there is any influence of trans-interaction or homozygosity. Since it would be necessary to study many hundreds of thousands of individuals to obtain sufficient homozygotes of even the most common haplotypes, we chose to examine the least polymorphic loci such as TCAP with only two alleles: 10 and 20. In the Mayura long-fed Wagyu data set, all 8 with Tm below 36°C are 20,20 homozygotes (see Figure 4.6). Using 37°C as a cut-off, 60% of 20,20 are lower compared with only 33% of the 10,10 homozygotes.

The effect of TCAP homozygosity is clear in a genetically very diverse group fed for 450 days. Only subcutaneous fat from over the rump was available. Although not comparable to other samples reported above, TCAP 10,10 homozygotes have higher Tm than 20,20 homozygotes (Table 4.4).

So as to address the issue in a very different data set, we analysed the Melaleuka Euro and crossbred cattle and found similar results. As shown in Figure 4.7, TCAP 10,10 and 20,20 homozygotes diverge after 50–100 DOF. After 100 DOF, 9 of 12 with TCAP 10,10 remain above 39°C compared with 3 of 12 with TCAP 20,20 ($P < 0\ 05$). The 20,20 homozygotes assume a faster trajectory to healthier fat indicating a quantitative regulatory effect (Figure 4.8).

A similar pattern is obtained when a model is constructed based on the slow induction of SCD (Figure 4.9).

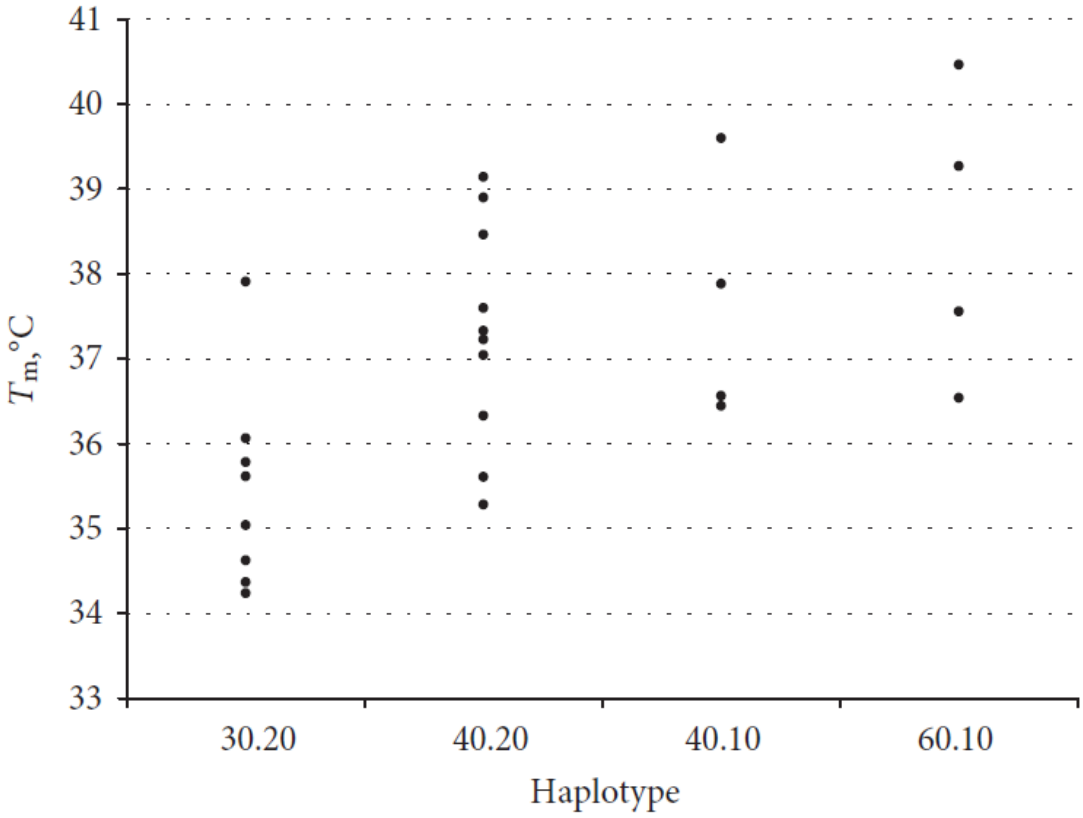


Figure 4.6 *Dot plots of Tm values in Wagyu 300±20 DOF in animals homozygous for the 5.24 Mb region MPRIP-TCAP*. Wagyu cattle bred on different Australian farms (Goorambat, Irongate Wagyu, Mayura, Rosevale, Peppermint Grove) were fed for 300 days at Mayura Station, South Australia, and beef/fat samples were assayed for C19 haplotypes and fat melting point Tm as indicated in Materials and methods. Each dot point represents a different homozygote. Homozygous haplotypes for MPRIP-TCAP region only. Students *t*-test for 30.20 v 40.20 yields p = 0.012.

Table 4.4 *In cross bred and purebred Wagyu fed for 450 days, those with homozygous TCAP 10 had higher Tm of the subcutaneous fat over the rump*. Breeds of dams include Brahman, Shorthorn and Angus. For simplicity, animals heterozygous at TCAP have been excluded from the table.

TCAP genotype	Subcutaneous rump Tm	N	SEM
10 10	35.7	4	0.46
20 20	33.2	22	0.75

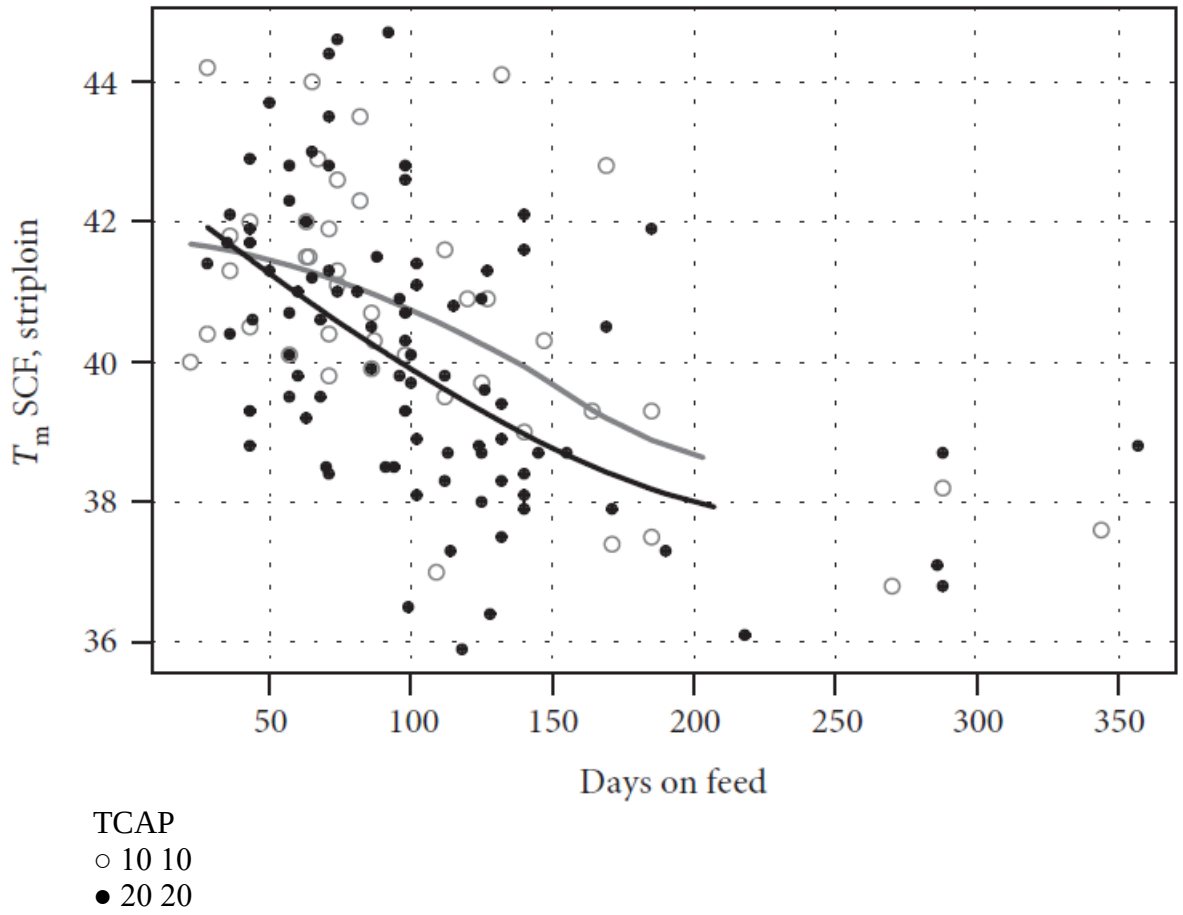


Figure 4.7 *TCAP 20 homozygotes achieve low Tm with less days on feed (Melaleuka Crossbreds).* Hollow circles show TCAP 10 homozygotes. The smoothed lines were calculated in R with a span of 100. Tm measured on various European and Japanese breeds and crossbreeds including Simmental, Gelbvieh, Black Wagyu, Red Wagyu. Subcutaneous fat samples were taken from the rump and front ends of striploins, with the Tm reported as the average of the two samples. Striploins were DNA tested to confirm a match to the animal sent to abattoir. 42 samples from TCAP 10 homozygotes and 87 samples from TCAP 20 homozygotes are shown. Tm measurements of 105 samples from TCAP 10 20 heterozygotes have been excluded from the graph for simplicity.

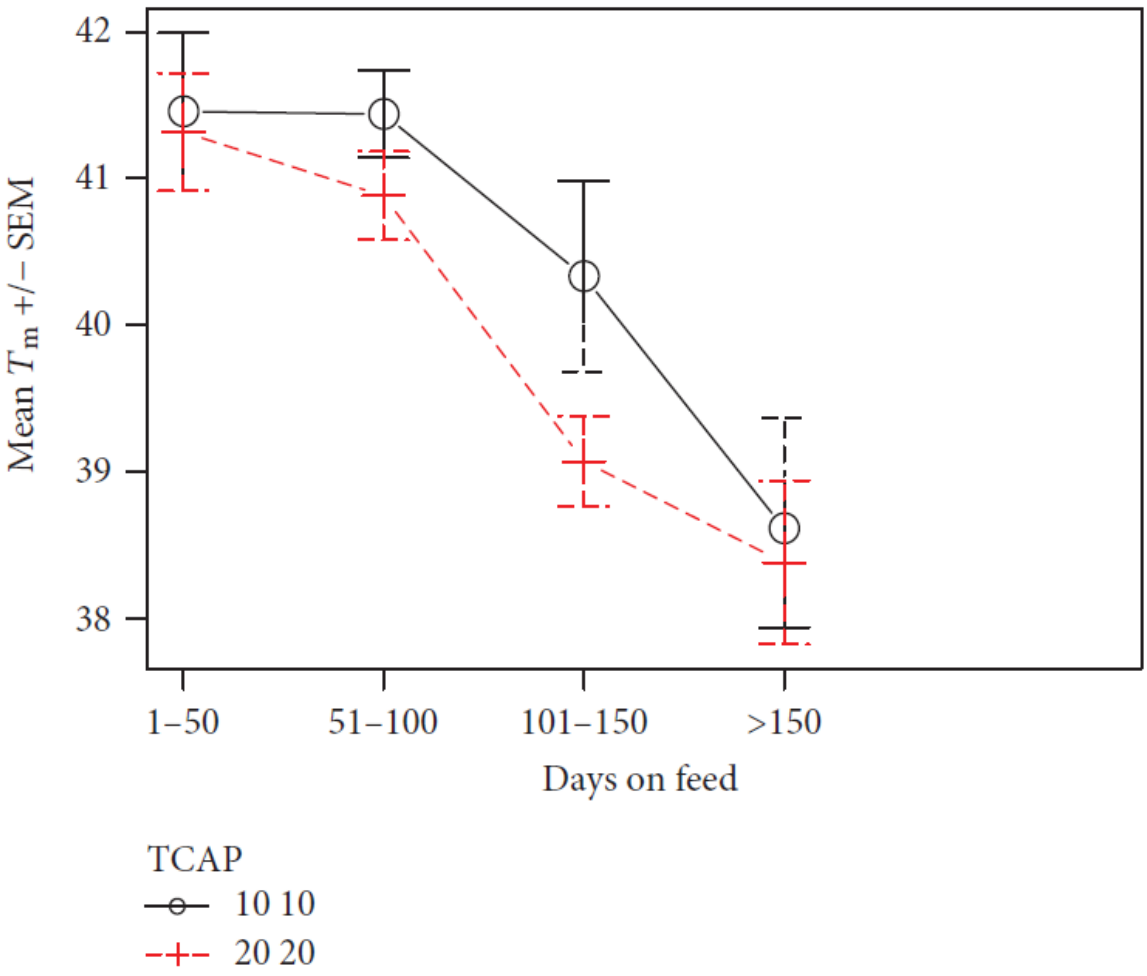


Figure 4.8 *TCAP 20 animals have significantly lower Tm for 50-150 days on feed.* The presence of homozygous 20 TCAP alleles makes the decrease in Tm occur at less days on feed. The initial Tm is similar between the two groups. The Tm in both groups appears to reach a minimum level with long feeding that is not affected by the TCAP allele.

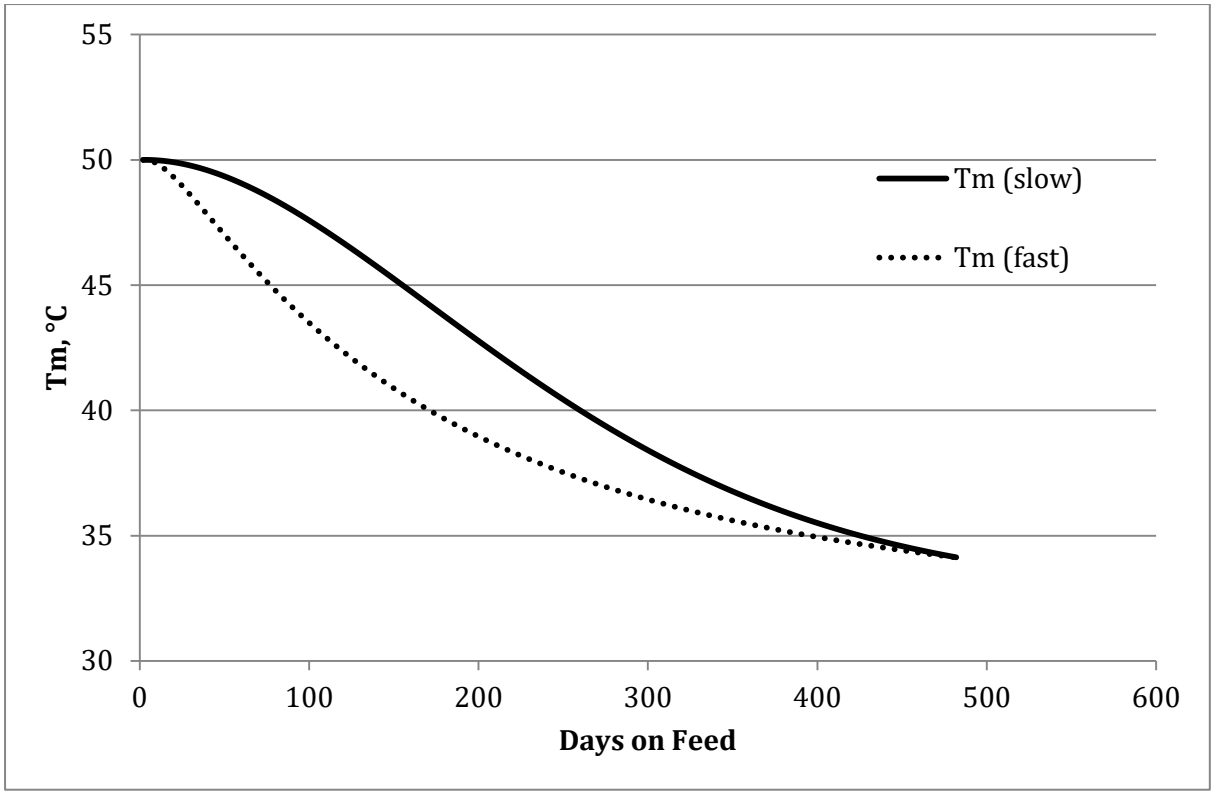


Figure 4.9 *Match to desaturation model of fat melting point*. The Tm decrease shown in Figure 4.7 can be described by the following mathematical model of fat production and desaturation. De novo fat production is controlled by FASN, in response to energy balance. This fat starts saturated. It is then desaturated by the SCD enzyme. The total fat present (F) can be described by dF/dt = a, where a is the rate of de novo fat production. The amount of unsaturated fat (U) increases in proportion to the amount of desaturation enzyme E as dU/dt = E. In this model, the amount of enzyme available is increased in response to the amount of saturated fat present, and also decays. The amount of enzyme is described by dE/dt = bS − cE, where b and c are constant control parameters and S = F − U is the amount of fat that remains unsaturated. The melting point decreases with increasing proportion of unsaturated fat. Tm decreases in proportion to the amount of unsaturated fat, Tm = TΔ and S/F + TU. The melting point curves resulting from this model have an initial high Tm decreasing slowly at first and then more rapidly as the desaturation enzyme builds up. This figure shows how the model reacts to parameter changes. The fast enzyme response with parameters b = 001 and c = 1 looks similar to the Tm curve shown in Figure 4.8, TCAP 20 homozygotes. A slower enzyme response has parameters of b = 0001 and c = 01 is more similar to the Tm curve for TCAP 10 homozygotes. TCAP is already known to be involved in pathways regulating the production of the SCD enzyme, so it influencing the Tm in this way is not surprising.

4.4 *Discussion*

The history of Japanese Wagyu or “Kobe” beef is intriguing. According to legend (Dawkins, 2015), Wagyu cattle were used for heavy burden although fed only with rice husks, suggesting that they may have been selected for efficient food conversion combined with an ability to utilise fatty acids as a direct energy source. Much later, after the eating of meat was permitted, selection (according to the Shogun’s preferences) must have led to enhanced flavour as found with fine marbling and low melting temperature intramuscular fat. Another consequence has been a tendency to muscle weakness attributed to the replacement of myocytes with adipocytes. Whatever the selection criteria, Wagyu have conserved the capacity to marble from their remote North Asian ancestors and seemingly still do so after crossing with diverse breeds now found worldwide. This history suggested that there must be highly conserved and penetrant haplotypes rather than recent random mutations.

The present study began with the seemingly simple aim of improving the healthiness of beef by crossing elite Japanese Wagyu with composite breeds able to survive in harsh conditions. The literature suggests that transfer of a Wagyu gene might improve the fat content thereby providing cholesterol-lowering, statin-sparing, tasty beef at reasonable cost. Experience has shown that improvement can occur but inconsistently and only after expensive grain-feeding thereby restricting worldwide access to potential benefits. Our interpretation is that the outcome depends on the particular mix of conserved haplotypes which exert quantitative control over all the multiple functions which shift the balance from muscle to lipid and especially MUFA.

As shown elsewhere (Lloyd et al., 2014b), it was necessary to develop a simple method of measuring the amount of beneficial fat. It transpires that heritability of the preferred fat can be demonstrated with Tm but not the standard visual method of quantifying marbling.

Hitherto, therefore, selection of sires must have suffered. This is important because, using Tm, the sire effect is evident within one generation, meaning that success or failure can occur rapidly, leading to the remarkable scatter as demonstrated in Figure 4.2. Fortunately, Tm measurements should lead to progressive improvement.

Given accurate and precise measurement of the endpoints, we have used four data sets.

Samples from a tasting competition reveal that some crossbreds can be as desirable as the most elite Wagyu. Here, we offer a strategy based on the observation that transmission of a certain Wagyu haplotype, such as 60.10.S.10, may dramatically improve quality. If confirmed, a homozygous bull will benefit all progeny and remove some of the existing inconsistency and expense. In an ongoing multicentre study, we hope to be able to evaluate the benefits of sires which are homozygous for 30.20.S.20 and 60.10.S.10 in their anticipated order of benefit. However, because of relatively low haplotype frequencies of say 0.05, the natural frequencies of homozygotes will be less than 0.0025, meaning that the value of such homozygotes will only be clear if all bulls are haplotyped. The solution to the lack of homozygotes for use today is being addressed through artificial insemination and embryo transfer. In the meanwhile, we are developing some hypotheses which recognize that evaluating all combinations of multimegabase haplotypes (as diplotypes) is impractical. The n values required for the comparisons are greater than the number of cattle alive today.

One of these hypotheses arose from the ability to compare two totally different data sets. The Mayura set standardises essentially all variables, other than the genetic differences between Wagyu. The Melaleuka set uses Tm/DOF as a measure to compare crossbreds.

Both show a remarkable effect of TCAP 10,10 versus 20,20. It should be emphasised that differences at TCAP itself cannot explain all findings such as the superiority of Wagyu over other breeds and the benefit of 60.10.S.10 in crossbreds.

Rather, taken together, the results argue for the importance of ancestral haplotypes rather than individual loci. This is illustrated in Figure 4.4. The alleles at GH and FASN are different depending upon the megabase haplotype, meaning that effects attributed to these individual alleles could reflect sequences more than 10 megabases away. Therefore, it is important to define the megabase haplotypes before sequencing or SNP analyses. No doubt, this explains why progress has been slow. Genomic analysis of polygenic traits like QTLs must consider the implications of synteny and the potential importance of paralogy, duplication, copy number, and retroviral regulators to list a few mechanisms (Dawkins et al., 1999; Shiina et al., 2004; Lecomte et al., 2010; McLure et al., 2013; Dawkins, 2015). As proposed here, duplication and copy number are more relevant than coding region differences; so too is regulation over extensive distances. Thus, SREBF1 is a master regulator which controls gene expression within the SREBF1 to FASN region via targeting transcriptional repressors of MPRIP, TCAP, and other muscle–related genes (BECN1, CACNB1, MYH2, MYH3, and MYH13); one of these repressors BHLHB 2 maps near FASN (Lecomte et al., 2010). Note also that muscular dystrophy, Limb-girdle type 2G is associated in humans with TCAP polymorphism (OMIM#604488) and the histopathology resembles marbling in some respects. Lipid accumulation has been observed in Limb-girdle muscular dystrophy (Grounds et al., 2014). Another unexpected recent finding is the location of UTS2R which is associated with development of type II diabetes and fat deposition (Sasazaki et al., 2014).

The balance between myogenesis and adipogenesis may be regulated in part by the TCAP-myostatin pathway in Wagyu as first suggested by Shibata et al. (2006).

We conclude that genomic differences regulate the amount of MUFA such as oleic acid in beef and determine the rate at which Tm falls with DOF. We regard this finding as indicating, possibly for the first time in the setting of genomics, that the genetic dissection confirms the expectation that the important differences are conserved and quantitative, rather than

qualitative, in keeping with our earlier studies on regulation of immunoglobulin and antibodies by MHC ancestral haplotypes (Cobain et al., 1983; Wilton et al., 1985; French and Dawkins, 1990; Dawkins et al., 1999).

Genomic markers for favorable rates of marbling will be of practical as well as theoretical interest. Reducing the time and cost on feed brings the potential to provide healthy beef to a wider sector of the world's population with the secondary benefits of reducing cardiovascular disease and the billions of dollars spent on cholesterol lowering statins.

One of the most important lessons from the present study is that a haplotype sequence of multiple genes and regulators can have complex associations and confounding effects. The 60.10.S.10 haplotypes is Wagyu specific, beneficial in upgrading non-Wagyu in the tasting competition, directly or indirectly associated with low Tm but only the 3rd or 4th best in the ranking of the Wagyu haplotypes. We believe that further data will show that, as in the case of the human MHC, diplotypes and their quantitative *cis*- and *trans*-interactions will be critical in explaining apparent contradictions.

In conclusion, we recommend combining an accurate measurement of the phenotype with a genomic description of the genotype allowing identification of megabase ancestral haplotypes.

4.5 *Materials and methods*

4.5.1 *Full-blood Wagyu with identified sires*

Two cohorts of Wagyu steers (n=128) and heifers (n=6) were fed for approximately 300±20 days on a proprietary ration within a commercial feedlot. One gram samples of meat and intramuscular fat were taken from each carcass from between the 10th and 11th rib. AUS-MEAT marbling score (MS) was scored between the 10th and 11th rib, with an average of 7.6

and a range from 2 to 11. Steers chosen for the sire comparison had their paternity confirmed via DNA testing (Lecomte et al., 2010).

4.5.2 *Branded beef competition*

Samples were obtained from 9 full blood, 16 crossbred, and 4 grass-fed entries to the Australian Wagyu Association's "Branded Beef Competition" from 2014 to 2015. Crossbred entries were F1, F2, F3, and F4 Wagyu. Feed composition and days on feed varied, but all entries had more than 25% intramuscular fat, as assessed by camera image analysis (Kuchida et al., 2000).

4.5.3 *Short-fed European and crossbreds*

The herd at Melaleuka Stud has a variety of European breeds including Simmental, Gelbvieh, and Angus. This herd was selected to produce high-quality beef on pasture, finished within 2 to 4 months of supplemental feeding. Black Wagyu have been introduced into the herd as full blood or pure bred and for crossing with some of the European breeds. Those designated 50% are the F1 progeny of a mating between Wagyu and European, whereas 25% and 75% are the second cross progeny of F1s mated with European and Wagyu, respectively.

Akaushi sires have recently been used over some European breed cows with European breed bulls continuing to be used over the remaining cows.

Calves stay on milk until 4 months of age when they are weaned, and male calves are castrated. After weaning, they continue grazing Kikuyu and Ryegrass pasture until they reach 300 kg. Their feed is then supplemented with 9mm EasyBeef pellets (Milne Feeds, Perth, Australia) *ad libitum.* The main ingredients of the EasyBeef pellets are lupins, barley, oats, wheat, and triticale. The nutritional composition, based on dry matter, is crude protein (min)

14.5%, metabolizable energy (est.) 11.0 MJ/kg, crude fiber (max) 20.0%, urea (max) 1.5%, and monensin 26.6 ppm.

The feeders are considered ready for slaughter when they reach a weight of 400 kg and are slaughtered to match demand. Some animals were kept on feed longer to test the effect of increased feeding on Tm and meat quality. The average live weight at slaughter for animals in this study was 461 kg, average age at slaughter was 15.4 months (range 8 to 23), and the average days on feed was 104 days (range 17 to 288). Body numbers from abattoirs were matched to farm records and pedigrees via their RFID tags, where possible identity was confirmed by inhouse proprietary DNA testing (Williamson et al., 2011).

Subcutaneous fat samples from the sirloin of these cattle were collected after boning and wet aging.

4.5.4 *Long-fed Wagyu crossbreeds with Bos indicus ancestry*

Fifty samples of subcutaneous fat were taken from the rump of crossbred Wagyu steers fed for 450 days in a commercial feedlot. Samples were collected from the abattoir and frozen immediately. DNA was extracted and tested for MPRIP markers, and triglycerides which separated from the adipose tissue during DNA extraction were tested for Tm. Wagyu ancestry ranged from 48% to 96% with the majority being F1 and average Wagyu ancestry at 57%. 19 of the 50 animals had some *Bos indicus* content. Other dam breeds included Shorthorn and Angus.

4.5.5 *DNA extraction and C19 haplotype testing*

Genomic DNA was extracted from all meat samples using the standard salting out method. Alleles of SREB, NT5M, MPRIP, TCAP, and GH markers were determined by PCR and capillary electrophoresis using the primers and method described in Lecomte et al. (Lecomte

et al., 2010). Alleles of FASN were determined using the primer and method described in Williamson et al., 2011. The FASN marker is more correctly known as SCT-FSN since the present marker is adjacent to the coding region for FASN, within the segmental duplication containing SECTM1. Haplotypes in heterozygous individuals were determined from segregation or alleles in related animals. The W plots were based on haplotype frequencies measured in Australian herds of registered Wagyu.

5 Carcass identification reliability: importance, impact and DNA contribution[3]

5.1 *Introduction*

Consistency in the eating quality of beef has become one of the main aims of the Australian meat industry. Accordingly, MLA (Meat & Livestock Australia) created a standardised grading system for carcass quality traits, implemented by MSA (Meat Standards Australia). The purpose of MSA grading is to predict the quality and therefore the value of each carcase.

According to the MSA Annual Outcomes Report 2008-2019, in Australia out of more than 8 million adult cattle slaughtered per year, 3.5 million (43%) are MSA graded.

In addition to the assessment of the individual carcase, the MSA grading information is used for calculating the genetic value of potential progenitors based on the performance of their offspring.

It is evident that MSA grading and the estimation of the EBVs based on the offsprings' performance are useful only if the information is accurate and reliable. The key point is that the information of the carcass must belong to the correct animal. If not, all the records in terms of breed composition, production performance, progeny and pedigree are lost or even worse, used to calculate the EVBs of a different animal. To avoid this issue and for other purposes, a strong traceability system becomes necessary.

Traceability can be defined as the ability to maintain credible custody of identification of products through various steps within the food chain. In this case, from birth to the retailer (McKean, 2001).

[3] Not to be published.

Different methods for animal identification and traceability have been applied for cattle, including external marking methods like ear notches, tattoos, hot branding, freeze branding, ear tags and biometric methods like muzzle or nose prints, coat colour patterns, iris patterns, and DNA markers (Petersen, 1922; Awad, 2016).

Since 1999, Australia uses The National Livestock Identification System (NLIS) for the individual identification and traceability of cattle, which expanded to sheep and goats in 2009 (https://www.nlis.com.au/NLIS-Information/)

5.1.1 *Nose prints*

In 1922 William Petersen raised the issue of animal substitution, and described the practicality of nose patterns to identify individuals as “no two animals have been found with the same design” and “the pattern remains the same throughout life”. He described six distinct patterns of lines starting from the centre of the nose, formed by the elevations and grooves created by the subcutaneous facial-nasal glands.

In 2012 Noviyanto and Arymurthy reinforced the need for a reliable system for animal identification to avoid adulteration of the identity of animals. Based on the same principle used by Petersen in 1922, they compared nose prints to fingerprints in humans and confirmed the practicality of the object recognition based method SURF (Speed-Up Robust Features) for automatic cattle identification based on muzzle photos.

In Japan, Wagyu producers have been aware of the importance of traceability and identification for a long time. For this purpose, the Japanese have been using the nose prints and including them in Wagyu pedigree certificates since at least 1944 (Bayard, 2020) (Figure 5.1), as the pattern is conserved as animals age (Petersen, 1922).

Figure 5.1 *Individual identification document used in Japan*. It shows the family tree of the animal and a nose print on the lower-left corner. With permission from Tatsuya, Japan (https://www.kobebeef.co.jp).

5.1.2 *NLIS and RFID*

In Australia, the NLIS stores in a central database the breeder, movements and death of all livestock (https://www.nlis.com.au/NLIS-Information/).

For this purpose, calves are ear-tagged, generally at young ages, with an RFID (Radio-Frequency Identification Device) linked to its individual NLIS number. This is mandatory before any change of location (new PIC), and from that moment, every movement is recorded on the database (Hossain and Quaddus, 2013).

RFIDs can be electronically read. They exist in two forms; ear tags and rumen bolus/visual ear tag combination, and should remain with the individual for the entire life (Tkachenko, 2017).

5.1.3 *DNA tracing*

Unfortunately, all the identification methods described above are only useful from birth to slaughter. At that last moment, external identification devices and marks are detached from the animal, and other characteristics like the coat and nose patterns are not useful anymore. This is a moment of high vulnerability of the system in terms of animal identification, and it is here where DNA testing becomes the most effective method of identification.

Identification of animals and animal products can be done through the study of the DNA, as it is contained in every cell of the body, it is hugely variable among individuals, is not alterable through the individual's life, and it's generally preserved through the food processes (Dalvit et al., 2007).

Various DNA polymorphisms, including microsatellites and SNPs, have been used as genetic markers for pedigree and traceability (Cunningham and Meghen, 2001). DNA and genetic fingerprints have been used for species identification, breed identification, pedigree verification and individual animal traceability (Dalvit et al., 2007).

One example of DNA tracing is SureTRAK® of Pfizer Animal Genetics, offering traceability from the meat product to the animal, using a 14-marker-panel test (https://www.zoetisus.com/_locale-assets/mcm-portal-assets/my-resources/genetics-pdf-attachments/suretrack/0007pag_suretrak_sell_sheet_na.pdf).

Here we show how the DNA polymorphisms used to determine the Bota C19 ancestral haplotypes involved in beef quality and healthiness can be used for traceability. In Melaleuka

Stud, those haplotypes are determined at birth; therefore, identification and traceability of the carcass or final products only require one extra sample at the end of the process.

5.2 *Material and methods*

In order to determine the ancestral haplotypes responsible for meat quality and intramuscular fat deposition described by J. Williamson in 2011, CY O'Connor Foundation DNA tests at birth every animal of Melaleuka Stud, using the tissue resulting from ear notching.

To guarantee that the correct carcass is received and identified, a small muscle sample is collected from the carcasses at the abattoir and the butcher, and from the meat products after delivery from the butcher.

We have used that information for comparing the genotypes of the slaughtered animals and the meat products, allowing us to detect inconsistencies.

The DNA extraction, PCR and capillary electrophoresis were executed according to the methods described in Chapter 4.

5.3 *Results and discussion*

Figure 5.2 shows an example of how the DNA information of slaughtered animals was compared to the DNA information of the carcasses, confirming and validating, in this case, the carcass information.

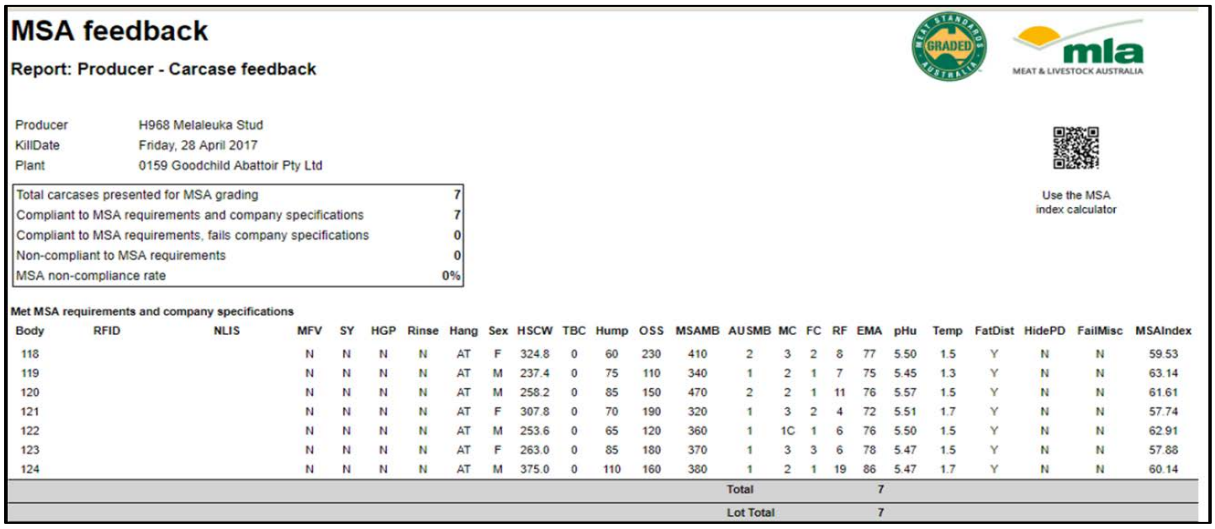
Melaleuka Stud Slaughtering date 16/06/2016										
Carcase alleles					Animal alleles					
Body N°	**SREB**	**NT5M**	**MRIP**	**TCAP**	**Tag**	**Hap**	**SREB**	**NT5M**	**MRIP**	**TCAP**
1	LL	10 20	30 30	20 20	L097	30.2; 30.6	LL	10 20	30 30	20 20
2	LL	20 20	40 60	20 20	K568	60.2; 40.2	LL	20 20	40 60	20 20
3	SL	20 20	30 40	10 20	L036	30.4; 40.3	SL	20 20	30 40	10 20
4	SL	10 20	10 60	10 10	L099	60.1; 10.1	SL	10 20	10 60	10 10
5	SS	20 20	40 40	20 20	L584	40.4; 40.4	SS	20 20	40 40	20 20

Figure 5.2 *Comparison of the DNA information of the carcasses with the DNA information of the animals sent to the abattoir for slaughter*. The alleles selected for DNA comparison are the ones described in Williamson et al. 2011., used for defining ancestral haplotypes in Bota 19. Note that SREB, NT5M, MRIP and TCAP alleles of the carcasses match perfectly the alleles of the individuals sent to the abattoir. In that case, the carcass information is validated and used for further analysis.

The most common mistakes at the abattoir and butcher were missing information, incorrect carcass labelling within the kill, and carcass substitution.

5.3.1 *Missing information*

In some cases, due to problems during slaughtering, the carcass information is assigned to the body number of the day, but the electronic ID tag of each body is not recorded, making it impossible to link the carcass information to a specific animal (Figure 5.3).



MSA feedback

Report: Producer - Carcase feedback

mla MEAT & LIVESTOCK AUSTRALIA

Producer H968 Melaleuka Stud
KillDate Friday, 28 April 2017
Plant 0159 Goodchild Abattoir Pty Ltd

Use the MSA index calculator

Total carcases presented for MSA grading	7
Compliant to MSA requirements and company specifications	7
Compliant to MSA requirements, fails company specifications	0
Non-compliant to MSA requirements	0
MSA non-compliance rate	0%

Met MSA requirements and company specifications

Body	RFID	NLIS	MFV	SY	HGP	Rinse	Hang	Sex	HSCW	TBC	Hump	OSS	MSAMB	AUSMB	MC	FC	RF	EMA	pHu	Temp	FatDist	HidePD	FailMisc	MSAIndex
118			N	N	N	N	AT	F	324.8	0	60	230	410	2	3	2	8	77	5.50	1.5	Y	N	N	59.53
119			N	N	N	N	AT	M	237.4	0	75	110	340	1	2	1	7	75	5.45	1.3	Y	N	N	63.14
120			N	N	N	N	AT	M	258.2	0	85	150	470	2	2	1	11	76	5.57	1.5	Y	N	N	61.61
121			N	N	N	N	AT	F	307.8	0	70	190	320	1	3	2	4	72	5.51	1.7	Y	N	N	57.74
122			N	N	N	N	AT	M	253.6	0	65	120	360	1	1C	1	6	76	5.50	1.5	Y	N	N	62.91
123			N	N	N	N	AT	F	263.0	0	85	180	370	1	3	3	6	78	5.47	1.5	Y	N	N	57.88
124			N	N	N	N	AT	M	375.0	0	110	160	380	1	2	1	19	86	5.47	1.7	Y	N	N	60.14
														Total				7						
														Lot Total				7						

Figure 5.3 *MSA Carcass Feedback Report with missing information*. In this case, the report contains the body number 118 to 124 of the day and the score of each carcass for every variable. In this report, the RFID and NLIS columns are blank, therefore is impossible under normal circumstances to link the carcass information to the right animal.

Figure 5.4 shows how C19 ancestral haplotypes were used to determine the correct animal for each body number and link it to the carcass data supplied by MSA.

Melaleuka Stud. Slaughtering date 28/04/2017										
Meat alleles						Animal alleles				
Body N	**SREB**	**NT5M**	**MRIP**	**TCAP**		**Tag**	**SREB**	**NT5M**	**MRIP**	**TCAP**
118	LL	20 20	30 40	20 20		K521	LL	10 20	10 30	10 20
119	LL	10 20	10 30	10 20		K543	LL	20 22	40 40	20 20
120	LL	20 20	30 40	10 20		K640	LL	10 20	30 40	10 10
121	LL	20 20	30 60	10 20		L614	LL	20 20	30 40	20 20
122	LL	10 20	30 40	10 10		L711	LL	20 20	30 60	10 20
123	LL	10 22	30 30	10 20		M032	LL	10 22	30 30	10 20
124	LL	20 22	40 40	20 20		M106	LL	20 20	30 40	10 20

Figure 5.4 *C19 ancestral haplotypes can be used to correct the information when the carcasses have not been assigned to the slaughtered animals.* The blue lines link each body number to the corresponding animal. Blue lines connect the carcass body number to the corresponding animal, defined by the combination of C19 alleles.

5.3.2 *Incorrect carcass labelling*

On some occasions, the C19 alleles of the meat products were not consistent with the live sample of the corresponding animals. In many cases, it was possible to identify a rearrangement within the batch and thus, match the samples to the correct animal (figure 5.5).

Melaleuka Stud. Slaughtering date 28/04/2017										
Carcase						Animal				
Body N	**SREB**	**NT5M**	**MRIP**	**TCAP**		**Tag**	**SREB**	**NT5M**	**MRIP**	**TCAP**
3	LL	20 22	30 40	10 10		M143	LL	20 22	30 40	10 10
4	LL	20 20	30 40	10 20		M142	LL	20 22	30 30	10 10
6	LL	20 22	30 40	20 20		M147	LL	20 22	30 40	20 20
7	LL	10 20	30 40	10 10		M160	LL	10 20	30 40	10 10
8	LL	20 22	30 30	10 10		M157	LL	20 20	30 40	10 20

Figure 5.5 *C19 ancestral haplotypes can be used to verify and correct mixed data.* The MSA report links body number 4 to the animal M142, and body number 8 to the animal M157, suggesting that the identification numbers of these two animals have been mixed. In those cases, fraud or substitution may be considered unlikely. Lines link the wrongly assigned carcasses to their correct animals.

5.3.3 *Substitution*

Finally, we identified cases where the alleles present in a carcass do not match any of the alleles previously identified in the group of slaughtered animals (Figure 5.6).

Melaleuka Stud. Slaughtering date 14/08/2014										
Carcase						Animal				
Body N°	**SREB**	**NT5M**	**MRIP**	**TCAP**		**Tag**	**SREB**	**NT5M**	**MRIP**	**TCAP**
101	LL	20 20	40 60	20 20	≠	J625	LL	20 20	30 40	10 20
						J618	LL	20 20	30 30	20 20
						J603	LL	20 20	30 30	10 20
						J628	LL	20 20	30 40	10 20
						J565	SL	20 20	30 40	20 20

Figure 5.6 *C19 ancestral haplotypes for detection of substitution.* The carcass number 101 has MRIP alleles 40 and 60, which are not present in the animal group. This is unequivocal evidence that one of the animals of the group has been substituted.

We found that for the years 2014 – 2017, 8.3% of the meat cuts did not match the corresponding slaughtered animal or it did not match any of the animals of the group delivered to the abattoir.

In the assessment of parental worth, particularly in the case of young new bulls, any inclusion of even a small number of substitutions will have an effect over the accuracy of the results. With the use of artificial insemination, it is possible to produce more than 300 doses of semen per ejaculation; therefore, the consequences of inaccuracies in the calculations of the genetic potential of some bulls will be magnified in the following generations.

At a simple but profound level, the success of breeding programs for meat quality depends on the ability to accurately match carcass data to individual animals and their pedigrees.

Therefore, here we propose C19 haplotyping as a single multipurpose test for;

i. Identification through life and beyond, including point of sale.

ii. Parentage, especially exclusion of sire or dam.

iii. Ancestry, in relation to lines such as Tajima.

iv. Breed composition, this topic was introduced in Chapter 4.

At one level, these issues can be addressed using a SNP or microsatellites approach; however, these tests can be more expensive and, unlike C19 ancestral haplotypes, do not target areas critical to meat quality.

6 Adipose invasion of muscle in Wagyu cattle: monitoring by histology and melting temperature[4]

The genetic component of marbling and Tm, and the genetic similarities between marbling and some muscular dystrophies discussed in chapters 3 and 4, leave open many questions.

Are marbling and muscular dystrophies similar processes, involving similar pathogeny or they just share similar genes? Does marbling share similar features with Limb-girdle muscular dystrophy at a cellular level? Is marbling really a passive process of steatosis (Smith and Johnson, 2014; Peletto et al., 2017), where degenerated myofibers are replaced by new adipocytes?

Muscular dystrophies are a large group of muscular degenerative conditions. The specific diagnosis relies on clinical signs, serology, DNA test and also in the combination of specific histological features. In the same way, to understand the nature of marbling, we need to understand and characterise the process at a microscopic level.

This work aims to investigate the histology of marbling and generate the knowledge that would allow to understand marbling in a greater perspective, to then compare it to other processes like human muscular dystrophies.

As the first author of this work, my contribution was:

i. Identification of the base of the tail and the *Ischiatic tuber* region as areas of particular interest for future muscle and fat biopsy.

ii. Development of the microscopic marbling scoring technique.

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[4]Published as: Valenzuela, J., Lloyd, S., Mastaglia, F, and Dawkins, R. (2020). Adipose invasion of muscle in Wagyu cattle: monitoring by histology and melting temperature. *Meat Science.* Volume 163, Article ID 108063, 10 pages. https://doi.org/10.1016/j.meatsci.2020.108063

iii. Sampling of fat and Tm measurements.

iv. Sampling and histological evaluation of muscle samples.

v. Data analysis.

vi. Writing the manuscript.

Here we characterise the process, describe the different patterns of intramuscular fat deposition, confirm the presence of intramyocellular fat droplets, prove the existence of different areas of the muscle for de novo adipocytes, and show different locations for marbling and Tm assessment.

This work validates an alternative area of measurement for marbling and Tm, which allows the monitoring of marbling and Tm in live animals through sequential biopsies at different stages of the production system.

6.1 *Abstract*

Remarkably, Wagyu cattle progressively desaturate intramuscular and subcutaneous fat leading to melting temperatures (Tm) well below 38ºC. In parallel, the adipose tissue expands, arborises and invades the muscle. The process is aggressive in that it leads to loss of myofibres resulting in much smaller fascicles and therefore fine marbling or snowflaking.

The “Microscopic score” appears to be an excellent measure of marbling especially for lesser and greater degrees which are not quantified reliably by other methods.

By comparing muscle groups, we conclude that the tailhead is a suitable site for sequential monitoring. Melting temperatures of intramuscular and subcutaneous tissue are also useful.

6.2 *Introduction*

The amount and type of intramuscular fat are increasingly important to cattle breeders because of the many connotations for human health. High concentrations of "healthy" monounsaturated fatty acids, such as oleic acid, are known to improve the cholesterol profiles of consumers (Mensink et al., 2003; Gilmore et al., 2011) and thereby reduce the need for expensive and sometimes detrimental statin therapy (Stroes et al., 2015; Simons, 2019). Fortunately, the same fats are associated with consumer preference (Pannier et al., 2014), suggesting that it may be simple to reduce cardiovascular complications with appropriate breed and sire selection.

Unfortunately, there is no agreed approach to the quantitation of marbling (Johnson et al., 1986; Cheng et al., 2015). In Australia, MSA MB (100 to 1190) and AUS MB (1 to 9) are scored by the naked eye. Current grading systems in Australia and the United States were not designed to grade carcasses with > 20% intramuscular fat in the loin muscle. An MSA MB of 500 corresponds to approximately 10% intramuscular fat w/w, 1190 to 20% intramuscular fat although there is considerable variation (Egarr, 2011; Frank et al., 2014). A camera is under trial (Kuchida et al., 2000)*. Other systems are used in Japan and the USA. Some studies have shown that the chemical measurement of intramuscular fat can be used (Cheng et al., 2015). Even more important than measuring the amount of marbling is determining the fatty acid profile most beneficial for health. In this respect melting temperature (Tm) is promising (Chung et al., 2006; Lloyd et al., 2017b). Further studies are required to compare the different methods, especially from the point of view of the health benefit to the end user.

* Since publication, progress has been made in the development of this technology, however, up to the date of submission of this thesis, there is not any camera approved by AUS-MEAT for the official grading of marbling.

In addition to measuring the amount of intramuscular fat, it is necessary to distinguish between coarse seams and the preferred fine marbling (Asa et al., 2017). This has been done by the naked eye or by camera although, again, the results differ (Peña et al., 2013).

Here we evaluate the utility of microscopic examination as an international benchmark for quantifying the amount of marbling. We have gathered histological samples from several sources that represent a wide range of marbling scores, cattle breeds and feeding regimes in order to describe a variety of marbling characteristics and to determine the utility of microscopic scoring of marbling. Further, we ask whether it is possible to explain the basis of fine and coarse marbling.

A major variable is the time on feed. As shown in Lloyd et al., (2017a, b) the time course is dependent upon breed in that Red Wagyu (Akaushi) begin to marble after only 50 days. By contrast, Black Wagyu follow a slower trajectory but tend to achieve much lower Tms and more, finer marbling after 300 to 500 Days On Feed (DOF).

An important aim of the current study is to understand the complex, and controversial processes involved in marbling. As shown in Lloyd et al. (2017a), there are indications of similarities between the genetic control of marbling and of human muscular dystrophy. In addition, there are histological similarities (Valenzuela et al., 2019). Dystrophy has been studied in great detail over many decades; there are many different forms and, undoubtedly, many processes that can result in dystrophy. In the present study we compare the histology and conclude that one major process in bovine marbling is more related to fatty invasion than simple accumulation by adipogenesis. However, there are significant issues with sampling depending upon location and muscle group. We show that it is practical to sample from the tail head, raising the possibility of sequential *in vivo* monitoring.

6.3 *Material and methods*

Post mortem samples of muscle and fat were taken from carcasses of animals harvested for routine food production. Therefore, ethics approval was not required.

6.3.1 *Animal breeds and feeding regimes*

Access to carcasses for this trial was provided by Melaleuka Stud, located in the Peel region of Western Australia, in Nambeelup, 100 km south of Perth. Melaleuka Stud produces two categories of beef: Cluster 2) “long fed Wagyu” sold at a premium and Cluster 1) a leaner beef with less grain feeding.

Melaleuka Stud has two calving seasons per year, starting in January and July. Within 2 days after birth, the calves are DNA tested, confirming dam and sire and allowing later traceability of the carcasses. The calves remain with their mothers until four to six months old.

At weaning the males are castrated and continue grazing kikuyu, ryegrass pasture and hay until they reach a weight of 300 kg, when they are fed pellets and ryegrass hay ad libitum. The pellets are 9 mm EasyBeef (Milne Feeds, Perth, Australia), containing lupins, barley, oats, wheat and triticale, with a nutritional composition based on dry matter of crude protein (min.) 14.5%, metabolizable energy (est.) 11.0 MJ/kg, crude fibre (max.) 20.0%, urea (max.) 1.5%, and monensin 26.6 ppm.

6.3.2 *Sources of material from minimal to extreme marbling*

Three clusters of were chosen in order to provide samples that represent a wide range of marbling scores for the purpose of describing the characteristics of marbling at different levels, and to demonstrate that the microscopic marbling scores and tail head biopsy site are informative over the full range of marbling.

Cluster 1 (lower marbling), fed for between 57 and 280 days (avg 116). N=44. 5 heifers and 39 steers, with MSA MB from 270 to 630 (avg 379). Black Wagyu content was generally less than 50%. Most were 50% or more Akaushi.

Cluster 2 (moderate to high marbling), N = 17, 10 Heifers and 7 steers with Wagyu content from 50% to 96% were fed for 350 to 500 days (avg 427) with MSA MB ranging from 330 to 1100 (avg 630).

Cluster 3 (high and extreme marbling). Full blood and crossbreed Wagyu sirloins submitted to the, 2015 Australian Wagyu Association's Branded Beef Competition from various producers around Australia. N = 13. IMF 11% to 54%.

6.3.3 *Sampling and analysis*

In Melaleuka Stud, a DNA sample is taken from each animal at birth and was available for this study. Postmortem, a 2-g muscle sample was taken from the tail of each carcass. C19 haplotypes were determined using PCR, following the method described by Williamson et al. (2011). The information resulting from this analysis was used to confirm the carcass identity and validate the data for each individual.

6.3.3.1 *Fat melting temperature*

2-gram subcutaneous fat samples were taken at the *Ischiatic tuber* (IT) region and at 10th–11th rib level and stored at 5°C for fat melting point analysis following the method described by Lloyd, Dawkins, & Dawkins, 2014.

6.3.3.2 *Muscle histology*

Muscle samples were taken from the muscle *Longissimus dorsi* (LD), at 10th–11th rib level and from *Sacrocaudalis dorsalis medialis* (SDM) muscle for Hematoxylin and Eosin (H&E)

and Oil red O staining, and were processed at the Histology Laboratory of Veterinary and Life Sciences, Murdoch University as follows:

i. H&E: 10 mm×10 mm×3 mm samples stored in formalin 10% and then processed as described in Suvarna, Layton, & Bancroft (2012). The information recorded was the location of adipose tissue (Perimysium, endomysium, epimysium), Adipocyte size and patterns of adipocyte accumulation were noted.

ii. Oil Red O: 3 mm × 3 mm × 3 mm samples, frozen in liquid nitrogen and OCT (Optimal Cutting Temperature Compound, Sakura Finetek USA) and processed following the method described in Kiernan (2015). The aim was to visualise and describe the presence of intramyocellular lipid droplets.

6.3.3.3 *MSA marble score (MSA MB)*

The official MSA grading score, provided by the abattoir and determined by naked eye following the procedures described in the Handbook of Australian Meat (Meat & Livestock Australia, 2005) was used for each carcass. MSA marble scores range from 100 to 1190 with a score of 1190 corresponding to approximately 20% intramuscular fat w/w.

6.3.3.4 *Microscopic scores*

H&E sections were read using a binocular microscope. The degree of marbling was scored from 0 to 10, taking into consideration the proportion of adipose tissue to muscle tissue. Since the samples had to be taken a day or more post mortem, the interpretation allowed for the expected and quantifiable artefacts of staining and shrinkage. In fact, these changes, which could have been dismissed as “artefacts”, are very useful because they identify the most vulnerable myocytes.

The scoring was done by two observers in turn: one blind evaluator and the other providing the samples and recording the scores. Reference samples were chosen for microscopic scores 1, 3, 5, 7 and 9 and used as blind duplicates during the scoring process.

Results were confirmed using Leica Application Suite version 4.12 software. The Area of Adipose Tissue (AAT) was selected by the software on the basis of colour. Connective tissue was excluded after visual assessment (see Fig. 2).

6.3.4 *Statistical methods*

Pearson's correlation coefficient was calculated for microscopic scores of marbling at the LD and SDM muscles against MSA MB in order to confirm the utility of the microscopic scoring at these locations in predicting macroscopic marble scores. Correlation was also calculated between the Tm of subcutaneous fat from the IT and that at the 11th–12th rib to establish the utility of monitoring Tm from this potential biopsy site. Cluster 1 and 2 results were combined as one group for this analysis. Fisher transformations were used to calculate 95% confidence intervals of these correlation coefficients.

Similarly, correlation coefficients were calculated between intramuscular fat Tm and subcutaneous fat Tm for cluster 3 samples to verify that subcutaneous fat could provide a useful indication of intramuscular fat composition.

$$r = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}$$

Comparing Cluster 1 results with Cluster 2 results was not the main purpose of this study, however, where p-values have been stated they were calculated using Fisher's exact test.

$$p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{a!\,b!\,c!\,d!\,n!}$$

6.4 *Results*

6.4.1 *Quantification by different methods*

Examples of degrees of marbling of the *Longissimus dorsi* are shown in Fig. 6.1 et seq. Microscopy of low MSA MB (Marble Score) (Fig. 1a) shows a few small areas of adipocytes surrounding neurovascular bundles but also some connective tissue adjacent to adipose tissue. We distinguish between the two microscopically and only consider the adipose tissue when allocating a "Microscopic score" and the "area of adipose tissue". Microscopic score is the histological assessment of the amount of fat within muscle, whether perimysial or endomysial.

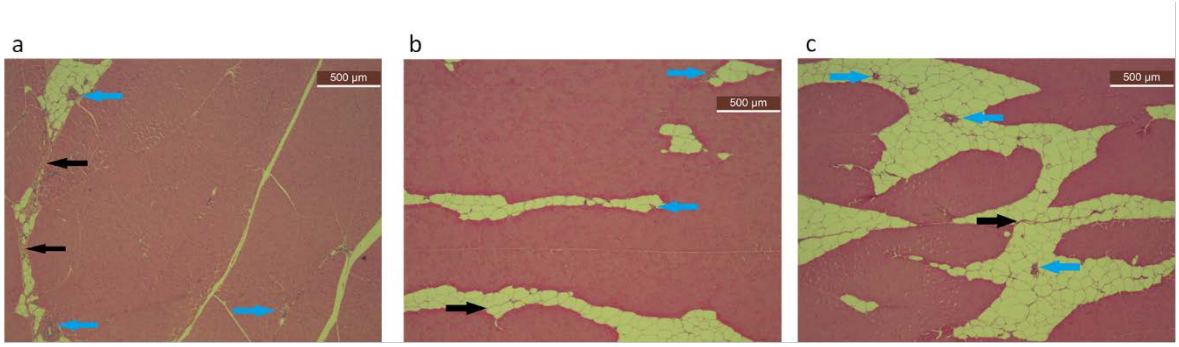

Figure 6.1 *Patterns of adipocytes within low, medium and high marbling muscle* Histological sections of *Longissimus dorsi* of three animals with increasing days on feed (DOF) and increasing marbling. Formalin-fixed Hematoxylin & Eosin. Blue arrows label examples of neurovascular bundles while black arrows label connective tissue. Figures selected as a representative portion of a bigger sample. (a) MSA MB 290, Microscopic score 1.5, AAT 3.0%, DOF 109. There are occasional adipocytes infiltrating some parts of the perimysium. CYO lab number Ch18/069P. (b) MSA MB 540, Microscopic score 4.5, AAT 10.7%, DOF 433. There is more extensive invasion of the perimysium. CYO lab number Ch18/031 K. (c) MSA MB 1100, Microscopic score 10, AAT 33.0%, DOF 471. CYO lab number Ch18/105X. There is extreme invasion creating the arborisation pattern of marbling, surrounding and separating most muscle fascicles. The adipocytes have increased in size as well as number. For example the maximum adipocyte diameter increases from 75 µm in (a) to 140 µm in (b) and 190 µm in (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

For MSA MB between 270 and 450 the AAT% was in the range 0.15% to 6.5%. As the marble score increases the area of adipose tissue increases, with more and larger adipocytes. For MSA MB between 450 and 700 the AAT% was in the range 7.5% to 25%. At high marble scores the area of adipose tissue extends (Fig. 1c). For MSA MB above 900 the AAT% was in the range 27% to 32%.

Fig. 2 compares the “Microscopic score” of the samples of *Longissimus dorsi* taken from clusters 1 and 2 with their MSA MB. The correlation between the two measurements (0.91, 95% confidence interval 0.85–0.94) is most apparent as marbling increases. For MSA MB below 400 the Microscopic score ranges from 0.5 to 2.0, suggesting that MSA MB is less discriminating.

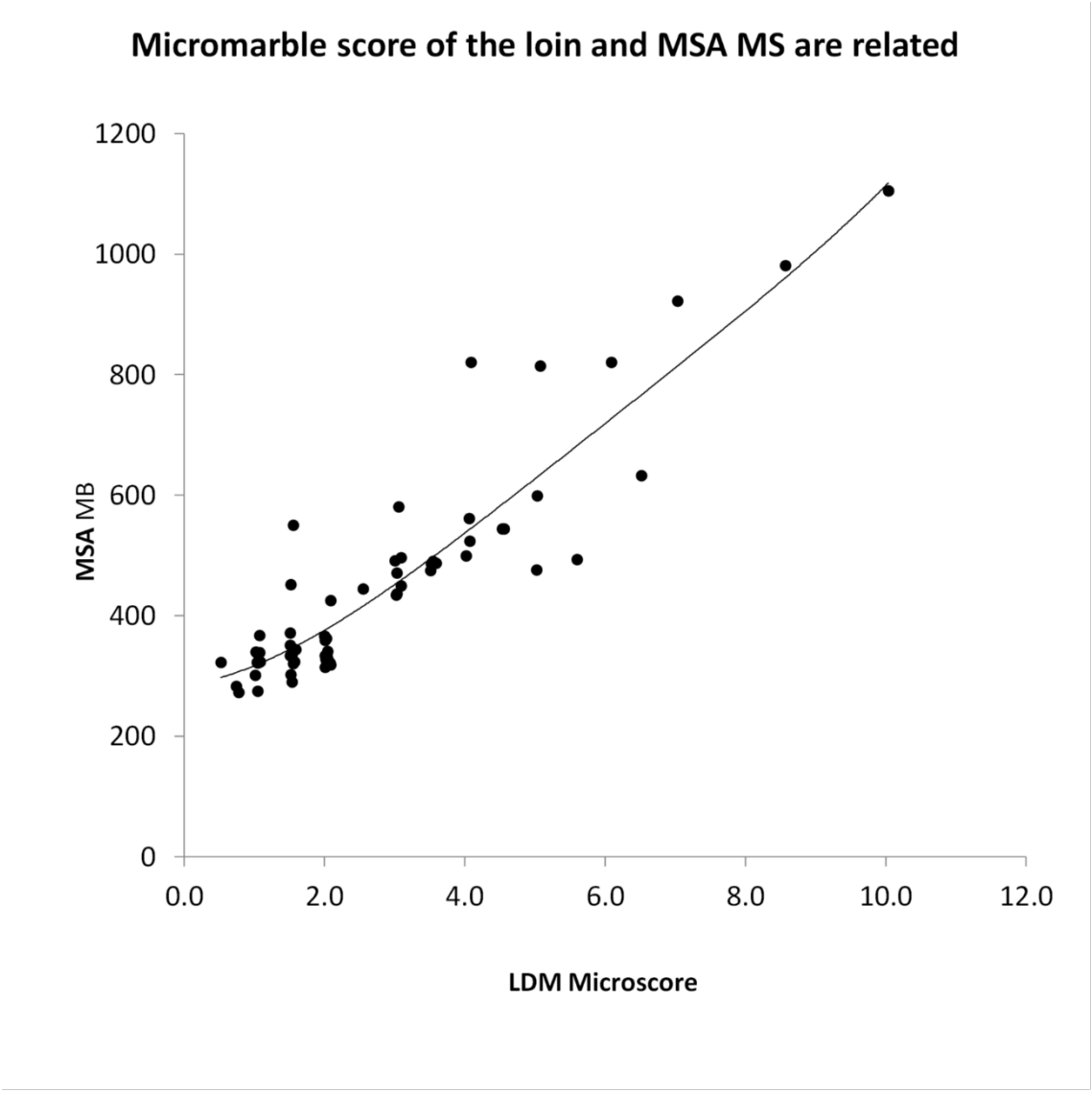

Figure 6.2 *Microscopic score of the loin and MSA MB are related.* Relationship between Microscopic score (X axis) and MSA MB (Y axis) of the *Longissimus dorsi* with increasing DOF. r = 0.91, 95% confidence interval 0.85 to 0.94. Plotted curve is best fit polynomial order 4.

Figure 3 compares histological sections of *Longissimus dorsi* of 2 animals so as to illustrate the risk of confusing perimysial connective tissue (Left) and true marbling due to adipocyte invasion (Right). Formalin-fixed Hematoxylin & Eosin. (A) MSA MB 290, Microscopic score 1.5, DOF 109. CYO lab number Ch18/069P. (B) MSA MB 360, Microscopic score 1, DOF 109. CYO lab number Ch18/71E.

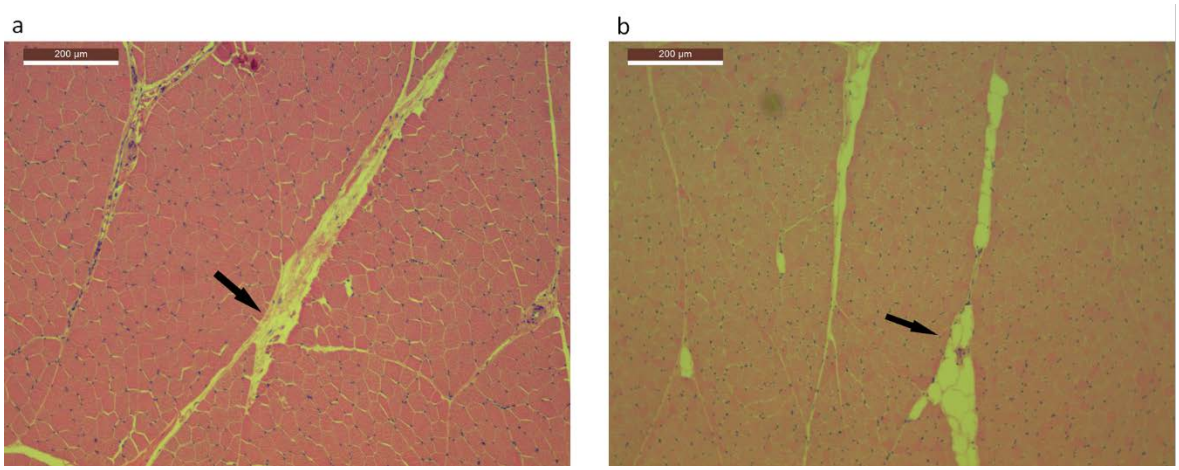

Figure 6.3 *Connective tissue and adipocytes can be distinguished microscopically*. Histological sections of *Longissimus dorsi* of 2 animals illustrate the risk of confusing perimysial connective tissue (Left) and true marbling due to adipocyte invasion (Right). Formalin-fixed Hematoxylin & Eosin. (A) MSA MB 290, Microscopic score 1.5, DOF 109. CYO lab number Ch18/069P. (B) MSA MB 360, Microscopic score 1, DOF 109. CYO lab number Ch18/71E.

6.4.2 *Mechanisms underlying IMF*

The progression of marbling, its characteristics and pattern are shown in Figure 4. It is possible to identify single perimysial adipocytes, generally located close to a neurovascular bundle (see also Figure 1). There are also lines of adipocytes along the perimysium between the muscle fascicles. In the centre of Figure 4, a wedge of adipocytes has formed pushing apart the adjacent muscle fascicles. Within the wedge there is considerable variation in adipocyte size. This suggests a process of progressive arborisation, whereby adipocytes invade and separate the fascicles. Another example of advanced arborisation can be seen in Figure 1c. At higher marbling the adipocytes are larger with more variation in size (Figure 1).

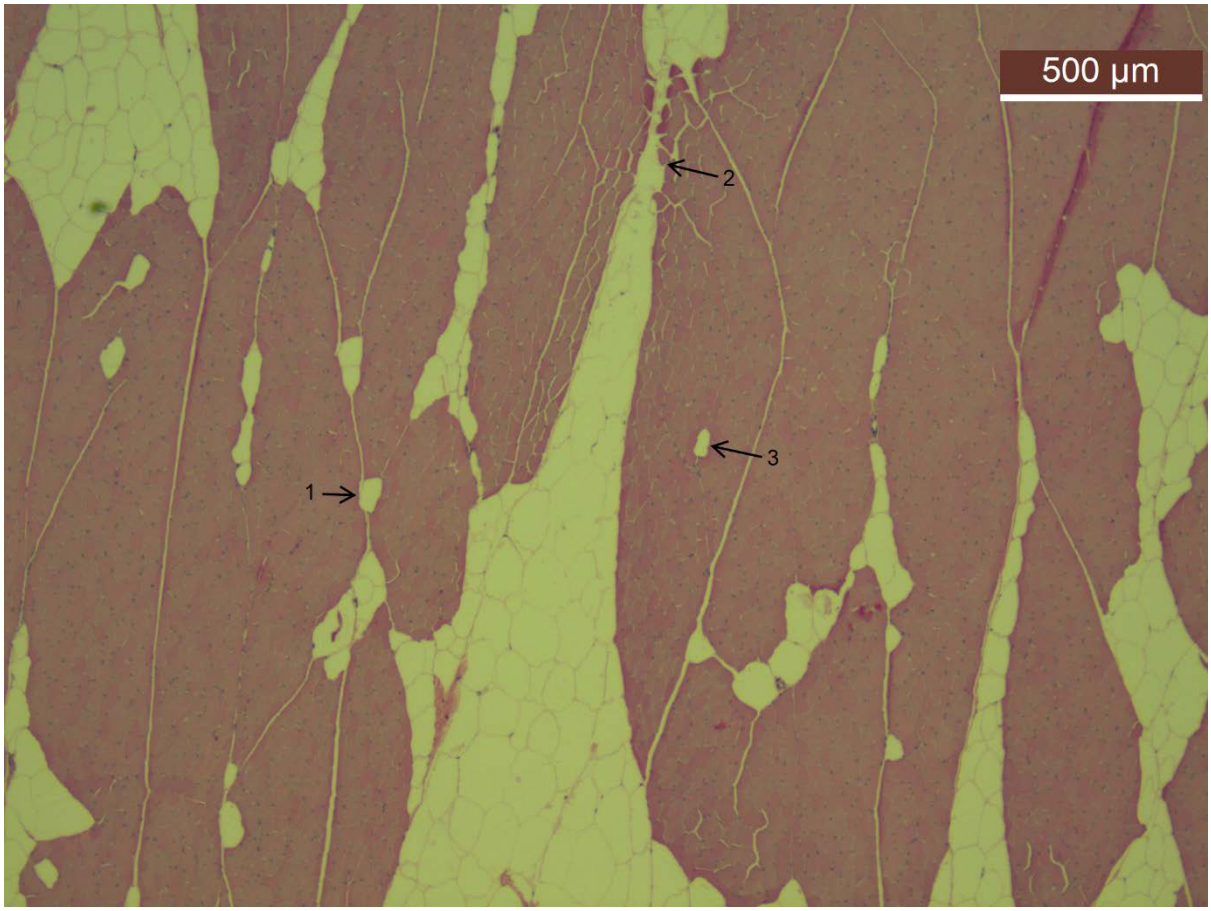

Figure 6.4 *Distribution of adipocytes in highly marbled Longissimus dorsi.* Histological section of *Longissimus dorsi* showing the presence of some apparently individual adipocytes. Some of these (1) are clearly within the perimysium, but others (3) appear to be isolated within the actual fascicles as might be expected if stem cells can follow the adipocyte pathway of differentiation. See also Fig. 6. Note also (2) the separation and eosinophilic staining of myofibres as invasion progresses. Formalin-fixed H & E, MSA MB 920. CYO lab number Ch18/045D.

At some boundaries between adipose and muscle bundles, the perifascicular myocytes are atrophic and show changes of shape and an increased affinity to eosin. This is most often observed near the ends of branches of adipocytes (Fig. 5). Islands of myocytes surrounded by adipocytes can be seen, suggesting that adipose tissue invades and isolates muscle (Fig. 5). The atrophy is especially apparent when fascicles are completely surrounded by adipocytes.

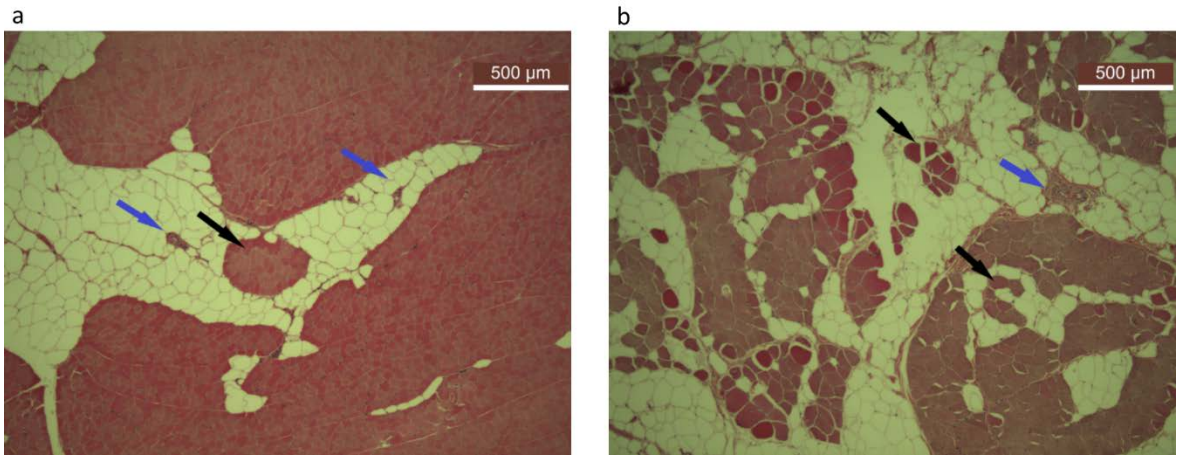

Figure 6.5 *Islands of myocytes surrounded by adipocytes.* Histological sections which illustrate the presence of neurovascular bundles (marked with blue arrows) and residual myofibres (black arrows as the arborisation advances. Formalin-fixed Hematoxylin & Eosin. A) *Longissimus dorsi* with MSA MB 580. Microscopic score 3, AAT% 17.1%, DOF 429. CYO Lab number Ch18/033Y. b) *Sacrocaudalis dorsalis medialis* of a high Wagyu content steer (88%) (wy63 ak25 dx13), MSA MB 1100, DOF 471. CYO lab number Ch18/110G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

In many highly marbled Wagyu, a few adipocytes were seen entirely within the endomysium, appearing to be singular (Fig. 4). To establish their complete independence from the perimysium, we examined serial sections of *Longissimus dorsi* at every 16 µm (Fig. 6). It is possible to see the beginning and the end of a region of endomysial adipose tissue, which confirms that this region is not connected to perimysial adipose tissue. The region was approximately 25 µm wide by 50 um long and may contain more than one adipocyte. In one highly marbled long fed Wagyu steer, we stained frozen sections with Oil red O and found the presence of intramyocellular lipid droplets (Fig. 7).

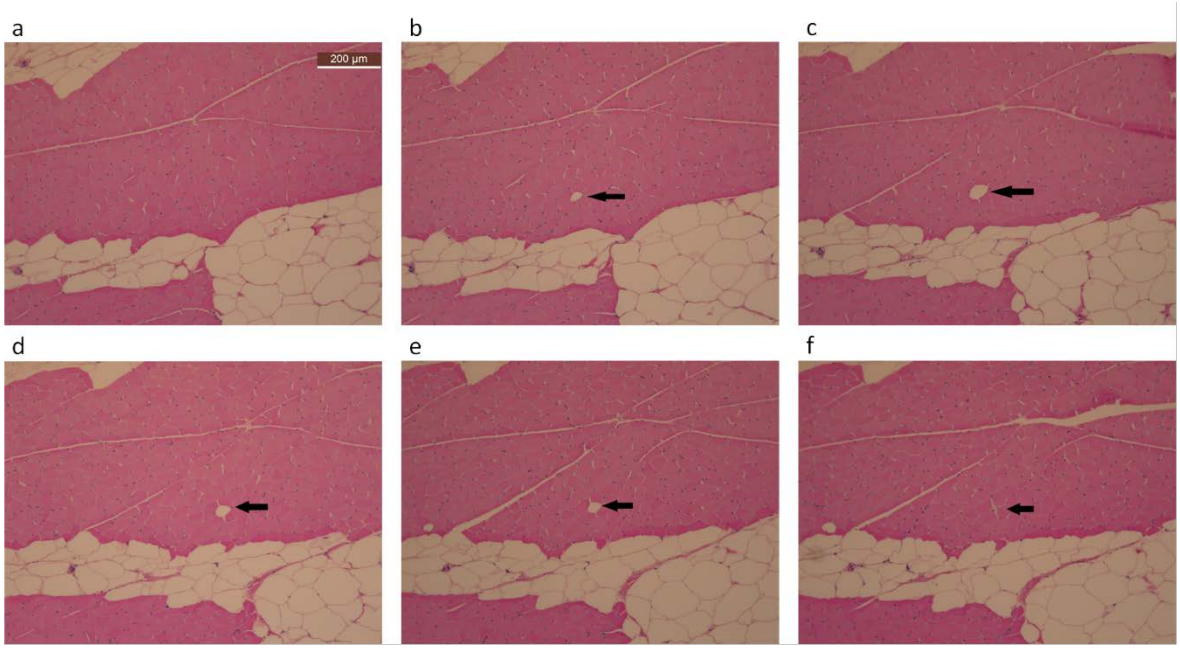

Figure 6.6 *Serial sections reveal genuine endomysial adipocytes.* Serial sections of *Longissimus dorsi* muscle of a 75% Wagyu, 25% Dexter heifer, 22 months old, DOF 443. MSA MB 920, Microscopic score 7. Levels shown at 0 µm, 16 µm, 128 µm,176 µm,188 µm and 200 µm. Formalin-fixed Hematoxylin Eosin. CYO lab number Ch18/045D. The arrows show the emergence and disappearance of an individual endomysial adipocyte without apparent connection to the perimysium. Note also the serration and moth-eaten appearance of the myofibres adjacent to invading adipocytes. By contrast, the borders are even where invasion is yet to occur.

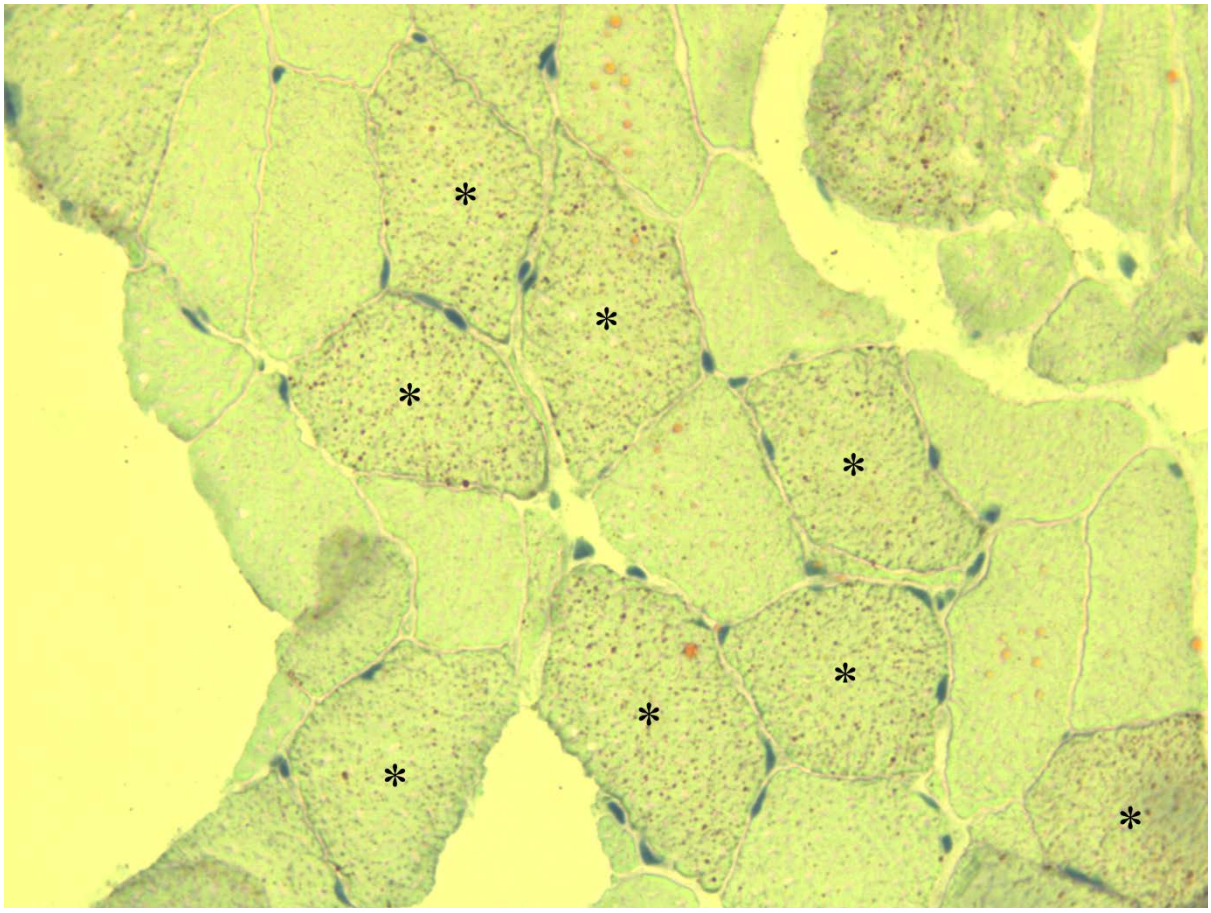

Figure 6.7 *Intramyocyte lipid droplets*. Histological frozen sections of *Longissimus dorsi* muscle of a full blood Wagyu steer, 35 months old, DOF 616, MSA MB 1080, Microscopic score 9.5. CYO lab N0 Ch18/119Q. Oil Red O stain. Marked fibres (∗) are positive to the presence of intramyocellular lipid droplets in keeping with type I or IIa myofibers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

6.4.3 *Subcutaneous v intramuscular*

In Fig. 8 we used 13 sirloins submitted to a Wagyu beef competition to compare the Tm of intramuscular fat with the Tm of the overlying subcutaneous fat. The Tms were clearly related, with a correlation of 0.85. This relationship prompts us to use Tm measurements of subcutaneous fat to monitor the intramuscular fat composition, when necessary.

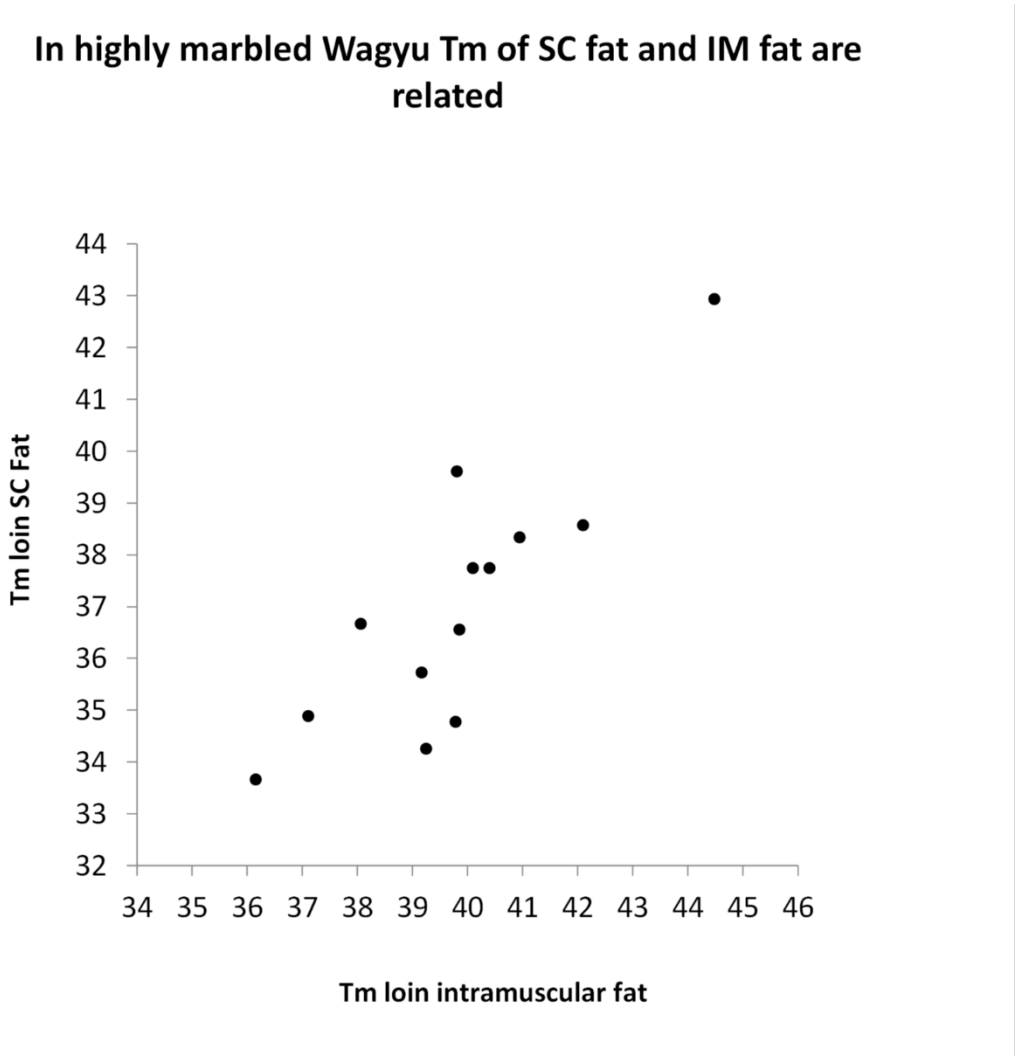

Figure 6.8 ***Tm of SC fat and IM fat are related.*** Comparison of Tm for subcutaneous and intramuscular fat of the loin of highly marbled Wagyu carcasses. r = 0.85, 95% confidence interval 0.55 to 0.95.

6.4.4 *Time course*

Variation of marbling with DOF is shown in Fig. 9. Animals with DOF less than 300 (Cluster1) had microscopic scores below 4, while animals with DOF higher than 350 (Cluster 2) presented microscopic scores from 3 to 10.

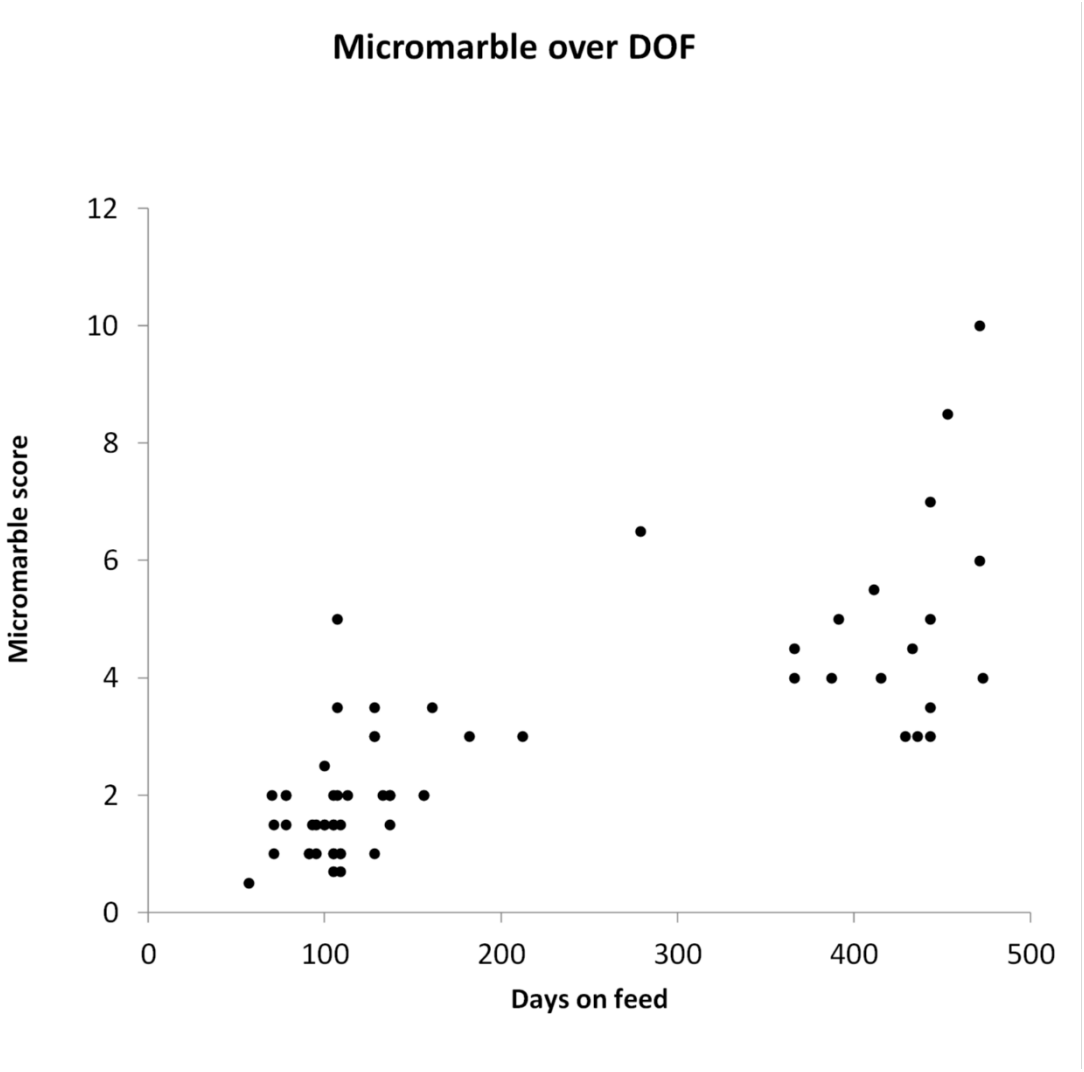

Figure 6.9 *Microscopic score demonstrates higher marbling in long fed cattle.* Comparison of Microscopic score for short fed (< 300 DOF) and long fed (> 350 DOF). r = 0.77, 95% confidence interval 0.64 to 0.86.

There were also qualitative differences. Cluster 1 had smaller adipocytes (less than 100 μm) located in the perimysium (Fig. 1a). Cluster 2 had bigger adipocytes (p-value<.005) and we observed greater variability of adipocyte size, more arborisation and lower proportion of connective tissue in relation to adipose tissue (Fig. 1c).

Despite a clear relationship between DOF and marbling, there is substantial variation between animals at similar DOF. Animals fed between 430 and 470 days have microscopic

scores varying between 3 and 10. An example is seen by comparing Fig. 1b with Fig. 1c. Genetic factors must be responsible (see legends).

The effects of DOF on Tm of subcutaneous fat is also shown in Fig. 10. Animals with less than 150 DOF have Tms more than 37ºC while animals with more than 350 DOF have Tms ranging from 34°C to 38°C. There is a clear trend of decreasing Tm with increased feeding and/or age but with substantial scatter (Fig. 10). It would be useful to know if:

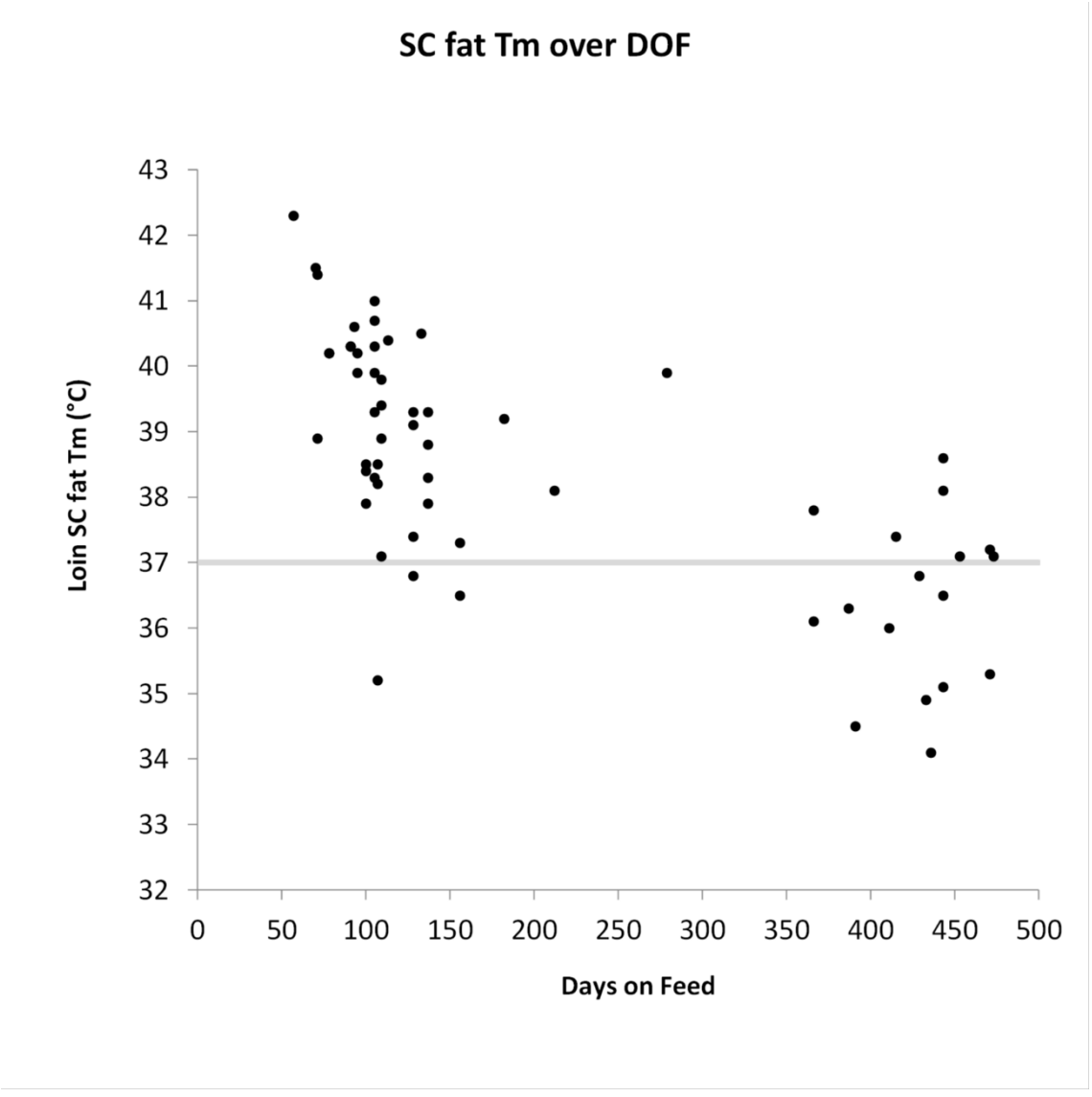

Figure 6.10 *Tm is lower in long fed cattle.* Comparison of Tm of the subcutaneous fat over the *Longissimus dorsi*. r = −0.70, 95% confidence interval − 0.81 to −0.55. Short fed (< 300 DOF) are generally above 37ºC whereas most long fed (> 350 DOF) are less than 37ºC. p < 0.0002 by Fisher's exact test.

It would be useful to know if :

(1) microscopic scores and Tm at lesser days on feed could predict future performance, after extended feeding, and.

(2) which additional genetic factors come into play. Therefore, we investigated sites for potential *in vivo* sequential monitoring.

6.4.5 *Potential for sequential biopsies*

Fig. 11 compares samples from *Sacrocaudalis dorsalis medialis* muscle (SDM) with *Longissimus dorsi* muscle (LD) for two different animals. Fig. 11 a and b were taken from a low marble score animal, which is reflected in both SDM and LD. Fig. 11c and d, from a higher marbled animal, show a proportionate increase in adipose tissue in both SDM and LD. The patterns of distribution and the abundance of adipocytes are similar between SDM and LD for both animals.

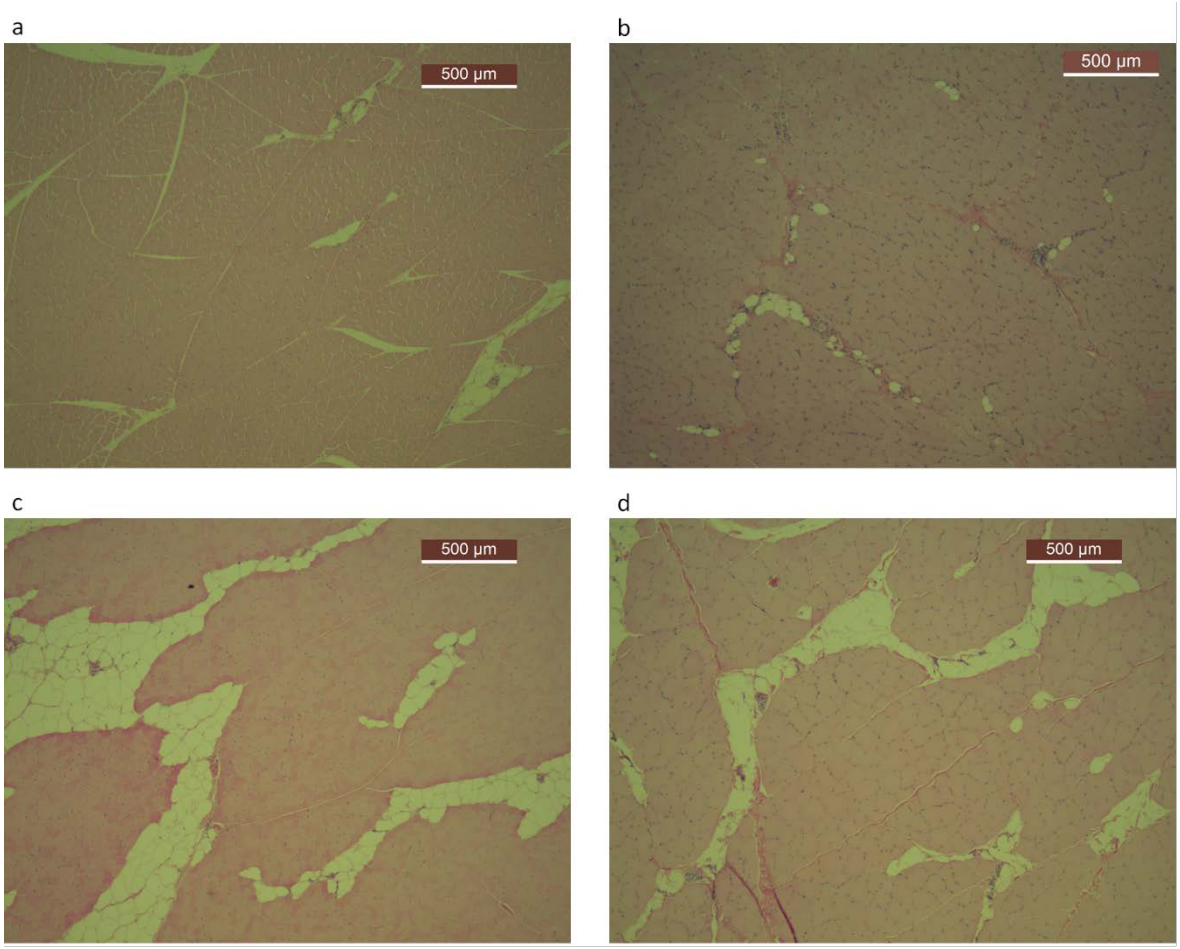

Figure 6.11 *Similar intramuscular fat deposition marbling at loin and tail.* Similar intramuscular fat deposition in *Longissimus dorsi* muscle (left) and *Sacrocaudalis dorsalis medialis* muscle (right) at two different marbling scores, MSA MB 330 (top) and MSA MB 560 (bottom). Formalin-fixed Hematoxylin & Eosin. Figures selected as a representative portion of a bigger sample. (a) MSA MB 330 *Longissimus dorsi* muscle. AAT 2.3%. Microscopic score 1.5. CYO lab No. Ch18/022Z. Maximum adipocyte diameter 120 µm (b) MSA MB 330 *Sacrocaudalis dorsalis medialis* muscle. AAT 1.3%. Microscopic score 1.5 CYO lab No. Ch18/014U. Maximum adipocyte diameter 80 µm (c and d) CYO lab No. Ch18/024M. MSA MB 560 (c) *Longissimus dorsi* muscle. AAT 19.3%. Microscopic score 4, maximum adipocyte diameter 150 µm. (d) *Sacrocaudalis dorsalis medialis* muscle. AAT 11.2%. Microscopic score 3, maximum adipocyte diameter 150 µm.

In Fig. 11a the area of adipose tissue for the LD is higher than the SDM for both animals. This trend was found in all other animals tested. Microscopic scores on SDM were assigned

taking this into account so that Microscopic scores on LD and SDM would cover the same range (0 to 10).

Figure 12 shows the relationship between the marbling in the loin, measured by MSA MB and the microscopic score of the SDM. Overall, there is an excellent correlation between microscopic score of the SDMM and the MSA MB (r = 0.89, 95% confidence interval 0.79 to 0.94).

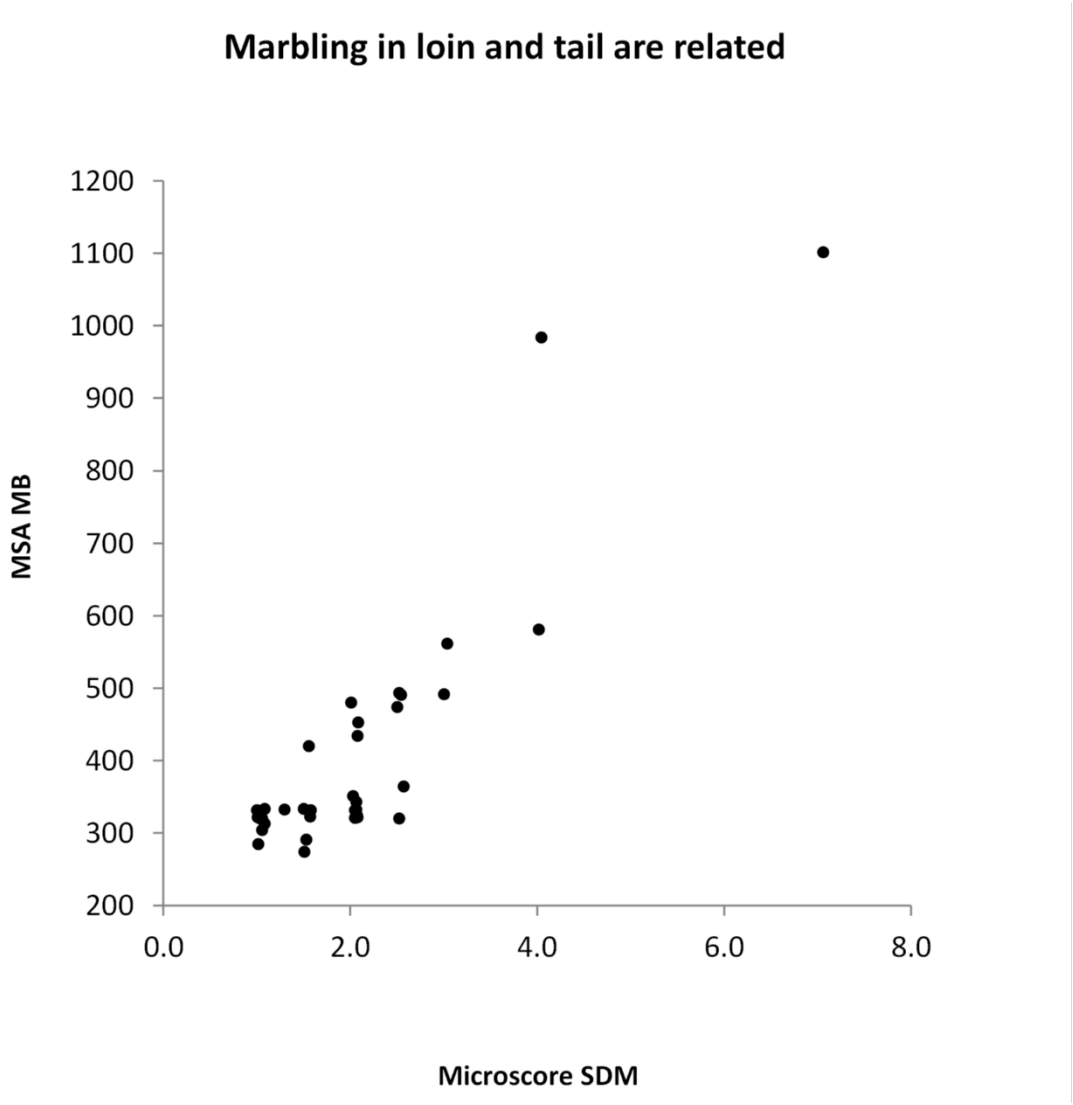

Figure 6.12 *Marbling in loin and tail are related.* Relationship between Microscopic score of *Sacrocaudalis dorsalis medialis* and MSA MB score assessed at *Longissimus dorsi* muscle. r = 0.89, 95% confidence interval 0.79 to 0.94.

The subcutaneous fat overlying the *Ischiatic tuber* (IT) also has potential for *in vivo* sampling to monitor changes in Tm during feeding. Fig. 13 compares the Tm of subcutaneous fat of the loin and the IT. The Tm at the IT is somewhat lower than that of the LD (r = 0.89, 95% confidence interval 0.81 to 0.93).

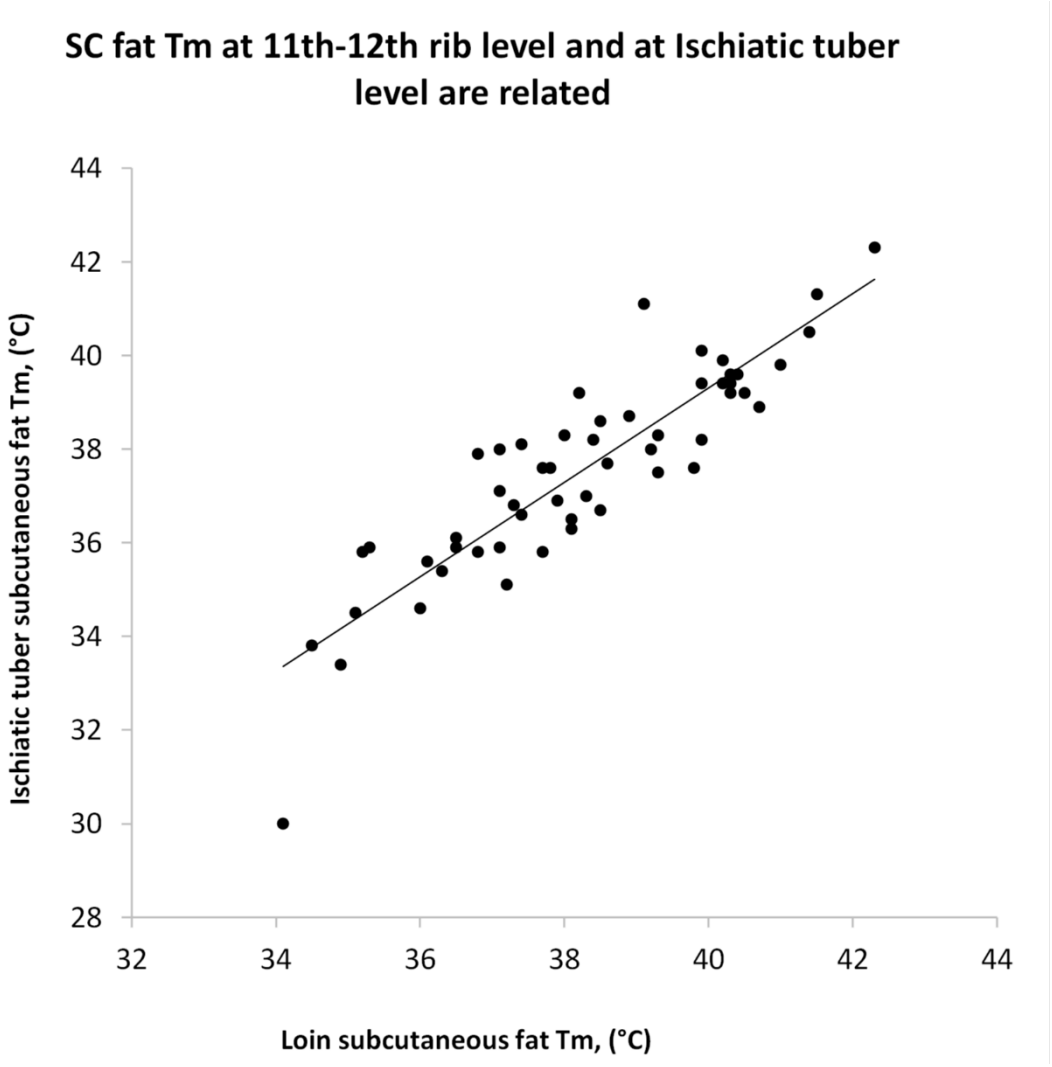

Figure 6.13 *SC fat Tm above loin and tail are related.* Relationship between the melting temperature (Tm) of the subcutaneous fat around the base of the tail (*Ischiatic tuber* fat) and at the loin (11th intercostal space fat), indicating that a biopsy of tail head fat can predict the standard result at slaughter. r = 0.89, 95% confidence interval 0.81 to 0.93.

6.5 *Discussion*

Histology has been widely used in human medicine, whether by biopsy or post mortem, to visualise, describe and understand morphological changes at a cellular level in muscle, for example in muscular dystrophies, which often involve excessive intramuscular fat deposition. We have applied the same techniques to bovine muscle to achieve similar goals, specifically to understand marbling.

6.5.1 *The process of IMF deposition*

A useful definition of marbling is the accumulation of perimysial fat in muscle (Smith et al., 2000; Brooks et al., 2011). An alternative process for intramuscular fat deposition in highly marbled Wagyu is mentioned by Smith and Johnson (Smith and Johnson, 2014); endomysial adipocytes replace myofibers. This process may occur in steatosis (Peletto et al., 2017).

Our observations show that marbling is predominantly perimysial fat as in the common definition. We also note that perimysial adipose tissue starts developing around neurovascular bundles and then expands through the surrounding perimysium. This abundance of perimysial adipose tissue in long fed Wagyu disrupts the structure of the intramuscular connective tissue leading to increasing tenderness with time on feed (Nishimura et al., 1999).

The predominance of perimysial fat is consistent with Moody and Cassens (Moody and Cassens, 1968) and Brooks et al. (Brooks et al., 2011). However, we also observed endomysial adipocytes (eg label 3 in Figure 4) which we confirm in Figure 6. These were only present in highly marbled Wagyu, as suggested by Chung et al. (Chung et al., 2007). Endomysial adipocytes formed a very small proportion of the intramuscular fat in all the animals we have examined.

We observed variations in adipocyte size and numbers, which indicate that the increase of intramuscular fat is due to the increase in adipocyte number as well as to hypertrophy of existing cells. In general, adipocyte diameters increased with marbling, but only to a limit of approximately 200 μm corresponding to a microscopic score of 6 or greater. Thus, extremes of marbling reflect increases in the number rather than the size of adipocytes. These findings on intramuscular fat are consistent with Brooks et al. (2011) but contrast with the notion that subcutaneous fat increases only by hypertrophy (Cianzio et al., 1985).

The origins of adipocytes are important to establish in order to understand the process of marbling in long fed Wagyu. Harper and Pethick (2004) present several possibilities for the origins of adipocytes in the muscle. They favour the differentiation of mesenchymal-like stem cells derived from the connective tissue. However, they also consider the potential for satellite cells dormant within the muscle fascicles to differentiate into adipocytes and for some contribution from stem cells arriving through the bloodstream to replenish the pool of available stem cells. Our observation that perimysial adipocytes start developing around neurovascular bundles (Figs. 1 and 5) is consistent with the majority of the adipocytes originating from stem cells arriving from the blood. However, the unequivocal existence of endomysial adipocytes in muscle tissue (Figs. 4 and 6), suggests satellite muscle cells also contribute.

Smith and Johnson (2014) raise the possibility that myogenic satellite cells within the fascicles can trans-differentiate into endomysial adipocytes. These muscle satellite cells are present between the basement membrane and the sarcolemma of the myocyte, where they are available for muscle repair. It is generally accepted that they are committed to the myogenic pathway (Starkey et al., 2011). However, there is evidence that they are able to differentiate into adipocytes (Asakura et al., 2001; Wada et al., 2002).

We observed that the vast majority of adipocytes, even in highly marbled Wagyu, were located in the perimysium. We propose that the main process is an active and aggressive expansion of adipose tissue rather than the passive steatosis-like replacement of muscle cells proposed by Smith and Johnson (Smith and Johnson, 2014). The process commences with the development of individual adipocytes next to neurovascular bundles, followed by arborisation along the perimysium (Figure 4.1).

The process of arborisation separates muscle bundles, in some cases leaving islands of muscle tissue completely isolated. In the highest grade of Japanese Wagyu, most muscle bundles may be reduced to isolated islands in a sea of adipocytes (Gotoh et al., 2018). This process resembles invasion as found within tumours (Baron et al., 2004) and should not be dismissed as artefactual.

Smith & Johnson (2014), propose that in Black Wagyu the considerable reduction of number of myofibers per bundle is due to the conversion or trans-differentiation of muscle cells to adipocytes. In contrast, we suggest that aggressive invasion by perimysial adipocytes causes adjacent muscle fibres to separate, atrophy/degenerate and ultimately disappear. Figure 5 is an example of muscle fibres in contact with invading adipocytes suffering changes in shape and an increased affinity for eosin, suggesting cellular injury and degeneration (Dubowitz et al., 2013). We propose that this degenerative process is the explanation for the number of adipocytes increasing, while the number of myofibers decreases. The decrease in number of muscle fibres is, therefore, a consequence of the invasive arborisation process, which acts as an aggressor to muscle cells. The increase in adipocytes is not a response to muscle fibre degeneration. Instead, adipocyte proliferation causes loss of muscle fibres.

As clearly shown here, invasive marbling is associated with concomitant decreases in Tm of fat, reflecting increasing Mono Unsaturated Fatty Acids and particularly oleic acid. We

therefore conclude that the two processes are directly related. At the simplest level, fatty acids with Tm below body temperature (37–39°C) are fluid, making the adipocytes more flexible and therefore able to penetrate. This permits active invasion resulting in fine marbling. This pragmatic explanation is consistent with historical practices such as the massaging of “soft” fat during the later stages of feeding of Wagyu. It also explains the fact that non-Wagyu, like Angus, retain higher Tms and are known for seam or coarse marbling.

We recognise that there is additional complexity. For example, there will be time-dependent induction of allelic gene products encoded within Wagyu specific haplotypes such as 60.1 and 30.4 (Williamson et al., 2011; Lloyd et al., 2017a). Foremost, is SREBF1 which regulates the Δ9-desaturase but also many other genes, including many known to be involved in muscle differentiation and other potentially relevant processes (Lloyd et al., 2017a, b). There are interactions between these adipogenic genes and various fatty acids such as oleic acid (Li et al., 2019), trans-10, cis-12 conjugated linoleic acid and sterculic acid (Kadegowda et al., 2013). The challenge is to unravel the multitude of interacting haplotypes, genes, products and regulators, especially because marbling is age and feed dependent. Experience with human muscular dystrophy teaches that current explanations for marbling are too simplistic. However, a strategy is suggested by

(1) the fact that these interactions must be represented within these two haplotypes (in contrast to non-Wagyu haplotypes),

(2) the opportunity to monitor activities quantitatively over time by sampling muscle and fat from the tail head, as shown above.

Such approaches will be valuable in translational studies to elucidate the pathogenesis of, and potential therapy, for human myopathies including dystrophies, statin (HMGCR inhibitors) myopathy, dermatomyositis and for obesity and other disorders of adipose tissue.

There is also the potential to monitor satellite and stem cells which apparently fail to repair the loss of myofibres during marbling. The origin and fate of the occasional endomysial adipocytes, as demonstrated here, require further investigation. There is also the opportunity to re-examine our conclusion that intramyocellular lipid droplets cannot explain marbling.

6.5.2 *Quantification of marbling*

We found that Microscopic score and MSA MB were well correlated for highly marbled beef, especially when coarse rather than fine. However, at the extremes we prefer the Microscopic score. In carcasses with MSA MB 400 and below, the correlation is not as strong. This can be explained by the macroscopic nature of MSA MB, restricted to the naked eye visualisation of white areas at least as large as 10 to 15 adipocytes (Harper, 2003). This limitation also applies to marbling assessments using a digital camera. Other types of connective tissue also appear white while the carcass is cold and are impossible to distinguish during marbling assessment (Harper and Pethick, 2004). In less marbled carcasses, where the proportion of connective tissue over fat is higher, marbling can be overestimated. In contrast, a microscopic assessment of intramuscular fat is able to distinguish these details, allowing differentiation of structures and levels of marbling more completely. Therefore, the Microscopic score is more accurate than MSA MB for the vast majority of standard, non-Wagyu carcasses.

Quantification by the naked eye can also be misleading with extreme degrees of marbling, in that it underestimates the more desired fine marbling. Some producers and some countries use an extended scale such as AUS MB 10-13. By whatever measure, however, the finer the marbling, the harder to quantify by the naked eye. Histological assessment will be helpful in developing measures of fineness by including the aggressiveness of the invasion and therefore the formation of residual islands of muscle and a snowflake appearance.

Quantitative microscopic measurements still need to be developed for adipocyte distribution and degree of arborisation, for adipocyte size variations and for proportion of connective tissue in order to determine the relative influences of environment and genetics on these factors.

6.5.3 *In vivo assessment of marbling*

Marbling and Tm change with DOF but are also significantly affected by genetics. It would be useful to have methods of monitoring amounts and composition of intramuscular fat in relation to age, DOF, breed composition, suitability for breeding and animal welfare.

Worldwide, marbling is assessed in the *Longissimus dorsi* muscle after quartering the carcasses, but marbling occurs at different rates depending upon the muscle sampled. Obviously, biopsies of the *Longissimus dorsi* would not be practical. Ultrasound scanning the LD has been found unreliable for assessing marbling in long fed Wagyu (Australian Wagyu Association, 2015) and, since the marble score does not predict fatty acid composition(Carvalho and Smith, 2018), it is even less useful for the live monitoring of Tm. The tailhead is more accessible than the LD, more practical for biopsies under epidural anaesthesia and therefore a potential site for *in vivo* monitoring. The question is: Is it possible to determine the amount and composition of intramuscular fat of an animal from a single site around the base of the tail?

We found the patterns of intramuscular fat deposition within LD or SDM to be very similar (Figure 11). The microscopic score of the SDM and the MSA MB are well correlated (r = 0.89, 95% confidence interval 0.79 to 0.94). Therefore, the SDM is a suitable site for monitoring progression of marbling.

The healthiness of beef fat, as measured by Tm, improves with feeding due to the desaturation of stearic acid into oleic acid. This process is driven primarily by the enzyme SCD (Bota 26) and regulated by SREBF1 (Bota 19) (Lloyd et al., 2017a). The same enzyme is involved in desaturation of both subcutaneous and intramuscular adipose tissue. Therefore, we expect to be able to monitor changes in Tm of intramuscular fat by sampling subcutaneous fat with a simple punch biopsy, as used in humans. A substantial deposit of subcutaneous fat over the *Ischiatic tuber* (IT) develops in "finished" cattle and provides a practical location for sampling.

We show in Figure 8 that intramuscular fat Tm of the loin is correlated with the Tm of the overlying subcutaneous fat ($r = 0.85$, 95% confidence interval 0.55 to 0.95). Interestingly, we also show in Figure 13, that the Tm of the subcutaneous fat over the IT is well correlated with the Tm of the subcutaneous fat over the loin. Therefore, samples from the IT fat deposit can be used to study the desaturation of IMF over time.

Turk and Smith (2009) tested fatty acid composition of subcutaneous fat overlying many beef cuts, showing the highest oleic acid concentration in the brisket and the lowest in the loin. This finding is consistent with a common view that the desaturation progresses from head to tail along with the development of marbling. However, to the contrary, the same authors found oleic acid concentrations increase from the loin to the tail (round or rump). The Tm results here confirm that finding, showing that on average, the Tm of the fat over the loin is higher than the subcutaneous fat at the base of the tail. Although not expected from the head to tail rule, it is clear that other variables contribute to oleic acid content and Tm.

In Figure 7 we confirm that intramyocellular fat droplets can be present in Wagyu cattle. This is of interest because excessive lipid accumulation within myocytes is found in some types of human muscular dystrophy where fatty replacement of muscles also occurs (Grounds et al.,

2014). Intramyocellular lipid droplets have also been found in periparturient dairy cows (Roberts et al., 1983). However, the relative importance of droplets has not been addressed in the current work. Further hypotheses will address this issue and its implications for fatty acid composition and healthiness of beef. Such studies are needed since intramyocellular lipid droplets can reach up to 20% of the intramuscular fat in normal healthy muscles (Listrat et al., 2016).

6.6 *Conclusions and implications*

Marbling is an invasive, hyperplastic and hypertrophic process of intramuscular fat development. De novo perimysial fat develops initially around neurovascular bundles and expands through the perimysium in a process we have called arborisation. As the Tm of the intramuscular fat decreases, the invasion becomes more aggressive. As a consequence, the myofibres in contact with the adipocytes suffer atrophy/degeneration and loss indicated by changes in shape and affinity to eosin. The consequence is fine marbling.

We recognise that the main contributor to intramuscular fat is perimysial fat. However, we have demonstrated the existence of endomysial adipocytes through careful histological sectioning of muscles at consecutive levels.

Finally, the level of marbling and fat desaturation of the loin is reflected at the base of the tail. The correlation between these two areas opens possibilities for new research to develop practical tools for *in vivo* testing in cattle. *In vivo* testing, for instance through sequential biopsies, will be invaluable to determine the performance and potential for marbling and healthy fat production of individual animals. At the same time, cattle provide an opportunity for translation to human dystrophy.

Supplementary data to this article can be found online at

https://doi.org/10.1016/j.meatsci.2020.108063.

7 Interspecies translation: Bovine marbling to human muscular dystrophy[5]

In Chapter 6 we characterised the process of intramuscular fat genesis at a cellular level, establishing the different patterns involved in the intramuscular fat deposition and describing the aggressive and invasive process of adipose arborisation, with the formation of islands of myocytes, the presence of true endomysial adipocytes and the existence of intramyocellular fat droplets.

In this chapter, with a better understanding of marbling and with the awareness of the microscopic changes that occur in the muscle, it was then possible to confirm some of our presuppositions and explain some intriguing situations.

For instance, it became evident why sometimes animals with expected great performance on marbling do not score well under the traditional method of marbling assessment. We present our new observations on fine marbling and our realisation that it will never be appropriately assessed under the current systems of marble scoring.

Considering the genetic similarities between marbling and muscular dystrophies showed in Chapter 4, and with the new microscopic findings presented in chapter 6, we asked whether the similarities are just genetic or there are other commonalities.

To answer that question, we compared the histology of the processes and analyse how the aggressiveness of marbling changes the structure of the muscle and muscle cells themselves.

Our deepest motivation was to assess the potential for cattle marbling to qualify as a model to contribute to the understanding of human muscular dystrophies.

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In this paper we present the comparison between relevant genes involved in bovine meat quality and the ones involved in the presentation of human muscular dystrophies, and a summary of the proteins of relevance in muscular dystrophies.

We show the formation of islands of myocytes during the process of marbling and how arborisation can make some changes that would not be found in normal muscle. We also present some common histological features between marbling and human muscular dystrophies.

As the principal author of this work, my contribution was:

i. Identifying histological similarities between marbling and muscular dystrophies.

ii. Sampling.

iii. Processing of frozen samples.

iv. Histological description and analysis.

v. Writing the manuscript.

This work contributes to establishing new possibilities for the interspecies translation of scientific findings. In this context, this work provides promising evidence for establishing bovine as a model for the understanding of muscular dystrophies and the development of new therapies.

7.1 *Abstract*

There are interesting similarities and differences when comparing the histopathology of bovine marbling and human muscular dystrophy. At the simplest level, both conditions are characterised by genetically controlled and more or less inexorable replacement of muscle

fibres with fat cells. At issue is whether an improved understanding of these two processes can lead to better outcomes for patients. There are many forms of dystrophy that differ in their genetics and their histopathology. There are also many forms of “marbling” ranging from the coarse to fine, epimysial, perimysial to endomysial and even to total replacement or steatosis. A detailed examination of marbling will provide a framework for further investigation of human dystrophy. Ultimately, the many genetic factors involved can be addressed through a better understanding of the metabolic pathways involved in marbling.

7.2 *Introduction*

The purpose of this review is to compare the genetics and histopathology of bovine marbling and human muscular dystrophy. Surprisingly, in spite of similarities, the literature suggests that marbling is a function of extreme adipogenesis, whereas dystrophy is a consequence of fundamental defects in muscle itself. In fact, completely independent studies, as summarised here, reveal that similar genes have been implicated in some selected situations. Further, it is clear that the histopathology of some forms of dystrophy can resemble some forms of bovine marbling.

7.3 *Marbling*

Marbling is the term used to describe the presence of macroscopically visible fat within muscle (Figures 7.1 and 7.2). Coarse marbling refers to white areas of fat through and around muscle bundles, generally as continuous bands arising from the subcutaneous adipose tissue. By contrast, fine or “snowflake” marbling is characterised by more even white flecks resulting in pink rather than red muscle.

Figure 7.1 *Loin at the eleventh intercostal level of a carcass of Melaleuka Stud steer M508 (wy63 ak25 dx13), MSA MB 1100, DOF 471*. There are extensive areas of fine marbling as indicated by pink muscle with fine flecks. Note 88% Wagyu (63% black, 25% red). See also Figure 7.5 for microscopic features.

Figure 7.2 *Loin at the eleventh intercostal level of carcass of Melaleuka Stud heifer M621 (wy75 dx25), MSA MB 920, DOF 443*. There is a predominance of fat arborizing from the subcutaneous tissue and creating coarse marbling. The muscle areas are dark red in comparison to Figure 7.1. Note lower MSA MB of 920 but similar days on feed (DOF).

These two forms may coexist but can be distinguished and quantified by skilled observers. Fine marbling is associated with improved taste and tenderness(Harper and Pethick, 2004). Further, it has been shown to relate to a preferred fatty acid profile. Accordingly, there is copious funding and now a substantial understanding of the environmental and genetic factors which favour fine rather than coarse.

7.4 *Interspecies translation*

Interspecies translation from cattle to man has unrecognised potential. Firstly, cattle are close to humans in evolutionary time and fall within that window of 50–100 million years of separation (or last common ancestor) which is characterised by very similar proteins but vastly different regulations of expression. The same window may explain the fact that the two species have synergised over some 40,000 years of contact and at least 7000 years of domestication. As one example, infections can be similar and, in some cases, are transmissible from one to the other, but close exposure to cattle is generally innocuous implying some form of immunity. As for example, in the case of pox and tuberculosis. We argue that cattle are both relevant and relatively safe for translational studies.

Secondly, domestic cattle are well maintained, closely observed and very well understood. There are huge databases and DNA banks which have been in existence for 50 years. Innumerable breeds can be compared often under different environmental conditions. Many of these breeds have been closed for hundreds of years and then intentionally crossed with each other. There is great potential for meaningful studies of population genetics and family and haplotype associations and, even more so, for structure-function genomics. Metabolic

and inflammatory pathways are relatively well understood and are supported by inestimable funding available to ensure future supplies of meat, milk, cheese, butter, leather, and fertiliser.

Thirdly, cattle are plentiful and even more so than humans. Because the generation time and life expectancy are much shorter, there are excellent opportunities to study and treat genetically determined diseases prospectively (Tan et al., 1997).

7.5 *Other instances of translation*

White muscle disease or selenium/vitamin E deficiency occurs quite commonly in livestock raised on leached soils. The pathology resembles dystrophy in some respects. A mutation in the selenoprotein N gene (SEPN1) is responsible for some types of congenital muscular dystrophies and myopathies (Ferreiro et al., 2004). Kakulas (Kakulas, 1966) demonstrated that dystrophy-like changes explained the weakness observed in quokkas on Rottnest Island. Importantly, the condition could be corrected by treating the deficiency, raising the possibility that human dystrophies could be reversible if the basic defect could be corrected.

7.6 *Genomic approach*

The term genome is used here to refer to the architecture of DNA sequences, whereas others have come to use the term in the context of single-nucleotide polymorphisms wherever they occur. The difference is fundamental to the discovery of gene clusters with coherent cis and trans interactions between conserved sequences known as ancestral haplotypes (Dawkins et al., 1999; Aly et al., 2006; Traherne et al., 2006; Williamson et al., 2011; Lloyd et al., 2015). Many studies have shown that the SNP approach in livestock and humans fails to identify these critical sequences and can be misleading at best (Alper et al., 2006). SNPs are neutral markers of parentage rather than functionally important (Dawkins et al., 2013).

One major benefit of ancestral haplotypes, as opposed to SNPs, is that it is possible to use interspecies translation. During mammalian evolution, polymorphic frozen blocks have diverged to some extent, although the functionally important sequences tend to be conserved.

As shown in Figure 7.3 and Table 7.1, there are similarities between genomic regions on Hosa 17 and Bota 19. Although there have been architectural changes such as insertions and transversions, the gene content has been preserved. Bota 19 was chosen as the reference because of its critical role in determining the degree of marbling between individuals of a breed, F1 crosses and between breeds (Williamson et al., 2011; Lloyd et al., 2017a, b; Valenzuela et al., 2018).

Hosa 17 was chosen for comparison because it contains some of the same genes, such as TCAP. Further analysis revealed an extraordinary degree of preservation or synteny in spite of an evolutionary separation time of at least 50 million years and therefore millions of generations. Implicit is that there are functional reasons for similarities in genomic architecture.

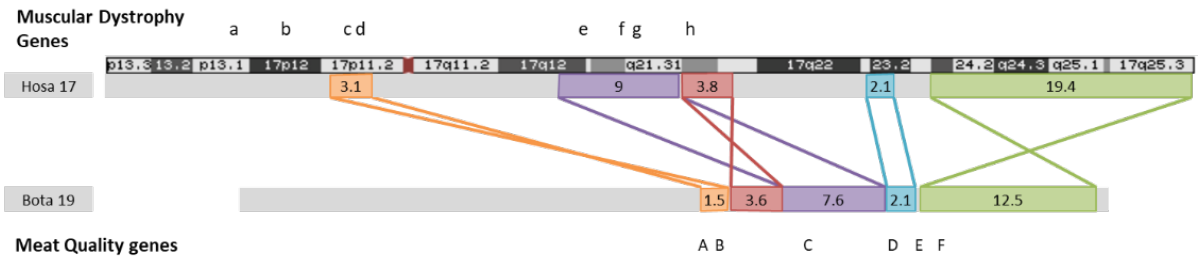

Figure 7.3 *Marbling and muscular dystrophy are syntenic on bovine Chromosome 19 (Bota 19) and human Chromosome 17 (Hosa 17).* Coloured boxes represent segments with the same gene content. Crossed joining lines indicate inverted translocations. Numbers represent Mb. Synteny was determined by the positions of homologous genes in the human assembly Hg 38 and bovine assembly BosTau8 located using the UCSC genome browser. Inverted sections and the exact location of boundaries between blocks were determined by dot plots comparing the two sequences. Adapted from: (Lloyd et al., 2017a) Locations of Muscular Dystrophy Genes: (a) MYH2, (b) PMP22, (c) TRPV2, (d) SREBF1, (e) TCAP, (f) CAVIN1, (g) BECN1, (h) SGCA

and Meat Quality Genes (A)SREBF1, (B) MPRIP, (C) TCAP, (D) GH, (E) UTS2R, (F) FASN shown here. See Table 7.1 for more information about these genes.

Table 7.1 *Details of relevant genes in Bota19 and Hosa17.*

Gene location	Description	Human muscular dystrophy	Meat quality trait
MYH2 Hosa 17p13.1 Bota Chr 19: 30.13Mb	MYH2 encodes the myosin heavy chain isoform that is expressed in fast type 2A muscle fibres.	Proximal myopathy and ophthalmoplegia is caused by heterozygous, compound heterozygous, or homozygous mutation in MYH2, leading to a lack of type 2a fibres (Tajsharghi et al., 2014).	In pork,IMF, water holding capacity, and meat color (Lim et al., 2015).
PMP22 Hosa 17p12 Bota Chr 19: 33.35Mb	Peripheral myelin protein-22.	Duplication of peripheral myelin protein 22 causes Charcot-Marie-Tooth disease type 1A (Nobbio et al., 2014).	
TRPV2 Hosa 17p11.2 Bota Chr 19: 33.816Mb	Transient Receptor Potential cation Channel, V2: Responds to heat and cations.	Muscular dystrophy is ameliorated in dystrophin-deficient mdx mice by dominant-negative inhibition of TRPV2 (Iwata et al., 2009).	
SREBF1 Hosa 17p11.2 Bota Chr 19: 35.23Mb	Sterol regulatory element-binding protein-1 controls cholesterol homeostasis by stimulating transcription of sterol-regulated genes.	Mutations of LMNA that cause Emery-Dreifuss muscular dystrophy (EDMD2-AD) and familial partial lipodystrophy (FPLD2) result in less binding of lamin A to SREBP1 (Lloyd et al., 2002).	SREBF1 is involved in adipogenesis and polymorphisms are associated with fatty acid composition of Japanese Black Cattle (Hoashi et al., 2007).
MPRIP Hosa 17p11.2 Bota Chr 19: 35.557Mb	Myosin phosphatase rho-interacting protein targets myosin phosphatase to regulate the phosphorylation of myosin light chain (Surks et al., 2005).		Haplotypes differentiated by polymorphisms in MRIP are associated with differences in intramuscular fat development in Wagyu (Dawkins et al., 2015).
SGCA Hosa 17q21.33 Bota Chr 19: 37.11Mb	Sarcoglycan, alpha Sarcoglycans form part of the dystrophin-glycoprotein complex.	Mutations in SGCA cause Limb-girdle muscular dystrophy type 2D. SGCB, SGCD and SGCG are associated with LGMD types 2E, 2F and 2C respectively (Trabelsi et al., 2008).	
TCAP Hosa 17q12 Bota Chr 19: 40.69Mb	Titin-Cap (telethonin) is a sarcomeric protein localised to the periphery of Z discs that define the border of the sarcomere as a structural anchor and signalling centre.	Limb-girdle muscular dystrophy type 2G (LGMD2G) is caused by mutations in the TCAP gene(Moreira et al., 2000).	A Polymorphism of TCAP is associated with IMF content and fatty acid composition of beef (Cheong et al., 2007; Lloyd et al., 2017a).
CAVIN1 Hosa 17q21.2 Bota Chr 19: 43.14 Mb	Cavin is an essential factor in the biogenesis of caveolae.	Congenital generalised lipodystrophy, type 4; (CGL4) is caused by mutations in CAVIN1 that result in CAV 3 deficiency (Hayashi et al., 2009).	
BECN1 Hosa 17q21.31 Bota Chr 19: 43.47Mb	Beclin-1 participates in the regulation of autophagy.	Expression of BECN1 was reduced in patients with muscular dystrophies BTHLM1 and UMCD1 which were caused by COL6A1 mutations (Grumati et al., 2010).	Involved in Proteolysis and beef aging (García-Macia et al., 2014).

GH1 Hosa 17q23.3 Bota Chr 19: 48.77Mb	Growth Hormone.	A Polymorphism of growth hormone is associated with fatty acid composition of Wagyu beef (Ardiyanti et al., 2009),
FASN Hosa 17q25.3 Bota Chr 19: 51.38 Mb	Fatty Acid Synthase the key enzyme of de novo lipogenesis to produce saturated fatty acids.	Fatty Acid Synthase is highlighted in GWAS for fatty acid content and composition of Wagyu and Hanwoo beef (Uemoto et al., 2010; Bhuiyan et al., 2018).
UTS2R Hosa17q25.3 Bota Chr 19: 50.81 Mb	A receptor abundant in heart and pancreas and responsive to Urotensin II which has potent vasoactive properties.	A polymorphism of UTS2R is associated with IMF content of Wagyu x Holstein beef (Adoligbe et al., 2016).

Yet further analysis suggests some explanations for the co-location of similar genes. Irrespective of cis and trans interactions between the protein products, there is evidence of co-regulation (see, e.g., SREBP). In this context, we conclude that, although products and their regulating transcription factors are preserved, separation has permitted the insertion of species-specific elements, which control the quantitative differences between humans and cattle.

Importantly, as shown in Figure 7.3 and Table 7.1, Hosa 17 contains multiple candidates for involvement in human muscular dystrophy. There is even more complexity in explaining the multiple candidates, as shown in Tables 7.2 and 7.3.

Thus, syntenic analysis has suggested a novel approach to identification of operative elements in marbling and in some forms of dystrophy.

Table 7.2 *Details of relevant genes outside of Hosa 17/Bota 19.*

Gene location	Description	Human muscular dystrophy	Meat quality trait
MSTN Hosa 2q32.2 Bota Chr 2: 6.21Mb	Myostatin	Muscle hypertrophy was caused by a homozygous mutation in myostatin. (Schuelke et al., 2004).	Mutations in myostatin cause double muscling in several cattle breeds (Wiener et al., 2009).

CAPN3 Hosa 15q15.1 Bota Chr 10: 37.8Mb	Calpains are nonlysosomal intracellular cysteine proteases. CAPN3 is a muscle-specific large subunit.	Limb-girdle muscular dystrophy type 2A (LGMD2A) is caused by homozygous or compound heterozygous mutation in CAPN3.	SNPs within CAPN3 are associated with tenderness in Bos Indicus cattle (Barendse et al., 2008).
CAPN1 Hosa 11q13.1 Bota Chr 29: 44.06Mb	m-Calpain.		Two CAPN1 genetic markers are associated with tenderness in Brahman beef (Casas et al., 2005).
DMD Hosa Xp21.2-.1 Bota Chr X: 115.34Mb	Dystrophin Maintains the structural integrity of myofibrils.	Duchene muscular dystrophy.	
LAMA2 Hosa 6q22.33 Bota Chr 9: 67.96Mb	LAMA2 gene encodes the alpha-2 chain of laminin-2. Laminin-2 (merosin) is the main laminin found in muscle fibres.	Congenital Merosin-Deficient Muscular Dystrophy Type 1A; MDC1A.	
MYOT Hosa 5q31.2 Bota Chr 7: 50.94Mb	Myotilin directly binds F-actin and efficiently crosslinks actin filaments prevents filament disassembly.	LGMD1A is caused by heterozygous mutation in the MYOT. It is characterised by adult-onset muscle weakness, progressing from the hip to the shoulder girdle.	SNPs in MYOT correlate with loin muscle area and intramuscular fat in Qinchuan cattle (Adoligbe et al., 2016).
CAV3 Hosa 3p25.3 Bota Chr 22: 17.83Mb	Caveolin 3.	Muscular Dystrophy, Limb-girdle, Type 1C; LGMD1C.	
SGCD *Hosa* 5q33.2-.3 Bota Chr 7: 69.59Mb	Sarcoglycan, Delta Is expressed in skeletal and heart muscle and to a lesser extent in smooth muscle. Delta-sarcoglycan is localised at the sarcolemma.	Muscular Dystrophy, Limb-girdle, Type 2F; LGMD2F.	
SGCE Hosa 7q21.3 Bota Chr 4: 11.84Mb	Epsilon-sarcoglycan.	Myoclonus-dystonia is a genetically heterogeneous disorder characterised by myoclonic jerks affecting mostly proximal muscles.	
SGCB Bota Chr 6: 69.53Mb	Beta-sarcoglycan.		
SGCG Bota Chr 12: 34.92Mb			
COL6A1 COL6A2 21q22.3	Collagen, Type VI, Alpha-1 and Alpha-2 Members of the collagen VI family, form distinct networks of microfibrils in connective tissue and interact with other extracellular matrix components.	Ullrich congenital muscular dystrophy 1 Bethlem myopathy 1.	
COL6A3 2q37.3	COLLAGEN, TYPE VI, ALPHA-3.	Ullrich congenital muscular dystrophy 1, Bethlem myopathy 1. Dystonia 27.	

ITGA7 12q13.2	The alpha-7 integrin is a specific cellular receptor for the basement membrane proteins laminin-1, -2 and -4. The alpha-7 subunit is expressed mainly in skeletal and cardiac muscle and may be involved in differentiation and migration processes during myogenesis.	Congenital muscular dystrophy.
EMD Xq28	Emerin is found along the nuclear rim of many cell types and is a member of the nuclear lamina-associated protein family.	Emery-dreifuss muscular dystrophy 1, X-LINKED; EDMD1.
ATP2A1 (SERCA-1) 16p11.2	Calcium-transporting ATPase lower cytoplasmic Ca(2+) concentration by pumping Ca(2+) to luminal or extracellular spaces. ATP2A1 is the ATPase type found in fast twitch muscles.	Brody myopathy.
DES 2q35	Desmin is the muscle-specific member of the intermediate filament (IF) protein family. It is one of the earliest myogenic markers, both in heart and somites, and is expressed in satellite stem cells and replicating myoblasts.	Myopathy, myofibrillar, 1.
PLEC 8q24.3	Plectin-1 is one of the largest polypeptides known and is believed to provide mechanical strength to cells and tissues by acting as a crosslinking element of the cytoskeleton.	Epidermolysis bullosa with muscular dystrophy Limb-girdle type 2Q.

Table 7.3 *Protein accumulations and deficits in dystrophy* (Adapted from Dubowitz et al., 2013 Table 6.3 dystrophy related protein changes detectable with immunohistochemistry).

Absent Protein	**Dystrophy type**	**Gene location**
Dystrophin	Xp21 muscular Dystrophies (Duchenne, Becker).	DMD Hosa Xp21.2-p21.1 Bota Chr X: 115,342,323-117,606,340
Sarcoglycans	Limb-girdle muscular dystrophies 2C-F.	SGCA Hosa 17q21.33 Bota 19 SGCB Bota Chr 6 SGCD *Hosa* 5q33.2 Bota 7 SGCE Hosa 7q21.3 Bota 4 SGCG Bota Chr 12
Dysferlin	Limb-girdle muscular Dystrophy 2B.	DYSF Hosa 2p13.2
Caveolin-3	Limb-girdle muscular dystrophy 1a, rippling muscle disease, hyper CK aemia.	CAV3 Hosa 3p25.3 Bota 22 CAVIN1 Hosa 17q21.2 Bota 19
Telethonin	Limb-girdle muscular dystrophy 2G.	TCAP Hosa 17q12 Bota 19
Laminin a2	MDC1A (‘merosin’ deficient congenital muscular dystrophy).	LAMA2 Hosa 6q22.33, Bota 9
Collagen VI	Ullrich congenital muscular dystrophy.	COL6A1&2 Hosa 21q22.3 COL6A3 Hosa 2q37.3
Integrin alpha7	Mild Congenital dystrophy/myopathy.	ITGA7 Hosa 12q13.2

Calpain-3 (easier to assess on immunoblots than sections)	Limb-girdle Muscular dystrophy 2A.	CAPN3 Hosa 15q15.1 Bota 10
Emerin	X-Linked Emery-Dreifuss muscular dystrophy	EMD Hosa Xq28
SERCA 1	Brody disease.	*ATP2A1 Hosa 16p11.2*
Plectin	Epidermolysis bullosa with muscular dystrophy, Limb-girdle dystrophy 2Q.	PLEC Hosa 8q24.3
LAMP-2	Danon disease.	LAMP2 Xq24
Accumulating Protein	**Dystrophy type.**	**Gene location**
Actin	Congenital actin myopathy/ nemaline myopathy.	ACTA1 *Hosa 1q24.13* TPM3 Hosa 1q23
Myosin	Myosin storage myopathy.	MYH7 Hosa 14q11
Myotilin	Myotilin-related myofibrillar myopathy.	MYOT Hosa 5q31.2
Desmin	Desmin myopathy.	DES Hosa 2q35 SEPN1 Hosa 1p36

7.7 *Histopathological approach*

The substantial range of changes found in the human dystrophies is illustrated in the study of Dubowitz et al. (Dubowitz et al., 2013).

We are fortunate in having histological muscle samples from cattle with degrees of marbling (Valenzuela et al., 2018). Some of these changes are illustrated in Figures 7.4–7.8 from three animals (M508, M621, and M129) fed a standard ration for 471, 443, and 481 days respectively. The macroscopic measure of marbling (MSA MB) ranged from high to moderate (1100, 920 and 820, respectively) as expected in high content Wagyu (88, 75, and 63%, respectively). A common feature is the invasion of adipose tissue between intact muscle fascicles (Figure 7.4). For the most part, the process extends along the perimysium leading to variation in fibre size, staining of myofibers (Figures 7.5 and 7.6), and the formation of residual islands of myofibers (Figure 7.7), which suggests an explanation for fine (see Figure 7.1) rather than coarse (see Figure 7.2) marbling; fine is due to more aggressive invasion reflecting quantitative differences in gene regulation.

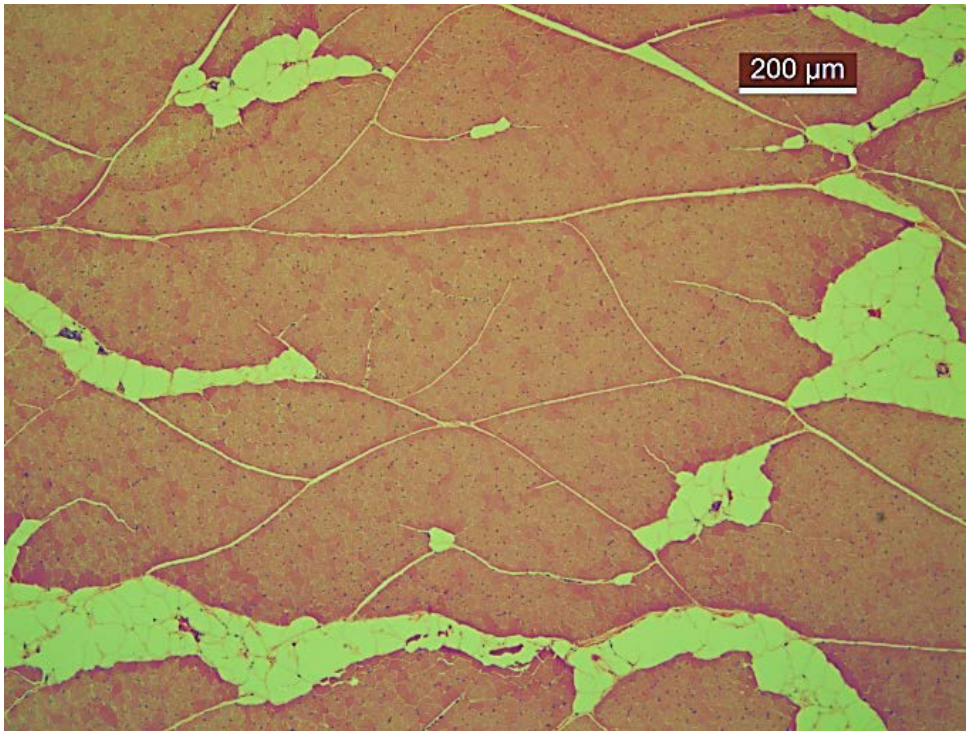

Figure 7.4 *Highly marbled loin muscle shows a pattern of fat arborisation and invasion with adipocytes predominantly in the perimysium, between muscle fascicles.* Note extensive vascularization centrally within the fat. M508 (wy63 ak25 dx13), MSA MB 1100, DOF 471. See also Figure 7.1.

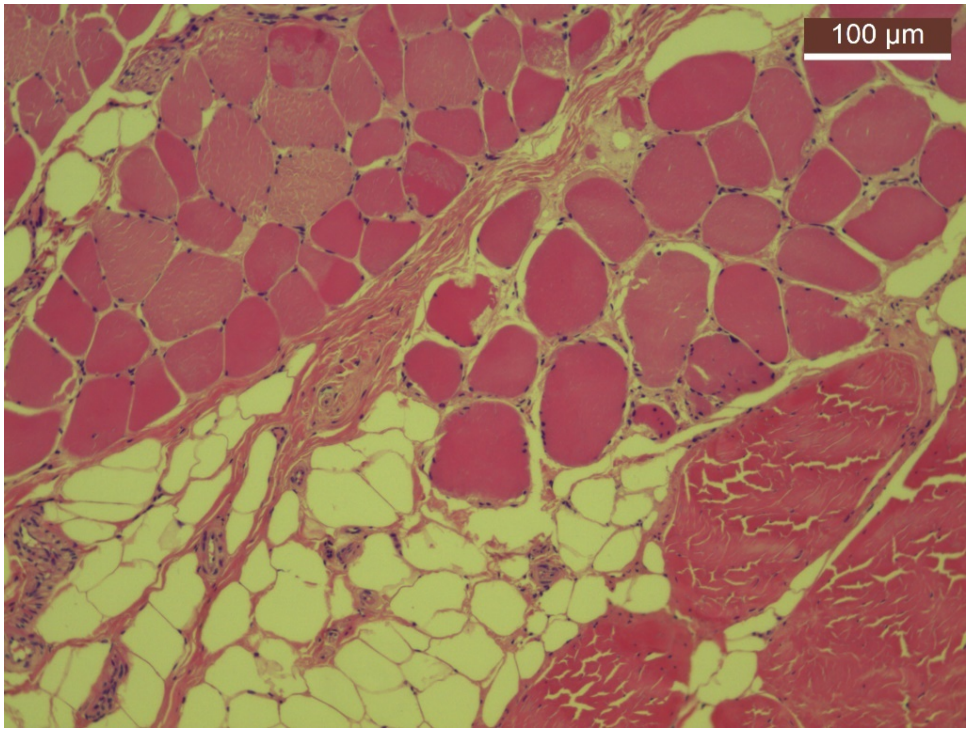

Figure 7.5 *Histological section of Sacrocaudalis dorsalis medialis of a highly marbled, high Wagyu content (88%) steer M508 (wy63 ak25 dx13), showing variation of fibre size, with the presence of rounded fibres, internal nuclei, abundant perimysial connective tissue, and considerable adipose tissue*. Formalin-fixed H & E, MSA MB 1100, DOF 471. CYO lab number Ch18/110G. See also Figure 7.1 for macroscopic comparison.

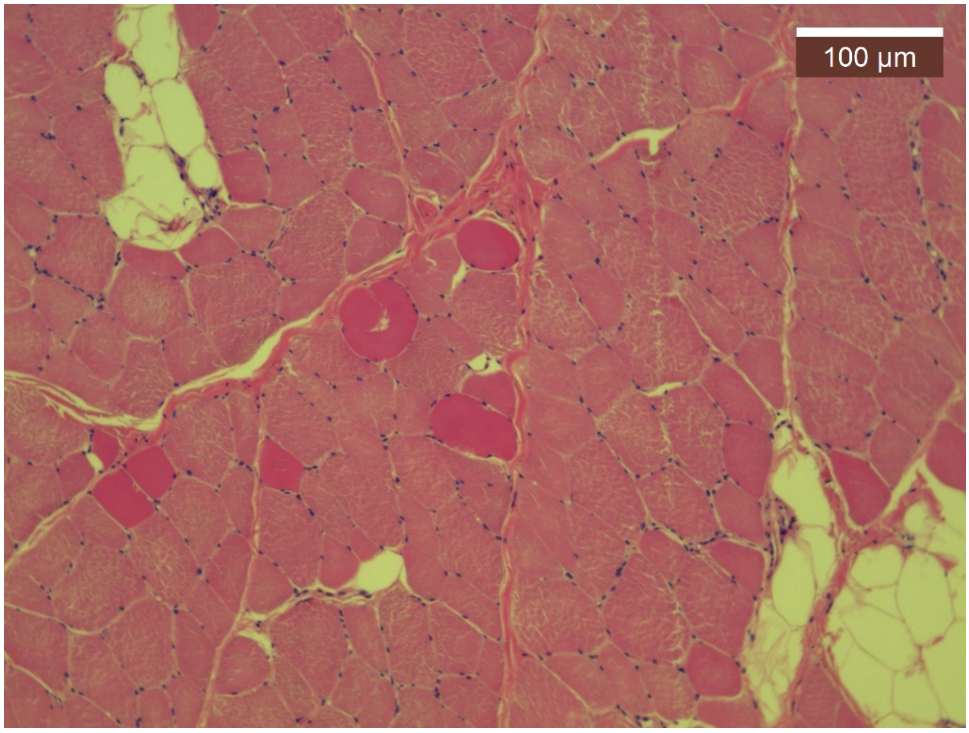

Figure 7.6 *Histological section of Sacrocaudalis dorsalis medialis of a highly marbled, high Wagyu content (75%) heifer M621 (wy75 dx25).* Field selected to show eosinophilic rounded fibres of variable size, with abundant perimysial connective tissue in their proximity. Formalin-fixed H & E, MSA MB 820, DOF 471. CYO lab number Ch18/109Y. See also Figure 7.2 for macroscopic features such as coarse marbling.

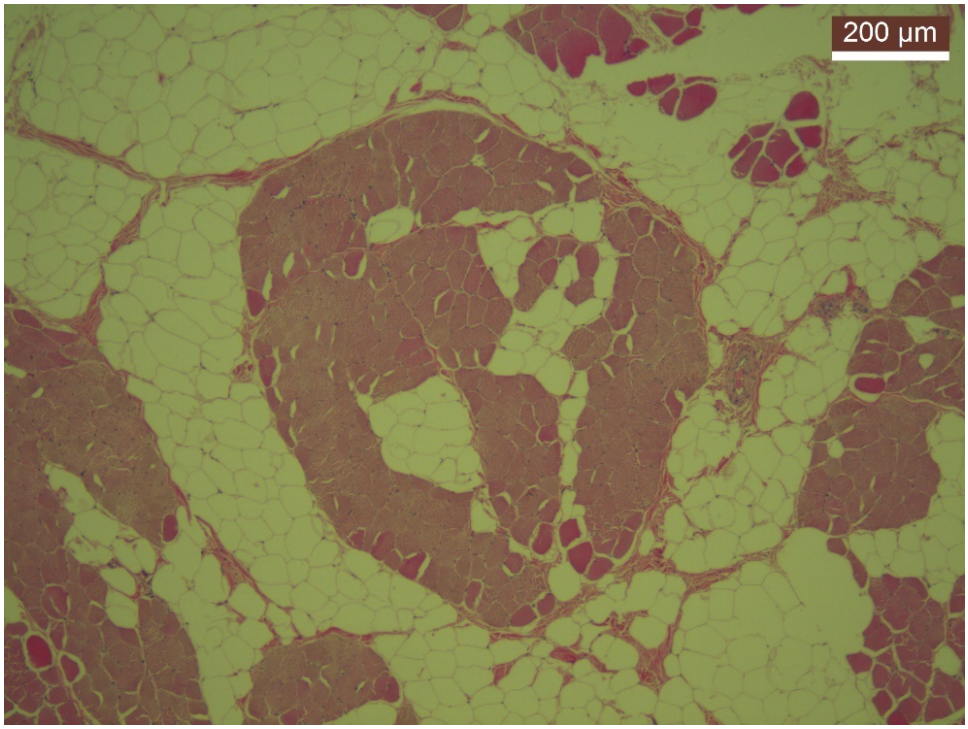

Figure 7.7 *Histological section of Sacrocaudalis dorsalis medialis of a highly marbled, high Wagyu content steer (88%) (wy63 ak25 dx13), showing aggressive adipose invasion, with abundant perimysial connective tissue and the generation of island-like areas of fibres with evident architectural changes including shrinkage of fibres as the front advances.* Formalin-fixed H & E, MSA MB 1100, DOF 471. CYO lab number Ch18/110G. See also Figures 7.1, 7.4, and 7.5.

In some fields, there are collections of nuclei, including intracytoplasmic (Figure 7.8).

These observations have led us to the conclusion that the extent and type of marbling is a function of the aggressive extension of the advancing adipocytes with secondary loss of myocytes.

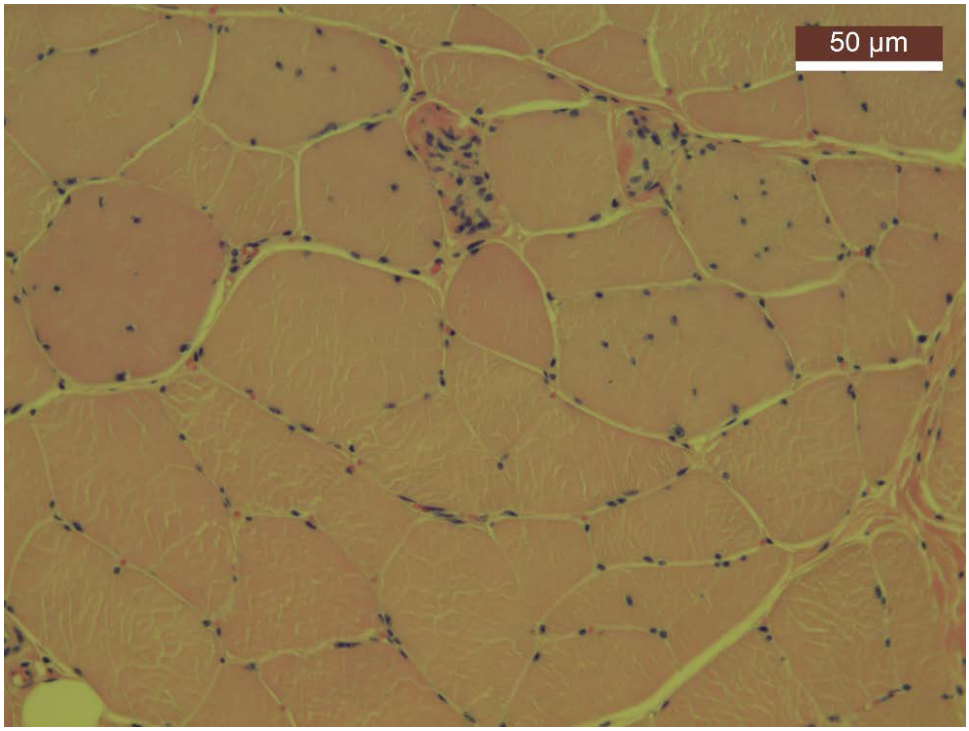

Figure 7.8 *Histological section of Sacrocaudalis dorsalis medialis of a highly marbled, high Wagyu content steer (63%), M129 (wy63 dx13).* Higher power selected to illustrate variability of fibre size, affinity for eosin, and the presence of intracytoplasmatic and interstitial nuclei. Formalin-fixed H & E, MSA MB 880, DOF 481. CYO lab number Ch18/135Z.

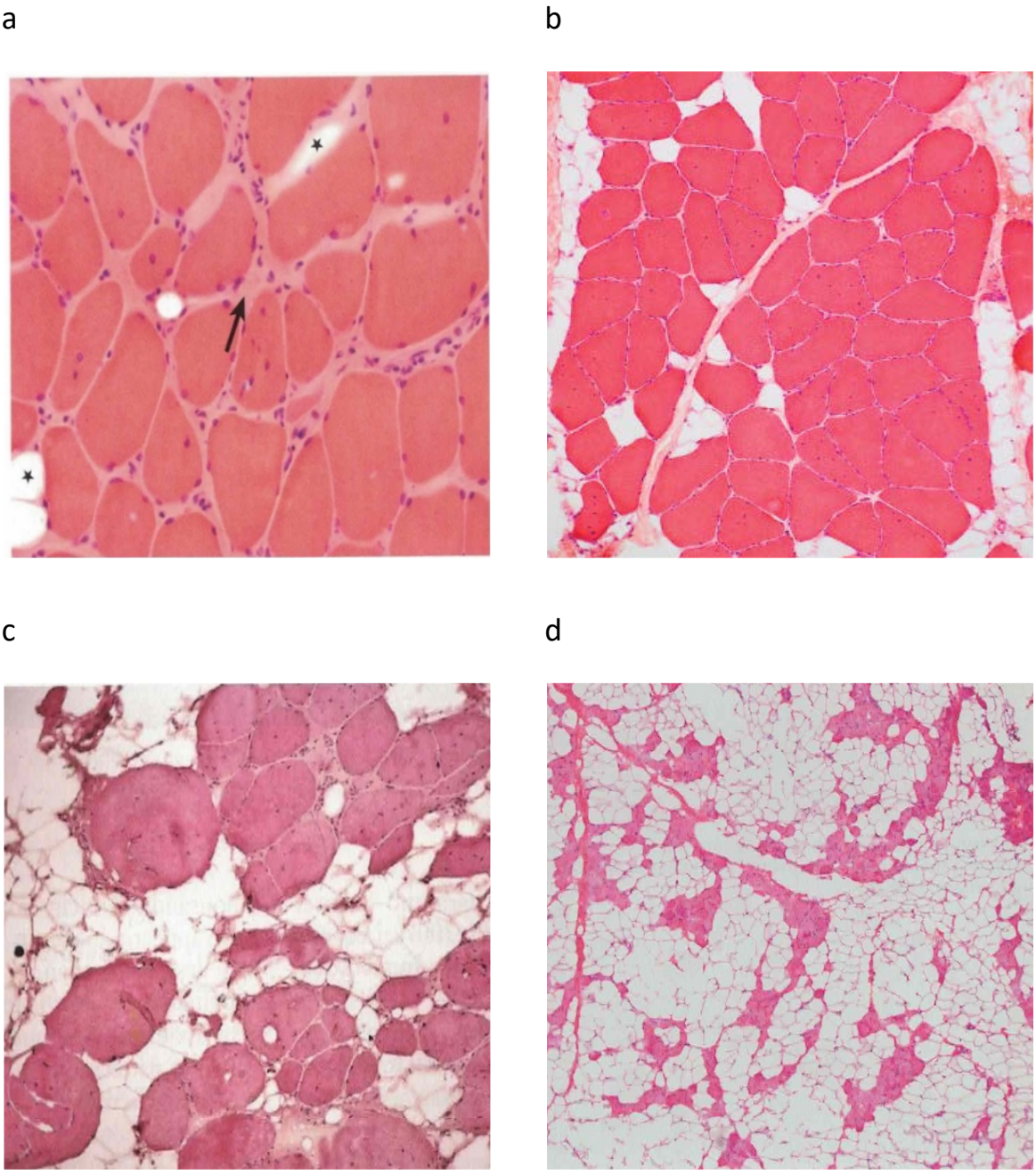

Figure 7.9 *Examples of adipocyte intrusion in human muscular dystrophy*. (a) Case of Limb-girdle muscular dystrophy showing most fibres surrounded by endomysial connective tissue with some adipocytes (*) (Dubowitz et al., 2013, Figure 11.4b). (b) From the deltoid muscle of a patient with ophthalmoplegia associated with a MYH2 mutation showing fatty infiltration, mild fibre atrophy, fibres with internal nuclei, an irregular myofibrillar network, and lobulated fibres (Dubowitz et al., 2013, Figure 15.27). (c) From the quadriceps of a patient with facioscapulohumeral dystrophy at 42 years showing pronounced proliferation of connective tissue and fat with a wide variation of muscle cell size and many internal nuclei ((Dubowitz et al., 2013), Figure 14.1b). (d) Low power view of a biopsy from a case of congenital muscular

dystrophy showing only islands of fibres in a vast amount of adipose tissue ((Dubowitz et al., 2013), Figure 4.30).

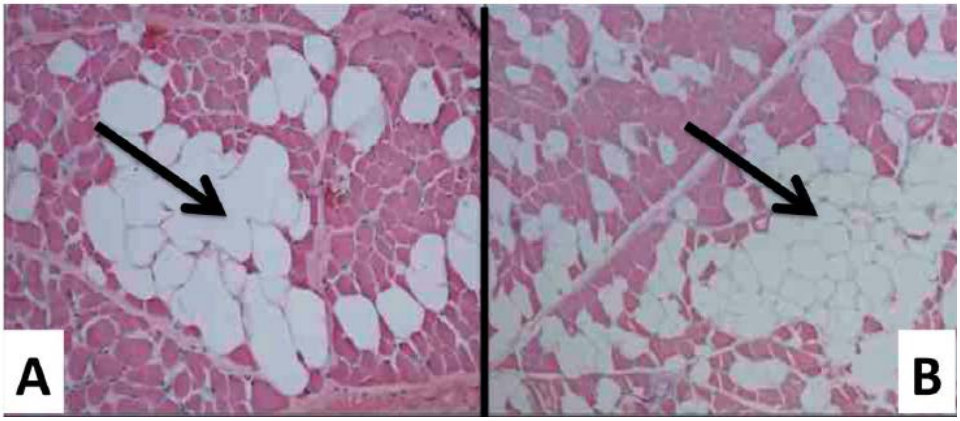

Figure 7.10 *Muscle samples taken from carcasses where steatosis was observed macroscopically at slaughter*. Fat infiltration occurs within the muscle fascicle, there are few adipocytes within the perimysium. Used with permission from Biasibetti et al., 2012.

Some forms of human dystrophy have very similar histopathology, for example, congenital myopathies as illustrated by Dubowitz et al. (Dubowitz et al., 2013) and reproduced here in Figure 7.9.

As in human dystrophies, there can be different degrees depending upon the muscle group and the field selected. Here, we focus on *Sacrocaudalis dorsalis medialis*, because it is convenient to biopsy, whereas the loin can only be accessed readily post-mortem.

Accordingly, it will be possible to undertake detailed time course studies so as to monitor sequential changes and eventually responses to therapy. Future studies should also address bovine steatosis. The pathology (Biasibetti et al., 2012; Peletto et al., 2017) is different from marbling. Adipocytes occur within rather than around fascicles (Figure 7.10) suggesting that the process may be a function of differentiation of stem cells, rather than invasion (Harper and Pethick, 2004).

7.8 *Conclusion*

In spite of similarities in pathology and genomics, there is more to learn before precise translation is possible. However, there are strong indications that such approaches could have important implications for human dystrophies and other muscle diseases. Moreover, a better understanding of the control factors and signals responsible for determining the relative proportions of muscle and adipose tissue in bovine muscles, and how they are coordinated, is fundamental and will be crucial to understanding more fully the significance of adipose tissue replacement in human dystrophies and to developing new therapeutic strategies for these diseases.

8 Comparison of feeds for marbling may be facilitated by haplotype pairing[6]

Along with the previous chapters, I have shown several aspects in which bovine intramuscular fat relates to human health, environmental and genetic factors which interact to determine intramuscular fat deposition and desaturation.

These complex interactions can make experiments aiming to determine the effectiveness of any specific treatment to increase marbling or desaturation more difficult. Traditionally large numbers would be required to find statistically significant effects and interactions. In Chapter 6, I validated a technique for sequential monitoring, which could help to elucidate these interactions. However, it also increases the incentives to reduce the numbers in each trial.

In this chapter, I implemented an alternative strategy for achieving good quality significant data with low number of animals, pairing animals for the known genetic factors identified in earlier chapters.

As a first author, my contribution to this work was:

i. Design the experiment and research plan.

ii. Execution and evaluation of the feed trials.

iii. Measurement of the different variables and generation of the data.

iv. Laboratory testing for Tm.

v. Data analysis.

vi. Writing of the manuscript.

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[6]Submitted to *Translational Animal Science* as: Jose L. Valenzuela, Sally S. Lloyd, Josh Sweeney, Roger L. Dawkins. (2020). Comparison of feeds for marbling may be facilitated by haplotype pairing

The results of this work are exciting, and the potential effect on research is huge.

With the use of this strategy and considering the reduction in the negative impacts, I expect more beef producers to be willing to collaborate with science. This approach would also favour the execution of experiments and trials designed for specific and perhaps reduced geographical areas, generating specific information for local conditions and needs.

8.1 *Abstract*

The amount of marbling in beef cattle is produced from complex interactions of genetics and environment. The interactions make it difficult for producers to select the best production system for their cattle. We fed a heterogeneous collection of heifers and steers on higher or lower energy rations with the intention of determining differences in weight gain and marbling without the need for a conventional feed trial. Depth and melting temperature of subcutaneous fat were also measured. Prior to staggered entry, the 78 animals were matched into 39 pairs based on sex, commencing weight and age, breed percentage and C19 haplotypes. One of each pair was then allocated to one or other ration randomly. When the two rations were compared, there was substantial scatter and significant differences between the two rations became clear only by comparing the two members of each pair. It was clear that the higher energy ration resulted in increased weight gain in 32 of 39 pairs (82%) and increased marbling in 24 of 34 pairs (71%). The strategy of pre-test pairing has the potential to facilitate comparisons of feed and other environmental factors whilst also identifying host factors such as sex, commencing weight and age, breed percentage and haplotypes. Major advantages include reduced numbers, time and cost, especially since the pairs were added as convenient and harvested as required for production.

8.2 *Introduction*

The amount of marbling is a function of genetics and environment. Elsewhere we have illustrated the importance of particular breeds and haplotypes in determining the fat melting temperature as a substitute for marbling (Williamson et al., 2011; Lloyd et al., 2017b). During these studies it became clear that feed was a confounding variable because of changes with season and supply. We, therefore, decided to compare two types of commercial pellets; the nutrient levels of each type are maintained within strict limits allowing meaningful comparisons over the months and even years required for consistent marbling.

Since the ultimate intention is to identify the genetic factors which facilitate marbling, it is necessary to compare animals which are relatively heterogeneous with respect to the markers to be evaluated. In many feed trials, environmental and genetic effects are either randomised between groups (Fluharty et al., 2000) or minimised across both groups (Gorocica-Buenfil et al., 2007). The first approach can require impractically large trials and the second gives results only applicable to a restricted set of environments and genetics. Only occasionally are multiple breeds of cattle used, with breed treated as an additional variable. Other approaches are possible. In one trial identical twin calves were created and paired to compare the effect of vitamin A on carcass composition (Nade et al., 2003).

Reducing the number of animals for research is important. Benefits are found in animal welfare, economic resources, lost production and time expended. Accordingly, we have evaluated a pairing strategy whereby each member of a matched pair can be compared. We have used sex, commencing weight and age, breed percentage and haplotypes to select the pairs and show here that there is a clear difference between the feeds in terms of weight gain and marbling. Such differences were not obvious when the groups were pooled, and the pairing was ignored.

8.3 *Materials and methods*

8.3.1 *Production system*

All the data used in this study came from retail carcasses supplied routinely by Melaleuka Stud, located in the Peel region of Western Australian, 100 km south of Perth. The animals used in this research were raised within commercial production systems and the animals were ethically and humanely treated in accordance with the accords with the guidelines described in Chapters 4, 5 and 6 of the Guide for the Care and use of Agricultural Animals in Research and Teaching. The feeds compared were both commercially available and used according to the manufacturers recommendation. No treatments differed from those used in routine production and any additional samples and measurements were taken post mortem therefore we did not seek ethics approval.

The dams are from a variety of European breeds and crossbreeds, including Simmental, Gelbvieh, and Angus and graze on mixtures of ryegrass, clover and kikuyu pastures. Meadow hay is fed in seasons where pasture growth is insufficient. Sires were mostly Red or Black Wagyu. Calves are weaned at approximately six months, having reached 180kg, then pasture fed to approximately 350kg before being paired and assigned to a feeding group. The target live weight at harvesting is 500kg achieved after 49 to 207 days on feed.

8.3.2 *Animals and pairing*

This study used a total of 78 heifers and steers of mixed breeds, including Wagyu, Akaushi, Simmental, Gelbvieh. The animals were sorted into similar pairs and then one animal from each pair assigned to a high or low energy diet. To isolate the effect of the feeds, after standardizing other environmental variation, we minimised the variability within pairs. To do so, we assigned every animal to a same sex similar pair before commencing the time on feed.

Then we considered live weight, followed by age, breed and genotypes (C19 haplotypes). An example of the pairing of a group of 10 animals at entry is shown in Table 1. Pairs were selected for slaughter at two weekly intervals to meet demand. Days on feed ranged from 50 to 200, but importantly, the pairs were harvested the same day. This system allows comparison of the rations even though heterogeneity is maintained. When it was not possible to match animals with the exact genotype, as illustrated in pair number 1 (Table 1), the selection was based on TCAP alleles, then SREB alleles and MRIP haplotypes (Williamson et al., 2011; Lloyd et al., 2017a).

Table 8.1 *Pairing of representative steers used in this study.*

EasyBeef								Vitalize						
Tag	Weight, kg	Age, mo	Breed	MRIP Haplotype	SREB	TCAP	Pair	Tag	Weight, kg	Age, mo	Breed	MRIP Haplotype	SREB	TCAP
M569	306	11.9	wy50 si25 aa13 gv13	30.2,60.1	S,L	10,20	1	M515	293	12.3	wy50 si50	30.2,60.1	S,L	10,20
M578	313	13	ak50 si25 aa13 gv13	30.2,30.2	L,L	20,20	2	M564	301	13.1	ak50 si38 aa13	30.2,40.2	L,L	20,20
L515	329	19.6	ak50 si50	40.1,30.2	L,L	20,20	3	L530	325	19.4	ak50 si50	30.2,30.2	L,L	20,20
M702	253	11.9	ak50 si50	40.1,30.3	L,L	10,20	4	M651	243	12.7	ak50 si25 gv25	30.6,60.7	L,L	10,20
M067	325	12.1	si47 wy25 dx25 aa3	40.3,30.3	L,L	10,10	5	M110	337	12.1	si37 wy25 dx25 ra13	40.3,40.3	L,L	10,10

wy=Wagyu; si=Simmental; aa=Angus; gv=Gelbvieh; ak=Akaushi; dx=Dexter; ra=Red Angus

8.3.3 *Feed types*

The two diets differed by Metabolizable Energy with the higher energy due to higher starch and reduced crude fibre. The “Low energy” diet consisted of EasyBeef pellets, compared to a “High energy” diet of Vitalize pellets. The EasyBeef and Vitalize pellets were produced by Milne Feeds, Welshpool, Western Australia. Both groups were given pellets *ad libitum* and had straw always available as roughage. Pelletised feed was used to ensure consistency in nutrient balance.

The main nutritional compositions of the two feeds are summarised in Table 2. Mineral and vitamin supplement was adjusted to maximise marbling and standardise the two feeds. This adjustment is shown in Table 3.

Table 8.2 *Feed composition.*

Component (dry matter basis)	High ME	Low ME
Metabolisable Energy (est)	12.5 MJ/kg	11.0 MJ/kg
NDF	25.25%	39.20%
ADF	12.20%	24.50%
Crude Fibre (max)	9.5%	20%
Fat	3.2%	3.0%
Crude Protein (min)	14.50%	14.50%
Urea (max)	1.5%	1.5%
Ionophore	Monensin 36ppm	Monensin 36ppm

Table 8.3 *Micronutrients adjusted from commercial mix to maximise marbling.*

Nutrients (as proportion of dry feed)	Experimental mix		Commercial mix	
Product	EasyBeef	Vitalize	EasyBeef	Vitalize
Vitamin B3 - Niacin (mg/kg)	500	500	0.0	5.0
Vitamin A (IU/kg)	500	500	9000	9000
Vitamin D (IU/kg)	250	250	2000	2000
Vitamin E (IU/kg)	90	90	27	27
Chromium picolinaete (mg/kg)	1.0	1.0	0.7	0.7

8.3.4 *Measurements*

Animals were weighed at the start of feeding, and then every 2 to 4 weeks through the feeding period. When both animals in a pair reached an acceptable weight and fatness, the

pair were harvested and the final weight and entry weights used to calculate an average daily gain (ADG).

Carcasses were graded at the abattoir by a Meat Standards Australia qualified assessor. Marble score (MSA MB) and subcutaneous fat depth (rib fat) from this grading were used in this study. Rib fat depth was measured at the 10th rib.

Subcutaneous fat overlying the anterior and posterior ends of the striploin (HAM number 2140) of these cattle was collected after boning and wet aging for 1 to 3 weeks. Neutral lipids were extracted, and their melting temperature (Tm) tested according to the method of (Lloyd et al., 2014b). The Tm reported is the average of the anterior and posterior ends.

8.3.5 *Statistics*

A paired T-test was applied to the difference between pairs to determine the significance of the effect of feeding. The t-statistic of this test is given by: $t = \frac{\bar{D}}{\sqrt{\frac{s_D^2}{N}}}$, where D is the pair difference, N is the number of pairs and s_D is the standard deviation of the pair differences.

The significance was compared with a Welch Two Sample t-test applied to the groups as if they had been randomised rather than paired. The t-statistic for this test is given by $t = \frac{\overline{X_1} - \overline{X_2}}{\sqrt{\frac{s_1^2 + s_2^2}{N}}}$, where X1 and X2 are the results for group 1 and group 2.

If the criteria used for pairing have no effect on the outcome, then we would expect $s_D^2 = s_1^2 + s_2^2$ and the t-statistic will not be increased by pairing.

For each test, the t-statistic and associated p-values were calculated within the program R (R Core Team, 2015).

8.4 *Results*

8.4.1 *Analysis of pairs*

The differences were significant for ADG and MSA MB Marbling.Those on the high energy diet had a growth rate 0.26 kg/day higher, equivalent to a 17% increase ($p < 0.005$) and a MSA MB score also higher by 55 ($p < 0.05$). By contrast, the subcutaneous fat depth and Tm varied substantially but were not affected by energy content of the diet.

The depiction shown in Figures 1 to 4 compares each individual against its paired equivalent. The figures show no significant difference between feeds for subcutaneous fat (Figure 1) and Tm (Figure 2), but a highly significant benefit of the higher energy feed for MSA MB ($p < 0.002$) (Figure 3) and ADG ($p < 0.001$) (Figure 4).

For rib fat, the proportion of pairs with higher subcutaneous fat was similar for the high and low energy rations. Of the 34 pairs with rib fat measurements, 14 measured higher for the high energy diet (41%), 13 lower (38%) and 7 (21%) were equal.

For the 28 pairs with Tm measurements, the high energy diet showed higher Tm in 10 pairs (36%) and lower in 17 pairs (61%). 1 pair (4%) measured equal Tm.

Of the 34 pairs with marble score, the high energy ration gave higher marble scores in 24 (70%) and lower in only 7 (20%).

The high energy ration resulted in increased ADG in 31 of the 39 pairs (79%), with 71% showing 10% or greater advantage for the high energy.

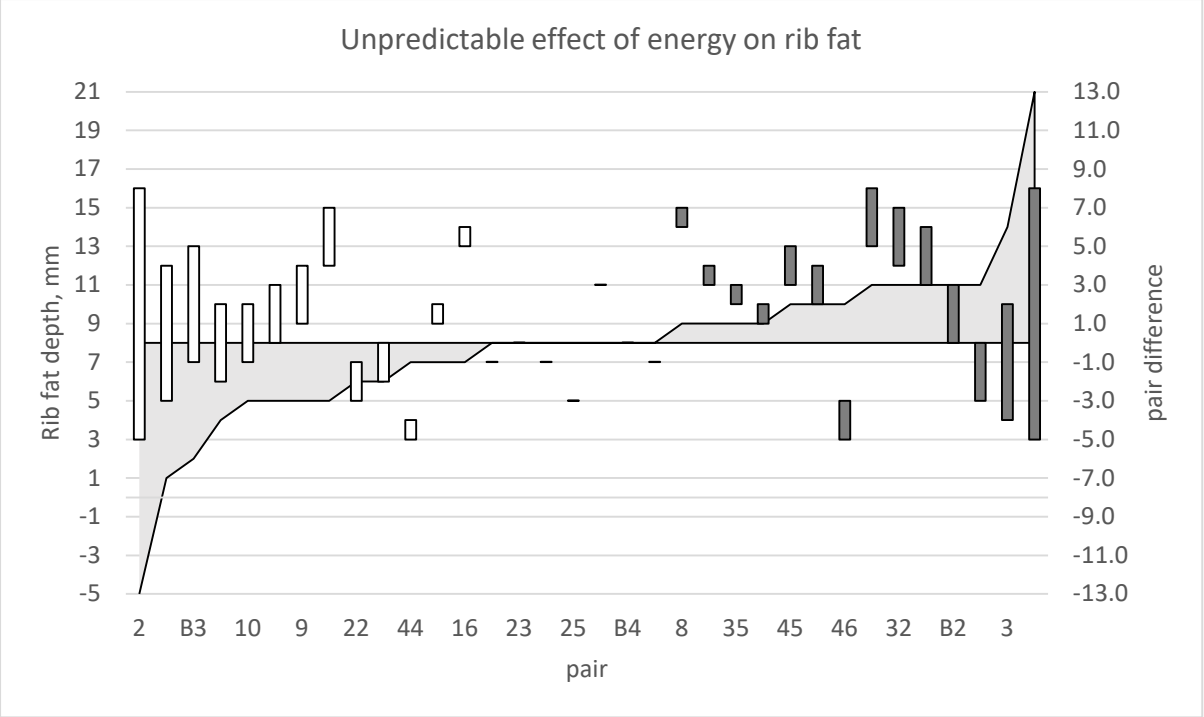

Figure 8.1 *Comparison between the effect of Feed 1 (low energy) and Feed 2 (high energy) over rib fat, showing the pair differences from left to right in order of increase with feed 2.* White bars represent when Feed 1 accumulated more rib fat than Feed 2. Black bars represent when Feed 2 accumulated more fat than Feed 1. The graph shows no differences between Feed 1 and Feed 2.

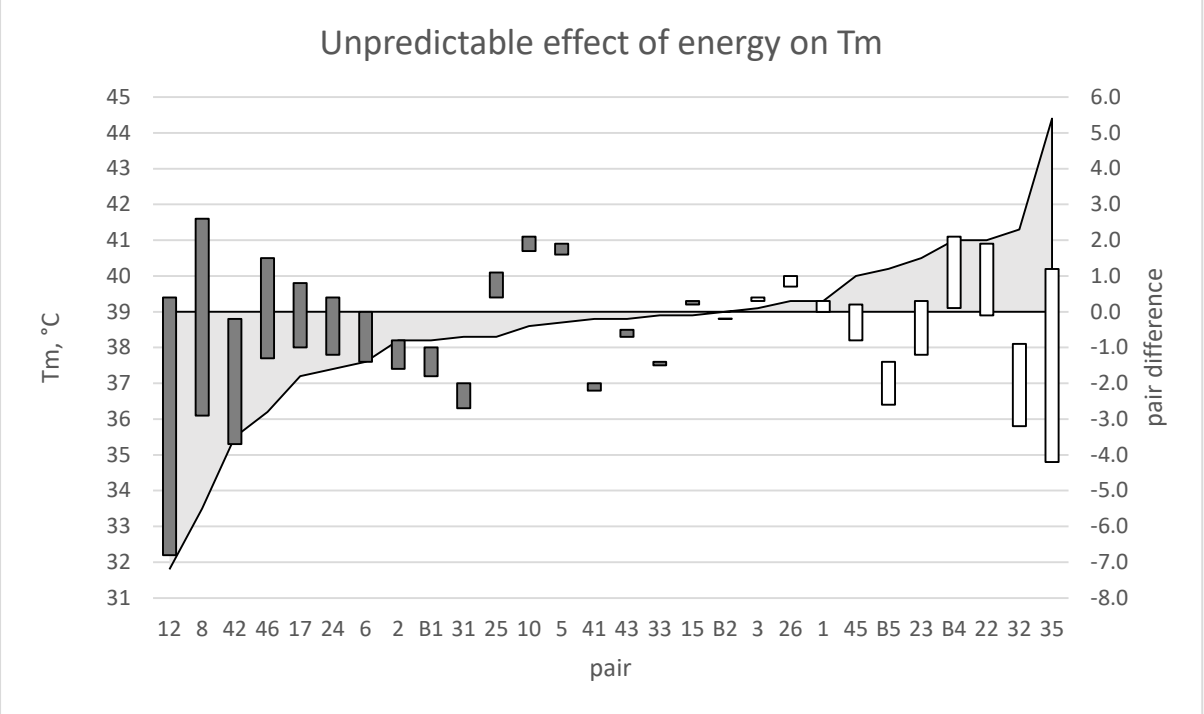

Figure 8.2 *Pairs comparison between the effect of Feed 1 (low energy) and Feed 2 (high energy) over Tm of subcutaneous fat from left to right in order of increase with feed 2.* Black bars represent the pairs where Feed 2 had lower Tm than Feed 1. White bars represent the pairs where Feed 1 had lower Tm than Feed 2. The graph shows no significant differences between Feed 1 and Feed 2.

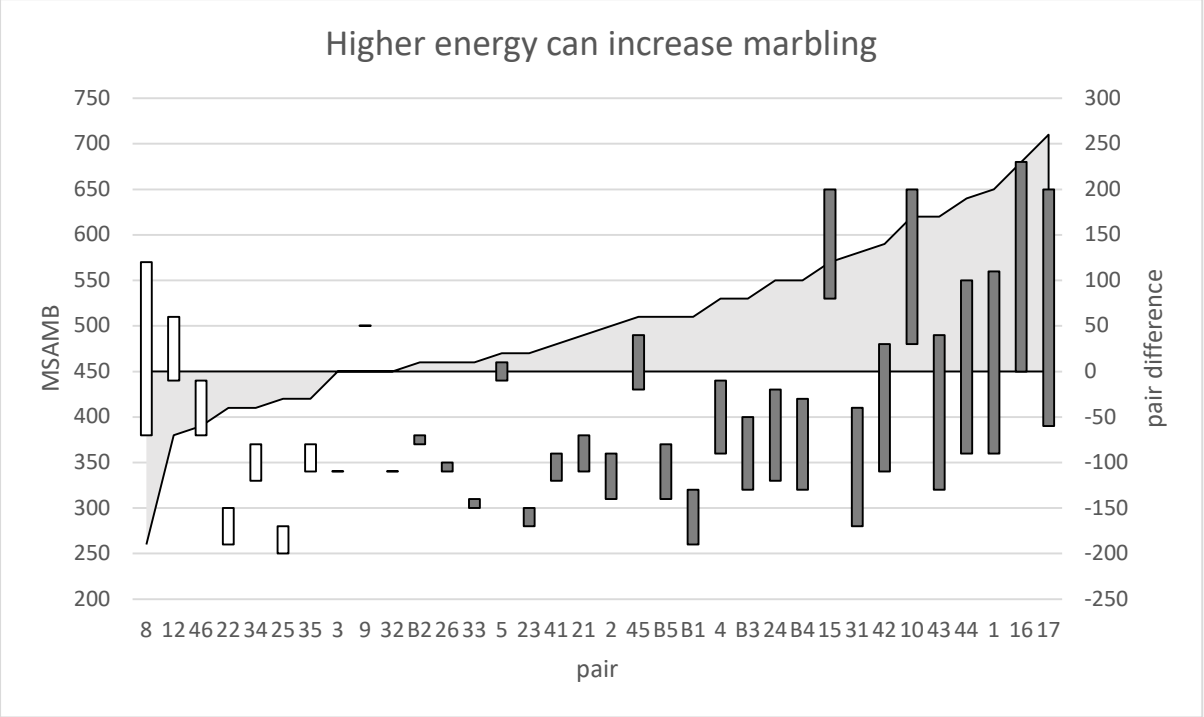

Figure 8.3 *Comparison between the effect of Feed 1 (low energy) and Feed 2 (high energy) over MSA MB in 34 pairs from left to right in order of increase with Feed 2, showing higher marbling with Feed 2 in 24 pairs.*

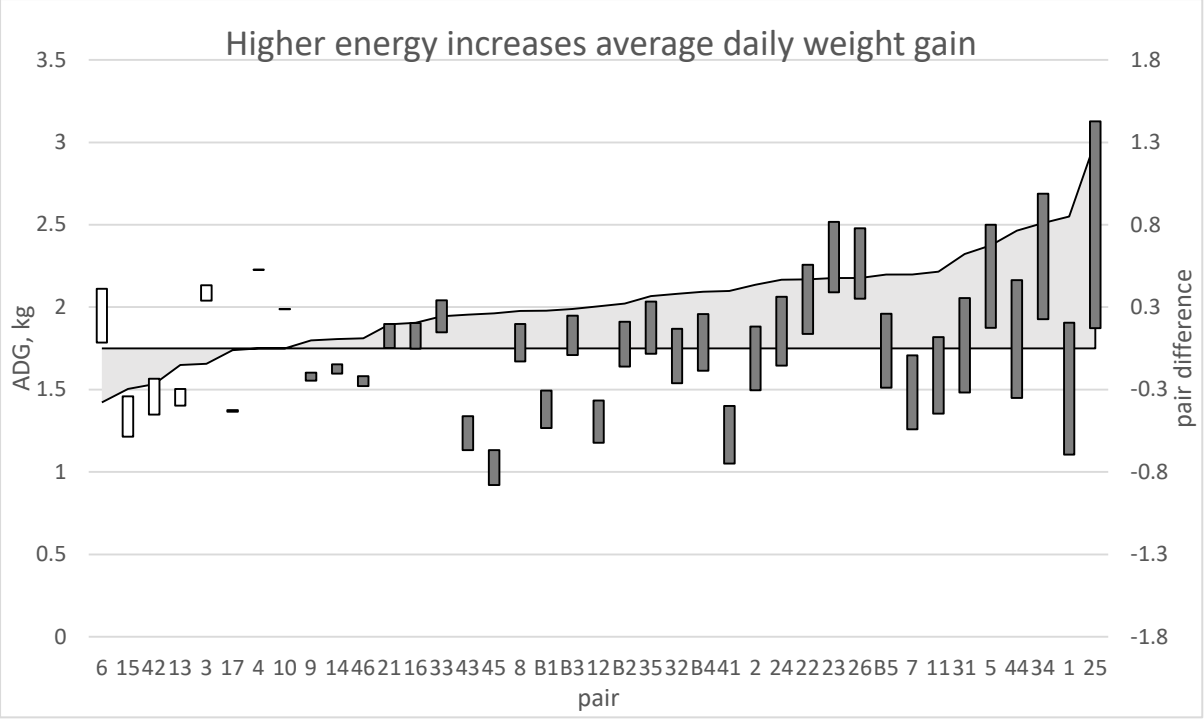

Figure 8.4 *Comparison between the effect of Feed 1 (low energy) and Feed 2 (high energy) over ADG in order of increase with feed 2 on pairs matched for sex, initial weight, breed percentage and haplotype.* 31 of 39 pairs reveal the superiority of feed 2. In 28 pairs there was an improvement of 10% or more.

8.4.2 *Analysis of groups without pairing*

In contrast to the results shown in Figures 1 to 4, when the comparisons are made as two groups rather than pairs (Figure 5), the differences between Feed 1 and Feed 2 are not apparent.

Figures 1 to 4 visually reveal the effect of pairing on the significance of the results, which is confirmed by the statistical analysis. The pair analysis gives considerably stronger significance than the group analysis for ADG and MSA MB. For these two variables, the variance of the pair differences is smaller than the sum of the variances of the groups, leading to higher t-statistic and stronger significance (see supplementary information).

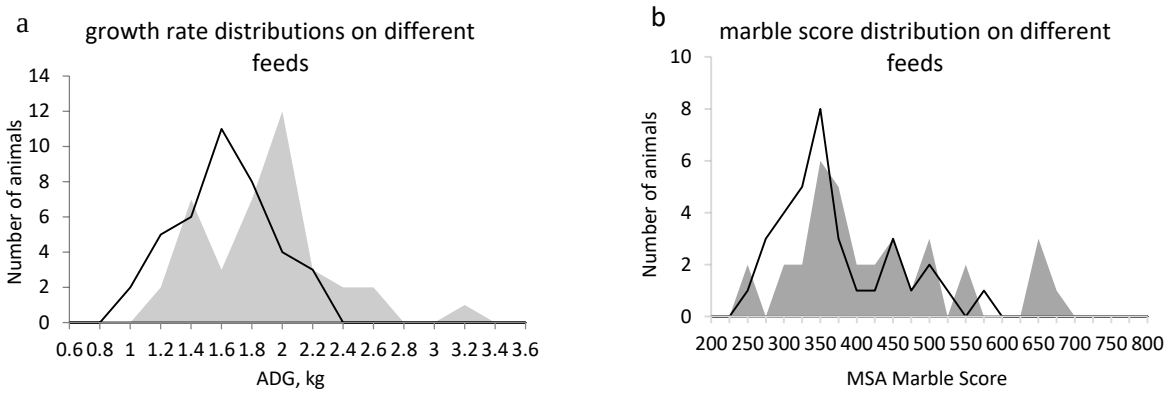

Figure 8.5 *Comparison between the effect of Feed 1 (low energy, shown by black line) and Feed 2 (high energy, shown by grey fill) over ADG (a) and MSA MB (b).* The graphs show little difference between the two groups, with substantial variation, especially with feed 2.

8.4.3 *Effect of genetic differences*

Given the evidence of the effect of the TCAP alleles over Tm presented in Lloyd 2017a, we analysed the influence of such alleles on the outcomes of this study. For the animals fed a high energy diet the results are consistent; the effect of TCAP 20,20 homozygosity was accompanied by a Tm below cattle body temperature (38.6ºC) in 10 of the 13 cases, whereas for non-homozygous 20, just a minority (5 out of 16) were within this range. The results are shown in table 4 and yield a p-value less than 0.02 by chi-square. Interestingly, TCAP alleles did not have the same effect on Tm on the animals fed a low energy diet. That can be explained by the higher marbling induced by the high energy diet.

Table 8.4 *TCAP 20 allows lower Tm with higher energy feed.*

		Vitalise. 12.5 MJ/kg TCAP 20 20	
		+	-
Tm < 38.6	-	3	11
	+	10	5
	Total	13	16

		Easy Beef. 11.0 MJ/kg TCAP 20 20	
		+	-
Tm < 38.6	-	8	10
	+	4	11
	Total	12	21

8.5 *Discussion*

Marbling is highly desirable not so much for taste as for the healthiness of the associated low melting temperature lipids. Premiums can be expected for even modest marbling of 470 MSA MB, as illustrated in Figure 6.

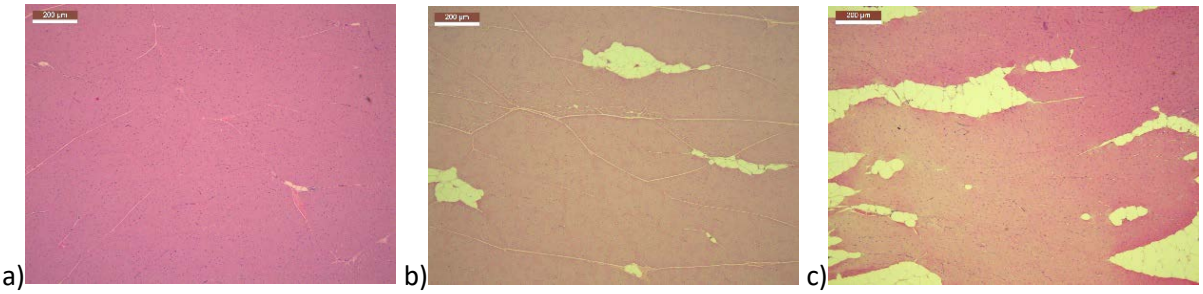

Figure 8.6 *Histological sections of Longissimus dorsi of three animals with increasing Marble Score (MSA MB).* Formalin-fixed Hematoxylin & Eosin. Figures selected as a representative portion of a bigger sample. a) MSA MB 270, AUS MB 0. There are very few clear spaces representing perimysial adipocytes, CYO lab number Ch18/044X, Melaleuka ID N034. b) MSA MB 470, AUS MB 2. There are collections of perimysial adipocytes of sufficient size and number to be readily visible to the naked eye. CYO lab number Ch18/042J, Melaleuka ID M625. c) MSA MB 630, AUS MB 4. There are areas of arborisation with evident separation of muscle bundles. CYO lab number Ch17/028P, Melaleuka ID M660.

Not surprisingly, producers tend to have their own proprietary approaches which are not subject to rigorous examination or improvement. In many cases, the animals are in feedlots for 2 years or even more, thereby making serious trials impractical. On the other hand, it is widely believed that such long feeding will not be possible in the future, given animal welfare, cost and shortages of grain, trade barriers etc. Nor should such long feeding be necessary if genetic factors were optimised and if the rate of marbling could be monitored sequentially (Lloyd et al., 2017b).

We are seeking a means of improving the reliability and cost effectiveness of potential improvements. In essence, the comparisons have to be continuous and a routine component of any production system. To this end, some of the variables need to be eliminated.

It has been known for decades that the SCD regulator SREBP1 and Growth Hormone are important in determining the degree if not the rate of marbling in Black Wagyu (Hoashi et al., 2007; Ardiyanti et al., 2009; Matsuhashi et al., 2011; Ladeira et al., 2016). They also contribute to finer marbling associated with very low melting temperatures approximating oleic acid. It is less well known that these genes are close together on BOTA 19 and, even more interestingly, are packed together with other relevant genes such as FASN, MPRIP, and TCAP all of which are highly pertinent to intramuscular lipids (Valenzuela et al., 2019). In fact, as we have shown repeatedly, alleles at these loci designate several haplotypes which are excellent markers for marbling even in crossbred animals (Valenzuela et al. 2019.; Lloyd et al., 2013; Lloyd et al., 2014a; Lloyd et al., 2017b).

These considerations led us to ask whether trials could be improved by matching for these haplotypes, thereby reducing genetic heterogeneity within each pair, but retaining differences between pairs. The present study is a pilot in the sense that additional animals will have to be recruited in order to test the large number of haplotype combinations.

We are greatly encouraged by the present results since we have demonstrated the value of pre-trial matching to identify similar pairs. Each member of the pair is allocated to one or other test group but otherwise not identified until harvest. Then the two members of each pair are compared. Since the scatter is much reduced conclusions were clear. The higher energy ration improves both weight gain and marbling. This result was not expected since many experts assert the weight gain comes at the cost of marbling and vice versa (Inoue et al., 2011). This could be true in some situations, but the present finding indicates that it is not necessarily the case.

It may be relevant that the degree of marbling is modest rather than extreme as might have occurred if higher Wagyu content and feeding time had been increased. However,

fortunately, such animals can now be added and compared. Similar considerations apply to Tm of the fat although it is encouraging that, irrespective of which ration, 25% did fall below human body temperature of 37°C. Also noteworthy is that all animals achieved a rib fat cover above the 3 mm minimum required and most achieved marbling and weight gain without excessive deposition of subcutaneous fat.

In this case, the fat content of the two diets is nearly identical, with the extra energy coming from the increase in starch which, combined with the tailored micronutrient premix and monensin, would lead to enhanced propionate production (Ellis et al., 2001). Propionate is known to provide glucose to muscles and favours intramuscular fat while circulating acetate and long chain fatty acids favour subcutaneous fat deposition (Smith and Crouse, 1984; Smith and Johnson, 2014; Ladeira et al., 2016; Park et al., 2018).

It was important to have a heterogeneous group since it is not yet known how sex, age, commencing weight and breed percentage interact with the BOTA 19 haplotypes. Since the study design allows pairs to be added and harvested at any time, further data can be accumulated without impacting upon welfare, production, cost and convenience.

8.6 *Supplementary info*

Paired t-test	ADG	MSA MB	SCF depth	TM
Total no. Pairs	39	34	34	28
mean pair difference	-0.26 kg	-55.29	0.15 cm	0.43 degrees
standard deviation of pair differences	0.30 kg	95.45	4.20 cm	2.39 degrees
t-statistic	-5.4196	-3.3779	0.20414	0.9473
Df	38	33	33	27
p-value	0.000004	0.001887	0.839500	0.351900

Welch Two Sample t-test	ADG		MSA MB		SCF depth		TM	
	Easy Beef	Vitalise	Easy Beef	Vitalise	Easy Beef	Vitalise	Easy Beef	Vitalise
N	39	39	34	34	34	34	28	28
Mean	1.53	1.79	370	425	9.4	9.2	38.7	38.3
Stdev	0.32	0.45	78	113	3.4	3.7	1.6	1.9
t statistic	-2.9091		-2.3384		0.17076		0.91358	
Df	68.993		58.728		65.702		51.852	
p-value	0.004873		0.02279		0.8649		0.3652	

9 General discussion

This thesis resolves itself into a series of questions and answers, which together make a strong case for sequential biopsies in order to improve the healthiness of beef.

Question 1: Do standardised and automated measurement of melting temperature of the fat reflect known differences between breeds and sires? The evidence presented in chapter 3 shows that the method can be standardised and is available for routine use. However, there are some qualifications deserving further study. The content of the lipid profile, and particularly the proportion of oleic acid in subcutaneous tissue differs. It is often claimed that the Tm increases as the samples taken from cranial to caudal. The experience here suggests that this is an oversimplification, and it is important to emphasize that changes can occur with time in addition to location. Given these complexities, the claim here is simply that melting temperature at one specified site can be compared across time and individual. The undoubtful influence of breed and sire on melting temperature was clearly demonstrated.

Question 2: Is it possible to use the information pertaining to syntenic regions in man so as to improve translation of findings in cattle?

The evidence presented in chapter 4 is persuasive. Although the components of Bota C19 are rearranged and separated by extensive insertion and translation, the fundamental structure remains the same, and it is this region which has been found to influence a form of human Limb-girdle muscular dystrophy. The potential importance of this discovery is that there has been extensive research in man, and many of the controlling factors have been identified. Thus future investigations can address whether these are involved in marbling in cattle.

This question led me to consider more fundamental aspects of marbling, as demonstrated by Smith and Johnson. Satellite cells are important and appear to be able to contribute to the

differentiation of adipocytes and myocytes; therefore, a key consideration is to understand the factors that drive the process in either of these two directions.

In addition to the genetic commonalities between marbling and Limb-girdle muscular dystrophy, chapters 6 and 7 present marbling as an invasive process, described here as “arborisation”, showing histological features remarkably common between the two processes: the separation of muscle fascicles, increment of perimysial connective tissue, changes in the affinity of myocytes to eosin, increased muscle cell size, cell shape variation, and the presence of intracytoplasmatic nuclei.

Genetic and histological findings make a strong case for translating findings between these two species.

One might speculate that a deficiency of TGF beta as found in double muscling might encourage in the development of the myocyte as opposed to the adipocyte. Note the interaction between the syntenic region and Myostatin, as illustrated in chapter 4. Contrariwise one might speculate that in Wagyu, genetic factors might inhibit the myogenic pathways and stimulate the adipogenic pathway via FASN or growth hormone. As noted by Shibata et al. (2006), myostatin levels in Wagyu are affected by TCAP genetic differences and influence marbling.

There is substantial potential for bovine research to inform understanding in human medicine, as illustrated in chapter 4.

Question 3: Is haplotyping valuable for maintaining Australia’s enviable reputation as a producer of healthy food?

There is a huge issue in traceability and fraud. Unequivocally haplotypes can identify and reduce this. The challenge now is to convert the procedure from one requiring a simple laboratory, to one applicable on a single machine.

Question 4: Does histological examination of marbling reflect the AUS meat marble scores?

Surprisingly there are many methods of measuring marbling. Japanese, Australian and US approaches are completely different, suggesting that they measure different aspects. This issue is important because ultimately we wish to quantify the healthiness of the product. For this reason, I included an analysis of melting temperature (see chapter 3), but given the findings of chapter 4, it became clear that we needed to improve the fundamental understanding of the processes involved in both, muscular dystrophy and bovine marbling. There are many issues to consider, given the vast differences in feeding, butchering, processing, cooking, etc., it seems unlikely that it will be possible to standardise and agree internationally. However, it is very clear that there are sequential changes that occur over time as marbling increases, and it is encouraging to note that these correlate to some degree with AUS MEAT marbling. The current work, therefore, justifies the ethical basis of sequential testing within feedlots in the hope that animal welfare and human health will benefit.

Question 5: Can haplotypes be used to obtain clearer results from feed trials intended to improve animal welfare?

The current practice is to use large groups for feed trials, and this leads to feed comparisons not studied. This has led to many feeding myths with secret recipes for Wagyu feeding and erosion of public confidence. We have identified the genetic components, and these haplotypes can be used to pair animals and identify differences between feeds with much smaller trials.

The Future

Collectively the discoveries described above define a growth plan based on

i. Sequential monitoring of marbling, Tm, consumer preference, health value and improved welfare.

ii. Provenance, traceability and branding.

iii. Interspecies translation.

iv. Understanding the four pathways to Marbling, their regulators and genetic controls.

v. Use of biopsies, whether for histology, histo-chemistry or other technologies.

vi. Improved access to the above information in retail and kitchen.

Intellectual Property

The IP described above is the property of the CYOEVF. The work described in this manuscript form part of a current patent application. As such, we would like this manuscript to be marked “Confidential and not for public disclosure” until further advised.

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