MOLECULAR AND IMMUNOHISTOCHEMICAL INVESTIGATIONS INTO FAT DEPOSITION IN HOLSTEIN AND CHAROLAIS CATTLE

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"We fat all creatures else to fat us, and we fat ourselves for maggots."

Shakespeare, W. (1603) Hamlet, Prince of Denmark Act IV, Scene iii

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein

ABSTRACT

Peroxisome proliferator-activated receptor (PPARγ) is a transcription factor that regulates adipogenic genes and preadipocyte factor-1 (pref-1) is a transmembrane protein that regulates preadipocyte differentiation. The role of PPARγ was investigated using reverse transcription polymerase chain reaction (RT-PCR) by measuring PPARγ mRNA levels in bovine adipose depots and *longissimus dorsi* muscle. No significant differences in PPARγ mRNA levels were observed between 10 Charolais and 10 Holstein cattle for either tissue. Differences were observed between depots within these breeds. Correlations were performed among PPARγ, carcass characteristics, and adipogenic genes. Pref-1 antibodies were used to immunolocate preadipocytes in bovine muscle tissue to the perimycium, near fat cells and blood vessels. The preadipocytes may exist in muscle tissue for short periods of time or may arise from a source external to the muscle. A computer image analysis program was developed for the quantification and characterization of intramuscular fat in whole muscle tissues.

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ABBREVIATIONS

ACS	acyl-CoA synthetase
AMSA	American Meat Science Association
aP2	adipocyte lipid binding protein
CBGA	Canadian Beef Grading Agency
CDN$	Canadian dollars
C/EBP	CCAAT/enhancer binding protein
CIA	computer image analysis
CFIA	Canadian Food Inspection Agency
CLA	conjugated linoleic acid
dlk	delta-like
ECM	extracellular matrix
EGF	epidermal growth factor
emcTAG	extramyocellular triacylglycerol
ERK-MAPK	extracellular-regulated mitogen-activated protein kinase
FA	fatty acid
FABP	fatty acid binding protein
FBN	Research Institute for the Biology of Farm Animals
FFA	free fatty acid
FSIS	Food Safety and Inspection Service
GAS1	growth-arrest specific protein 1
GLUT	glucose transporter
h	hour

HRP	horseradish peroxidase
HSL	hormone sensitive lipase
IGF	insulin-like growth factor
i.m.	intramuscular
imcTAG	intramyocellular triacylglycerol
KB	ketone body
LinCo	Line Coincidence
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
min	minute
MLA	Meat and Livestock Australia
mo	month
mRNA	messenger ribonucleic acid
MSA	Meat Standards Australia
MU	*longissimus* muscle
NBQA	National Beef Quality Audit
NCBA	National Cattleman's Beef Association
NEFA	non-esterified fatty acid
Ob	leptin gene (obese)
Ob-R	leptin receptor
O/E-1	Olf-1/early B-cell factor
OM	omental fat
PBS	phosphate buffered saline

PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor-response element
PR	perirenal fat
pref-1	preadipocyte factor-1
RIA	radioimmunoassay
RT-PCR	reverse transcription polymerase chain reaction
RXR	*cis*-retinoid X receptor
SC	subcutaneous fat
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFA	saturated fatty acid
sn	stereospecific numbering
SREBP/ADD	sterol regulatory element binding protein/adipocyte determination and differentiation factor
SV	stromal-vascular
TAG	triacylglycerol
TIFF	tagged image file format
TNF	tumour necrosis factor
TSA	tyramide signalling amplification
U.S.	United States
US$	United States dollars
USDA	United States Department of Agriculture
VBM	value-based marketing
VFA	volatile fatty acid
VLDL	very low-density lipoprotein

WAT	white adipose tissue

1. INTRODUCTION

The cattle industry is a major contributor to the North American economy. For example, in 2001, the Canadian cattle industry earned CDN$6.6 billion in farm cash receipts. One trait that leads to higher sale values for beef carcasses in both the Canadian and American beef markets is increased amounts of marbling, which is the presence of a discernible amount of intramuscular (i.m.) fat in beef cattle muscle and is usually assessed by a visual inspection by trained inspectors. The tenderness, juiciness and flavour of the meat has a low association to the amount of marbling in meat (Wheeler et al., 1994; Jeremiah, 1996). In addition, during a recent marbling symposium in Australia, Johnston (2003) reported significant genetic correlations between i.m. fat and many eating quality characteristics. Campion et al. (1975) suggested that grading factors accounted for no more than 10% of the variation in tenderness, but, marbling was the most important grading trait for predicting eating quality. The Canadian meat packing industry is beginning to employ value-based marketing strategies whereby carcasses containing above-average marbling deposits are rewarded with higher prices whereas lower meat-yielding and poor marbling carcasses are discounted among other carcass traits that results in rewards or discounts.

The amount of marbling that an animal has depends upon its genetic composition, length of time on feed, kind of feed (ratio of roughage to concentrate), degree of maturity (age), and gender. The deposition of i.m. fat in cattle is a continuously varying trait exhibiting a high degree of heritability with levels of up to 0.65 when evaluated in individual cattle at a constant age (Marschall, 1999). The relatively high level of

heritability, along with the poor association between the deposition of i.m. fat and fat in other adipose depots, opens up the opportunity to select for the marbling traits using breeding schemes (Kinghorn and Simm, 1999). Studies have therefore been conducted to look for trends between cattle carcass characteristics and fat in the intramuscular (i.m.) adipose depots.

One of the objectives of animal scientists who investigate production animals is to focus on the multi-faceted mechanisms of fat production (triacylglycerol biosynthesis), new adipocyte development (hyperplasia) and fat deposition into adipocytes (hypertrophy) in order to contribute to the future development of meat animal production. One facet of my research was to investigate the expression of peroxisome proliferator-activated receptor γ (PPARγ) in cattle, one member of a family of transcription factors known to play a role in the differentiation of preadipocytes into mature lipid-filled adipocytes (Houseknecht et al., 2002). The mRNA expression level of PPARγ was investigated in four adipose depots and one muscle from Holstein and Charolais cattle. Initial comparisons were performed within and between the different cattle breeds with subsequent analyses correlating mRNA expression levels with other previously investigated adipogenic genes such as leptin.

Microscopically, i.m. fat comprises a true adipose tissue being composed of adipocytes embedded in a connective tissue matrix and occurring in close proximity to a blood capillary network (Hood and Allen, 1973). Even though staining methods to characterize fully-differentiated adipocytes have long been developed (such as Oil Red O and Sudan Black), undifferentiated adipocytes (i.e. preadipocytes) cannot be distinguished solely on the basis of their morphology. Therefore attempts were

undertaken to develop immunohistochemical techniques to locate and determine the number of preadipocytes in order to assess the size of the preadipocyte population in different tissues. Thus, a second goal of my research was to develop a method to immunolocate bovine preadipocytes in muscle tissue in order to determine the adipocyte developmental potential of muscles in the bovine. Preadipocyte factor-1 (pref-1), a transmembrane protein known to be expressed abundantly in preadipocytes, and characterized by complete abolishment of expression during adipocyte conversion, with constitutive overexpression blocking adipogenesis, was used as a marker of preadipocytes (Smas and Sul, 1993). Anti-pref-1 antibody was tested in a cell culture of bovine preadipocytes and used to immunolocate preadipocytes in bovine *longissimus dorsi* muscle from Holstein and Charolais cattle.

In addition to the microscopic analysis of i.m. fat development, macroscopic analysis techniques were also employed to characterize the development of marbling fat. Grading is done on the *longissimus dorsi* muscle between the 12^{th} and 13^{th} ribs with the assessment of marbling taking into account the amount of fat deposits, the distribution of fat deposits and the size of the deposits with comparisons made to marbling reference standards. The final facet of my research was to develop an objective computer image analysis program for characterizing the quantity and the distribution of i.m. fat in muscle tissue. The fat in thin slices of whole muscle was stained to amplify the visible fat deposits and digital images of the stained muscle tissue were obtained. The digital images were imported into the computer program and analyzed for various quantitative traits of i.m. fat deposition. The program development and analysis process is outlined in Appendix 1.

2. Literature Review

2.1 The North American Cattle Industry - Inspecting and Grading Beef

Cattle are an important contributor to the North American food production and manufacturing industry. In 2000, there were 12.8 million cattle and calves on 121,375 ranches across Canada earning CDN$6.6 billion in farm cash receipts (Canadian Agri-Food Trade Service, 2002). In the same year, Canada exported 446,288 tons of beef and beef products valued at CDN$1.9 billion, to the United States, Japan, Mexico, South Korea, Taiwan and Hong Kong (Canadian Agri-Food Trade Service, 2002). In 2002, the United States (U.S.) had a total of 105.2 million cattle and calves (National Agricultural Statistics Service, 2002) on over 1.3 million farms (Profile of Farms with Livestock in the United States, 2002) with cash receipts from cattle and calves at US$40.4 billion (2001 Cash Receipts for Meat Animals in Montana and the U.S., 2002). With such a large number of cattle serving a major part of the food manufacturing industry, both Canada and the U.S. have developed meat inspection agencies or departments that are responsible for ensuring that safe and wholesome meat reaches the consumer.

The responsibility for meat inspection in Canada lies with the Canadian Food Inspection Agency (CFIA). The CFIA is a consolidation of four federal departments that deliver all federal food, animal and plant health inspection programs (The Canadian Food Inspection Agency, 2002). Overall their role is to enforce the food safety and nutritional quality standards established by Health Canada and to set standards and carry out enforcement and inspection for animal health and plant protection (The Canadian Food Inspection Agency, 2002). In the U.S., responsibility for meat inspection lies with the

Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) and plays a similar role to the CFIA (Food Safety and Inspection Service, 2002). Inspections in both countries are mandatory, whereas in the U.S. quality grading of meat is optional.

Quality grading is intended to place carcasses into uniform groups of similar quality, yield, and value, in order to facilitate marketing and production decisions. In Canada, grading is the responsibility of the Canadian Beef Grading Agency (CBGA), which is a private, non-profit corporation accredited by the CFIA to deliver grading services for beef in Canada (Canadian Beef Grading Agency, 2002). The Canadian Federal government sets the grade standards that are based on recommendations from the Industry/Government Consultative Committee on Beef Grading (Canadian Beef Grading Agency, 2002). In the U.S., the USDA's Agricultural Marketing Service is the agency responsible for grading meat (Food Safety and Inspection Service, 2002). After meat inspection, grading entails an evaluation of traits somewhat related to tenderness, juiciness and flavour of the meat along with measurements of meat yield. One trait that plays a role in determining the degree of juiciness and tenderness in beef is marbling.

Marbling is fat deposited within bovine muscle tissue (see muscle images in Figure 1 for examples). In Canada, the CBGA uses three of the ten copyrighted marbling standards (slightly abundant, small, and slight; see Figure 2) developed by the USDA when grading carcasses (Food Safety and Inspection Service, 2002). Grading is done on the *longissimus dorsi* muscle between the 12^{th} and 13^{th} ribs (see Figure 3) with the assessment of marbling taking into account the amount of fat deposits, the distribution of fat deposits and the size of the fat deposits visible in the muscle (Canadian Beef Grading

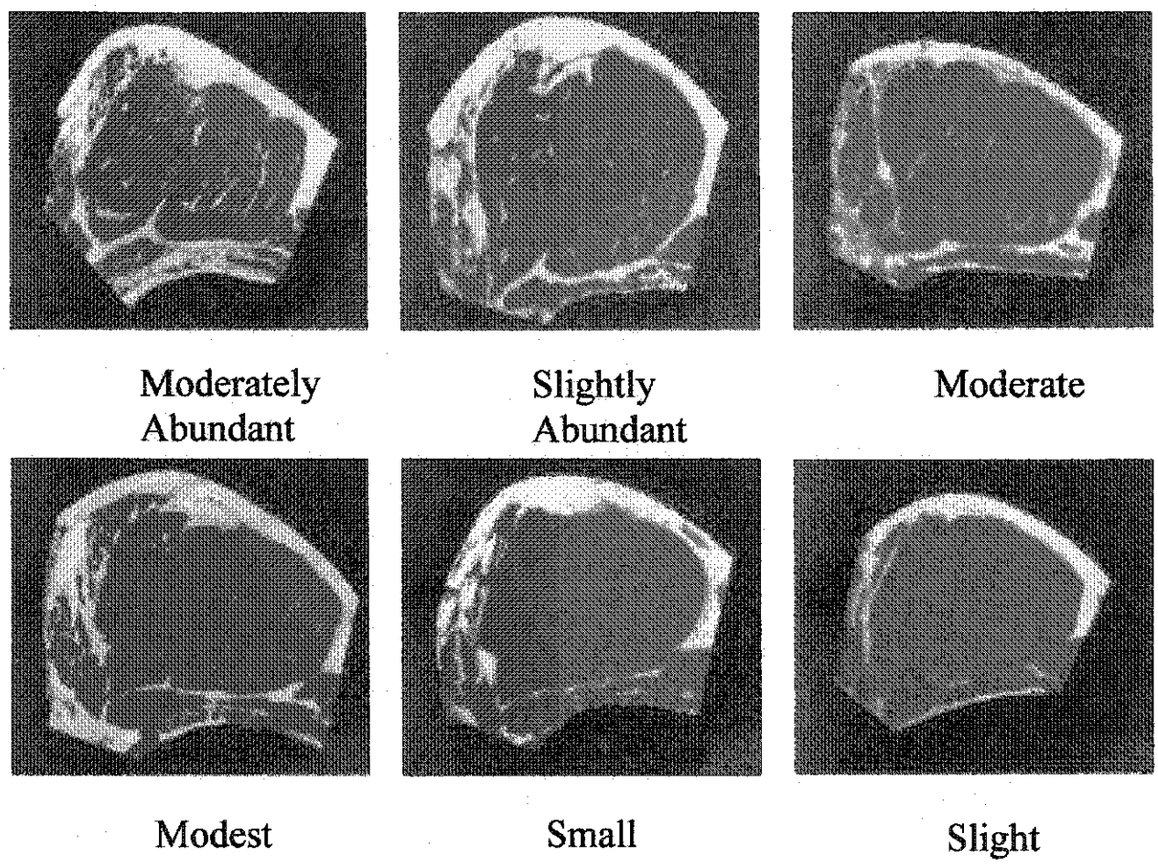

Figure 1. Marbling standards from the USDA used in the quality grading of beef in the U.S. Images obtained from the USDA. The marbling grade depends on the amount and distribution of intramuscular fat in the *longissimus dorsi* muscle with each standard representing the lower end of each respective marbling score range. Represented are six of the ten marbling standards used by the USDA.

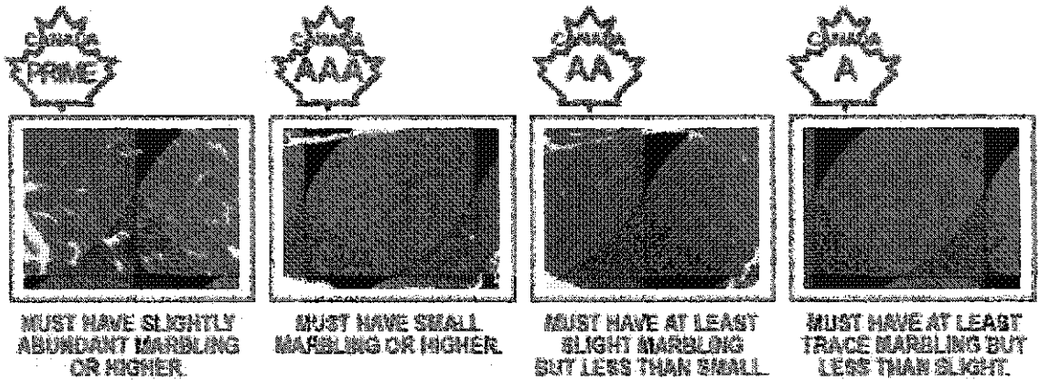

Figure 2. Marbling grades from the (CBGA) used in the quality grading of beef in Canada. The four grades correspond to the USDA grades slightly abundant, small, slight, and trace, respectively. Images obtained from The Beef Information Centre (http://www.beefinfo.org/marbling.html).

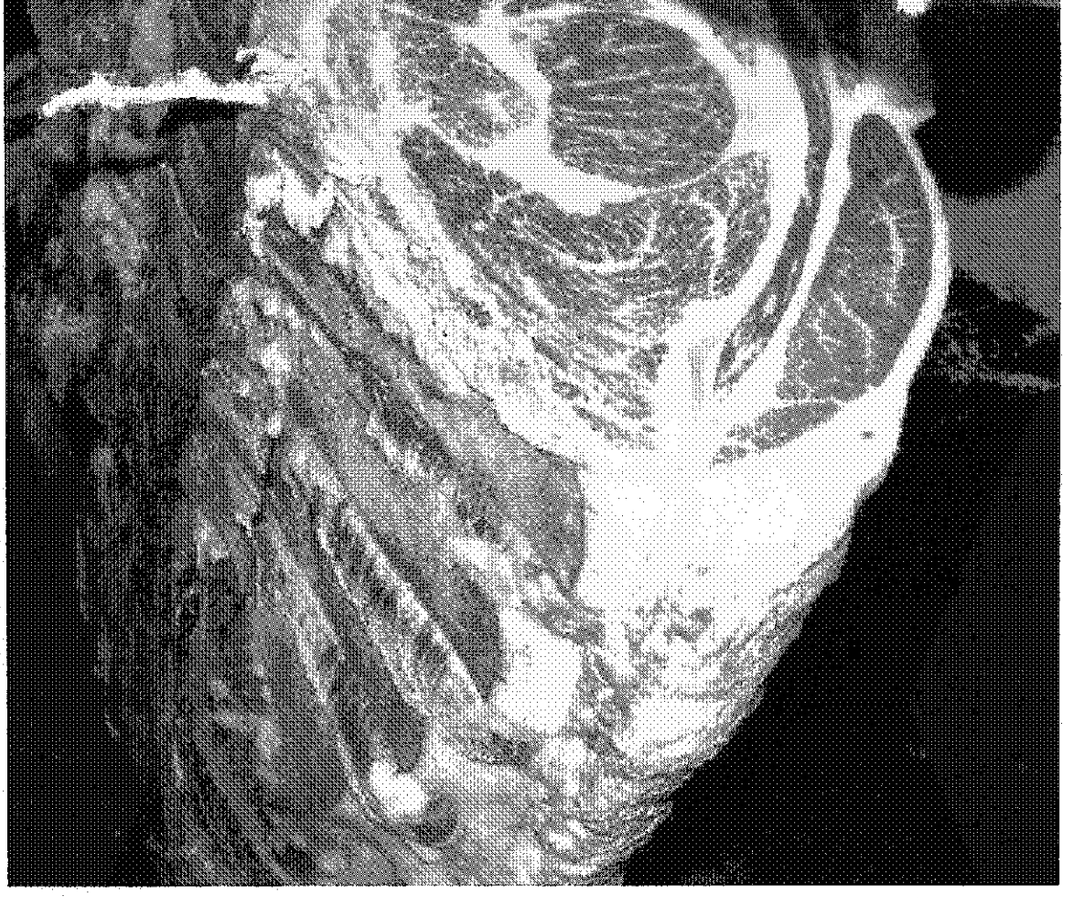

Figure 3. Cross-section of Japanese Black (Wagyu) beef in a Japanese abattoir demonstrating high degree of marbling typical for this breed. In addition, this is the view obtained during inspection when the carcass in sectioned at the 12^{th} to 13^{th} rib. Image obtained from Dr. Takafumi Gotoh of the Kuju Agricultural Research Center, Kyushu University, Japan.

Agency, 2002). If a very low degree of marbling is visible, a grade of trace is given, whereas the grades slightly abundant, small and slight are given subjectively depending on their similarity to the USDA pictorial reference standards (Canadian Beef Grading Agency, 2002) (Figure 2). The four marbling standards slightly abundant, small, slight and trace respectively correlate with the following quality grades: Canada Prime, Canada AAA, Canada AA, and Canada A (Canadian Beef Grading Agency, 2002). It should be noted that the pictorial standards indicate the minimum level of marbling required for the standard (Canadian Beef Grading Agency, 2002).

For grading purposes, the marbling level is established corresponding to the official CBGA categories. For research purposes, however, the grader often provides marbling scores. Currently, in Canada, two marbling score systems are being used: the inverse 10-point scale and the more popularly applied American Meat Science Association (AMSA) scale (see Table 1). Unfortunately the inverse scale complicates statistical analyses and therefore the AMSA scale is currently being adopted across Canada by researchers interested in the statistical analysis of marbling (Personal communication, Dr. Wayne Robertson, Agriculture and Agri-Food Canada).

2.2 The Economics of the Beef Carcass

Marbling has been shown to have a low association with meat palatability, tenderness and juiciness (Wheeler et al., 1994; Jeremiah, 1996). In addition, Campion et al. (1975) suggested that grading factors accounted for no more than 10% of the variation in tenderness, but, marbling was the most important grading trait for predicting eating quality. Recently, during a marbling symposium in Australia, Johnston (2003) reported

Table 1. Marbling score system in use by North American researchers for the purpose of statistical analysis of marbling in cattle. The inverse 10-point scale is reported to one decimal place whereas the AMSA system is recorded in increments of 10.

Description	Inverse 10-point scale	AMSA system
Devoid (D)	11	100
Practically Devoid (PD)	10	200
Traces (T)	9	300
Slight (Sl)	8	400
Small (Sm)	7	500
Modest (Mt)	6	600
Moderate (Md)	5	700
Slightly Abundant (SlA)	4	800
Moderately Abundant (MdA)	3	900
Abundant (Ab)	2	1000
Very Abundant (VAb)	1	1100

statistical correlations between i.m. fat and the following Meat Standards Australia (MSA) eating quality characteristics in temperate breeds: MSA – flavour score (r = 0.75), MSA – juiciness score (r = 0.69) and MSA – tenderness score (r = 0.73). During the same symposium, Thompson (2003) reported a strong effect of chemical fat percentage on both sensory juiciness and flavour scores after adjusting for shear force. Savell and Cross (1989) found that a minimum of 3% i.m. fat was necessary for quality palatability whereas a maximum of 7% i.m. fat would be the limit of current recommendations for the daily caloric restrictions for fat in the diet. Results from Wheeler et al. (1994) indicated that the 3 to 7% i.m. fat 'Window of Acceptability' proposed by Savell and Cross (1989) would provide a balance between the positive contribution of marbling to carcass quality along with the negative contribution of increased dietary fat intake. Nevertheless, marbling continues to be used in major beef producing countries such as Japan (Japan Meat Grading Association, 1988), the U.S. (Food Safety and Inspection Service, 2002), Canada (Canadian Beef Grading Agency, 2002) and Australia (MLA, 2003) as one of the main factors in determining beef quality grades.

Product enhancement research funded by the American National Cattlemen's Beef Association (NCBA) demonstrated that consumers (72.6%) visually preferred low marbled steaks, but, on average, higher marbled steaks were rated more juicy and more desirable in flavour and overall acceptability than low marbled steaks by consumers (Product Enhancement Research for 2001, 2003). In addition, consumers were willing to pay more for highly marbled steaks. A market therefore exists for the development of highly marbled steaks that do not visually appear to contain large amounts of fat but contain evenly distributed i.m. fat deposits.

In an effort to assess the status of the quality of steers and heifers in the U.S. cattle industry, the NCBA funded National Beef Quality Audits (NBQAs) in 1991, 1995, and most recently in 2000 [see (NBQA 2000) for the full report and (McKenna et al., 2002) for the summary]. Results from the NBQA-2000 indicated that producers faced a lost opportunity of US$100.10 for each steer or heifer harvested in 2000 (NBQA 2000). Value losses are down from the 1991 value of US$277.81 and the 1995 value of US$135.88 (NBQA 2000). In 2000, total waste accounted for US$50.96 of the total 'value-loss' with US$42.80 and US$8.16 accounting for excess fat (consisting of excess external fat, excess seam fat, and fat in excess of 20% beef trimmings) and inappropriate muscling (too light plus too heavy), respectively (NBQA 2000). Taste accounted for US$24.45 of the total lost opportunity per animal with US$2.39 for inadequate palatability (viz. tenderness), US$20.96 for insufficient marbling, US$0.63 for hardboned carcasses (hard bones occur in beef that is very advanced in maturity), and US$0.47 for bullock carcasses (a designation for young beef bulls with slightly lower and more variable palatability). The additional characteristics involved in total value-loss were management aspects (e.g. hide damage, carcass condemnations, offal condemnations, injection-site lesions, bruises, dark cutters and blood splashes, callused ribeyes and yellow fat) accounting for a loss of US$18.23 and inappropriate carcass weights (too light and too heavy) accounting for a loss of US$6.46 (NBQA 2000). Over the years that the audit was conducted, the value-loss for insufficient marbling decreased by only US$0.72 from 1991 with an increase to US$28.41 reported in the 1995 NBQA. Overall, the results indicated that insufficient marbling is highly variable and is an area where major improvements can be made in the U.S. cattle industry. One of the 'Top Ten Quality

Challenges' set out in the NBQA-2000 was to 'improve the level of marbling to better meet the demand for Choice and Prime carcasses' (NBQA 2000). Researchers have thus been challenged to determine the causes of marbling and develop methods to control marbling in cattle. The next NBQA in the U.S. will take place in 2005 (McKenna et al., 2002).

The Canadian beef quality audit conducted in 1995-96 (Van Donkersgoed et al., 1997) indicated that quality defects cost the Canadian beef industry CDN$70.52 per head or CDN$189.6 million annually. Most recently, in 1999, the second such audit was conducted and indicated a loss of CDN$82.62 per head or CDN$273.7 million annually (Van Donkersgoed et al., 2001). The average grade losses for steers and heifers amounted to CDN$23.11 per head, and for cows and bulls was CDN$15.26 per head, indicating a major area where the Canadian beef industry could improve upon carcass value lose by enhancing marbling. Even though 27% of the steers were AAA and 40% of the heifers were AAA, only 2% of the heifers and 0.9% of the steers were Canada prime (Van Donkersgoed et al., 2001). The next Canadian beef quality audit is planned to take place in 2003/04 pending funding from the National Beef Industry Development Fund (personal communication, Rob McNabb, P. Ag. Canadian Cattleman's Association).

In 2001, the National Renderers Association reported that 49% of a slaughtered cow was used for meat production, whereas the other 51% was used in rendering (National Renderers Association, 2002). Even though rendering provides products ranging from animal feeds to consumer products such as candles and tires, this represents a major economic loss for beef producers who would prefer to increase the conversion of feed to well-marbled meat. As such in 2001, over 18.2 billion pounds of rendered

products were produced in the U.S. with an estimated production value of US$2.7 billion for the rendering industry.

2.3 The Importance of Marbling in the Canadian Cattle Market

Once cattle are determined to be ready for slaughter, they may be sold using two methods. First, cattle can be sold for a price that has been determined by the current market trend that is based on the daily average. This method does not require the individual producer to know the yield or quality of their animals and does not reward or penalise carcasses with high or low quality grades. A second method incorporates value-based marketing (VBM) of the animals and depends on the marbling grade, the yield grade and the slaughter weight of the animal (Alberta Agriculture, 2003). By applying VBM, carcass merits may reward or penalise the cattle. VBM therefore creates an opportunity for producers to be rewarded for using above-average genetics, with control over the price a particular animal may receive being placed directly in the hands of the producer with less emphasis on current market trends. For example, Cargill Foods at High River, Alberta, Canada, in an alliance with Western Feedlots, has a base for premiums of Y2/AA with the highest price rewarded to a Y1/AAA carcass (Alberta Agriculture, 2003). Carcasses with low meat yields and/or poor marbling are discounted under the grid system. The base of the price grid system is determined based on the sales from the previous week (personal communication, Cargill Foods). Current pricing schemes are available from the individual packer and it is recommended to producers to obtain such intion. Gracey (2003), however, stated that grids have two major flaws: by basing the price on the previous week's live prices, market movement is being ignored,

and, the cattle that are presently being graded do not figure into the price discovery process. Similar grid systems also exist for XL Beef and Lakeside – IBP, both packing companies in Alberta. Thus, the packers reward producers supplying high yielding and good marbling cattle (among other desirable traits such as carcass weight) with higher payoffs, but improvements need to be made to reflect daily changes in the market. See Table 2 for a sample grid.

2.4 Digestive Physiology in Ruminant Animals

Ruminants have stomachs that consist of four compartments: the rumen, reticulum, omasum and abomasum. The rumen and reticulum are often considered together as the reticulorumen because of their unclear separation (Whitehead, 2000). Within the rumen a large population of bacteria (25 – 50 billion L^{-1}), protozoa (200 – 500 thousand L^{-1}) and anaerobic fungi secrete enzymes that carry out partial food digestion representing the first stage of digestion (Whitehead, 2000). Microbial digestion of cellulose and hemicellulose (from roughages) and starch (from grains) results in the production of energy rich by-products called volatile fatty acids (VFAs) which are absorbed by the animal through the rumen wall providing a major source of energy for the animal (Whitehead, 2000). Some starch is not digested in the rumen and is passed on to the abomasum and small intestine (Whitehead, 2000). To further aid in digestion, the ruminant will 'chew their cud' whereby they re-chew food products that are not fully digested thus increasing the surface area for ruminant digestion.

Table 2. Value-based marketing grid sample used to grade carcasses in Canadian packing plants. Sample grid is in Canadian dollars. Those values in brackets indicate a discount to the base price, whereas values not in brackets indicate a reward for higher quality carcasses. Carcass weight, cwt (a measure of per 100 lb. carcass weight). For example, the reward for a carcass with AAA marbling, weighing 880 lb. is calculated as follows: $13.00 - $5.00 = $8.00/cwt reward, therefore the total reward would be $8.00 * 8.8 = $70.40.

Base Price	Optimum carcass weight range 550 to 850 lb.		
$165	Y-1	Y-2	Y-3
Prime	$19.00	$16.00	$13.00
AAA	$13.00	$10.00	$7.00
AA	$3.00	Base	($3.00)
A	($1.00)	($4.00)	($7.00)
< 550 lb. Discount –$ 20/cwt for each cell			
> 850 lb. Discount – $ 5/cwt for each cell			
> 950 lb. Discount – $ 30/cwt for each cell			

The second stage of digestion, which occurs in the abomasum and the intestines, is similar to simple-stomached animals and is performed by enzymes secreted by the ruminant itself (Whitehead, 2000). Substrates for these enzymes consist of partially digested dietary materials and microbial biomass synthesised in the rumen (Whitehead, 2000). Three advantages arise from the ruminant two-stage digestion: (i) diets that consist entirely of fibrous material can be utilised, (ii) nutrients that are inadequate in the diet, such as B vitamins and individual amino acids are synthesised by rumen microorganisms, and (iii) non-protein forms of N and S are incorporated into the proteins of the microorganisms' biomass enabling these forms of N and S to be utilised during the second digestion stage (Whitehead, 2000).

Digestion begins with chewed food being mixed with saliva produced in amounts upwards of 150 L day^{-1} (Maekawa et al., 2002), which facilitates swallowing. Saliva contains bicarbonate and phosphate ions that buffer rumen pH (between 5.5 and 7.2), and provides the necessary fluid volume for fermentation to occur in the rumen. Soluble compounds produced in the rumen are absorbed directly from the rumen into the bloodstream or are incorporated into the microbial biomass (Whitehead, 2000). The microorganisms digest the plant fibre and produce VFAs. Partially digested food passes to the omasum where water is removed and then to the abomasum where gastric juices (containing hydrochloric acid) are secreted thus reducing pH and activating the digestive enzyme pepsin. Secretions from the pancreas and liver neutralise the acid and partially digested proteins are hydrolysed to amino acids. Soluble products of digestion are absorbed into the bloodstream after enzyme secretion occurs into the small intestine from the pancreas, gall bladder and intestinal walls. Water and bicarbonate absorbtion occurs

in the large intestine with little digestion occurring. As a general rule, the digestibility of plant material declines as the content of lignin in the cell wall increases. Thus, the physical breakdown of the plant cell wall has a direct impact on the availability of nutrients from the cytoplasm. Whitehead (2000) conducted a thorough review of the metabolic requirement for nutrient elements as well as their homeostatic control. Vernon (1980) and Hocquette and Bauchart (1999) have published reviews that outline, in detail, the partitioning, origin and fate of the various classes of fatty acids between the major body tissues.

2.5 Bovine Fat Depots

During times of decreased food availability or during extended periods away from an immediate source of food, animals must have a source of energy that is both light and compact. Triacylglycerol (TAG) serves this purpose because it is energy dense and hydrophobic, as one gram of TAG contains virtually no water whereas a gram of glycogen contains upwards of 75% water. The basic structure of TAG consists of a glycerol backbone to which three fatty acid moieties are esterified at the *sn*-1, *sn*-2 and *sn*-3 carbon positions of the glycerol backbone (Gurr and Harwood, 1991). The glycerol molecule does not exhibit rotational symmetry and thus carbon atoms are classified by stereospecific numbering (*sn*). TAG can be stored in virtually any cell of the body (Pond, 1992). Lower vertabrates store the majority of their TAG in skeletal muscle and liver whereas higher vertebrates have evolved a speciality type of cell known as the adipocyte to store TAGs. Hence, the evolutionary development of TAG storage in adipocytes is restricted to higher animals. Adipocytes are the major cellular component of adipose

tissue with the other major cellular components being connective tissue and preadipocytes (also known as fibroblast-like mesenchymal cells). Adipose tissue can be either white or brown with the latter performing a role in non-shivering thermogenesis (Pond, 1999). White adipose tissue (WAT) is characterized by an abundance of spherical adipocytes that each contain a unilocular drop of lipid and a small volume of cytoplasmic material at the cell periphery along with the cell nucleus (Figure 4). Growth of adipose tissue must account for both hyperplastic (increases in cell number) and hypertrophic (increases in cell volume) growth. Hyperplasia refers to the differentiation of preadipocyte cells found throughout the adipose tissue, whereas hypertrophy mainly involves the accumulation of intracellular lipids.

The evolution of homeothermy freed animals from certain environmental constraints but caused an increased metabolic strain on the animal (Pond, 1992) that is reflected in the complex arrangement of adipose depots in mammals and birds. Anatomical studies by Pond (1986) showed that there are some 16 adipose depots in mammals and birds in the abdominal cavity, within the musculature and under the skin. In a study of cattle adipose tissue growth from 15 to 65% mature weight, Robelin (1981) demonstrated that the largest depot is located intermuscularly accounting for about 47% of total adipose tissue fresh weight. The internal adipose depots (pelvic, channel, kidney, peritoneal, mesenteric and epicardial fat) (Figure5) account for 37% of total adipose tissue fresh weight whereas subcutaneous fat (16% total adipose tissue fresh weight) accounts for the rest of the weight (Robelin, 1981). The study by Robelin (1981) did not account for the deposition of marbling fat, but later studies indicated that this depot represents the second least (of 6 adipose depots) amount of fat in the adipose depots of

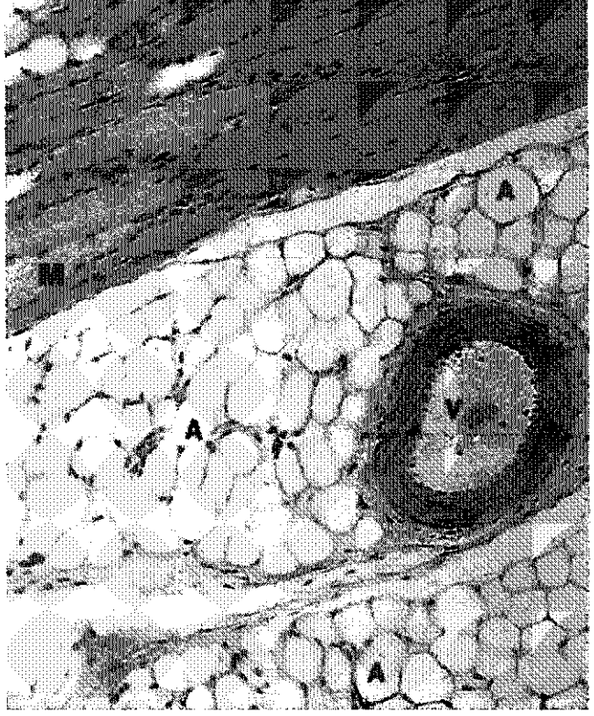

Figure 4. Histochemical section of mature adipose tissue (A – adipocyte, V – blood vessel, M – muscle fibre). Fat cells are unilocular (contain one large droplet of fat per cell) with the fat within the adipocyte making up the major volume of the cell with the aqueous cytoplasm surrounding the droplet and the nucleus located in the periphery of the cell. Adipose tissue remains highly vascularized fulfilling the requirement for rapid removal of lipid from the circulation or addition to the circulation for energy needs. Image adapted from Colour Atlas of Basic Histology, Ed. I. Berman, Appleton & Lange, 1993.

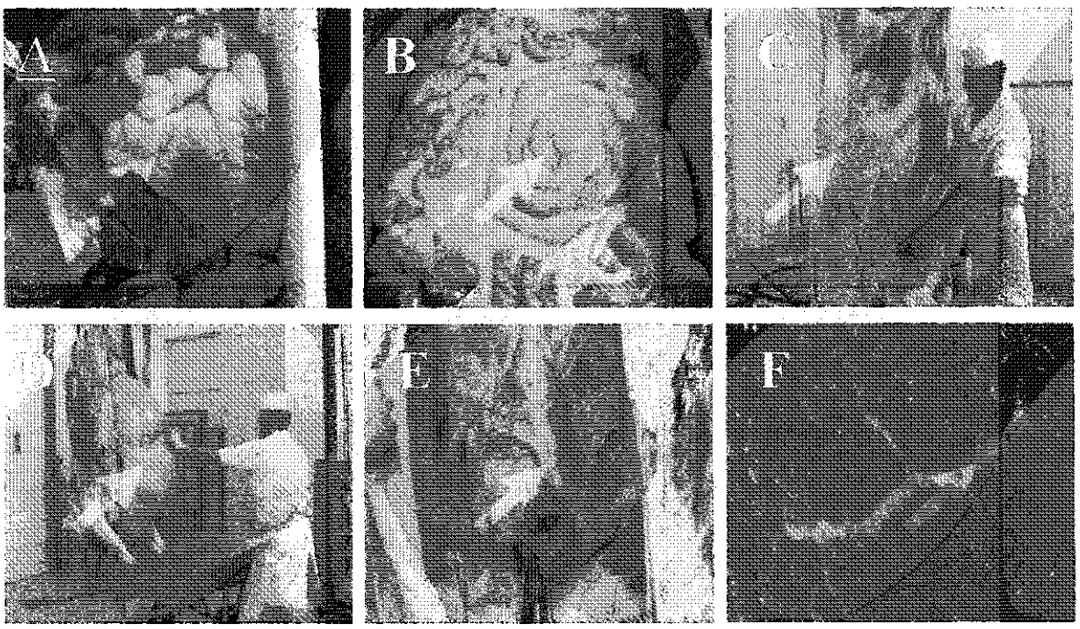

Figure 5. Distribution of adipose tissue in different adipose depots in cattle. Arrows indicate the respective location of: A, epicardial adipose tissue; B, mesenteric (intestinal) adipose tissue; C, subcutaneous adipose tissue; D, omental adipose tissue; E, perirenal adipose tissue; and F, intramuscular adipose tissue. Images taken at the Research Institute for the Biology of Farm Animals (FBN), Dummerstorf, Germany.

the bovine carcass (Cianzio et al., 1985).

The foundational research by Hammond (1955) in bovine adipose tissue development demonstrated that the adipose depots in cattle are deposited in the following general order (all with a certain degree of overlap): perirenal, intermuscular, subcutaneous, and intramuscular. Each adipose depot, however, grows at a different rate and differently with respect to hypertrophy or hyperplasia. As a general rule, during the finishing phase, the early developing depots (i.e. intermuscular, perirenal and mesenteric fat) have completed their hyperplastic development and thus deposit fat by hypertrophy, whereas the subcutaneous and i.m. depots continue to develop by hyperplasia and hypertrophy of already recruited adipocytes (May et al., 1994; Sainz and Hasting, 2000). In addition, Wegner et al. (1998) clearly outlined i.m. adipocyte hyperplasia in four breeds of cattle over a 24 month period demonstrating that new adipocytes arise in muscular tissue with age.

Adipocyte hyperplasia in cattle has been shown to occur in the subcutaneous and i.m. adipose depots after 45% mature weight. This is accompanied by a stabilization of adipocyte hypertrophy in early developing depots (Robelin, 1981). In addition, many other investigations have shown apparent subcutaneous (Cianzio et al., 1985; Truscott et al., 1983) and intramuscular (May et al., 1994) adipocyte hyperplasia in cattle. Stabilization occurred at a mean adipocyte cell size of 50×10^4 μm^3 (cellular diameter of 100 μm) indicating that a critical cell size leads to the redirection of energy from adipocyte hypertrophy to hyperplasia as indicated by new adipocyte formation (Robelin, 1981). A relationship among adipose depots for cell size and total mass has led to the subcutaneous adipose depot being viewed as an indicator of overall adipose tissue growth

in the animal (Robelin, 1981). The majority of research in cattle has therefore focussed on the subcutaneous adipose depot for the reasons outlined above and the i.m. adipose depot as an indicator of beef quality. In addition, investigations into bovine subcutaneous fat development have shown that adipocyte differentiation precedes the induction of a number of lipogenic genes (Smith et al., 1984). Stearoyl Coenzyme A desaturase was shown in subcutaneous fat to indicate the extent of terminal differentiation in the bovine (Martin et al., 1999).

Neurogenic muscular steatosis is a muscular condition characterised by a replacement of muscle fibres by adipocytes (Ohfuji, 1999). The condition principally affects feedlot cattle and may be indicated prior to slaughter by an abnormal gait, but is usually only found after the animal has been slaughtered. The condition is characterised by a posterior weakness and almost all skeletal and diaphragmatic muscles of the carcass contained considerable adipose tissue with some fascicles having complete exchange of muscle fibres with adipocytes (Ohfuji, 1999). A study by Link et al. (1967) also indicated that biopsy results in the occurrence of steatosis in bovine skeletal muscle. Anecdotal evidence from Swatland (2003) has indicated that muscular steatosis occurs in cases of muscle damage or denervation brought about by strenuous muscle exertion, particularly in the muscles used when an animal rears on its hind legs. Inspection protocols of the FSIS of the USDA have indicated that the condition does not affect the carcass therefore the affected area is removed and the carcass is passed for food (Cattle Head Inspection, 2003). Difficulties arise during slaughter because there is no clear scientifically determined line between excessive i.m. fat deposition and steatosis, with the only restriction being that steatosis may occur in only one muscle group whereas carcasses

with excessive marbling have this characteristic in all muscle groups (Swatland, 2003). Steatosis is also observed in the liver (Schouvert, 2000) and in the pancreas (Kelley et al., 1996) of cattle. Research has yet to be carried out to determine if cells that have undergone steatosis are adipocytes that have arisen from preadipocytes or if the native cells (i.e. muscle fibres) have been converted to adipocytes.

In addition to the function of adipose tissue as a source of energy and its use by the animal in insulation and shock protection, recent research has been conducted investigating the role of adipose tissue as a an endocrine organ (reviewed in Vernon and Houseknecht, 2000 and Ahima and Flier, 2000a). Briefly, adipose tissue is known to metabolize sex steroids and glucocorticoids, produce and secrete inflammatory cytokines (i.e. tumour necrosis factor α), regulate proteins involved in coagulation and fibrinolytic pathways and express proteins of the renin-angiotensin system.

2.6 The Search for Predictors of Intramuscular Fat Deposition in Cattle

In order for selection based on marbling to occur, biochemical and/or genetic markers must be discovered that can be used as indicators of the degree of marbling in an animal prior to slaughter. Studies have therefore been conducted to look for trends between cattle carcass characteristics, or genetic and biochemical measurements and intramuscularly deposited fat. Studies have included the analysis of lipid biosynthetic enzymes (Middleton et al., 1998), the use of image analysis (Kuchida et al., 2000), ultrasound evaluation (Hassen et al., 1999), correlation studies of the fatty acid composition of meat (Kazala et al., 1999) subcutaneous fat thickness (Jeremiah, 1996) and serum hormones levels (Wegner et al., 2001).

In addition, Australian researchers have marketed the first commercially based genetic test for marbling entitled geneSTAR marbling; a genetic test that measures the allelic frequency of the thyroglobulin gene (Genetic Solutions, 2002). The same group also released the geneSTAR tenderness test that detects variants of the bovine gene for the enzyme calpastatin (Genetic Solutions, 2002). These tests are currently being used in the selection and breeding programs of Australian farmers with markets in 2002 opening up in South Africa (Genetic Solutions, 2002) and in the U.S..

Research work conducted by Fitzsimmons et al. (1998) demonstrated variants in the obese gene that led to the development of a DNA-based test for marbling by Quantum Genetics Inc., an Alberta/Saskatchewan, Canada-based company (Thomas, 2002). The test essentially looks for the presence of a thymine residue, which increases fat deposition, whereas the presence of a cytosine residue produces a leaner carcass. Breeders and producers alike can thus use DNA-based analysis to develop cattle that deposit greater amounts of marbling fat in order to supply consumer demands.

Contrary to popular perceptions, beef is a valuable nutritional product. Beef is an important source of bioavailable Fe, Zn and other trace element such as Cu, and Se, along with B and D vitamins. Current medical recommendations for fat intake state that energy intake from fat should not exceed 30-35% of the total energy intake, and that saturated fatty acids (SFAs) should not exceed 10% of total energy intake (Moloney et al., 2001). Currently, SFAs represent less than half of the total FAs in beef, with 30% of that represented by stearic acid, a FA known to be neutral in its effects on plasma cholesterol levels (Bonanome and Grundy, 1988).

With the current global emphasis on genomics, the potential to select animals for specific genetic traits has led to one of the greatest revolutions in animal agriculture since Robert Bakewell began selecting cattle for milk or meat production (MacLachlan, 2001). With the advancement in scientific knowledge dealing with the control of livestock traits, a balance must be drawn between advancements obtained through genetic selection and potential drawbacks. Certain breeds have already been selected for high muscle development (Belgian Blue) based on the expression of the myostatin gene (McPherron and Lee, 1997). These double-muscled cattle have reduced meat quality due to the prevalence of glycolytic fibres, accelerated post-mortem glycolysis (which causes rapid falls in post-mortem pH with rapid rises in lactic acid) and a greater sensitivity to stress (Hocquette et al., 1998b). The genes controlling other traits, such as fat deposition, are under investigation.

2.7 Microscopic and Histochemical Investigations of Intramuscular Fat

Microscopically, i.m. fat comprises a true adipose tissue being composed of adipocytes embedded in a connective tissue matrix and occurring in close proximity to a blood capillary network (Hood and Allen, 1973) (see Figure 6). In addition, i.m. adipocytes are generally smaller in diameter (Cianzio et al., 1985) and generally occur in clusters of 15 to 20 adipocyte cells (Figure 7) deposited between bundles of muscle fibres in the perimycium (Nishimura et al., 1999). Mature adipose tissues have been investigated using histochemical techniques for many years (Gurr and Kirtland, 1978). Techniques for staining adipocytes are varied and many staining protocols, such as Oil Red O and Sudan Black B, have been developed to preferentially stain adipocytes

(Hausman, 1981). Additional staining methods focus on staining cells and structures surrounding adipocytes while leaving adipocytes unstained (see Figure 7). Nevertheless, the limitation of these procedures is that the objects that are stained must contain structures that can bind to the stain in use and the objects must be visible using conventional microscopy. Therefore, only differentiated adipocytes can be identified. Undifferentiated adipocytes (i.e. preadipocytes) however, cannot be distinguished solely on the basis of their morphology. Attempts were therefore undertaken to develop immunohistochemical techniques to locate and determine the number of preadipocytes in order to assess the size of the preadipocyte population in different tissues.

Histochemical studies with rats indicated that S-100 protein, thought to be a carrier of fatty acids in the lipolytic mechanisms of adipocytes (Haimoto et al., 1985), is present in normal, early, and late preadipocytes present in mature epididymal adipose tissue *in vivo* and *in vitro* (Cinti et al., 1989). Additional studies indicated that S-100 protein is expressed in preadipocyte cells of human embryonal subcutis origin (Atanassova, 2001). Both studies indicated that S-100 protein might be used as a reliable biochemical and immunocytochemical marker to distinguish early preadipocyte cells from fibroblast cells. S-100 protein was expressed, however, in mature adipocytes, thus limiting the use of S-100 protein as a preadipocyte-specific marker.

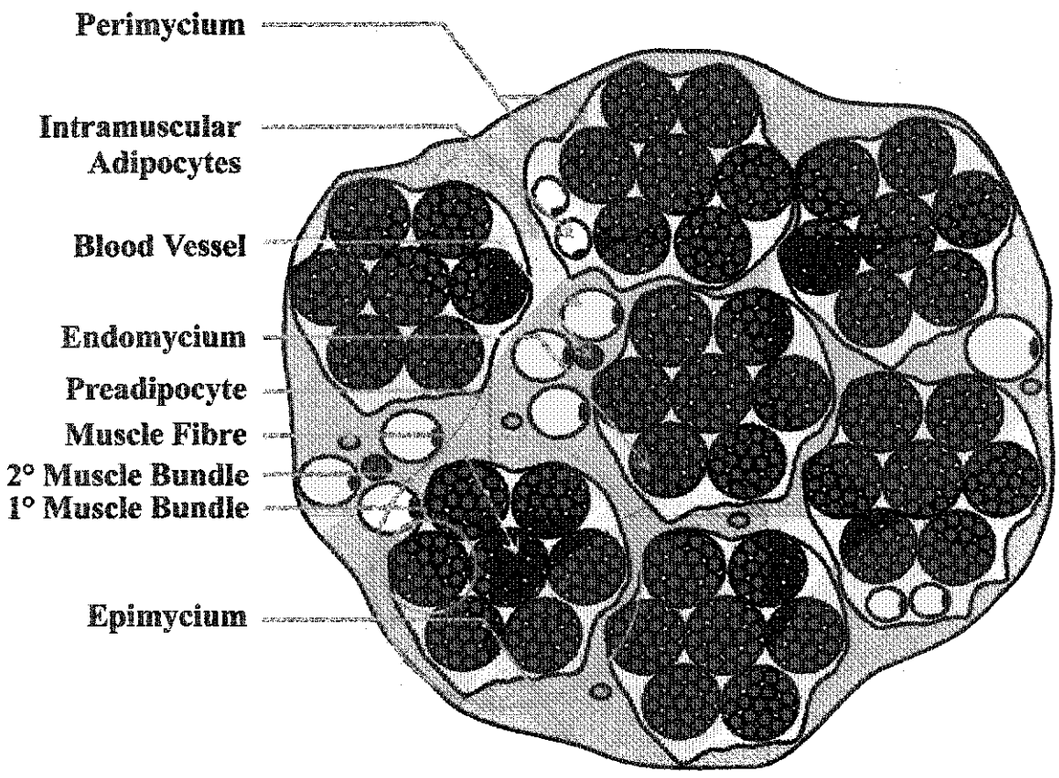

Figure 6. Muscle fibre structure indicating the location of major components of muscle fibres along with extramyocellular adipocytes. Intramyocellular triacylglycerol is not indicated in the individual muscle fibres.

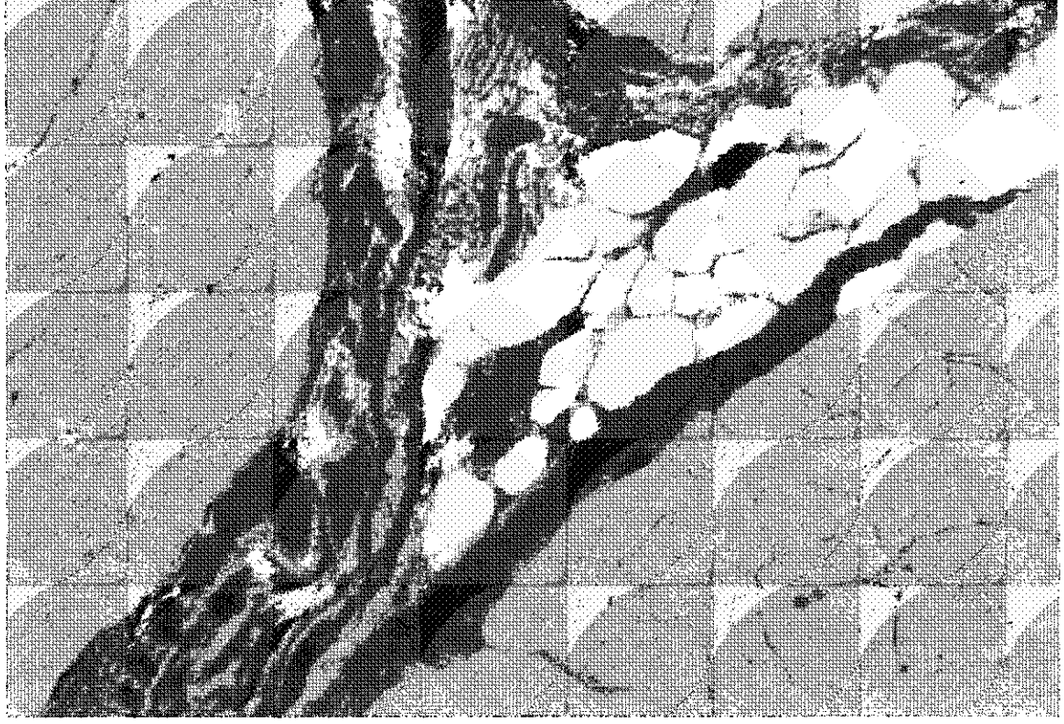

Figure 7. Histochemical section of bovine *longissimus dorsi* muscle. The section was stained to differentiate among connective tissue (blue), muscle fibres (orange), and intramuscular adipocytes (white). Image obtained from Dr. Jochen Wegner (http://www.fbn-dummerstorf.de/fb6/wegner/microphoto.htm) of the Research Institute for the Biology of Farm Animals, Dummerstorf, Germany.

Analyses by Wright and Hausman (1990) also indicated that certain cell surface components could be used as targets for differentiating between immature and mature adipocytes. The monoclonal antibodies AD-1 and AD-2 were developed by Wright and Hausman (1990), and later Yu et al. (1997) developed the AD-3 antibody, to an unidentified cell surface protein, as a marker of preadipocytes.

Structural and histochemical analyses by Hausman and Thomas (1986) indicated that a positive relationship existed between adipocyte cluster size and the proximity to the entry point of large (parent) blood vessels in both perirenal and subcutaneous adipose tissues. Such a relationship indicates an apparent dependence on blood flow for lipid deposition and capillary development. In addition, the supply of blood vessels within 1° and 2° muscle bundles has been shown to result in the formation of i.m. adipose tissue in Japanese Black cattle (Hoshino et al., 1987). Results indicated that adipose tissues were formed around the vessels within the 2° muscle bundles. Thus, the supply of nutrients to the muscle tissue may stimulate the formation of i.m. adipocytes.

2.8 Investigations into the Deposition of Muscular Fat

From a meat producer perspective, fat deposited in the muscle serves the function of increasing the juiciness, improving the flavour and slightly increasing the tenderness of the meat that they produce. i.m. fat may or may not serve a physiological function, however, because i.m. adipocytes may arise from muscular repair mechanisms. In addition, in cases such as Duchenne and Becker muscular dystrophy, i.m. adipocytes may arise from the lack of certain integral proteins. Therefore, i.m. adipocytes may be considered as a vestigial tissue or may arise as a supplementary depot for FAs not

oxidized by muscle fibres. Investigations have been conducted to elucidate the biological purpose and function of skeletal muscle lipid deposition and metabolism.

Skeletal muscle serves four main physiological functions: growth, posture, physical activity and thermogenesis (Hocquette et al., 1998b). The energy requirements of each of these functions are fulfilled by extramuscular sources that include non-esterified fatty acids (NEFAs), TAG, VFA and ketone bodies (KBs) and intracellular storage molecules (glycogen and TAG) (Hocquette et al., 1998b). The partitioning, metabolism and incorporation pathways for the different FA classes have been thoroughly reviewed (Hocquette and Bauchart, 1999). Briefly, the majority of acetate and KBs extracted by muscle is directly and immediately oxidized whereas 75% of the NEFAs extracted by muscles enters the i.m. pool of TAG (consisting of both i.m. adipocytes and TAG in muscle fibres). NEFA muscle uptake is most likely controlled by the differential partitioning among binding sites in the blood (albumin), plasma membranes and intracellular fatty acid binding protein (FABP). The FABP may transfer FA to oxidation or storage (esterification) sites (Veerkamp, 1995), however, because i.m. adipocytes express a different FABP, marbling scores are not related to muscle FABP activity levels (Moore et al., 1991).

When examining fat in muscle tissue, the situation is complex because TAG is found in muscle tissue in both intramyocellular and extramyocellular locations (Guo, 2001). Intramyocellular TAG (imcTAG) is found within individual muscle fibres, and is strongly correlated with insulin resistance in human obesity and diabetes (Storlien et al., 1991). imcTAG is highly variable among muscle fibre types with type I (slow-twitch oxidative) > type IIA (fast twitch oxido-glycolytic) > type IIB (fast-twitch glycolytic)

(Guo and Jensen, 1998; Malenfant et al., 2001a). Variability in imcTAG content also exists among muscles and organisms (reviewed in Guo, 2001). Reports have also shown that imcTAG is depleted after exercise in highly trained athletes (Johnson et al., 2002), but not in obese human subjects after diet and exercise training (Malenfant et al., 2001b), possibly indicative of defective lipid oxidation or differences in lipid metabolism occurring after exercise in obese subjects. In addition, imcTAG depletion, resulting from dietary lipid deprivation, has recently been shown to reverse insulin resistance in morbidly obese humans (Greco et al., 2002). In humans, imcTAG content is about 1% of the total TAG content of muscle (Figure 8) (Gorski, 1992), indicative of the importance of extramyocellular TAG (emcTAG) in muscle as it relates to the storage lipid as an energy source.

Adipocytes that develop within muscle tissue (i.m. adipocytes) are believed to develop for the purpose of providing an immediate supply of energy to the muscle cells. The fatty acids released by hormone sensitive lipase (HSL) (Kazala et al., 2003; Langin et al., 1996) and other lipases from i.m. adipocytes may be a physiological insignificant energy source due to low interstitial concentrations of albumin in skeletal muscle (Martin, 1996). Nevertheless, no conclusive evidence exists for any physiological advantage to animals that develop i.m. adipocytes. I.m. adipocytes may serve as storage site for FAs that are not oxidized by muscle fibres (personal communication, Dr. Gregory Harper, Fat Deposition Project Leader, CSIRO Livestock Industries, Australia) but FAs from i.m. adipocytes may have to be shunted into circulation to serve as a source of energy. As such, investigations into skeletal muscle lipid metabolism have been undertaken to characterize how bovine muscle differentially metabolizes energy from a TAG

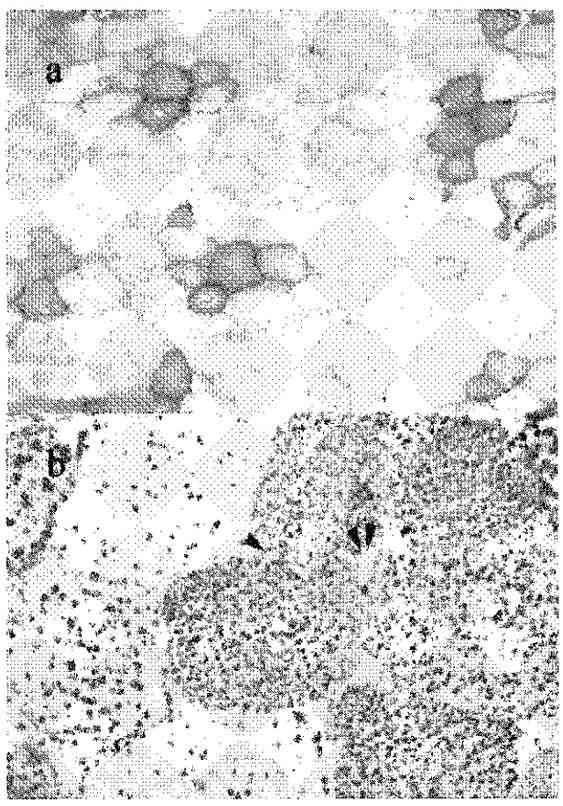

Figure 8. Intramyocellular triacylglycerol (TAG) in **a**, porcine muscle fibres stained with Sudan Black B and **b**, human m. *vastus lateralis* stained with oil red O to emphasise the location of TAG in the sarcoplasm. In **b**, the single arrowhead indicates the lumen of a blood vessel lacking oil red O staining, while the double arrowhead indicates the extramyocellular space lacking oil red O staining. The muscle fibres containing the greatest amount of lipid droplets are type I red, slow-twitch oxidative fibres. Image **a** was adapted from the homepage of Dr. Swatland (http://www.aps.uoguelph.ca/~swatland/ch7_2.htm) and image **b** was obtained from Koopman et al. (2001).

source (both imcTAG and emcTAG) as compared to from a glycogen source (Hocquette et al., 1998b).

Investigations analysing the relative contribution of the three major lipogenic precursors, acetate, lactate, and glucose, showed that glucose provided 50-75% of the acetyl units in the i.m. fat depot whereas acetate provided 10-25% (Smith and Crouse, 1984). Results also indicate that i.m. fat may be manipulated without any additional deposition of fat to other adipose depots. A bovine i.m. preadipocyte cell line was developed by Aso et al. (1995) from Japanese Black cattle that displayed a transient increase in glucose uptake during the first 5-day period of differentiation with acetate being preferentially used as the source of carbon for TAG formation throughout the 5-day trial. Additionally, only glucose transporter (GLUT)-1 and not GLUT-4 were detectable during differentiation. GLUT-4 is considered to be a major glucose transporter protein in muscle and adipose tissue and is insulin dependent, whereas GLUT-1 is responsible for transporting glucose across the blood-brain-barrier and is independent of insulin (Peters et al., 2002) and may explain the insulin-independent adipocyte differentiation described by Suryawan and Hu (1995).

A stem cell population that is maintained by 'asymmetric' division in muscle tissue is believed to serve as the source of preadipocytes in humans (Grounds, 1999). As such, for each cell that differentiates into an adipocyte, another daughter cell is returned to the stem cell pool creating a possibly endless source of i.m. adipocytes. Additionally, some stem cells in muscle come from a bone marrow source via the blood stream (Grounds, 1999) possibly indicating that quantification of adipocyte precursor cells in muscle tissue may not be a viable indication of marbling potential in an animal.

2.9 The Importance of Lipoprotein Lipase in Muscle Fat Accretion

The hydrolysis of plasma TAG in chylomicrons and very low-density lipoproteins (VLDLs) is catalysed by the capillary-bound enzyme lipoprotein lipase (LPL) that has a role in the regulation of plasma TAG concentrations and TAG fatty acid assimilation into the muscle. Investigations have been undertaken to investigate the relationship between LPL expression and activity with the deposition of muscular fat in ruminants. In a study analysing the partitioning of free fatty acids (FFAs) in adult sheep, 55-60% of FFAs originate from hydrolysis of circulating TAG catalyzed by LPL with utilization by skeletal and heart muscle amounting to approximately 40% (Pethick and Dunshea, 1993). Hocquette et al. (1998a) investigated the differential expression of LPL in bovine muscle and showed that LPL transcript levels were higher in bovine heart and oxidative skeletal muscle fibres which preferentially use FFA as a source of energy than in fast-twitch glycolytic muscle fibres which preferentially use glucose as a fuel source. LPL activity has been shown to parallel the imcTAG content levels observed by Guo and Jensen (1998) and Malenfant et al. (2001a). Hocquette et al. (1998a) also indicated that higher LPL activity levels were related to a larger size of adipocyte. An increase in the activity level of muscle LPL may lead to a greater deposition of i.m. fat. In addition, investigations have shown that muscle LPL and adipose LPL are reciprocally regulated suggesting that muscle and adipose tissue communicate with respect to FA distribution.

In an investigation of TAG clearance in rats fed ^{14}C-labelled triolein, Bessesen et al. (1995) demonstrated that muscle tissue was the primary site for TAG clearance. Microanatomical studies have shown that no adipocytes are found in the interfascicular space (between muscle bundles) or in the intrafascicular space (between muscle fibres) in

three rat muscles (Guo et al., 2001). These findings indicate that rat muscles (and perhaps other rodent muscles) are not good systems to investigate the deposition of i.m. fat. Conversely, investigations with rat muscle may be useful in identifying factors associated with the deposition of emcTAG. As such, no investigation has been undertaken to determine if rat muscle contains preadipocyte cells to confirm the microanatomical studies. Nevertheless, skeletal muscle, through the action of LPL, has the potential to strongly influence TAG trafficking, storage and oxidation. Similar investigations in ruminants have yet to be undertaken.

It may be possible to improve the deposition of i.m. fat by controlling lipase activity once TAG has been initially deposited in the muscle. Katamoto et al. (1996) reported that Japanese Black cattle exhibiting fat necrosis had higher LPL levels than a control group. In addition, serum concentrations of TAG, cholesterol, and total lipid in α-lipoprotein were lower as a result of the increased LPL activity. Because LPL controls the partitioning of TAG between muscle and adipose tissues, the enzyme has been shown to have a direct role in controlling fat necrosis.

2.10 Current Systems Utilized in the Analysis of Adipogenesis and Adipocyte Metabolism

Some of the complexities of adipocyte and adipose tissue development have been investigated through the use of cell culture systems that facilitate the analysis of preadipocytes progressing into mature adipocytes without the influence of other cell types in the developmental environment. The models used most frequently are subclones of the 3T3 cell line (Green and Kehinde, 1974) or the ob17 cell line (Negrel et al., 1978).

Plaas and Cryer (1980) and Cryer et al. (1984) investigated the development of bovine adipocytes in cell culture systems in serum-containing media. Cultures were developed from the stromal vascular fraction obtained from bovine subcutaneous adipose tissue. LPL activity levels were low prior to confluence (reviewed in Suryawan and Hu, 1995). After differentiation, cells produced substantial amounts of lipid and LPL activity. The inclusion of insulin, however, did not affect LPL activity, as in humans and rats, indicating that bovine preadipocytes do not require insulin to undergo cellular differentiation (Suryawan and Hu, 1995). Insulin effects in cattle may be breed dependent, however, as observed by Miller et al. (1991) in tissue explants from subcutaneous and i.m. adipose depots.

To advance our knowledge of cellular development in living systems, co-culture systems have been developed whereby two different cell types are cultured together in order to better mimic the cellular interactions that may occur *in vivo*. In order to investigate the regulation of fat and muscle deposition in meat animals, Dodson et al. (1997) developed the first muscle and fat co-culture system using sheep myogenic and preadipocyte stem cells, and demonstrated that each cell type may influence the expression of Insulin-like Growth Factor (IGF) binding proteins. Co-culture systems may also facilitate investigations aimed at characterizing factors involved in the migration, proliferation and differentiation of satellite cells in muscle tissue. Membranes currently in use to physically separate the cells may be detrimental to cellular integrity as well as limit cellular differentiation (Dodson et al., 1997). Additional investigations using muscle and fat co-culture systems in humans have shown that the release of fat cell factors (such as

troglitazone) induce insulin resistance in human skeletal muscle cells (Dietze et al., 2002).

Methods for studying adipose tissue metabolism *in vivo* can be divided into the following four areas: (i) whole-body turnover measurements, (ii) measurements of arteriovenous differences, (iii) microdialysis measurements, and (iv) adipose tissue blood flow measurements (Frayn et al., 1997). In human research, measurements of arterio-venous differences are at the forefront, because it is possible to catheterise the venous drainage of the subcutaneous abdominal depot (Frayn, 1999), but not intra-abdominal adipose tissue.

The biological importance of WAT has been better characterized with the development of a line of transgenic mice that do not develop WAT (Moitra et al., 1998). Mice lacking WAT had enlarged internal organs, steatosis of the liver, were diabetic, and had reduced leptin. Elevated serum glucose, insulin, FFAs and TAG also demonstrated the importance of WAT in growth, glucose metabolism, reproduction and toleration of fasting (Moitra et al., 1998).

2.11 The Transition from Fibroblast-like Preadipocytes to Adipocytes

Current systems to investigate adipogenesis have provided researchers with detailed information about how adipocytes form. What follows is an explanation of the current known stages of adipocyte differentiation. Nnodim (1987) provided an exhaustive historical review of the development of adipose tissue detailing original research as far back as 1845, whereas Gregoire et al. (1998) provided a more up to date outline of

adipocyte differentiation processes. The reader is referred to Figure 9, which outlines the major differentiation events.

The origin of the adipocyte precursor cell is still under debate. Research using multipotent clonal cell lines, however, has suggested that the lineage of preadipocytes originate from embryonic stem cell precursors that have the capacity to differentiate into adipocyte, chondrocyte, osteoblast and myocyte mesodermal cell types (Gregoire et al., 1998). Conversely, a bone marrow stromal cell type has been shown to give rise to adipogenic and osteogenic cells (Gregoire et al., 1998). The majority of research dealing with adipogenesis, however, has used preadipocyte cell lines already committed to an adipocyte lineage.

Preadipocytes must initially undergo growth arrest and not cell confluence or cell-cell contact to begin adipocyte differentiation (Gregoire et al., 1998). The low expression levels of adipogenic transcription factors in preadipocytes has been shown to cooperatively cause growth arrest (Altiok et al., 1997). The subsequent differentiation process comes about after growth arrest at confluence when preadipocytes receive both mitogenic and adipogenic signals. Growth-arrested cells undergo at least one round of DNA replication and cell doubling leading to clonal amplification of committed cells (Gregoire et al., 1998). Early gene expression changes include alterations of LPL transcript levels, and may reflect the growth-arrest stage of adipogenesis (Amri et al., 1986). In addition different isoforms of the peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer binding proteins (C/EBP) transcription factors (see below) are induced with PPAR transcription factors in response to hormonal differentiation induction (Brun et al., 1997).

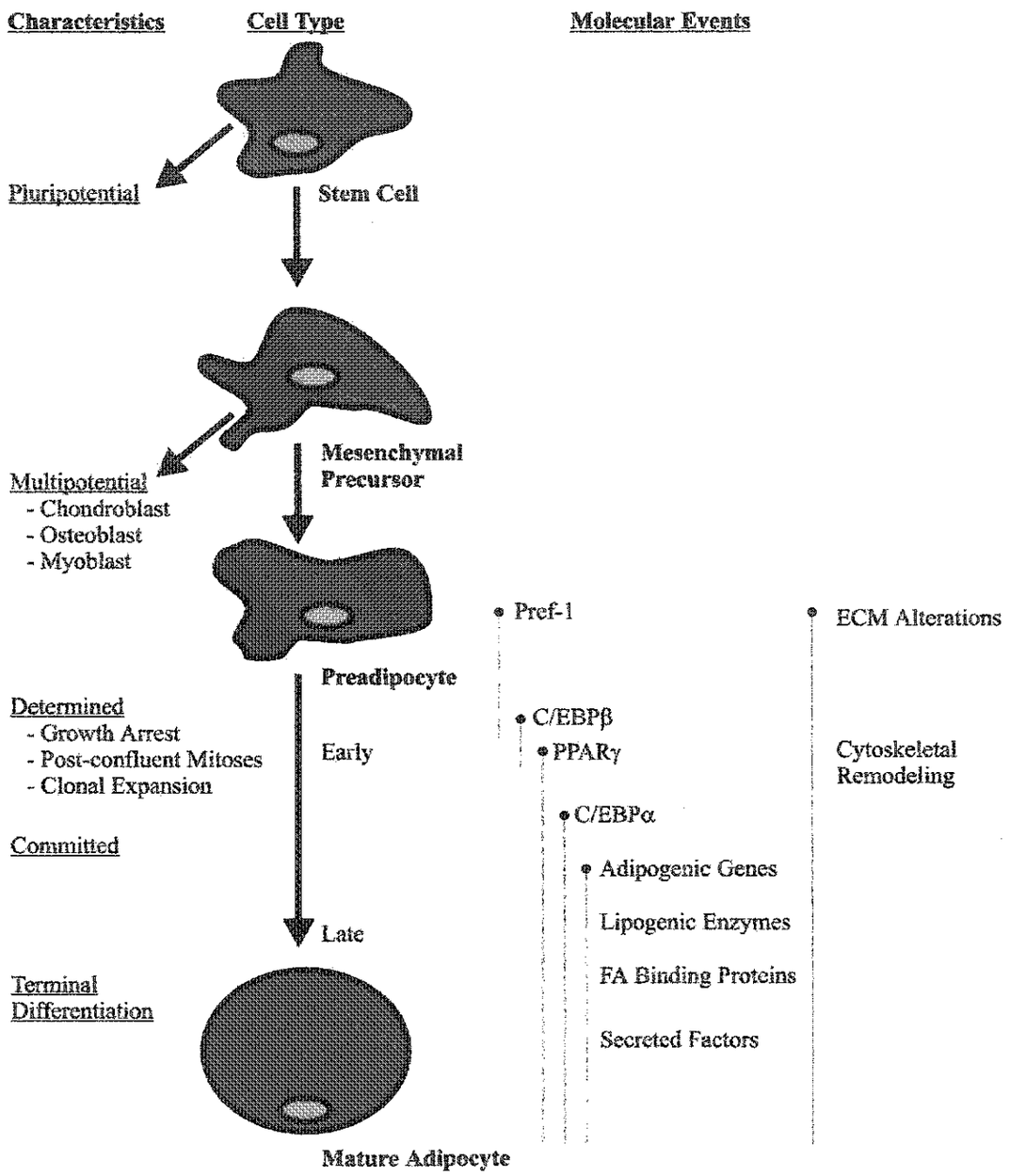

Figure 9. Adipocyte differentiation stages. Figure adapted from Gregoire et al. (1998). Abbreviations are as follows: Pref-1, preadipocyte factor-1; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; FA, fatty acid; ECM, extracellular matrix.

Also expressed early is the sterol regulatory element binding protein-1c (SREBP-1c)/adipocyte determination and differentiation factor 1 (ADD1) transcription factor involved in cholesterol metabolism and adipocyte gene expression (Rangwala and Lazar, 2000). Early changes in gene expression result in the conversion of cells from a fibroblastic to a spherical shape with concomitant changes in cell morphology, cytoskeletal changes and modifications in extracellular matrix (ECM) components (Hausman et al., 1996). Spiegelman and Farmer (1982) demonstrated that ECM modifications might be the signal for the induction of adipocyte-specific genes. Finally, early morphological changes have been shown to occur concurrently with dramatic decreases in preadipocyte factor-1 (pref-1) expression levels (see below and Smas and Sul, 1993). Late events during adipogenesis include dramatic increases in *de novo* lipogenesis, an acquisition of insulin sensitivity along with increases in the expression of TAG metabolism components (see Figure 10) (Gregoire et al., 1998). The precise stage of terminal differentiation, however, has not been clearly determined as evidence exists of cell division in partially differentiated human preadipocytes (Prins and O'Rahilly, 1997), along with the dedifferentiation of mature adipocytes (Sugihara et al., 1986). Gregoire et al. (1998) have done an elegant review of the hormones, cytokines, and growth factors that further modulate adipogenesis.

Previous analyses have indicated that once preadipocytes are shunted into the adipogenic development pathway their fate has been determined. Nonetheless, preadipocytes have been shown to rapidly and massively acquire high phagocytic activity, essentially converting to macrophages in a macrophage environment (Charriere et al., 2003).

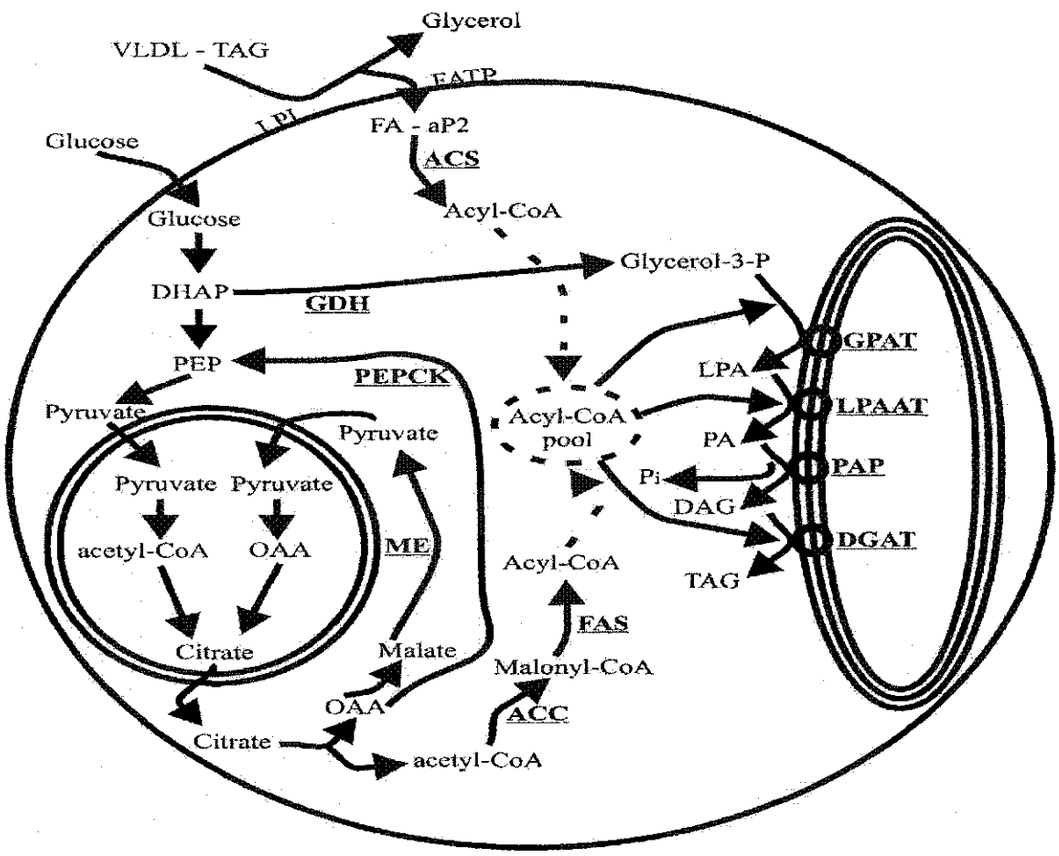

Figure 10. The fatty acid metabolic pathways in adipocytes. Double line signifies the mitochondrial compartment and the triple line signifies the endoplasmic reticulum. Abbreviations are as follows: VLDL, very low-density lipoprotein; TAG, triacylglycerol; FATP, fatty acid transport protein; LPL, lipoprotein lipase; FA, fatty acid; aP2, adipocte lipid binding protein; ACS, acyl-CoA synthetase; DHAP, dihydroxyacetone phosphate; PEP, phospoenol pyruvate; OAA, oxaloacetate; ME, malic enzyme; PEPCK, phosphoenolpyruvate carboxy kinase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; PAP, phosphatidate phosphatase; DGAT, diacylglycerol acyltransferase; LPA, lysophosphatidate; PA, phosphatidate; DAG, diacylglycerol; GDH, glycerol-phosphate dehydrogenase.

Results of this recent study have strengthened the connection between adipose tissue and the immune system and have also indicated that preadipocytes have greater plasticity than previously believed.

In addition to the formation of adipocytes from preadipocyte precursor cells, myoblasts have been shown to have the potential for adipogenesis. Myoblasts are satellite cells that reside between the sarcolemma and basal lamina of muscle fibres (Snow, 1977). These cells are known to proliferate, migrate to a site of injury, fuse with existing muscle fibres and thus regenerate damaged or degenerating fibres (Bischoff, 1994). In cell culture systems, however, myoblasts have been shown to convert into adipocyte-like cells by treatment with thiazolidinediones, potent activators of PPARγ (a known activator of adipocyte-specific gene expression, see below) (Grimaldi et al., 1997). Even though Lapsys et al. (2000) demonstrated that PPARγ is a normal regulator of FA metabolism in muscle tissue, overexpression of PPARγ and C/EBPα (another potent activator of adipocyte-specific genes) have been shown to stimulate the transdifferentiation of C2C12 myoblasts (Hu et al., 1995). Additionally, Taylor-Jones et al. (2002) demonstrated that adipogenic markers were expressed in myoblasts from older mice that were simultaneously undergoing myogenic fusion thus increasing the adipogenic potential of these cells. This may explain why muscular steatosis is observed in older animals. Interestingly, PPARγ1 and γ2 (see below) were both expressed in older myoblasts, concomitant with no repression of myogenic gene expression. Results indicated that signalling pathways in myoblasts inhibit specific functions of PPARγ contrary to previous reports that showed an inhibition of myogenic differentiation by this transcription factor class (Hu et al., 1995; Lecka-Czernik et al., 1999). Overall, results

from these investigations have suggested that, with aging, mesenchymal stem cell populations are replaced with cells that have increased adipogenic potential. Conversely, aging mesenchymal cells may inherently activate adipogenic gene programs, thus stimulating adipogenesis.

2.12 The Role of Leptin in Controlling Fat Accumulation

Once fat has been deposited in an animal, a mechanism must exist by which the adipose tissue informs the body of the energy storage status. As early as 1953, Kennedy proposed the lipostat theory whereby fat storage information is provided to the body by ways of a humoral feedback loop. In 1994, leptin, the 16-kDa protein hormone product of the *ob* gene, was discovered in mice (Zhang et al., 1994). In monogastrics, leptin has been shown to contribute to the regulation of body energy homeostasis, appetite suppression and energy expenditure (see reviews by Ahima and Flier, 2000b, Reidy and Weber, 2000 and Wauters et al., 2000 along with references therein). Leptin has been proposed to be the main factor linking metabolic status to reproduction (Barash et al., 1996; Cheung et al., 1997). Recently, Williams et al. (2002) reviewed leptin's role in the regulation of cattle reproduction. Briefly, leptin in adipose tissue and plasma leptin concentrations are positively related to the amount and status of the adipose energy store with nutritional regulation mediated by insulin. Other less characterized roles for leptin, include modulation of the reward circuitry for feeding, glucose metabolism, lipid oxidation, substrate partitioning and adipocyte apoptosis (Ahima and Flier, 2000b). Studies in humans and rodents have dominated the leptin research field. This is primarily due to the increased prevalence of obesity in humans along with the use of readily

available mouse lines that are either severely leptin resistant (*Agouti yellow*, A^Y/a) (Halaas et al., 1997) or leptin-deficient (*ob/ob*).

Analysis of bovine leptin began in 1997 after the bovine leptin mRNA and gene were sequenced (Tellam, 1995; Tellam, 1996). Research was bolstered by the development of a commercial 'multi-species' radioimmunoassay (RIA) for leptin (Linco Research, St. Charles, MO, USA). Leptin is mainly secreted by WAT, but is also secreted by placental and fetal tissues, mammary glands (Smith and Sheffield, 2002), stomach, muscles and brown adipose tissue (Andrews, 1998). Initial reports demonstrated that no differences existed in leptin mRNA expression among subcutaneous, perirenal and omental adipose tissues (Ji et al., 1998). Kim et al. (2000), however, reported higher leptin mRNA levels in perirenal adipose tissue, moderate levels in abdominal, subcutaneous and intermuscular adipose tissue, and low levels in i.m. adipose tissue. Ren et al. (2002) recently reported higher leptin mRNA levels in perirenal and omental AT depots with lower levels in subcutaneous fat. Breed differences were also observed.

With the close association between leptin and body fat stores observed in humans and rodents (Friedman, 2002), pigs (Robert et al., 1998) and horses (Braissant and Wahli, 1998), animal scientists sought to determine if similar relationships existed in cattle. Initial studies reported relationships between leptin and muscle marbling and body fatness, respectively, in cattle (Minton et al., 1998; Tokuda et al., 1999). Furthermore, Tokuda et al. (1999) reported increases in plasma leptin levels with increases in carcass fattening from 14 to 22 mo of age. Chilliard et al. (1998) reported a curvilinear relationship between plasma leptin and subcutaneous adipocyte size. Moreover, plasma

leptin was linearly related to fatness in both growing and adult cattle (Ehrhardt et al., 2000).

Investigations into the relationship between leptin and bovine fat development continued with the use of Japanese Black (Wagyu) cattle, a breed known to deposit large amounts of i.m. fat (Zembayashi and Lunt, 1995). Contrary to previous investigations, Kawakita et al. (2001) reported no correlation between backfat thickness or lipid content of *longissimus* m. with plasma leptin concentrations but both fat characteristics were correlated with the rate of increase of plasma leptin. Other investigations using crossbred Wagyu cattle reported a positive relationship in *longissimus* m. and *pars costalis diaphragmatis* m. between i.m. fat and plasma leptin among three Wagyu crosses when analysed together (Wegner et al., 2001). In the same study, a positive relationship was observed in cattle with no Wagyu genetics, no relationship in cattle with 50% Wagyu genetics, and a negative relationship with 75% Wagyu genetics possibly indicating breed-related differences in leptin regulation of i.m. fat deposition. In addition, Geary et al. (2003) recently reported a relationship between circulating serum leptin and both marbling scores and kidney, pelvic and heart fat amount. The investigators proposed that the lack of a relationship between serum leptin and marbling observed by Kawakita et al. (2001) may have resulted from the period of time between serum collection and marbling score determination at slaughter. There is no doubt that further investigations are required to elucidate the true nature of the relationship in cattle between leptin and fat deposition. Investigations involving the BM1500 microsatellite and R4C (Fitzsimmons et al., 1998; Buchanan et al., 2002), showed an association with these genotypes and carcass fat measures.

Overall, regulation of leptin in the short-term involves interactions with blood metabolites (such as glucose and ketone bodies), and hormones (such as insulin, growth hormone, or catecholamines). In the medium-term, regulation involves feeding level and daylength. Long-term regulation involves increased leptin synthesis from increases in adipocyte cell size and number. The multiple aspects of regulation were elegantly outlined in a review by Chilliard et al. (2001). Furthermore, two research groups have recently developed bovine-specific leptin RIA (Delavaud et al., 2000; Ehrhardt et al., 2000). No commercially available enzyme-linked immunosorbent assay or RIA, however, is currently available for bovine leptin, which may be due to the low immunogenicity of ruminant leptin (Dr. Elizabeth Adkisson, Linco Research, Inc., personal communication).

Leptin protein is believed to regulate energy and appetite via leptin receptors (Ob-R) and leptin-receptor-immunoreactive neurones located in the hypothalamus (reviewed in Jequier, 2002). Leptin receptors have also been located in the anterior pituitary gland, adipose tissue, mammary gland and adrenal medullary cells (see Chilliard et al., 2001 for references). Currently, six splice variants (a-f) have been cloned for Ob-R and, of these, only the long form of the receptor (Ob-Rb), and possibly the short form (Ob-Ra), are capable of signalling and, thus, are believed to be responsible for mediating the biological effects of leptin (Lee et al., 1996). The effects of dietary fat in Ob-Rb function have recently been reviewed (Heshka and Jones, 2001).

Even though current strategies in livestock research focus upon increasing i.m. fat while concurrently reducing the size of extramuscular adipocyte depots, current conventional treatments for human obesity via caloric restriction attempt to reduce the

overall amount of fat in the body. Unfortunately, with caloric restriction methods of weight reduction, the adipocytes retain their lipogenic enzymes (Zhou et al., 1999) increasing the risk of TAG reaccumulation upon reinitiation of former lifestyle practices. Adenovirus-induced hyperleptinemia, however, has been shown to deplete adipocyte fat content while down-regulating lipogenic enzymes, reducing PPARγ, adipocyte lipid binding protein (aP2), and tumour necrosis factor (TNF)-α and leptin levels, and increasing fatty acid oxidation enzymes (Zhou et al., 1999). Pharmacological drug treatments are currently limited because most obese patients are known to be leptin resistant (Jequier, 2002).

2.13 The Role of Peroxisome Proliferator-Activated Receptors (PPARs) in Fat Accumulation

In general, research into adipogenesis has focussed on a few important regulators involved in the control of lipid metabolic genes: PPARγ, SREBP1/ADD1, and C/EBPs (recently reviewed in Rosen et al., 2000) and pref-1 (Laborda, 2000). Nonetheless, researchers continue to characterize new regulators of adipogenesis on a regular basis. For example, recent studies with Olf-1/early B-cell factor (O/E-1) have indicated expression in preadipocyte cell lines augments adipogenesis and expression in non-committed fibroblasts leads to the initiation of adipocyte differentiation (Akerblad et al., 2002).

Peroxisome proliferators regulate gene transcription by activating a class of nuclear receptors, the peroxisome proliferator-activated receptors (PPAR), which are related to receptors for thyroid and steroid hormones (Chawla et al., 1994) and are

members of the nuclear hormone receptor superfamily (Escher and Wahli, 2000). Synthetic PPAR ligands include peroxisome proliferators, hypolipidemic, anti-inflammatory and insulin sensitising compounds whereas natural PPAR ligands include medium and long-chain fatty acids and eicosanoids (Escher and Wahli, 2000; Willson and Wahli, 1997). Three PPAR isoforms, designated α, β and γ, have been characterised in a wide variety of species, with each isoform encoded by a separate gene and demonstrating ligand-selectivity (Grimaldi, 2001). For example, Belury et al. (2002) recently demonstrated that conjugated linoleic acid (CLA) is an activator and ligand for both PPARα and PPARγ. CLAs are a mixture of positional and geometric isomers of octadecadienoic acid (C18:2). Of the nine known isomers, the *cis*-9, *trans*-11 form, also known as rumenic acid, is the most common natural form with biological activity (Moloney et al., 2001). CLA has been shown to have anti-carcinogenic, anti-atherogenic, lean-body mass enhancing and anti-diabetic properties (reviewed in McGuire and McGuire, 1999). The highest concentrations of CLA are found in the fat from ruminant animals where it is produced in the rumen from linoleic acid (reviewed in Bauman et al., 1999). Ruminants themselves synthesise rumenic acid from *trans*-11 octadecenoic acid via the enzymatic action of Δ^9-desaturase normally present in mammary tissue and adipose tissue (Bauman et al., 1999).

Octanoate has recently been shown to inhibit the expression of PPARγ, C/EBPα and SREBP-1c at both the mRNA and the protein level along with diminishing the expression of the adipogenic differentiation markers (Han et al., 2002). Sato et al. (1996) reported that during their determination of the optimum culture conditions for bovine i.m. adipocyte differentiation, octanoate was an essential factor for differentiation. It is more

likely, however, that a metabolite of octanoate may stimulate adipogenesis whereas octanoate itself attenuates differentiation. Both the amount of PPARγ, and the ability to produce natural ligands have been shown to lead to depot- and age-specific differences in preadipocyte differentiation (Soret et al., 1999).

Structurally, the amino-terminal domain of PPARs contains a ligand-dependent transactivation function, which is poorly conserved between the three isomers, whereas the central domain is for DNA binding and the carboxy-terminal domain contain the ligand-dependent transactivation function (Grimaldi, 2001). PPARs heterodimerize to *cis*-retinoid X receptors (RXR) with this complex binding to PPAR-response elements (PPREs) (Schulman et al., 1998). These PPREs are imperfect direct repeats of six nucleotides with one spacing base (Grimaldi, 2001). Functional PPREs have been characterized for several genes involved in lipid metabolism, including FA translocase, FA transport protein, FA binding protein, aP2, acyl-CoA synthetase (ACS) and acyl-CoA oxidase, adipocyte FA binding protein, phosophoenol pyruvate carboxykinase and LPL (Grimaldi, 2001).

PPARα has been shown to be expressed in cells that have high rates of FA oxidation and high peroxisome-dependent activities including liver and oxidative muscles (Escher and Wahli, 2000). In addition, PPARα has been shown to regulate mitochondrial FA metabolism, the β-oxidative medium chain acyl-CoA dehydrogenase gene along with other genes involved in the uptake, activation and degradation (β-oxidation) of FAs (reviewed in Escher and Wahli, 2000).

PPARβ may have a limited role in the development of the rat central nervous system by influencing cell proliferation or differentiation transcriptional control (Escher

and Wahli, 2000). Additionally, Braissant and Wahli (1998) have described a membrane lipid synthesis and turnover control function for PPARβ.

PPARγ was first shown by Chawla et al. (1994) to be highly expressed in adipose tissue and induced early during adipocyte differentiation. The bovine PPARγ isoforms γ1 and γ2 were isolated, characterized and assigned to bovine chromosome 22 (Sundvold et al., 1997). The PPARγ1 and PPARγ2 isoforms have been isolated from many bovine tissues including muscle, spleen, lung, ovaries and mammary glands (Sundvold et al., 1997). Nevertheless, expression is greatest in WAT. PPARγ may also have a role linking nutritional status to reproduction (Viergutz et al., 2000).

The role of PPARγ in adipogenesis has been exhaustively reviewed (Auwerx, 1999; Grimaldi, 2001; Houseknecht et al., 2002). Briefly, ectopic expression of PPARγ has been shown to convert preadipocytes (Chawla et al., 1994), fibroblasts (Tontonoz et al., 1994) and myoblasts (Grimaldi et al., 1997; Hu et al., 1995) into adipocytes. In addition, co-expression of PPARγ with C/EBPα, another potent stimulator of adipogenesis, has been shown to reinforce the potency of PPARγ (Brun et al., 1996). The currently proposed model of adipogenic transcription factor initiation involves initiation by hormonal stimulation of C/EBPβ and δ. These factors stimulate PPARγ expression, (via promoter binding) and PPARγ heterodimerizes to RXRα, which in turn stimulates C/EBPα along with known adipogenic genes containing PPREs (thoroughly reviewed in Houseknecht et al., 2002 and Rosen and Spiegelman, 2000). Recent investigations have also shown that PPARγ has a role in FA uptake and TAG storage through the regulation and expression maintenance of aP2, ACS, LPL, HSL and perilipin (Tamori et al., 2002).

Investigations using bovine adipose tissue-derived preadipocytes have shown that the expression of PPARγ via stimulation with a known synthetic ligand resulted in preadipocyte conversion to adipocytes (Ohyama et al., 1998). Retinoids were shown to inhibit bovine adipogenesis, however, possibly explaining why Japanese beef cattle showing low serum retinol levels exhibit highly marbled beef (Torii et al., 1996). Similar investigations (Wu et al., 2000) demonstrated that VLDL was a potent stimulator of bovine preadipocyte proliferation and differentiation. Additionally, Torii et al. (1998) isolated fibroblast-like cells from bovine skeletal muscle and, using a stimulator of PPARγ, demonstrated in cell culture that bovine skeletal muscle contain a population of cells that can differentiate into adipocytes.

In addition to the importance of PPARγ in adipogenesis, RXRα also plays a contributory role. Recently, Imai et al. (2001) reported that under a high fat diet, mice lacking RXRα had smaller adipocytes than wild type mice indicating that hypertrophic growth of adipocytes and fat accretion during adipocyte differentiation may be mediated by RXRα-PPARγ heterodimers. In addition to the well-characterized role of PPARγ and PPARα in adipogenesis and FA oxidation, these receptors have been shown to have roles in atherosclerosis associated with controlling plasma levels of cholesterol and TAG (reviewed by Plutzky, 2000, Marx, 2002 and Barbier et al., 2002).

Finally, PPARγ has been implicated in having a role in the adipocyte apoptosis of large and fully differentiated cells (Okuno et al., 1998; Sorisky et al., 2000). Auwerx (1999) proposed that the combined effect of adipocyte apoptosis and preadipocyte differentiation by PPARγ may favour the replacement of large adipocytes with smaller adipocytes, potentially leading to a state of PPARγ resistance.

2.14 The Role of Preadipocyte Factor-1 in Fat Accumulation

Mature adipocyte development from preadipocyte cells is accompanied by the significant changes in the expression level of over 100 proteins (Sidhu, 1979). Adipogenesis also has been shown to involve the change in expression of approximately 2000 different genes and very few of those genes have been characterized for their respective function in differentiation (Guo and Liao, 2000). One such protein was shown to be expressed abundantly in preadipocytes, characterized by complete abolishment of expression during adipocyte conversion, with constitutive overexpression blocking adipogenesis and therefore was named preadipocyte factor-1 (pref-1) (Smas and Sul, 1993).

The protein encoded by the pref-1 gene is a transmembrane protein that possesses six Epidermal Growth Factor (EGF)-like sequences at the extracellular domain, a single transmembrane domain and a short intracellular tail (reviewed by Laborda, 2000). Pref-1 contains two proteolytic processing sites, that upon cleavage by an uncharacterized protease, result in 50 and 25 kDa soluble proteins (Smas et al., 1997). In addition, four major splicing products (pref-1A – pref-1D) are the result of an in-frame deletion of the juxtamembrane region of the sixth EGF-repeat (Mei et al., 2002), where pref-1A and pref-1B lead to the generation of both soluble proteins, whereas pref-1C and pref-1D produce only the smaller protein. Recently, Mei et al. (2002) reported that the 50 kDa protein, and not the transmembrane or the small protein, inhibited adipocyte differentiation. Thus, Mei et al. (2002) proposed that pref-1 does not regulate adipogenesis in a juxtacrine fashion, but the membrane-bound form may have a role in a cell growth function. Interestingly, pref-1 has been discovered by six different research

groups and thus has been given six different names (pG2, FA-1, pref-1, SCP-1, ZOG, and dlk) (Laborda, 2000).

Even though *Dlk1* has been accepted as the official name (Lee et al., 1995), researchers focussed on elucidating the relationship to adipogenesis still use the term pref-1 (Laborda, 2000). The gene for pref-1 has also been shown to be expressed in animals from birds to mammals, but is absent from lower animals suggesting that a function specific for higher animals may be possessed by pref-1 (Laborda et al., 1993). Pref-1 upregulation also plays a role in bone marrow stromal cells by modifying their adipogenic potential and regulating the growth of lymphocyte precursors (Laborda, 2000), and recently has been shown to function in maintaining mouse blastema cells in a dedifferentiated state during regeneration after physical wounding (Samulewicz et al., 2002). Pref-1 may function in maintaining the state of preadipocyte cells (Mei et al., 2002), and has thus been used as a marker for preadipocytes (Zhou et al., 1999). In addition, pref-1 null mice have been shown to be obese, exhibit growth retardation and skeletal malformations, and have increased serum TAG, FA and cholesterol along with a fatty liver (Moon et al., 2002).

Fahrenkrug et al. (1999) have cloned and genetically mapped the bovine pref-1 gene to the telomeric end of BTA 21. Analysis revealed a splice-form, apparently unique to the bovine pref-1 gene, which was referred to as splice-form E. The exon/intron junctions of the bovine pref-1 gene were the same as those observed for mice. The 'suppression in adipocyte differentiation' sequence, previously identified by Smas et al. (1998), was conserved. In addition, Minoshima et al. (2001) confirmed the location of the pref-1 gene to bovine chromosome 21q24 and, via mapping of the regulatory region,

demonstrated two regions upstream of the putative transcription start site were sufficient to confer promoter activity. Most importantly, however, was the lack of pref-1A or C2 expression observed in adult bovine subcutaneous fat by Minoshima et al. (2001). Pref-1 expression is known to be restricted to the stromal-vascular (SV) fraction, but not the adipocyte fraction of adipose tissue (Mei et al., 2002), possibly explaining the lack of pref-1 expression in adult tissues reported by Minoshima et al. (2001) due to a lack of enriched SV cells in their preparation.

The potential mechanisms for pref-1 action have recently been reviewed (Laborda, 2000, dlk therein). Two mechanisms of cell differentiation have been proposed: lateral specification and inductive signalling. Lateral specification involves ligand and receptor signals in equivalent cells being expressed equally. Modifications in the respective ligand and receptor expression levels are caused by random fluctuations in signal intensity, and thus, one cell becomes a ligand-expressing cell, while the other becomes a receptor-expressing cell leading to a spaced pattern of cells in the tissue.

Inductive signalling results from signals being transmitted between non-equivalent cells; thus, once one cell differentiates, it signals to its neighbouring cells to differentiate. Laborda (2000) proposed that inductive signalling is the most likely mechanism by which adipocyte differentiation occurs with pref-1 participating in the signalling role. Currently, the literature points to pref-1 functioning as a receptor, a ligand and possibly as an inhibitor of adipogenesis with experimental evidence strong for all three cases.

Working under the assumption that inductive signalling leads to adipose cell differentiation, Baladron et al. (2002) have attempted to clarify which factors are

involved in the initial signal that leads to differentiation. Initial experiments by Shugart et al. (1995) reported a strong expression of growth-arrest specific protein 1 (GAS1) during the initiation of adipogenesis. GAS1 is a membrane glycosylated phosphatidylinositol anchor-linked protein whose expression causes growth arrest and was shown to decrease in expression upon completion of differentiation thus following a similar expression pattern as pref-1 (dlk therein). Baladron et al. (2002) proposed that a potential interaction between pref-1 and GAS1 might modulate clonal expansion and growth arrest during adipocyte differentiation.

Recent research has indicated one mechanism by which pref-1 modulates the adipocyte differentiation signalling pathway (Ruiz-Hidalgo et al., 2002). In 3T3-L1 preadipocytes, IGF-1 and insulin have been shown to trigger a cascade resulting in the expression of adipogenic transcription factors (Hausman et al., 2001; Smith et al., 1988). One of the cascades is the Ras-Raf-1-Mitogen-Activated Protein Kinase pathway (MAPK (Hu et al., 1996). Ruiz-Hidalgo et al. (2002) demonstrated that different levels of pref-1 (referred to as dlk therein) expression correlated with changes in the level and activation kinetics of Extracellular-Regulated Kinase (ERK)-MAPK in response to insulin and IGF-1 activation, and thus the extent of adipocyte differentiation. Therefore, a certain level of pref-1 expression is necessary for adipocyte differentiation to occur that may be mediated by ERK-MAPK activation. Hence, increases in pref-1 expression reported by Hansen et al. (1998) may result from a necessity for greater interaction between pref-1 and stimulators of adipogenesis.

Tumor necrosis factor α (TNFα) is a polypeptide hormone with pleiotropic effects on cellular differentiation and has been shown to inhibit and reverse adipocyte

differentiation (Torti et al., 1989). Analyses by Xing et al. (1997) have indicated that TNFα treatment in differentiating preadipocytes reduces PPARγ, but has no effect on pref-1 mRNA levels. Additionally, the authors proposed that pref-1 expression, once down-regulated, is non-reversible by their observation that TNFα treatment in fully differentiated adipocytes results in rapid down-regulation of PPARγ mRNA and protein levels without any effect on pref-1 mRNA expression. Han et al. (2002) observed no effect on pref-1 levels during attenuation of adipogenic transcription factors and differentiation markers through the exposure of adipocytes to octanoate.

Adenovirus-induced hyperleptinemia (Zhou et al., 1999), which has been shown to convert adipocytes to post-adipocytes (previously mature adipocytes that have lost their lipogenic enzymes and adipocyte markers), resulted in a 41-fold increase in pref-1 levels. Therefore, pref-1 expression may also be a marker for post-adipocytes, contrary to the proposal by Xing et al. (1997).

The above results, in addition to the pref-1 analysis by Minoshima et al. (2001), create a dilemma in the analysis of bovine i.m. adipocyte development. Morphological and histochemical analysis has clearly demonstrated that i.m. adipocytes develop by hyperplastic growth throughout the life of many animals including pigs, cattle, chickens and rabbits. Minoshima et al. (2001) have shown, however, that pref-1 mRNA was undetectable in abdominal fat samples from 30-month old cattle, suggesting that pref-1 may be expressed in an age- and/or depot-specific manner. Therefore two important unanswered question relating to bovine adipogenesis arises: first, at which stage does preadipocyte development in cattle cease in each respective adipose depot, and second,

do newly developing adipocytes arise from cells present in the depot or do they arise from a separate source?

3. Expression of Peroxisome Proliferator-Activated Receptor (PPARγ) mRNA in Adipose and Muscle Tissue of Holstein and Charolais Cattle

3.1 Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) regulates adipogenesis and lipid metabolism-related gene transcripts. The role, however, of PPARγ in different adipose depots and muscle in secretion type (Holstein) and accretion type (Charolais) cattle is still unclear. We used 20 animals (10 from each breed) for semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to measure PPARγ mRNA levels in subcutaneous (SC), perirenal (PR), omental (OM), and intramuscular (IM) adipose depots as well as *longissimus* muscle (MU). IM fat was dissected from muscle tissue in MU. The Holstein were characterized by their higher OM ($P < 0.01$) and PR ($P < 0.05$) fat weights while the Charolais had a higher body weight ($P < 0.001$) and a larger *longissimus* muscle area ($P < 0.001$). The IM fat content and marbling scores tended to be higher in the Holstein. No significant differences in PPARγ mRNA expression were observed between different these two breeds for any tissue. In both breeds, MU PPARγ had the lowest expressed transcript ($P < 0.05$). In the IM fat depot, expression was higher ($P < 0.05$) than MU, but lower than the SC, PR, and OM fat depot PPARγ mRNA levels. Only OM PPARγ mRNA levels were higher ($P < 0.05$) than SC and PR in the Charolais cattle. To characterize the role of PPARγ mRNA in bovine adipogenesis, correlations were performed among PPARγ mRNA, carcass characteristics, and adipogenesis-related genes. A high expression of MU PPARγ mRNA in Charolais cattle was correlated significantly ($P < 0.05$) with a high deposition of fat (SC, $r = 0.78$; PR, $r = 0.74$; and IM fat in the

semitendinosus muscle, r = 0.66). A high expression of IM PPARγ mRNA was correlated with high SC leptin mRNA expression in both Holstein and Charolais cattle (r = 0.86 and r = 0.85, respectively). Correlations were also observed between SC PPARγ mRNA and SC lipoprotein lipase (LPL) mRNA in both Holstein (r = 0.58) and Charolais cattle (r = 0.70). PPARγ may have a role in regulating increased fatty acid oxidation in SC fat through the action of LPL. Leptin mRNA in the SC, PR, and OM fat depots was positively correlated with SC PPARγ mRNA and negatively associated with Charolais cattle OM PPARγ mRNA. In the Charolais cattle, a high level of PPARγ mRNA in SC was correlated with a low expression in OM (r = -0.87) and a high expression in IM (r = 0.87). Fat content in cattle may therefore be regulated by the PPARγ transcription factor in a depot-specific manner.

3.2 Introduction

Selective breeding programs have, over time, produced cattle with an enhanced ability to metabolize their intake into either muscle (accretion breeds) or as milk (secretion breeds). The Holstein breed of cattle are known for their ability to excrete a relatively large proportion of their nutrient intake as milk while also producing muscle. In contrast, the Charolais breed deposit large amounts of muscle, and less fat, while producing little milk (for a review see Kühn et al., 2002). The molecular mechanisms that regulate adipogenesis in different adipose depots and muscle of accretion and secretion breeds is still unclear.

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor hormone superfamily and are essential for cellular differentiation and

lipid accumulation during adipogenesis (Lapsys et al., 2000). Three PPAR isoforms, PPARα, PPARγ, and PPARδ, are subject to similar regulation mechanisms. PPARs are a class of transcription factor that must heterodimerize with another nuclear hormone receptor, Retinoid X Receptor, to bind to DNA to be transcriptionally active (Rosen et al., 2000). The three γ isoforms of PPAR (γ1, γ2, and γ3) are generated by alternative promoter usage and alternative splicing (Auwerx, 1999) and have tissue-specific distribution patterns in humans, mice, and rats (Mukherjee et al., 1997; Lapsys et al., 2000). Bovine PPARγ mRNA expression has been observed in spleen, ovary, fat, lung, mammary gland, small intestine, kidney, muscle and heart, along with faint signals in the liver, in an isoform-specific manner (Sundvold et al., 1997). Expression of PPARγ is higher in adipose tissue than in other bovine tissues. PPARγ1, the most commonly expressed isoform (Tontonoz et al., 1995), makes up the majority of the total expressed PPARγ in expressing tissues with the only exception being adipose tissue where PPARγ2 makes up approximately 20% of the total PPARγ mRNA (Auboeuf et al., 1997). PPARγ3 mRNA gives rise to an identical protein product as PPARγ1 mRNA (Auwerx 1999).

The understanding of PPARγ's role in bovine adipogenesis is still emerging but its role in the mammalian adipogenic process has been largely characterized thanks to work on cell culture systems, particularly murine 3T3-L1 cells. During cell culture adipogenesis, PPARγ expression has been shown to increase dramatically early, and, thereafter, remain at high levels in fully differentiated adipocytes, suggesting that it is at least partly responsible for maintaining the differentiated state of the cell (Cowherd et al., 1999; Lowell 1999). As well, ectopic expression of PPARγ results in the conversion of fibroblast cells (Kubota et al., 1999), 3T3-L1 preadipocytes (Tontonoz et al., 1994), and

myoblasts (in the presence of C/EBPα) (Hu et al., 1995) into adipocytes. PPARγ is, therefore, thought to be a key factor involved both in the stimulation of adipogenesis as well as the maintenance of adipocytes once they have differentiated. Evidence is also emerging that PPARγ plays a key role in apoptosis of fully differentiated adipocytes. Auwerx et al. (1999) theorize that PPARγ may be also responsible for the "remodelling" of adipose tissue resulting in many smaller adipocytes with the end result being adipocytes which are resistant to PPARγ activation.

The objective of the current study was to examine PPARγ mRNA expression levels in different tissues of two metabolic types of cattle and determine if PPARγ mRNA levels may be indicative of the amount of adipose tissue present in a depot. We used reverse transcriptase-polymerase chain reaction (RT-PCR) to measure the expression of PPARγ transcript levels in subcutaneous (SC), perirenal (PR), omental (OM), and intramuscular (IM) fat, and *longissimus* muscle (MU) tissues of Holstein and Charolais bulls. The relationship between PPARγ transcript levels and previously investigated adipogenic genes (leptin and lipoprotein lipase), as well as between PPARγ transcript levels and several carcass characteristics, were investigated to determine if regulatory relationships exist.

3.3 Materials and Methods

3.3.1 Animals and Diet

An unrelated parental generation of Holstein (n = 10) and Charolais (n = 10) bulls were used in the investigation. Animals were cared for according to the regulations of the Animal Protection Committee from the German Ministry for Consumer Protection, Food

and Agriculture. Calves were fed with a milk replacer diet up to 4 mo of age. After weaning, the bulls received a body weight-related diet consisting of concentrates based on barley, beet pulp and soybean extract meal (92.8% organic matter, 15% crude protein, and 9% crude fibre) and hay. All animals were reared using a tethering system in individual pens and were fed the same diet (Table 3).

3.3.2 Sample Collection

All animals were slaughtered at 18 mo of age in the Dummerstorf Research Institute's abattoir. The SC, PR, and OM fat, and the MU (containing IM fat) were collected within 30 min of slaughter. The SC fat was sampled from a depot overlaying the *semitendinosus* muscle. PR fat was from around the kidneys and OM fat was from around the gut. All tissues were immediately frozen in liquid nitrogen and stored at –70°C until RNA extraction. Samples of MU were magnified 20x using a dissection microscope (AskAnia GSZ 2T, Germany) while IM fat was separated from the MU that was held on dry ice. Respective samples of MU and IM fat were stored in RNAlater (Ambion, Inc. Austin, Texas) at –20°C until RNA was extracted within a 2 week period.

3.3.3 Measures of Carcass Quality

After slaughter, body weight, and the PR, OM, and SC fat weights were estimated. The *longissimus* muscle cross-sectional area was measured using the Interactive Measurements/Area function of the ImageC program for WinNT (Version 2.52a (Level 510), Aquinto AG, Berlin, Germany). Marbling scores were based on the German 6-point system where a score of 1 equates to low marbling and a score of 6 to high marbling. The IM fat content from *longissimus* and *semitendinosus* muscle was obtained via the Soxhlet

extraction method (AOAC, 1984) using petroleum ether as the solvent and determined gravimetrically after evaporating the extraction solvent.

3.3.4 RNA Extraction, cDNA Synthesis and Polymerase Chain Reaction

Total RNA was isolated from the SC, PR, and OM fat samples using the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA from IM and MU samples was isolated using TRIzol reagent (Life Technologie GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. RNA yields and purities were assessed by absorbance at 260 and 280 nm using a RNA/DNA calculator (Pharmacia GeneQuant^{TM}, Erlangen, Germany). Ratios of absorbance (260/280 nm) of all samples were between 1.7 and 2.0.

cDNA was synthesized from 1.0 µg of total RNA from each sample. One unit of DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) was added, to eliminate residual genomic DNA from the RNA sample, prior to RT reactions, and incubated at 37°C for 30 min followed by heat inactivation of the enzyme at 75°C for 5 min (Huang et al., 1996). Denaturation of the RNA samples occurred by a 65°C incubation for 15 min and were subsequently placed on ice for 5 min before RT. The final reaction volume of 25 µL contained 1x reaction buffer, 5 mM $MgCl_2$, 1 mM of dNTPs, 3.2 µg of random hexamer primers, 50 units of RNase inhibitor, 0.01 mg/mL gelatin and 20 units of AMV reverse transcriptase (1st Strand cDNA Synthesis Kit, Roche Diagnostics GmbH). PCR conditions were as follows: 25°C for 10 min, 42°C for 60 min, 99°C for 5 min (for enzyme inactivation), and 4°C for 5 min. The RT products were used immediately for PCR or stored at –20°C.

The PCR reaction was performed in a 50 μL reaction volume containing 2 μL of tissue-specific cDNA (equivalent to 80 ng starting RNA), 1.5 mM $MgCl_2$, 2 units of *Taq* DNA polymerase (Roche Diagnostics GmbH), 0.2 mM of dNTPs and 0.4 μM of each primer. For amplification of PPARγ, the following primer pair was used: forward 5'-GAC TTG AAC GAC CAA GTA ACT C-3' (nt 1200 – 1221) and reverse 5'-CTC TGC TAA TAC AAG TCC TTG TAG-3' (nt 1711 – 1688), which are specific for both bovine PPARγ1 and γ2 mRNAs (GenBank accession numbers Y12419 and Y12420, respectively) and amplify a 467 bp fragment (Wu et al., 2000).

The primers span exons 5 and 6 and thus are specific to bovine PPARγ mRNA (See Appendix 3, Figures 24 and 25 for the PPARγ1 and γ2 gene sequences, respectively). To identify the exponential phase of amplification during PCR, the cycle numbers were first tested for the target gene. Plotting of PPARγ PCR signal intensity (expressed as net intensity) against the number of amplification cycles revealed a linear relationship between cycles 28 and 34 ($r^2 = 0.96$).

Quantum RNA™ 18S primer and competimer (Ambion, Inc., Huntingdon, UK) were used as internal controls for target gene amplification at a ratio of 2:8. The primer pair amplifies a 324 bp fragment. Amplifications were performed in a Biometra Personal Cycler (Biomedizinische Analytik GmbH, Göttingen, Germany). The following cycle parameters were used: 60 s at 94°C, 34 cycles at 94°C for 40 s, 55°C for 40 s and 72°C for 50 s. Each reaction was followed by 7 min at 72°C for extension and held at 4°C. After amplification, 10 μL of the PCR products were used for electrophoresis on a 2% agarose gel to compare the relative signal intensity.

Table 3. Feed and carcass characteristics from Holstein and Charolais cattle.

	Holstein[z]	Charolais	Significance[y]
Age (months)	18	18	None
Concentrates (kg)	2829±69 (218)	2758±52 (166)	None
Hay (kg)	962.7±19.7 (62.4)	929.1±17.0 (53.8)	None
Live Weight (kg)	662.8±15.1 (47.7)	744.4±16.4 (51.7)	***
longissimus Area (cm^2)[x]	78.24±2.94 (9.29)	122.50±3.93 (12.42)	***
Subcutaneous Fat (kg)	20.42±1.80 (5.70)	21.28±1.14 (3.62)	None
Perirenal Fat (kg)	18.95±1.64 (5.18)	14.67±0.94 (2.99)	*
Omental Fat (kg)	20.30±1.67 (5.29)	14.10±0.76 (2.41)	**
semitendinosus IM Fat (%)[w]	1.91±0.26 (0.81)	1.48±0.34 (1.08)	None
longissimus IM Fat (%)	4.35±0.64 (2.03)	3.14±0.68 (2.15)	None
Marbling[v]	2.80±0.20 (0.63)	2.30±0.21 (0.67)	†

[z] The values listed in each column represent the mean of n = 10 samples including ± standard error of the mean and (standard deviation) of Holstein and Charolais cattle.

[y] Significance = †($P < 0.10$), *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$). t-test calculated using the SAS System for Windows v. 8.02 (SAS Institute, Inc. 1999).

[x] The *longissimus* muscle cross-sectional area was measured using the Interactive Measurements/Area function of the ImageC program for WinNT (Version 2.52a (Level 510), Aquinto AG, Berlin, Germany).

[w] The IM fat content from *longissimus* and *semitendinosus* muscle was obtained via the Soxhlet extraction method using petroleum ether as the solvent and determined gravimetrically after evaporating the extraction solvent.

[v] German marbling score system based on a 6-point scale with a score of 1 indicating a carcass devoid of marbling and a score of 6 indicating very abundant marbling.

Data for lipoprotein lipase (LPL) and leptin mRNA transcript levels, used for correlation analysis in this study, were obtained from a previous study by our research group on the same animals (Ren et al., 2002).

3.3.5 Semi-Quantification of PCR Products (Image Analysis)

Gels were stained with ethidium bromide and analyzed using the Kodak DS Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA). The net intensities of individual bands were measured and the ratio of the target gene to that of the internal control band (QuantumRNA™ 18S) was calculated prior to statistical analysis. To minimize the between-assay error, samples from the two cattle breeds were always processed in parallel.

3.3.6 Statistics

Data were analysed using the SAS System for Windows v. 8.02 (SAS Institute Inc. 1999). Significance was calculated using a pair-wise t-test. Significance levels were: $^{\dagger}(P < 0.10)$, $*(P < 0.05)$, $**(P < 0.01)$, $***(P < 0.001)$. The experimental units were bulls of two breeds. The statistical aim was to test for differences in PPARγ mRNA levels between the breeds and tissues, and for associations between PPARγ mRNA levels and adipogenesis-related gene transcripts as well as carcass characteristics. Data were submitted to a simple linear correlation analysis (Pearson product moment correlation) using SAS. Correlations were performed within the individual breeds.

3.4 Results

Animals used in this study were a subset of the Segregating Families (SEGFAM) herd currently under investigation at the Research Institute in Dummerstorf (Kühn et al., 2002). Carcass traits for both metabolic types of cattle are summarized in Table 3. Charolais, as a beef type cattle, with a higher capacity to deposit nutrients as muscle as opposed to fat, exhibited a higher body weight ($P < 0.001$) and *longissimus* muscle area ($P < 0.001$) than Holstein bulls. The Holstein cattle, however, had higher PR ($P < 0.05$), OM ($P < 0.01$) fat weights and a slightly higher ($P < 0.10$) marbling score indicating a greater ability to deposit nutrients preferentially as fat. The amount of the SC and IM fat depots were not significantly different between the breeds.

Using semi-quantitative RT-PCR analyses we measured PPARγ mRNA levels in 5 different tissues from Holstein and Charolais bulls. To identify depot- and breed-specific differences in PPARγ mRNA, expression was first corrected for the expression of 18S rRNA. No significant differences in PPARγ mRNA expression were observed between the two cattle breeds for any tissue, whereas expression differed based on tissue type (Figure 11). In Holstein no differences were observed between SC, PR, or OM fat depots while PPARγ mRNA expression in IM fat was lower than each of these tissues ($P < 0.05$). The lowest ($P < 0.05$) expression was observed in MU. In Charolais cattle, PPARγ mRNA expression was higher ($P < 0.05$) in OM than in SC and PR fat whereas IM fat was lower ($P < 0.05$) and, as in Holstein cattle, MU PPARγ mRNA was the lowest ($P < 0.05$).

In order to identify any relationships in PPARγ mRNA expression between the respective tissues or with previously investigated adipogenic-related genes (Ren et al.,

2002) and carcass traits, we performed Pearson product moment correlations within the breeds. The relationships among PPARγ, leptin, and LPL mRNA expression levels in the adipose depots and muscle tissue are shown in Table 4. Correlations within PPARγ mRNA expression levels among the five tissues showed a significant ($P < 0.05$) relationship between SC PPARγ mRNA and both OM PPARγ mRNA ($r = -0.87$) and IM PPARγ mRNA ($r = 0.87$).

In order to determine whether carcass fat characteristics influence, or were influenced by PPARγ, we investigated the relationships between carcass characteristics of the animals and mRNA expression of PPARγ. Significant correlations were observed between MU PPARγ and OM fat ($r = -0.62$) in Holstein bulls, and between MU PPARγ and SC fat ($r = 0.74$), PR fat ($r = 0.74$), and *semitendinosus* IM fat ($r = 0.66$) in Charolais cattle. In addition, IM PPARγ correlated significantly with *longissimus* IM fat ($r = 0.91$) in Holstein, and OM PPARγ and *longissimus* IM fat ($r = 0.68$) in Charolais cattle, respectively.

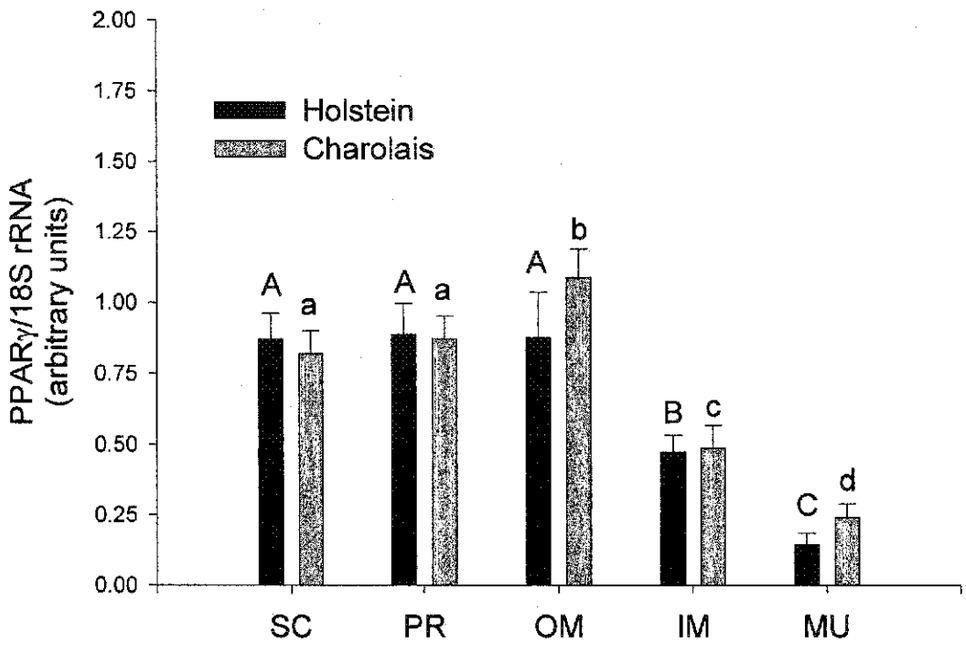

Figure 11. Depot- and breed-specific PPARγ gene transcript expression. PPARγ reverse-transcriptase polymerase chain reaction (RT-PCR) products from subcutaneous (SC), perirenal (PR), omental (OM), and intramuscular (IM) adipose depots as well as *longissimus* muscle (MU) in Holstein (GH; n = 10, except for IM, where n = 4) and Charolais (CHA; n = 10, except for IM, where n = 7) bulls were normalized to the internal 18S rRNA standard. Columns show means with vertical bars indicating standard error of the mean. Means with different superscripts are significantly different ($P < 0.05$). Uppercase (A, B, C) letters pertain to Holstein and lower case (a, b, c, d) to Charolais. No significant differences were observed between the breeds in any tissue analysed.

Table 4. Simple linear correlation coefficients between PPARγ mRNA and fat-related gene mRNAs in 5 different bovine tissues.

a. Holstein Bulls

	PPARγ (PPARγ/18S rRNA)				
	SC	PR	OM	IM	MU
Leptin SC	0.29	- 0.28	- 0.44	0.86 *	- 0.29
Leptin PR	- 0.22	0.01	0.04	- 0.62	- 0.04
Leptin OM	- 0.13	0.14	- 0.10	- 0.74	- 0.09
LPL SC	0.58 *	0.06	0.15	0.04	- 0.42
LPL PR	- 0.31	0.36	0.16	- 0.23	- 0.33
LPL OM	0.27	- 0.73 *	0.05	0.37	- 0.65 *

b. Charolais Bulls

	PPARγ (PPARγ/18S rRNA)				
	SC	PR	OM	IM	MU
Leptin SC	0.85 *	- 0.24	- 0.92 *	0.85 *	0.54
Leptin PR	0.68 *	0.12	- 0.63 *	0.86 *	0.50
Leptin OM	0.66 *	- 0.17	- 0.56 †	0.77	0.50
LPL SC	0.70 *	- 0.32	- 0.69 *	0.69	- 0.13
LPL PR	0.23	0.20	- 0.23	0.53	- 0.41
LPL OM	0.22	- 0.22	0.11	- 0.29	- 0.18

Significance = † ($P < 0.10$), *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

3.5 Discussion

The Charolais cattle's higher body weight, and *longissimus* muscle area are indications of the breed's ability to deposit their nutrient intake as muscle rather than milk. Conversely, Holstein cattle tended to have higher marbling scores ($P < 0.10$), as well as higher OM and PR fat weights, an indication of this breed's genetic predisposition to fat deposition. In our previous investigations in these and other animals, a higher insulin level previously observed in Holstein was one reason postulated for the breed's higher fat content, but cannot completely explain the differences in fat development between the breeds (Ren et al., 2002). In this study we focussed on the fat deposition in Holstein and Charolais cattle by investigating PPARγ mRNA expression in these two breeds and its relationship to fat deposition.

No differences in the expression of PPARγ mRNA between the breeds for each of the 5 respective tissues were observed suggesting production of PPARγ mRNA levels are produced similarly in the two breeds. Results indicate that PPARγ do not cause the observed differences in fat depots between the two cattle breeds. In human studies comparing obese, nonobese and postobese patients, no differences were observed in PPARγ mRNA levels between SC and OM fat depots (Krempler et al., 2000).

PPARγ mRNA levels were also independent of the amount of fat in the adipose depot under investigation. Therefore, PPARγ mRNA levels cannot be used as a measure of the amount of fat in the adipose tissue. In addition, investigations in rats demonstrated that PPARγ mRNA expression was independent of the fat state (Shimoike et al., 1998) supporting our observations.

In their investigations of human PPARγ mRNA, Vidal-Puig et al., (1997) reported a drop in PPARγ levels after fat loss. Levels of PPARγ rebounded to earlier pre-treatment levels indicating that a steady-state expression may exist independent of food intake or body weight. Because short-term weight loss did not result in a decrease in the overall level of PPARγ (Vidal-Puig et al., 1997), perhaps PPARγ mRNA levels are used as a signal for the maintenance of the depot size. PPARγ mRNA levels may be resistant to change with short-term modification of nutrient intake but may be able to change with long-term modifications to diet.

The SC depot is one of the final adipose depots to be filled in ruminants (Mtenga et al., 1996) and is known to have the smallest adipocytes in the carcass (Mendizabel et al., 1999). SC adipocyte hyperplasia is observed as early as 5 mo of age and up to as old as 16 mo of age (Martin et al., 1999). Therefore, the expression of PPARγ in our animals may stem from the development of new adipocytes as well as the maintenance of the current size and number of adipocytes already developed. Martin et al. (1999) theorized that the total adiposity of growing cattle may be especially sensitive to both dietary and hormonal manipulations. The equivalent PPARγ expression, along with the equivalent amount of SC fat deposited, may result from the equivalent long-term, weight-related diet fed to the Holstein and Charolais cattle. As SC is the first depot to be used in cases of nutritional deficiency (Mervyn and Leat 1983), the level of PPARγ mRNA in SC fat may be indicative of, as well as sensitive to, nutritional levels.

We demonstrated a significant positive correlation of SC fat PPARγ mRNA expression with SC fat LPL in Holstein and Charolais. As LPL contains a functional PPAR response element (Schoonjans et al., 1996), PPARγ may have a role in regulating

the increase in fatty acid oxidation in SC fat through the action of LPL. Lapsys et al. (2000) also observed a positive correlation between PPARγ and LPL mRNA transcript levels in human muscle tissue.

A significant correlation was observed only in Charolais cattle, between SC fat PPARγ mRNA and leptin mRNA in the SC, PR, and OM fat depots. Bellmann et al. (2002) theorized, based on their observations of the same research animals, that cattle which are genetically selected to transform nutrients into muscle components are more regulated by leptin. This was also observed in an analysis of crossbred Wagyu cattle by Wegner et al. (2001). Evidence of a regulatory role of PPARγ in leptin expression first came from the localization of a putative binding site for PPARγ in the leptin promoter of rats (Hollenberg et al., 1997). Furthermore, obese human carriers of a silent PPARγ polymorphism had higher plasma leptin levels than wild-type subjects (Meirhaeghe et al., 1998). A study with rats demonstrated that leptin injection increased the expression of PPARγ protein in adipocytes (Qian et al., 1998). A leptin-induced signal might cause PPARγ phosphorylation, thus resulting in adipose-apoptotic signal transduction (Qian et al., 1998). We therefore hypothesize that PPARγ and leptin may directly regulate each other and that this regulation can be observed in breeds that are selected for muscle rather than fat deposition.

PR fat is an internal depot that protects and supports the kidneys, and is known to be the first depot to develop in ruminants (Mervyn and Leat 1983). In contrast to SC fat, PR fat hyperplasia is completed by 8 mo of age with further growth due mainly to hypertrophy (Hood and Allen 1973; Truscott et al., 1983; Mendizabal et al., 1999). The equivalent levels of PPARγ mRNA measured for both breeds in the PR depot may result

from the maintenance of the adipocytes at their current size, but more likely could indicate that a pool of PPARγ mRNA is maintained at equivalent concentrations in the adipose depots.

OM fat is an internal depot responsible for the protection and support of the intestines. As it is considered to be a commercially valueless depot, little attention is given to its characterization. OM fat is observed in all newborn cattle and growth is observed until at least 24 mo of age (unpublished observations). Growth of the OM fat depot, as in PR fat, is mainly due to hypertrophy (Mendizabal et al., 1999). Our observed equivalent PPARγ mRNA levels between Holstein and Charolais cattle is not a reflection of the amount of OM fat in the carcass as Charolais cattle had less ($P < 0.01$) fat in the OM depot than did the Holstein cattle. The higher expression of PPARγ mRNA in OM fat in Charolais cattle is thought to be a reflection of development potential of the different breeds as well as the different depots. Mtenga et al. (1996) observed in goats that OM is one of the last depots to develop and fully mature. Adipocyte hyperplasia may therefore be ongoing in the Charolais cattle at slaughter age and is reflected in the slightly higher PPARγ mRNA levels in this depot. Observations in human subjects have exhibited contrasting results. Montague et al. (1998) and Krempler et al. (2000) reported no differences in the level of PPARγ mRNA between SC and OM fat while Lefebvre et al. (1998) demonstrated a two-fold higher level of PPARγ mRNA expression in SC as compared to OM adipose tissue. Therefore, in human subjects, as in cattle, PPARγ mRNA levels do not appear to reflect the amount of adipose tissue in the depot.

Deposition of IM fat has been observed in very young cattle, but the majority of development occurs very late in life (Mervyn and Leat 1983; Albrecht et al., 1996;

Nishimura et al., 1999). IM fat may serve as an immediate energy source for muscles due to its immediate proximity to muscle cells and IM fat may also serve as a depot for fatty acids not oxidized by muscle fibres. The deposition of IM fat is closely linked to breed (Zembayashi et al., 1995; Herring et al., 1996), the sire used (Vieselmeyer et al., 1996), and the finishing system (Zembayashi 1994; Allen et al., 1996) and is also associated with excessive deposition of fat in other depots of the body, particularly the SC depot (Mervyn and Leat 1983; May et al., 1992). IM hyperplasia has been observed at 14 mo of age (Allen 1976; Hood 1977; Enser and Wood 1978) and results from our lab have shown hypertrophy up to 24 mo of age (Albrecht et al., 1996). Indeed, Nishimura et al. (1999) demonstrated that *longissimus* IM fat depots have only reached 40% of their maximum size by the typical age of slaughter in Europe (18 mo of age). The low ($P <$ 0.05) PPARγ mRNA levels observed in the IM fat depot suggest that this depot has only partially matured and still has great potential to develop IM adipocytes. May et al. (1995) concluded that IM fat is a less mature depot than SC fat at the time of slaughter in conventional management regimens, confirming our observations.

We demonstrated a significant correlation of IM fat PPARγ expression with leptin in the SC fat depot for both Holstein and Charolais bulls. The lipostat theory (Rousseau et al., 1997) states that leptin serves as a signal reflecting the state of energy stores (including body fat, cell size and number) in the fat depot. The authors also state that leptin expression is under nutritional (both quantity and quality) control. We hypothesize that the SC fat depot may influence IM fat development through leptin. Qian et al. (1998) previously demonstrated that injection of leptin results in an increase in PPARγ levels, giving credence to our theory. Smith and Crouse (1984), however, have shown that IM

fat development is independent of the development of other fat depots. The significant correlation observed in Holstein between IM PPARγ and *longissimus* IM fat might indicate that PPARγ could serve as a marker for IM fat development because through biopsy measurements of mRNA expression level may be obtainable early in the life of the animal. A clear understanding, however, of why the relationship was only observed in one breed must be obtained prior to further interpretation.

Torii et al. (1998) demonstrated that bovine skeletal muscle contains fibroblast-like cells that possess functionally active PPARγ and suggested that these cells contribute to the deposition of IM fat. Other reports have described PPARγ expression in human skeletal muscle (Lapsys et al., 2000), fibroblast cells adjacent to murine skeletal muscle (Löhrke et al., 2000), and human vascular smooth muscle cells (Benson et al., 2000). Using immunohistochemistry, PPARγ expression was observed within the nuclei of human *quadriceps* muscle, rat *soleus* muscle, and adjacent vascular endothelial cells (Zierath et al., 1998). The origin of the cells expressing PPARγ in MU is not completely clear. One potential source is a population of mesenchymal stem cells resident in rat skeletal muscle, observed in both neonatal and adult animals (Pate et al., 1993; Lucas et al., 1995). Conversely, the expression of PPARγ in muscle may stem from endothelial cells that have also been shown to express PPARγ (Marx et al., 1999). In our investigation, PPARγ mRNA expression in MU was lower ($P < 0.05$) compared to SC, PR, OM, and IM fat depots, as we used whole *longissimus*, separated from IM fat, as the source of the mRNA for our analysis. Torii et al. (1998) observed only slightly lower PPARγ protein level in the bovine skeletal muscle-derived fibroblast-like cells when

compared to PR fat. Differences in protein level and mRNA expression profiles may account for this difference.

An alternate source of PPARγ mRNA is the muscle fibres themselves. Zierath et al. (1998) clearly demonstrated that PPARγ is expressed in the nuclei of muscle fibres. The number of muscle fibres expressing PPARγ was not measured, so the primary source of PPARγ in muscle is not currently known. The fibroblast-like cells expressing PPARγ in skeletal muscle most likely contribute to the formation of adipocytes (Torii et al., 1998), whereas PPARγ expressed in the muscle fibres contributes to the sensitization of insulin in insulin-resistant cells (Zierath et al., 1998). In addition, Boelsterli and Bedoucha (2002) have shown that prolonged activation of PPARγ in liver has been shown to result in steatosis. PPARγ in the muscle may therefore be a target used in the prevention of steatosis in cattle. The significant correlations we observed between MU PPARγ mRNA levels and fat carcass characteristics may result from signals between fat depots and fibroblast cells in the muscle, which possess the ability to differentiate into adipocytes. A better understanding of the respective roles of hyperplasia and hypertrophy in the tissues could be obtained if the expression of PPARγ that caused adipogenesis could be differentiated from the expression of PPARγ that maintains the adipocyte. Further investigation in this area could shed new light on the process of bovine adipogenesis.

3.6 Conclusion

The absolute expression level of PPARγ mRNA may be an indication of the extent of adipocyte development in cattle. As expression of PPARγ was equivalent in SC and PR

depots, and only slightly higher in Charolais bulls for OM fat, the formation of new adipocytes in these depots had most likely finished while hypertrophy continued until slaughter as long as sufficient nutrients were available. On the other hand, IM fat hyperplasia is still active while adipocyte hyperplasia in MU tissue is at an early stage of development. MU PPARγ expression most likely originates from both muscles and mesenchymal cells resident in the skeletal muscle. Leptin expression from the SC fat depot may have a role in the regulation of PPARγ expression in the IM fat depot, resulting in the development of marbling fat in the muscle. The nutritionally controlled SC fat depot, therefore, may have a role in the determination of IM fat development through PPARγ and leptin. This could have an impact on breeding strategies aimed at increasing IM fat formation while decreasing other depot fat stores for better meat quality and greater production efficiency.

3.7 Acknowledgements

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4. Immunohistochemical Localisation of Preadipocyte Factor-1: Potential Marker of Preadipocytes in Bovine Muscle Tissue

4.1 Abstract

The objective of the present study was to develop an immunohistochemical technique to locate and quantify preadipocytes in bovine muscle tissue. Preadipocyte factor-1 (pref-1) is a transmembrane protein that possesses six Epidermal Growth Factor (EGF)-like sequences at the extracellular domain, a single transmembrane domain and a short intracellular tail and is part of the family of EGF-like repeat-containing proteins that are involved in cell fate determination. Pref-1 is highly expressed in preadipocytes, but expression is completely abolished during differentiation into an adipocyte. In the present study, reverse transcription polymerase chain reaction demonstrated the bovine intramuscular adipose tissue contains the three splice forms of pref-1 (A, C2, and E) previously demonstrated to be expressed in bovine adipose tissue. As well, Western blots were used to confirm that the protein for pref-1 was expressed in intramuscular adipose tissue. The polyclonal antibodies against pref-1 were then tested against a cell culture of bovine preadipocytes from an embryo source to confirm that the antibody would function at immunolocating bovine preadipocytes. The antibody was tested in sections of *longissimus dorsi* muscle from Charolais and Holstein cattle. Immunohistochemical results showed that pref-1 is expressed in the perimycium near mature adipocytes and near blood vessels. The pool size of preadipocytes appeared to be low. Previous reports, however, have demonstrated that preadipocytes are known to divide providing an endless source of adipogenic precursor cells.

4.2 Introduction

During times of decreased food availability or during extended periods away from an immediate source of food, animals must have a source of energy that is both light and compact. Triacylglycerol (TAG) serves this purpose because it is energy dense and hydrophobic, as one gram of TAG contains virtually no water whereas a gram of glycogen contains upwards of 75% water. TAG can be stored in virtually any cell of the body (Pond, 1992). Lower vertebrates store the majority of their TAG in skeletal muscle and liver whereas higher vertebrates have evolved a speciality type of cell, the adipocyte, to store TAG. Adipocytes are the major cellular component of adipose tissue with the other cellular components including blood vessel and lymph channel cells along with connective tissue and preadipocytes (also known as fibroblast-like mesenchymal cells).

Anatomical studies by Pond (1986) showed that there are some 16 adipose depots in mammals and birds in the abdominal cavity, within the musculature and under the skin. The intramuscular (i.m.) adipose depot comprises a true adipose tissue being composed of adipocytes embedded in a connective tissue matrix and occurring in close proximity to a blood capillary network (Hood and Allen, 1973). In cattle, adipocytes from the i.m. depot are generally smaller in diameter (Cianzio et al., 1985) and generally occur in clusters of 15 to 20 adipocyte cells. The smaller i.m. adipocyte size and their proximity to blood vessels in muscle tissue suggests that they serve as an immediate source of energy for muscle fibres (Pond, 1992).

The development of large deposits of i.m. adipocytes in cattle is called marbling. Marbling has been shown to have a low association with meat palatability, tenderness and juiciness (Wheeler et al., 1994; Jeremiah, 1996; Johnston, 2003). Marbling is an

important trait to the meat packing industry because carcasses containing greater amounts of marbling are given a higher sale value. In addition, the deposition of i.m. fat in cattle is a continuously varying trait exhibiting a high degree of heritability with levels of up to 0.65 when evaluated in individual cattle at a constant age (Marschall, 1999). The relatively high level of heritability, along with the poor association between the deposition of i.m. fat and fat in other adipose depots (Cianzio et al. 1985), opens up the opportunity to select for the marbling traits using breeding schemes (Kinghorn and Simm, 1999).

The differentiation process of a preadipocyte to an adipocyte, known as adipogenesis, involves the change in expression of approximately 2000 different genes (Guo and Liao, 2000). Of those genes, very few have been characterised for their respective function in the differentiation process as over 100 expressed sequence tags representing uncharacterised genes were expressed only in preadipocytes or in adipocytes (Guo and Liao, 2000). In addition, unlike adipocytes, preadipocytes are capable of undergoing cell division making attempts at quantification of the adipocyte developmental potential in tissue difficult (reviewed by Gregoire et al., 1998).

Numerous methods to stain for and quantify fully mature adipocytes in muscle tissue have been developed and validated (Preece, 1972; Hausman, 1981). These procedures have been established because the major component of adipocytes, TAG, is easily visualised microscopically with the use of stains such as Oil Red O and Sudan Black B. Undifferentiated adipocytes, or preadipocytes, however, cannot be distinguished solely on the basis of their morphology. Attempts therefore have been undertaken to develop immunohistochemical techniques to locate and determine the number of

preadipocytes in order to assess the size of the preadipocyte population in different tissues. Hollenberg and Vost (1968) first demonstrated the existence of preadipocytes in adult tissue through studies in rat adipose tissue using thymidine incorporation into DNA. The origin of these preadipocytes, however, is still under debate. Research using multipotent clonal cell lines has suggested that the lineage of preadipocytes originate from embryonic stem cell precursors that have the capacity to differentiate into adipocyte, chondrocyte, osteoblast and myocyte mesodermal cell types (Gregoire et al., 1998). Conversely, a bone marrow stromal cell type has been shown to give rise to adipogenic and osteogenic cells (Gregoire et al., 1998) thus suggesting an unlimited supply of progenitor adipocyte cells.

Histochemical studies with rats indicated that S-100 protein, thought to be a carrier of FAs in the lipolytic mechanisms of adipocytes (Haimoto et al., 1985), is present in normal, early and late preadipocytes that are found in mature epididymal adipose tissue *in vivo* and *in vitro* (Cinti et al., 1989). Additional studies indicated that S-100 protein is expressed in preadipocyte cells of human embryonal subcutis (mesenchymal cells differentiating into fibroblasts and fibrocytes and endothelial cells) origin (Atanassova, 2001). Both studies indicated that S-100 protein might be used as a reliable biochemical and immunocytochemical marker to distinguish early preadipocyte cells from fibroblast cells. S-100 protein was expressed, however, in mature adipocytes thus limiting the use of S-100 protein as a preadipocyte-specific marker. Analyses by Wright and Hausman (1990) also indicated that certain cellular cell surface components could be used as targets for differentiating between immature and mature adipocytes. The monoclonal antibodies AD-1 and AD-2 were developed by Wright and Hausman (1990), and later Yu

et al. (1997) developed the AD-3 antibody, used as a marker of preadipocytes, which immunolocates an unidentified cell surface protein.

Preadipocyte factor-1 (pref-1), also known as dlk (Lee et al., 1995), is a transmembrane protein that possesses six Epidermal Growth Factor (EGF)-like sequences at the extracellular domain, a single transmembrane domain and a short intracellular tail (reviewed by Laborda, 2000). Pref-1 contains two proteolytic processing sites, that upon cleavage by an as yet uncharacterised protease, result in 50 and 25 kDa soluble proteins (Smas et al., 1997). Pref-1 is abundantly expressed in preadipocytes, characterised by complete abolishment of expression during adipocyte conversion, with constitutive overexpression blocking adipogenesis (Smas and Sul, 1993). Results therefore indicated that pref-1 may serve as a potential marker of preadipocytes. The current study demonstrates an immunohistochemical method for immunolocalizing preadipocytes in tissue using pref-1 as a marker of preadipocytes.

4.3 Materials and Methods

4.3.1 Animals and Diet

German Holstein and Charolais bulls (*Bos taurus*) were used in the investigation. Animals were cared for according to the regulations of the Animal Protection Committee from the German Ministry for Consumer Protection, Food and Agriculture. Calves were fed with a milk replacer diet up to 4 mo of age. After weaning, the bulls received a body weight-related diet consisting of concentrates based on barley, beet pulp and soybean extract meal (92.8% organic matter, 15% crude protein, and 9% crude fibre) and hay. All

animals were reared using a tethering system in individual pens and were fed the same diet. The dietary intake of the study animals in described in Kühn et al. (2002).

4.3.2 Muscle Sample Collection

All animals were slaughtered at 18 mo of age in the Research Institute's abattoir. Muscle samples were obtained immediately *post mortem* from the central surfaces of *longissimus dorsi* muscles of German Holstein or Charolais cattle. The samples were immediately flash frozen in liquid nitrogen and stored at -80°C until sectioning or RNA/protein isolation.

4.3.3 RT-PCR

Total RNA was isolated from the i.m. fat samples using TRIzol reagent (Life Technologie GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. Samples of muscle tissue were magnified 20x using a dissection microscope (AskAnia GSZ 2T, Germany) while the i.m. fat was separated from the muscle tissue that was held on dry ice. I.m. fat was stored in RNAlater (Ambion, Inc. Austin, Texas) at –20°C until RNA or protein extraction within a 2 week period.

RNA yields and purities were assessed by absorbance at 260 and 280 nm using a RNA/DNA calculator (Pharmacia GeneQuant™, Erlangen, Germany). Ratios of absorbtion (260/280 nm) of all samples were between 1.7 and 2.0.

The cDNA was synthesised using 1.0 µg of total RNA from each sample. To eliminate residual genomic DNA from the RNA sample, 1 unit of DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) was added, prior to the reverse transcription (RT) reaction, and incubated at 37°C for 30 min followed by heat inactivation of the enzyme at 75°C for 5 min (Huang et al., 1996). RNA samples were denatured at 65°C for

15 min and placed on ice for 5 min before RT. The final reaction volume of 25 µL contained 1x reaction buffer, 5 mM $MgCl_2$, 1 mM of dNTPs, 3.2 µg of random hexamer primer, 50 units of RNase inhibitor, 0.01 mg/mL gelatin and 20 units of AMV reverse transcriptase (1st Strand cDNA Synthesis Kit, Roche Diagnostics GmbH). The reaction was performed at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min for enzyme inactivation, and 4°C for 5 min. The RT products were either stored at –20°C or used immediately for polymerase chain reaction (PCR).

The PCR reaction was performed in a 50 µL reaction volume containing 2 µL of tissue-specific cDNA (equivalent to 80 ng starting RNA), 1.5 mM $MgCl_2$, 2 units of Taq DNA polymerase (Roche Diagnostics GmbH), 0.2 mM of dNTPs and 0.4 µM of each primer. For amplification of pref-1 the following primer pairs were used: forward 5'-GGT GAC TTC CGC TGC CGT TGC-3' and reverse 5'-GAT GGT GAA GCA GAT GGC CTG, the latter of which brackets the alternative splice-donors and splice acceptors and are both specific for bovine pref-1 (Figure 12) (Minoshima et al., 2001; Fahrenkrug et al., 1999). After amplification, 10 µL of the PCR products were used for electrophoresis on a 2% agarose gel. Gels were stained with ethidium bromide and imaged using the Kodak DS Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA). For the control samples, 2 µL of water was used instead of the tissue-specific cDNA.

4.3.4 Western blotting

Protein expression was analysed using Western blotting. Equivalent concentrations of i.m. fat protein (50 µg) were resolved by SDS-PAGE (Laemmli, 1970) with a separating gel consisting of 12% acrylamide/bis (12% total concentration; 1.1%

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M  A  A  T  A  A  L  L  P  A  L  L  L  L  L  A  F  G  R  S
atggccgcgaccgcagccctcctgcccgccctcttgctcctgctggctttcggccgcagt          60
A  H  G  A  E  C  F  P  A  C  H  P  E  N  G  F  C  D  D  D
gcccatggagctgaatgcttcccggcctgccaccctgaaaatggattctgcgacgatgac          120
S  V  C  R  C  Q  P  G  W  Q  G  P  L  C  D  Q  C  V  T  F
agtgtgtgcaggtgccagcctggctggcagggtcccctgtgtgaccagtgcgtgaccttt          180
P  G  C  V  N  G  L  C  V  E  P  W  Q  C  I  C  K  D  G  W
cccggctgtgtgaacggcctctgcgtggagccatggcagtgcatctgcaaggacggctgg          240
D  G  H  L  C  D  L  D  I  R  A  C  T  S  T  P  C  A  N  N
gacggacacctctgtgacctagacatccgggcttgcacctcgacccccctgcgccaacaac          300
G  T  C  L  N  L  D  D  G  Q  Y  E  C  S  C  A  P  G  F  S
ggcacctgcctgaacctcgatgacggccagtacgagtgctcctgcgcccccgggttctca          360
G  K  D  C  Q  E  M  D  G  P  C  V  V  N  G  S  P  C  Q  H
ggaaaggattgtcaggaaatggatgggccctgcgtggtgaatggctcgccctgccagcac          420
G  G  S  C  V  D  D  E  G  R  A  P  H  A  V  C  L  C  P  P
ggaggcagctgcgtggacgatgagggccgggccccccacgctgtctgcctgtgccccct          480
G  F  S  G  N  F  C  E  I  V  T  N  S  C  I  P  N  P  C  E
ggcttctcgggcaacttctgcgagatcgtgaccaacagctgcatccccaacccgtgcgag          540
N  Q  G  I  C  T  D  I  G  G  D  F  R  C  R  C  P  A  G  F
aaccagggcatctgcaccgacatcgggggtgacttccgctgccgttgccccgccggcttc          600
M  D  K  T  C  S  R  P  V  N  T  C  T  S  E  P  C  L  N  G
atggacaagacctgcagccgcccggtgaacacctgcaccagcgagccgtgcctcaacggc          660
G  T  C  L  Q  H  S  Q  V  S  F  E  C  L  C  K  P  A  F  T
ggcacctgcctgcagcactccCAGG▲C2tgagcttcgagtgtctgtgcaagcccgcgttcacc      720
G  P  R  C  G  R  K  R  A  A  G  P  Q  Q  V  T  R  L  P  S
ggcccccggtgtggccggaagcgcgcggcgggcccccagCAGG▲Etcacccgtctgcccagc       780
G  Y  G  L  T  Y  R  L  T  P  G  V  H  E  L  P  V  P  Q  P
ggttacgggctgacctaccgcctgaccccctggggtgcacgagctgccggtgccgcagccc          840
E  H  R  V  L  K  V  S  M  K  E  L  N  K  S  T  P  L  L  S
gagcaccgcgtcctgaaggtgtccatgaaggagctcaacaagagcactccgctcctctcc          900
E  G  Q  A  I  C  F  T  I  L  G  V  L  T  S  L  V  V  L  G
gagggaCAGG▲C2/ECCATCTGCTTCACCATCctgggcgtgctcaccagcctggtggtcctgggc    960
T  M  G  I  V  F  L  N  K  C  E  A  W  V  S  N  L  R  Y  N
accatgggcatcgtcttcctcaacaagtgcgaggcctgggtgtccaatctgcgctacaac         1020
H  M  L  R  K  K  K  N  L  L  L  H  Y  N  S  G  E  E  L  A
cacatgttgcgcaagaagaagaacctgctgctgcactacaacagcggggaggagctggcc         1080
V  N  I  V  F  P  E  K  I  D  M  T  T  F  T  K  E  A  G  E
gtcaacatcgtcttcccggagaagatcgacatgaccaccttcaccaaggaggccggcgag         1140
E  E  I  *
gaggagatctga                                                         1152
```

Figure 12. Nucleotide and deduced amino acid sequence for bovine pref-1A. The forward (single underlined nucleotide sequence) and reverse (double underlined nucleotide

sequence) primer sequences are noted. The reverse primer sequence is broken into two segments due to the predicted alternative splicing events. The symbols $\blacktriangle_{C2}$ and $\blacktriangle_{E}$ denote the alternative splice sites that produce the C2 an E splice forms. Beginning at the translation start site, the underlined amino acid sequence denotes the respective location of the signal peptide, epidermal growth factor (EGF)-like repeats 1, 2, 3, 4, 5, and 6, and the transmembrane region.

cross-linker). Protein was obtained after RNA extraction following the TRIzol reagent protocol (Life Technologie) according to the manufacturer's instructions for protein extraction. The gel was electroblotted onto nitrocellulose, and placed in 1x TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 20 min. The blot was blocked for 60 min at room temperature with shaking in a 3% (w/v) solution of gelatin in TBS. Immunoblotting was performed following the protocol for the goat anti-rabbit IgG (H and L) horse radish peroxidase (HRP) Immun-Blot Assay Kit (Bio-Rad Laboratories, München, Germany) using a pref-1 antibody concentration of 1:500. The antibody was designed based on both the intracellular and extracellular regions of the rat pref-1 sequence and is described by Carlsson et al. (1997).

4.3.5 Immunocytochemical reaction with pref-1 antibody

Bovine embryonic muscle preadipocytes were acquired from the Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany. Cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Prior to the immunocytochemical reaction, cells were fixed in 4% paraformaldehyde at 4°C for 20 min. Cell permeabolization was performed using 0.2% Triton X-100 in 1x phosphate buffered saline (PBS) (500 µM KH_2PO_4, 5 mM Na_2HPO_4, 68.5 mM NaCl, 1350 µM KCl, pH 7.4) for 20 min at room temperature. Cells were centrifuged at 310 x g for 5 min at room temperature, rinsed with 1x PBS for 5 min, and centrifuged again. The immunocytochemical reaction followed the protocol for the Tyramide Signalling Amplification (TSA) Kit #19 (Molecular Probes, Eugene, OR, USA) with a few modifications. Procedures were performed at room temperature except where stated. Endogenous peroxidase activity was blocked with 1% H_2O_2 for 60 min and

cells were subsequently spun at 310 x g for 5 min at room temperature. Cells were incubated in 1% blocking agent for 60 min at room temperature, incubated with pref-1 antibody diluted 1:30 in blocking agent overnight at 4°C, rinsed once with 1x PBS for five min, and incubated with a 1:100 dilution of HRP conjugate working solution for 60 min. Cells were rinsed once for 5 min in 1x PBS. Tyramide labelling of the cells followed the manufacturers recommended protocol. Counter-staining was performed using 1mg/mL bis-benzimide (Hoechst, see below) (Sigma-Aldrich, Taufkirchen, Germany) to stain for all cell nucleic acid for a 10 min incubation period. Cells were spun as above, plated on SuperFrost*/Plus slides (Menzel-Gläser, Karlsruhe, Germany) previously treated with 0.1% poly-L-lysine (Sigma), cover-slipped with an aqueous-based mounting media, and viewed with the fluorescent microscope.

4.3.6 Immunohistochemical reaction with pref-1 antibody

Ten micron thick transverse muscle sections were cut using a Cryostat 2800N Frigocut (Reichert-Jung, Leica, Bensheim, Germany) set at -21°C. Sections were placed on SuperFrost*/Plus slides (Menzel-Gläser) previously treated with 0.1% poly-L-lysine (Sigma). Sections were allowed to sit at room temperature to dry, fixed in 2% paraformaldehyde, 0.1 M L-lysine, 0.1 M sodium meta-periodate in 0.1 M Na_2HPO_4, (pH 7.4 (PLP) solution (made immediately prior to use) for 20 min at 4°C and rinsed three times for five min in 1x PBS. Antigen retrieval was performed by microwaving the sections two times for five min each in Coplin jars each time in 0.05 M glycine-HCl buffer (pH 3.5) that was changed every five min. Sections were rinsed three times in 1x PBS for five min, permeabolized with acetone for 20 min at -20°C, and rinsed once with 1x PBS for five min.

The immunohistochemical reaction followed the protocol for the TSA Kit #19 (Molecular Probes, Eugene, OR, USA). Endogenous peroxidase activity was blocked with 1% H_2O_2 for 60 min. Briefly, sections were incubated in 1% blocking agent for 60 min at room temperature. The tissue was incubated with pref-1 antibody diluted 1:20 in blocking agent for 60 min, rinsed three times with 1x PBS for five min each and incubated with a 1:100 dilution of HRP conjugate working solution for 60 min. Sections were rinsed three times for 5 min each in 1x PBS. Tyramide labelling of the sections followed the manufacturers recommended protocol. Table 5 provides an overview comparing the immunocytochemical and immunohistochemical staining procedures. Controls for cell culture and tissue staining consisted of the respective immunostaining protocol with the absence of pref-1 antibody.

4.3.7 Nuclear localisation and adipocyte staining

Bis-benzimide (Hoechst, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used to stain for all cell nucleic acid. Stock bis-benzimide was made by dissolving 0.001 g of bis-benzimide in 1 mL of deionised water. A sufficient aliquot of the stock to cover the sections was incubated for 10 min at room temperature after which the sections or cells were rinsed once for 5 min in water.

A stock solution of Oil-Red O (Chroma GmbH+Co. KG, Munster-Roxel, Germany) was prepared by adding 0.50 g oil red O in 0.10 L isopropanol (CHROMA GmbH+Co. KG). Prior to staining, 12 mL oil red O stock was diluted in 8 mL deionized water and left to stand for 20 min. The diluted oil red O solution was filtered through Whatman paper number 42 (Whatman, Maidstone, UK) to remove any crystallized oil red O. Only muscle sections were immersed in the final working solution for 10 min at room temperature and

washed three times using deionised water for 5 min each. Muscle sections and plated preadipocyte cells were covered with a coverslip using MOWIOL (Sigma).

4.3.8 Image acquisition

Cells and sections were visualised using a Microphot-SA fluorescence microscope (Nikon, Europe B.V., The Netherlands) equipped with 3-CCD colour camera (Sony, Japan) attached to a Pentium III 700 MHz PC computer. Immunostained sections were examined in brightfield as well as in epifluorescence using a FITC/Auramine excitation filter (420 – 490 nm). Digitally captured images were processed using Image C software (version 2.52, Level 510, Aquinto, Berlin, Germany). Images containing positive reactions for pref-1 antibody were captured from different areas of multiple sections. All images (768 x 576 pixels) were saved as TIFF files to maximize image quality.

4.4 Results

4.4.1 Expression of pref-1 mRNA

The coding sequence for full length pref-1A shown in Figure 12 was obtained from Fahrenkrug et al. (1999) along with the derived information outlined in Vuocolo et al. (2003). The PCR primers used to amplify the three characterized bovine splice forms (pref-1A, pref-1E, and pref-1C2) are noted along with the deduced amino acid sequence and the location of the signal peptide, six EGF-like repeats and the transmembrane region. RT-PCR was used to amplify the splice forms from bovine i.m. adipose tissue, identified based on the size of the migrated bands (Figure 13). Results indicated that i.m. adipose tissue in cattle contain the mRNA for the three splice forms (two analogous to

Table 5. Summarized immunocytochemical and immunohistochemical staining protocol for preadipocyte factor-1 (pref-1). All protocols were performed at room temperature unless otherwise stated. [a] This procedure has also been performed using 0.8 M urea with a lesser degree of permeation. [b] This procedure has also been performed using Triton X-100 for bovine muscle tissue and was unsuccessful due to the delipidising effect of Triton X-100.

Treatment	Bovine Preadipocyte Cell Culture	Bovine Muscle Tissue
Fixation	4% paraformaldehyde, 4°C, 20 min	PLP, 4°C, 20 min
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Pretreatment [a]	-	Microwave, 0.05 M glycine-HCl (pH 3.5), 2x, 5 min
Rinsing	-	1x PBS, 3x, 5 min
Permeabolization [b]	0.2% Triton X-100 (in 1x PBS), 20 min	Acetone, -20°C, 20 min
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Peroxidase Quench	1% H_2O_2, 60 min	1% H_2O_2, 60 min
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Blocking	1% Blocking Agent, 60 min	1% Blocking Agent, 60 min
Incubation	Pref-1 Ab 1:30, overnight at 4°C	Pref-1 Ab 1:20, 60 min,
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Incubation	Horse Radish Peroxidase conjugate 1:100, 60 min	Horse Radish Peroxidase conjugate 1:100, 60 min
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Incubation	Tyramide labelling, 1:100, 10 min	Tyramide labelling, 1:100, 10 min
Rinsing	Centrifuged 310 x g 5 min, rinsed 1x PBS, 5 min, recentrifuged	1x PBS, 3x, 5 min
Staining	Bis-benzimide, 10 min	Bis-benzimide, 10 min
Staining	-	Oil-Red O, 10 min
Rinsing	deionised water, 5 min	deionised water, 5 min
Mounting	MOWIOL	MOWIOL

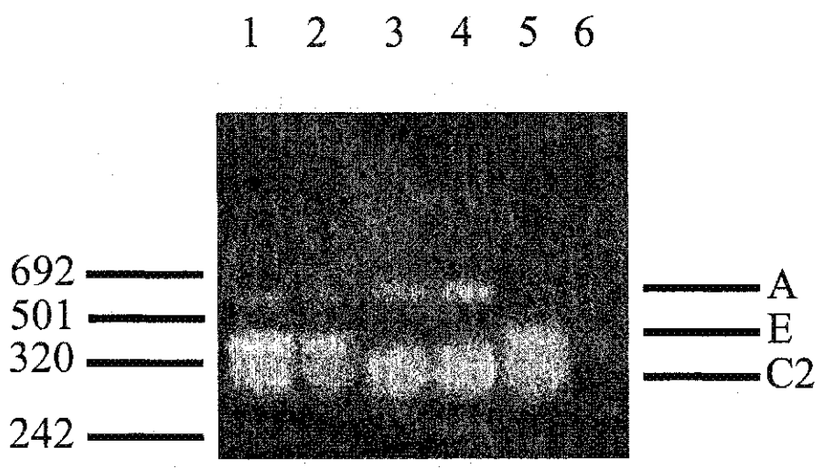

Figure 13. The expression of pref-1 mRNA in the intramuscular adipose depot from Holstein and Charolais cattle. Total mRNAs were extracted, sampled after slaughter, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a pair of bovine-specific pref-1 primers. PCR products resolved by electrophoresis on 2% agarose gels for the purpose of confirming the presence of pref-1 mRNA in the intramuscular adipose depot. The bp ladder is denoted to the left of the gel whereas the proposed splice forms of pref-1 are denoted on the right. Lanes 1 and 2 are from Holstein whereas 3, 4 and 5 are from Charolais, with lane 6 as a negative control (water replaced cDNA in the reaction). The letters A, E and C2 designate the respective splice forms known to exist in cattle.

the human and mouse forms and one unique cattle form (E)) identified previously by Fahrenkrug et al. (1999).

4.4.2 Expression of pref-1 protein

To confirm that pref-1 protein was present in bovine i.m. adipose tissue, Western analysis of isolated protein was conducted. Use of pref-1 specific antibody detected multiple forms of pref-1 in the range of 45 to 60 kDa indicating that bovine i.m. adipose tissue contains cells expressing pref-1 protein (Figure 14).

4.4.3 Immunolocalization of pref-1 in bovine preadipocytes

To confirm that the pref-1 antibody would immunolocate bovine preadipocytes, a bovine preadipocyte cell culture was used as a positive control. The preadipocyte cells were used within a 24 hour period of culturing because any delay resulted in the development of triacylglycerol (TAG) droplets in each cell due to the presence of fetal bovine serum in the culture medium stimulating adipogenesis, thus indicating that cellular differentiation had occurred. In the culture, pref-1 immunoreactivity was localized to the cellular surface of each preadipocyte. The morphological sign of the immunocytochemical reaction was a fluorescent green stain in pref-1 positive cells, whereas nucleic acid was morphologically fluorescent blue. The pref-1 positive cells were round in shape with centrally located nuclei (Figure 15). Most of the positive cells in culture displayed strong staining (Figure 15A) to the pref-1 antibody, but not all cells in culture were positive (Figure 15B). All controls appeared negative (see Appendix 2, Figure 23). A permeabolization step was required for staining to occur as attempts at staining using only the TSA procedure resulted in no staining (data not shown).

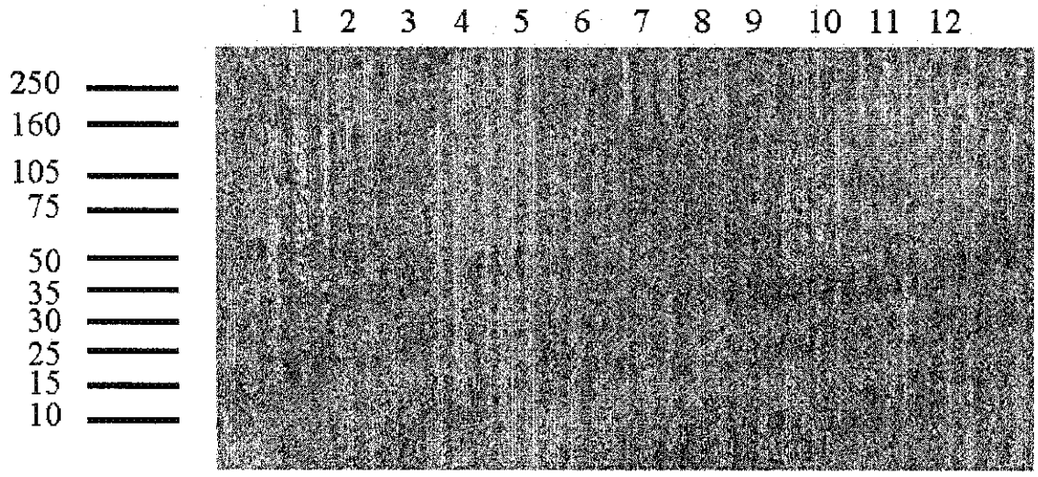

Figure 14. The expression of pref-1 protein in the intramuscular adipose depot from bulls. Total protein was extracted, sampled after slaughter, and applied to a SDS-PAGE (50 μg per lane) using a separating gel consisting of 12% acrylamide/bis (12% total concentration; 1.1% cross-linker). The gel was electroblotted onto nitrocellulose and immunoblotted using antibodies against pref-1 (1:500 dilution) for the purpose of confirming the presence of pref-1 protein in the intramuscular adipose depot. Lanes 1 through 6 denote protein from six different German Holstein bulls, whereas lanes 7 through 12 denote protein from six different Charolais bulls. Western blots performed with no antibody did not exhibit any banding.

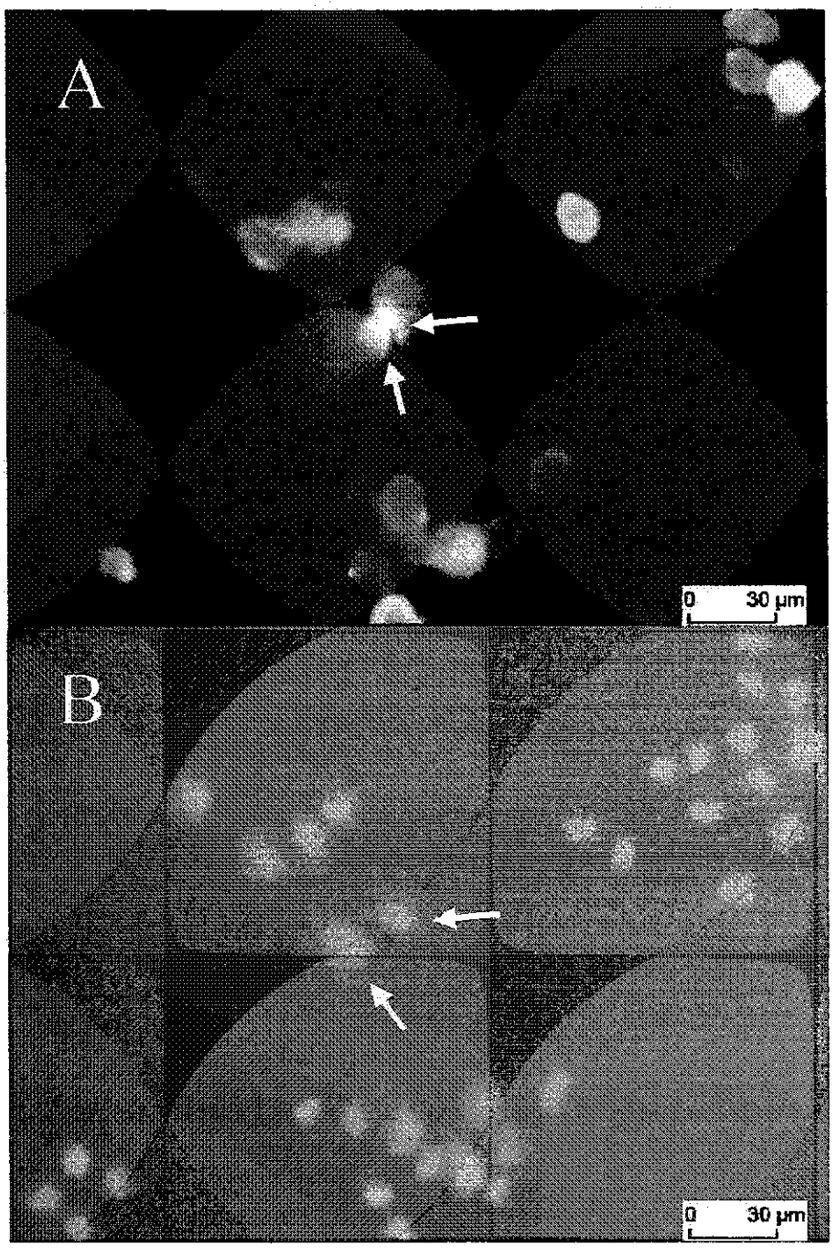

Figure 15. Immunocytochemical reaction (arrows) for pref-1 (A) and corresponding nucleic acid stain (B) in bovine embryonic muscle preadipocytes. Results confirm that the antibody employed in the present study, developed from the rat pref-1 sequence (Carlsson, et al., 1997), was capable of immunolocating bovine preadipocytes.

4.4.4 Immunolocalization of pref-1 in muscle tissue sections

The pref-1 immunostaining protocol was applied to muscle sections. Muscle sections were fixed in PLP to preserve tissue morphology and antigenicity. Permeabolization was performed using acetone rather than Triton X-100 due to the delipidizing effect of Triton X-100 on adipocytes in the muscle section. Positive reactions for pref-1 were localized to two regions: in the perimycium near fully differentiated adipocytes (Figure 16) and in the perimycium near blood vessels (Figure 17). No differences were observable between either cattle breed, thus, all experimental results were considered together.

4.5 Discussion

Previous investigators have developed methods to quantify preadipocytes (Kirkland et al., 1994), but the tissue morphology was destroyed during processing. Thus, no information about preadipocyte localization was obtained. In order to develop an immunohistochemical procedure to localize and quantify preadipocytes in undisturbed whole muscle tissue, we used antibodies against pref-1 to immunolocate bovine preadipocytes. The present study suggests that preadipocytes are expressed in low numbers in the perimycium of bovine skeletal muscle and are located near mature adipocytes and blood vessels.

In the present study, RT-PCR analysis demonstrated that bovine i.m. adipose tissue contains the three splice forms previously reported for bovine pref-1 (Fahrenkrug et al., 1999). Different expression levels of each isoform are visible in the gel, but quantification of each isoform was not performed. Minoshima et al. (2001) demonstrated

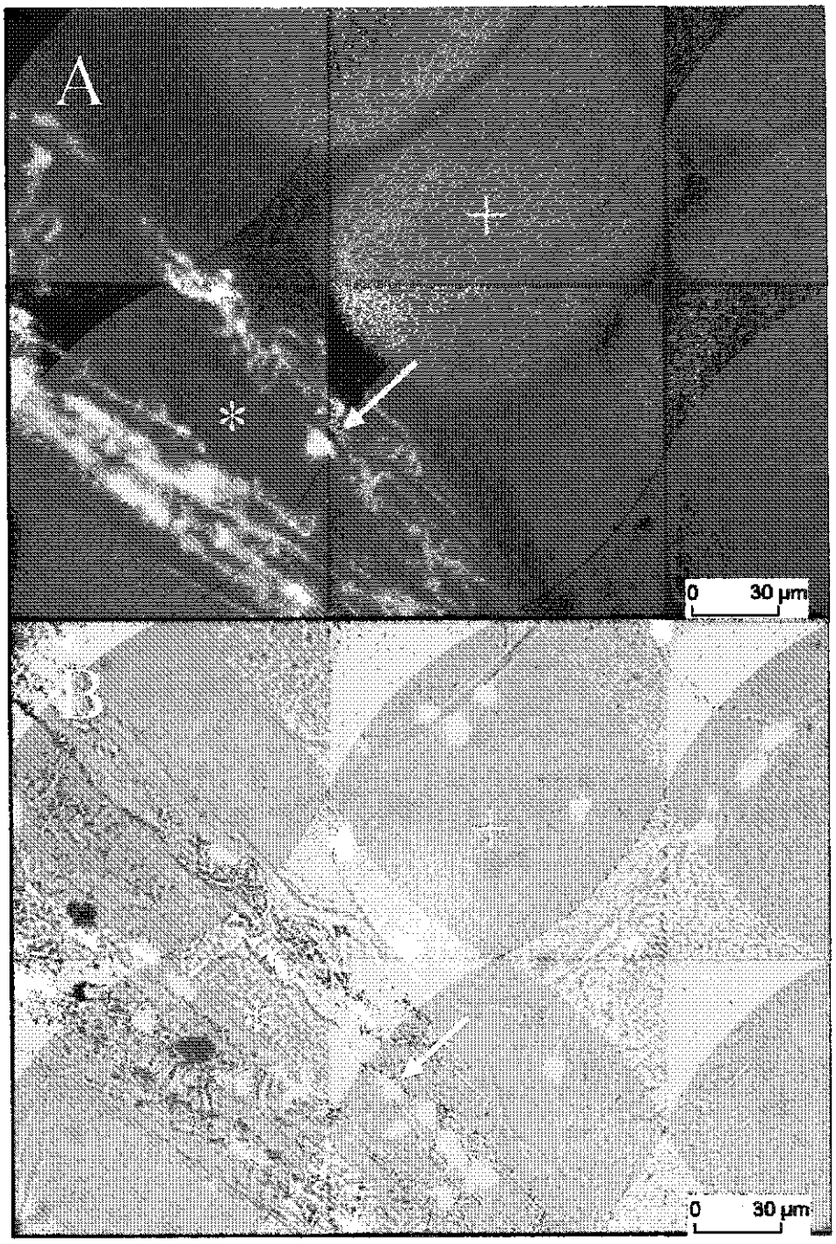

Figure 16. Immunohistochemical reaction (arrow) for pref-1 (A) and corresponding nucleic acid stain (B) in bovine *longissimus dorsi* muscle; *, Adipocyte, +, Muscle fibre. A positive reaction for pref-1 in the perimycium may indicate the location of a developing preadipocyte.

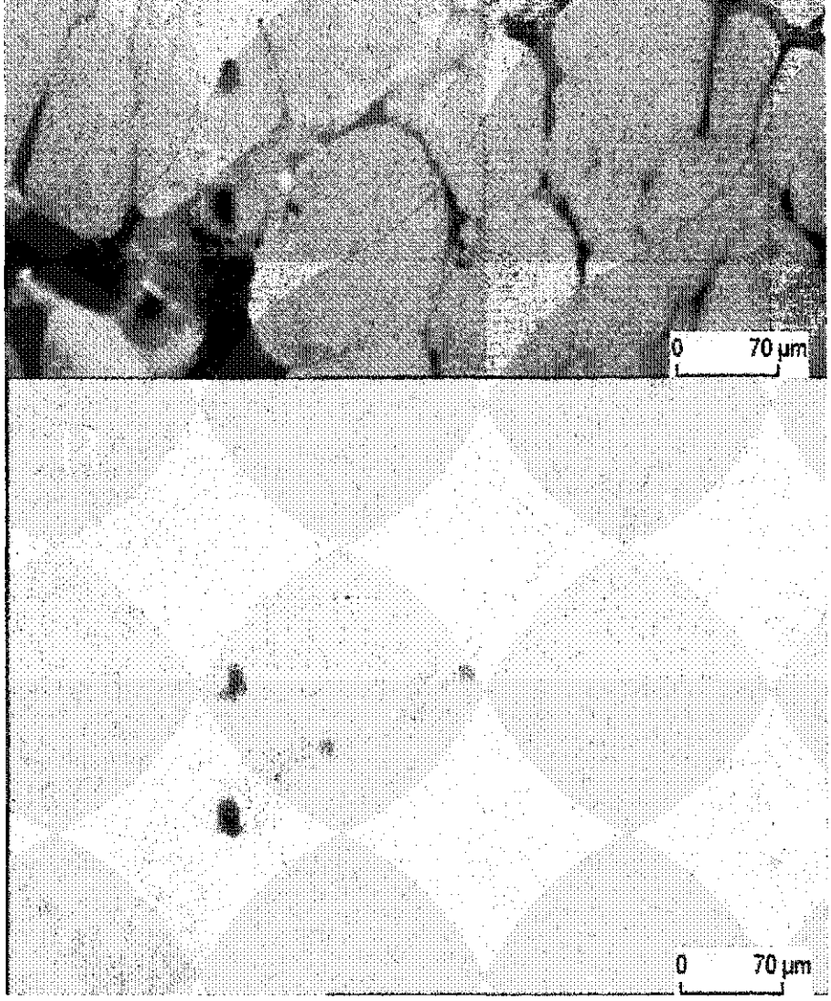

Figure 17. Immunohistochemical reaction (solid line arrow) for pref-1 in bovine muscle tissue near blood vessels (dashed line arrow) (A) and corresponding nucleic acid stain (B) in bovine *longissimus dorsi* muscle; +, Muscle fibre. A positive reaction for pref-1 in the perimycium may indicate the location of a developing preadipocyte.

that the pref-1A and pref-1C2 splice forms are present in neonatal fat tissue, but no splice forms were detectable in adult abdominal fat tissue (sampled at 30 mo of age), thus suggesting that pref-1 expression was suppressed in an age-dependent manner. Pref-1 expression is known to be restricted to the stromal-vascular (SV) fraction, but not the adipocyte fraction of adipose tissue (Mei et al., 2002), possibly explaining the lack of pref-1 expression observed by Minoshima et al. (2001) due to a lack of enriched SV cells in their preparation or possibly due to the use of a detection technique with low sensitivity.

The present study also indicated differences in the expression of pref-1 splice forms in different breeds of cattle. Results suggest that bovine pref-1 splice forms may be expressed in a breed-specific manner.Vuocolo et al. (2003) demonstrated expression of the pref-1C2 splice form in bovine cardiac, kidney and omental fat and in cardiac, *semitendinosus* and *longissimus dorsi* muscle using RT-PCR and quantitative real time RT-PCR, techniques known to have greater sensitivity. The use of whole skeletal muscle tissue as opposed to isolated i.m. fat may explain the expression of only one splice form of pref-1 as compared to the present study. Vuocolo et al. (2003) were unable to detect expression in subcutaneous or brisket adipose depots in the two animals used (one at 24 mo of age and one of unspecified age) and suggested that these depots have little capacity for further adipogenesis. Robelin (1981), Cianzio et al. (1985) and Truscott et al. (1983) have all shown apparent subcutaneous adipose tissue hyperplasia in mature animals, but no animals over 20 mo of age were sampled. Results suggest that adipocyte hyperplasia in the subcutaneous adipose depot may be age-dependent or adipocyte cell size dependent as suggested by Robelin (1981).

Each adipose depot grows at a different rate and differently with respect to hypertrophy or hyperplasia. As a general rule, during the finishing phase, the early developing depots (i.e. intermuscular, perirenal and mesenteric fat) have completed their hyperplastic development and thus deposit fat by hypertrophy, whereas the subcutaneous and intramuscular depots continue to develop by hyperplasia and hypertrophy of already recruited adipocytes (May et al., 1994; Sainz and Hasting, 2000). In addition, Wegner et al. (1998) clearly outlined i.m. adipocyte hyperplasia in Wagyu crossbreeds over a 24 month period in *longissimus dorsi* demonstrating that new adipocytes arise in muscular tissue with age. Nishimura et al. (1999) provided anecdotal evidence of adipocyte hyperplasia by demonstrating significant increases in crude fat content after 24 mo of age in Japanese Black steers. Results indicated that the i.m. adipose depot may develop at a later stage of bovine development and, thus, the expression of pref-1 mRNA may be different in this depot. In addition, Aso et al. (1995) demonstrated that the intramuscular adipose tissue from 24 mo old Japanese Black cattle contain a pool of preadipocyte cells that can differentiate into adipocytes.

The high sequence similarity among bovine, mouse, human and rat dlk proteins has been described previously (see Figure 2 from Laborda, 2000). Alignment analysis of the protein sequences from four mammalian species demonstrated a high level of homology among a variety of species. Results indicated that antibodies developed from a rat source might be used to immunolocate bovine pref-1 protein. Western blot analysis confirmed that the rat pref-1 antibody used in this study was capable of detecting the immobilized blotted antigen.

The low number of pref-1 positive cells in the cell culture may be due to a delay in the harvesting of the cells. Bovine serum is known to stimulate preadipocytes to differentiate into adipocytes (Torii et al., 1996). In the present study, differentiation was visualized by the development of small TAG droplets in the cytoplasm of the differentiating cells. The first stage in the development of an adipocyte is known to be the slight increase in the expression of pref-1, shortly followed by the complete loss of pref-1 (Hansen et al., 1998). Therefore, those cells not expressing pref-1 protein have most likely begun the differentiating steps leading to the formation of an adipocyte. Nevertheless, a positive reaction for pref-1 in the bovine preadipocyte culture demonstrated that the antibody in use has the potential to immunolocate bovine preadipocytes. In addition, the expression of pref-1 in preadipocyte cells suggests that it has a role in preadipocyte differentiation.

Structural and histochemical analyses by Hausman and Thomas (1986) indicated that a positive relationship existed between adipocyte cluster size and the proximity to the entry point of large (parent) blood vessels in both perirenal and subcutaneous adipose tissues. This relationship suggested that lipid deposition and capillary development depended on blood flow. In addition, the supply of blood vessels within 1° and 2° muscle bundles has been shown in Japanese Black cattle to result in the formation of i.m. adipose tissue (Hoshino et al., 1987). Results indicated that adipose tissues were formed around the vessels within the 2° muscle bundles. Thus, the supply of nutrients to the muscle tissue is directly related to the formation of i.m. adipocytes. Results from the present study demonstrated the expression of pref-1 near blood vessels in bovine skeletal muscle, but the expression level of pref-1 was low based on the number of pref-1 positive cells in

the immunohistochemical image. Nevertheless, a stem cell population that is maintained by 'asymmetric' division in muscle tissue is believed to serve as the source of preadipocytes (Grounds, 1999). As such, for each cell that differentiates into an adipocyte, another daughter cell is returned to the stem cell pool creating a possibly endless source of i.m. adipocytes. Histochemical images from the present study suggest that preadipocytes arise near mature adipocytes. Thus, the population of preadipocytes present in the i.m. adipocyte depot may arise from the same preadipocyte cell. Therefore, detection of one preadipocyte may indicate the location of a newly developing i.m. adipocyte population and may explain why i.m. adipocytes arise in clusters of 15-20 cells. Additionally, some stem cells in muscle are derived from a bone marrow source via the blood stream (Grounds, 1999). These results, in addition to the observations in the present study, possibly indicate that quantification of adipocyte precursor cells in muscle tissue may not be a viable indication of marbling potential in an animal.

Taylor-Jones et al. (2002) demonstrated that adipogenic markers were expressed in myoblasts from older mice that were undergoing myogenic fusion thus increasing the adipogenic potential of these cells. The authors' results may explain an additional source of adipocyte precursor cells in skeletal muscle tissue from older cattle not observed in the present study. Also, PPARγ1 and γ2 (transcription factors that regulate the expression of several genes involved in lipid metabolism and adipogenesis) were both expressed in older myoblasts, concomitant with no repression of myogenic gene expression. Results indicated that signalling pathways in myoblasts inhibit specific functions of PPARγ. Previous reports, however, have demonstrated inhibition of myogenic differentiation by this transcription factor class (Hu et al. 1995; Lecka-Czernik et al., 1999). Overall, results

from these investigations have suggested that, with ageing, mesenchymal stem cell populations are replaced with cells that have increased adipogenic potential. Conversely, ageing mesenchymal cells may inherently activate adipogenic gene programs.

It should also be noted that the Callipyge mutation, causing muscle hypertrophy and leanness, is co-located to the same chromosomal region as pref-1 (Vuocolo et al., 2003) and that sheep containing the Callipyge mutation over-express pref-1 (dlk therein). They propose that pref-1 overexpression may indicate a precursor cell population that contributes that abnormal muscle hypertrophy. Conversely, enhanced pref-1 expression may suppress muscle differentiation, resulting in abnormal muscle cell growth.

4.6 Conclusion

The following study demonstrates that pref-1 may be used as a biochemical and immunohistochemical marker for preadipocytes in bovine muscle tissue. Results, however, demonstrated that the number of preadipocytes present in the perimycium is low suggesting that the preadipocyte population is small, that i.m. preadipocytes arise from a cell population external to the muscle, or that the i.m. adipocyte population arise from the same preadipocyte precursor cell. Interestingly, Mei et al. (2002) recently demonstrated that only the large soluble form of pref-1 inhibits adipocyte differentiation. Thus, the generation of antibodies directed towards each respective protein isoform synthesized from the spliced mRNA may further characterize their respective roles in bovine adipogenesis.

4.7 Acknowledgements

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5. Conclusions and Implications

The absolute expression level of PPARγ mRNA may be an indication of the extent of adipocyte development in the animal, but protein expression levels would better characterize such a relationship. A pool of PPARγ mRNA may have been measured that explains the equivalent levels observed among some of the depots. We hypothesize, based on the low PPARγ mRNA levels that IM fat hyperplasia is still active while adipocyte hyperplasia in MU tissue is at an early stage of development. MU PPARγ expression most likely originates from both muscles and mesenchymal cells resident in the skeletal muscle. The SC fat depot may have a role in the determination of IM fat development. Breeding strategies aimed at increasing IM fat formation while decreasing other depot fat stores may potentially apply knowledge about the adipocyte development potential of the respective depots.

The immunohistochemical technique to localize preadipocytes may be used as a biochemical or histochemical marker to determine the location and number of preadipocytes in tissue. The application of the technique may be widespread in that the determination of the preadipocyte population may be an indication of the adipocyte developmental potential of a tissue. By determining the potential to which a particular animal may deposit adipocytes in the muscle, cattle breeders may exploit this trait to amplify the marbling potential of their animals through the use of immunohistochemical tests on biopsy sections. Conversely, breeders attempting to develop a leaner animal with a limited amount of adipocytes deposited in the muscle may wish to know the extent to which their animals may develop i.m. fat deposits and 'breed-out' this trait. In either case, cattle breeders will benefit by knowing their animal's adipocyte developmental potential.

The development of an applicable test, however, is many years away as evidence has shown that preadipocytes may have an extramuscular origin, not accounted for using the method presented.

In addition, with the significant increase in the number of overweight and obese people in society, testing the adipocyte developmental potential of an individual may be an indication of their respective risk at developing obesity, or weight-related disorders such as arteriosclerosis or diabetes. Nevertheless, because preadipocytes may arise from external sources to the tissue under investigation, the extent to which results of preadipocyte number may be applied, is limited.

The image analysis computer program to determine the amount of i.m. fat in whole muscle tissue is in use by Dr. Luigi Faucitano from Agriculture and Agri-Food Canada. Results may potentially better demonstrate the effect of different feeding systems or better characterize the effect of circulating hormone levels on the deposition of i.m. fat in muscle tissue. It is expected that this program will be substantially improved upon based on the experience and requirements of initial users.

6. Future Directions

The following section describes recommended directions that the research outlined in this thesis may take based on my experiences while conducting this research project. The suggestions may serve as the impetus for additional projects or directions that current projects may take. The section is divided into the three main subject areas investigated in this study.

6.1 Investigations into PPARγ Expression in Cattle

The interpretation of the research results were limited in this part of the thesis study because mRNA expression levels do not always reflect the level of protein in a biological system. Quantification of PPARγ protein levels would therefore be a natural extension of the investigation. As such, the significant experimental correlations observed herein would be reinforced by significant correlations that may be observed among PPARγ protein levels, carcass characteristics and protein levels of other adipogenic genes. In addition, real time RT-PCR measurements would reinforce our semi-quantitative RT-PCR results. Another extension of the research would involve the search for DNA polymorphism in the promoter region of PPARγ. Such results would show whether variation in the promoter region of PPARγ may result in variations observed in the cattle population.

6.2 Investigations into pref-1 Expression in Cattle

Adaptations to the methodology presented may indicate the predominant pref-1 splice form expressed in bovine tissue. The antibody used in the present thesis

immunolocates both the intra- and extracellular portions of the pref-1 protein. Therefore, the use of splice-form specific antibodies would produce information about the degree of differential splice-form expression. Furthermore, a time-course application of the procedure to bovine preadipocyte cell culture systems, from the preadipocyte stage to a fully differentiated adipocyte, may allow for the visualization of pref-1 expression throughout adipocyte differentiation. A better understanding of the slice form expression may provide information about each splice form's respective role in bovine adipogenesis. Quantification of the respective splice form expression using real time RT-PCR may also provide information about the respective role of each splice form.

Through the use of muscle biopsy techniques, currently in use at the Research Institute for the Biology of Farm Animals in Dummerstorf, Germany, samples of muscle tissue throughout the life of the same animal may be obtained. Therefore, through the application of the technique described in this thesis, measurements of preadipocyte number over an animal's lifetime may be obtained.

In situ hybridisation analysis of muscle tissue to characterize the mRNA expression of pref-1 would also be a future direction that the research may take. Currently, a pref-1 cDNA clone obtained in Japan is available to the researchers in Germany. Attempts to locate the mRNA for pref-1 would potentially confirm the protein expression results and possibly shed light on the location of other potential preadipocytes.

The additional results presented in the Appendix demonstrate that pref-1 may also be expressed in a muscle fibre type specific manner. Serial sections that respectively stain for pref-1 and for the three different muscle fibre types may allow for the characterization

of pref-1 in muscle tissue. In addition, Western blot expression or RT-PCR analysis would corroborate the histochemical sections already obtained.

6.3 Computer Image Analysis of Visible Intramuscular Adipose Tissue in Muscle

The current computer program may benefit from a number of additions. For example, one important aspect of the deposition of i.m. fat as it relates to a marbling grade is the distribution of the fat islands throughout the muscle. Currently, the program does not produce any data that describes the distribution of fat deposits, but this may be incorporated into the program. The ImageC program will allow each individual pixel in an image to be weighted based on the colour intensity of the respective pixel. Using such a function, the muscle's virtual centre of gravity may be determined thus allowing the muscle to be divided into four equilateral sections. Thus, the quantity of visible fat deposits in each quadrant may be determined after which the deviation among the four quadrants can be calculated and the degree of deviation from an equal distribution among the four quadrants determined. Results obtained from the current program, however, would allow for correlation analysis to feeding systems, the deposition of fat in other adipose depots, circulating hormone levels such as leptin or insulin along with other carcass characteristics.

Similar image analysis programs have also been used in the objective analysis of marbling in a slaughterhouse environment. The only limitation to such an application would be the necessity to section and stain the muscle, which may result in a greater amount of work than necessary for the information obtained. The application may however be viable without the use of staining via the inclusion of UV lighting, a light

source known to illuminate connective tissue thus allowing for the subtraction of the effect of the white connective tissue from the i.m. fat.

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APPENDIX 1

DEVELOPMENT OF A COMPUTER IMAGE ANALYSIS PROGRAM FOR THE QUANTIFICATION AND CHARACTERIZATION OF INTRAMUSCULAR ADIPOSE TISSUE

Introduction

In order to be able to objectively characterise the visible adipose tissue that is deposited in muscle tissue, computer image analysis (CIA) programs have been developed. Currently, marbling in the slaughterhouse is subjectively given a marbling score based upon scales respective to each country's standards (Canada, Canadian Beef Grading Agency, 2002; USA, Food Safety and Inspection Service, 2002; Japan, Japan Meat Grading Association, 1988). Many research groups are using CIA programs as a method to obtain data that is used to evaluate feeding studies, evaluate carcasses, and characterize adipose tissue deposition based on the amount of fat deposited in the intramuscular (i.m.) depot (Albrecht et al., 1996; Karnuah et al., 2001; Kuchida et al., 2000). A CIA program was written that is able to characterise adipose tissue deposited intramuscularly from connective tissues that often appear as white as the deposited fat. The following methods and computer program were developed to evaluate the fat content in bovine and porcine muscle tissue samples. The program is currently being evaluated.

Sample Preparation and Staining

A visual outline of muscle sample preparation and the staining protocol is shown in Figure 18. A 1.5 to 2 cm slice of the 24 h *post mortem* muscle tissue under investigation was obtained (Figure 18A). The slices were fixed in 5%

formaldehyde/calcium for 1 to 3 days and stored in 5% formaldehyde until further processing. The variability in the length of time in the 5% formaldehyde/calcium solution depended on the thickness of the section. Using any standard universal meat slicing machine (Figure 18B), 1 to 2 mm thick slices of the fixed muscle section were obtained (Figure 18C) and placed in distilled water for 12 h. Oil-Red O (Chroma GmbH+Co. KG, Munster-Roxel, Germany) was dissolved to make a stock solution by adding 0.50 g oil red O in 0.10 L isopropanol (CHROMA GmbH+Co. KG). Prior to staining, 12 mL oil red O stock was diluted in 8 mL deionised water and left to stand for 20 min. The diluted oil red O solution was filtered through Whatman paper number 42 (Whatman, Maidstone, UK) to remove any crystallised oil red O. The muscle slices were placed in the staining solution (Figure 18D) for 6 to 8 h and rinsed in distilled water overnight. The stained slices were then placed in a 70% isopropanol solution for 2 to 4 h under constant motion. Samples are rinsed for a minimum of 12 h and then stored in 5% formaldehyde until image acquisition (Figure 18E). Figure 19 provides a close-up view of the final stained muscle section prior to image acquisition.

Image Acquisition

Muscle sections were visualised using a Kodak DS 120 digital camera attached to a standard camera stand (Eastman Kodak Company, Rochester, NY, USA). Digital images were imported into a Pentium III 700 MHz PC computer. All images (768 x 576 pixels) were saved as TIFF files to maximize image quality.

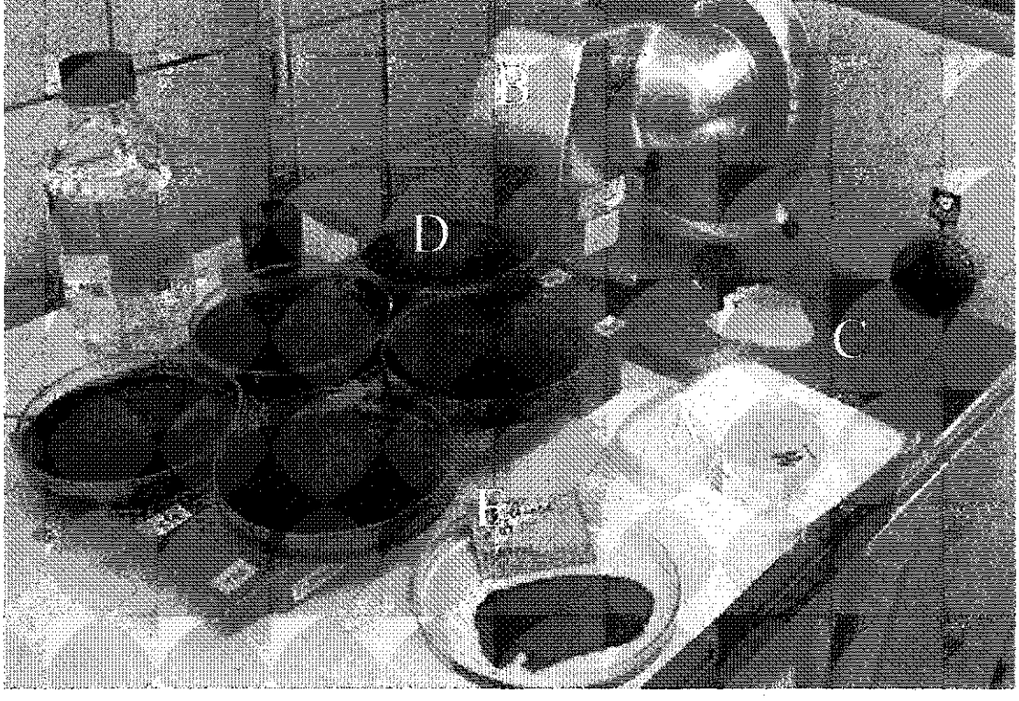


Figure 18. General set up and equipment for staining whole muscle sections for computer image analysis. A, Muscle tissue after treatment with formaldehyde/calcium; B, meat slicer; C, muscle section after slicing; D, muscle section immersed in oil red O solution on shaker, E, muscle section after staining procedure is completed.

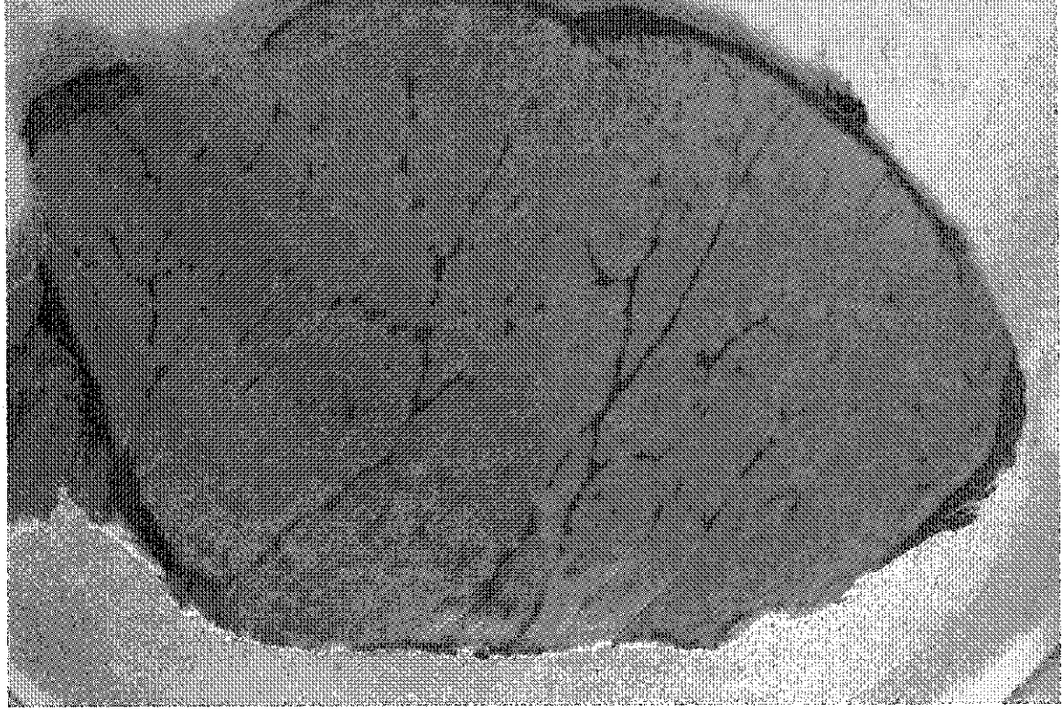


Figure 19. Close-up image of whole muscle tissue section after oil red O staining procedure is completed. The muscle tissue section at this stage is ready for computer image analysis.

Image Analysis

The program for the CIA was written in the Visual Basic Language, a 32 bit application development language, for the ImageC Image Analysis Program (version 2.52, Level 510, Aquinto, Berlin, Germany). The Visual Basic program integrated CIA components of the ImageC program allowing for the development of an individualised program. The complete program is as follows:

```
'#Reference {8E7DF211-6FA5-11D0-9B10-
0000C06606DF}#1.0#0#C:\Programme\IMTRONIC\ImageC\Bin\WIMCDSP.dll#Wimc
dsp
'#Reference {DDE31561-056F-11D0-9AA8-
0000C08206DF}#1.0#0#C:\Programme\IMTRONIC\ImageC\Bin\WIMCDEVX.dll#wim
cdevx
'#Reference {BF5ACDC1-0164-11D0-9A99-
0000B4235D27}#1.0#0#C:\Programme\IMTRONIC\ImageC\Bin\WIMCBAS2.dll#Wim
cbas2
Option Explicit

Sub Main( )
Dim Anw As Object
Dim AreaMuscle As Double
Dim Count As Long
Dim stat As New Wimcdsp.StatisticX
Dim ds As Wimcbas2.AtmDataSource
Dim Buffer As Variant
Dim AreaPercentage As Double
Dim FatLength As Double
Dim area3biggest1 As Double
Dim area3biggest2 As Double
Dim area3biggest3 As Double
Dim length1island As Double
Dim length2island As Double
Dim length3island As Double
Dim zaehler1 As Integer
Dim zaehler2 As Integer
Dim zaehler3 As Integer
Dim areabig As Double
Dim anzahldichte1 As Double
Dim anzahldichte2 As Double
Dim anzahldichte3 As Double
```

```
Dim areawhole1 As Double
Dim areawhole2 As Double
Dim areawhole3 As Double
Dim flächendichte1 As Double
Dim flächendichte2 As Double
Dim flächendichte3 As Double
Dim gesamtflächendichte As Double
Dim lean As Double
Dim fatweight As Double
Dim leanweight As Double

Set Anw = GetObject( "", "wimc.Anwendung" )
Anw.AnsichtListe.Eintrag( 0, 0, "Image" ).Aktivieren
Anw.Operations_ClearOverlay
Anw.DokumentListe.Öffnen "D:\Huff\Muskel\mucelmethod.osz"

Measure 240,256,True

Set ds = Anw.DokumentListe.Eintrag(0, "ObjectData").Data
Count = ds.GetNumberOfRecords (Wimcbas2.ADS_SRC_OBJ)
Buffer= ds.GetDataArray (Wimcbas2.ADS_SRC_OBJ,"Area$", 1, Count)
stat.Create
stat.PutData Buffer
Dim Results As Variant
Results = stat.StatisticParameters()
AreaMuscle =  Results(1)*Results(2)
stat.Delete

'////////////////////////////////////////////////////////////
'End of MuscleArea

Anw.AnsichtListe.Eintrag( 0, 0, "Image" ).Aktivieren
Anw.Operations_ClearOverlay
Anw.Operations_SeparateChannels 0, 1, 0, 0, 0, 0, 1
Anw.AnsichtListe.Eintrag( 0, 0, "Image" ).Ansicht_Farbtabellen 0
FilterX
Anw.AnsichtListe.Eintrag( 0, 0, "ObjectData" ).Aktivieren
Anw.DokumentListe.Eintrag( 0, "ObjectData" ).Specimen_DeleteAllData -1
Anw.AnsichtListe.Eintrag( 0, 0, "ObjectData" ).AktuellesBild 0

Measure 0,50,False

Count = ds.GetNumberOfRecords (Wimcbas2.ADS_SRC_OBJ)
Buffer= ds.GetDataArray (Wimcbas2.ADS_SRC_OBJ,"Area$", 1, Count)

stat.Create
```

```
stat.PutData Buffer
stat.Sort True

area3biggest1=stat.GetData(1)
area3biggest2=stat.GetData(2)
area3biggest3=stat.GetData(3)


zaehler1 =0
areabig =0

Do
zaehler1= zaehler1 + 1
areawhole1 =areawhole1 +areabig
areabig = stat.GetData(zaehler1)
Loop Until areabig <= 21400000
'areawhole1 = areawhole1 -areabig
zaehler1=zaehler1-1

zaehler2=zaehler1
areabig =0

Do

zaehler2= zaehler2 + 1
areawhole2=areawhole2+areabig
areabig = stat.GetData(zaehler2)


Loop Until areabig <2700000

'areawhole2=areawhole2-areabig
zaehler2=zaehler2-1

zaehler3=zaehler2
zaehler2=zaehler2-zaehler1
areabig =0

Do
zaehler3= zaehler3 + 1
areawhole3=areawhole3+areabig
areabig = stat.GetData(zaehler3)


Loop Until Count=zaehler3
```

```
areawhole3=areawhole3-areabig
zaehler3=zaehler3-zaehler2-zaehler1
Results = stat.StatisticParameters()
Dim AreaFat
AreaFat =  Results(1)*Results(2)
stat.Delete
AreaPercentage =AreaFat /AreaMuscle * 100


Buffer= ds.GetDataArray (Wimcbas2.ADS_SRC_OBJ,"Faserlaenge$", 1, Count)

stat.Create
stat.PutData Buffer
stat.Sort True

length1island= stat.GetData(1)
length2island= stat.GetData(2)
length3island= stat.GetData(3)

Results = stat.StatisticParameters()
FatLength =Results(7)/10000

Dim xl As Object
Set xl =xlS

'umrechnen

lean = (AreaMuscle-AreaFat)
fatweight =((AreaFat*0.94)/((AreaFat*0.94)+(lean*1.07)))
leanweight =((lean*1.07)/((AreaFat*0.94)+(lean*1.07)))
lean =lean/100000000

flächendichte1= areawhole1/AreaMuscle *100
flächendichte2= areawhole2/AreaMuscle *100
flächendichte3= areawhole3/AreaMuscle *100

AreaMuscle= AreaMuscle/100000000
AreaFat=AreaFat/100000000

length1island=length1island/10000
length2island=length2island/10000
length3island=length3island/10000

anzahldichte1 = zaehler1/AreaMuscle
anzahldichte2 = zaehler2/AreaMuscle
anzahldichte3 = zaehler3/AreaMuscle
```

```
area3biggest1 = area3biggest1 /100000000
area3biggest2 = area3biggest2 /100000000
area3biggest3 = area3biggest3 /100000000

xl.cells(1,1).value="Muskelfläche:"
xl.cells(1,2).value =Format(AreaMuscle,"0.00")
xl.cells(1,3).value="qcm"
xl.cells(2,1).value="Fettfläche:"
xl.cells(2,2).value=Format(AreaFat,"0.00")
xl.cells(2,3).value="qcm"
xl.cells(3,1).value="Fettanteil:"
xl.Cells(3,2).Value =Format(AreaPercentage,"0.00")
xl.cells(3,3).value="%"
xl.cells(4,1).value="Fettstranglänge:"
xl.Cells(4,2).Value =Format(FatLength,"0.00")
xl.cells(4,3).value="cm"
xl.cells(5,1).value="Anzahl Fettinseln:"
xl.cells(5,2).value=Format(Count,"0")
xl.cells(6,1).value="Größte Fettinsel:"
xl.cells(6,2).value=Format(area3biggest1,"0.00")
xl.cells(6,3).value="qcm"
xl.cells(7,1).value="2größte Fettinsel:"
xl.cells(7,2).value=Format(area3biggest2,"0.00")
xl.cells(7,3).value="qcm"
xl.cells(8,1).value="3größte Fettinsel:"
xl.cells(8,2).value=Format(area3biggest3,"0.00")
xl.cells(8,3).value="qcm"

xl.cells(10,1).value="längste Fettinsel:"
xl.cells(10,2).value=Format(length1island,"0.00")
xl.cells(10,3).value="cm"
xl.cells(11,1).value="2längste Fettinsel:"
xl.cells(11,2).value=Format(length2island,"0.00")
xl.cells(11,3).value="cm"
xl.cells(12,1).value="3längste Fettinsel:"
xl.cells(12,2).value=Format(length3island,"0.00")
xl.cells(12,3).value="cm"


xl.cells(13,1).value="Gesamtanzahldichte:"
xl.cells(13,2).value=Format((Count/AreaMuscle),"0.00")
xl.cells(13,3).value="x/qcm"
xl.cells(14,1).value="Anzahldichte >21.4:"
xl.cells(14,2).value=Format(anzahldichte1,"0.00")
```

```
xl.cells(14,3).value="x/qcm"
xl.cells(15,1).value="Anzahldichte 21.4>= x >=2.7:"
xl.cells(15,2).value=Format(anzahldichte2,"0.00")
xl.cells(15,3).value="x/qcm"
xl.cells(16,1).value="Anzahldichte <2.7:"
xl.cells(16,2).value=Format(anzahldichte3,"0.00")
xl.cells(16,3).value="x/qcm"


xl.cells(19,1).value="Flächendichte >21.4:"
xl.cells(19,2).value=Format(flächendichte1,"0.00")
xl.cells(20,1).value="Flächendichte 21.4>= x>=2.7:"
xl.cells(20,2).value=Format(flächendichte2,"0.00")
xl.cells(21,1).value="Flächendichte <2.7:"
xl.cells(21,2).value=Format(flächendichte3,"0.00")

xl.cells(24,1).value="Lean:"
xl.cells(24,2).value=Format(lean,"0.00")
xl.cells(25,1).value="Fatweight:"
xl.cells(25,2).value=Format(fatweight,"0.00")
xl.cells(26,1).value="Leanweight:"
xl.cells(26,2).value=Format(leanweight,"0.00")

End Sub


Sub Measure(ByVal thrs1 As Long,ByVal thrs2 As Long,ByVal Inverse As Boolean)
  Dim Anw As Object
  Set Anw = GetObject("","Wimc.Anwendung")
  Dim s As String
  s ="ObjectData"
  On Error GoTo M1
      Anw.AnsichtListe.Eintrag( 0, 0, "ObjectData" ).AktuellesBild 0
  GoTo  M1
  s = "Objektsuche"
  M1:
      Anw.AnsichtListe.Eintrag( 0, 0, s ).AktuellesBild 0
      Dim o As Object
      Set o = Anw.AnsichtListe.Eintrag( 0, 0, s ).SearchOptions
      o.Thrs2 = thrs2
      o.Thrs1 = thrs1
      o.Inverse = Inverse
      Anw.Stop
      Anw.DokumentListe.Eintrag( 0, s ).Search_SearchOptions.Beenden
      Anw.AnsichtListe.Eintrag( 0, 0, s ).NewField
```

```
        Anw.AnsichtListe.Eintrag( 0, 0, s ).AutomaticalSearch
End Sub

Function xlS() As Object
 Dim x As Object
 Set x = CreateObject("Excel.Application")
 x.Visible =True
 Set xlS=x.WorkBooks.Add ' "test.xlt"
 Set xlS = xlS.Sheets(1) ' xlS.Sheets("Tabelle1")
End Function

Sub FilterX
 Dim Anw As Object
 Set Anw = GetObject("","Wimc.Anwendung")
 Anw.AnsichtListe.Eintrag( 0, 0, "Image" ).Aktivieren
 Anw.Filter_FiltersExtended
 Anw.Filter_FiltersExtended.Aktivieren
 Anw.Filter_FiltersExtended.FilterFromName "Median (5x5) circle", 1, False
 Anw.Filter_FiltersExtended.Beenden
End Sub
```

Digital Image Processing

The stained muscle tissue image was imported as a TIFF file and given a calibration value to allow absolute data values to be calculated. Using the Micro : Line Coincidence (LinCo) 2 method of the ImageC program, the image was converted to gray scale and a threshold function was performed. The threshold was then set for the entire image (0 – 256) so that the entire muscle was selected in order to determine the Muscle Area. The program allowed for interactive adjustment of the threshold to account for minor differences between samples. A Median (5x5) Circle filter was then performed followed by a Green Channel selection on the original colour image. The green component was used for binarization because the variance of the green channel was the largest for fat in meat samples. The (LinCo) 2 method was again performed resulting in a grey scale image of the filtered original image. A threshold was set to select for the fat flecks and the following group of marbling characteristics was calculated: marbling fleck area (cm^2); proportion (%) of areas of fat flecks as the ratio between the total fat areas and the muscle area; number of marbling flecks as a global count of fat flecks on the muscle surface; number of marbling flecks divided by the total muscle area ($flecks/cm^2$); total length of marbling flecks (cm); size (cm^2) of the first, second and third largest marbling fleck area (cm^2); proportion (%) of the three largest fat areas compared with the total fat area; length of the first, second and third longest marbling fleck length (cm).

In order to account for the effects of marbling abundance (density), a slightly modified model from Gerrard et al. (1996) was applied. According to this model, marbling flecks were classified into two arbitrary categories according to their area (A):

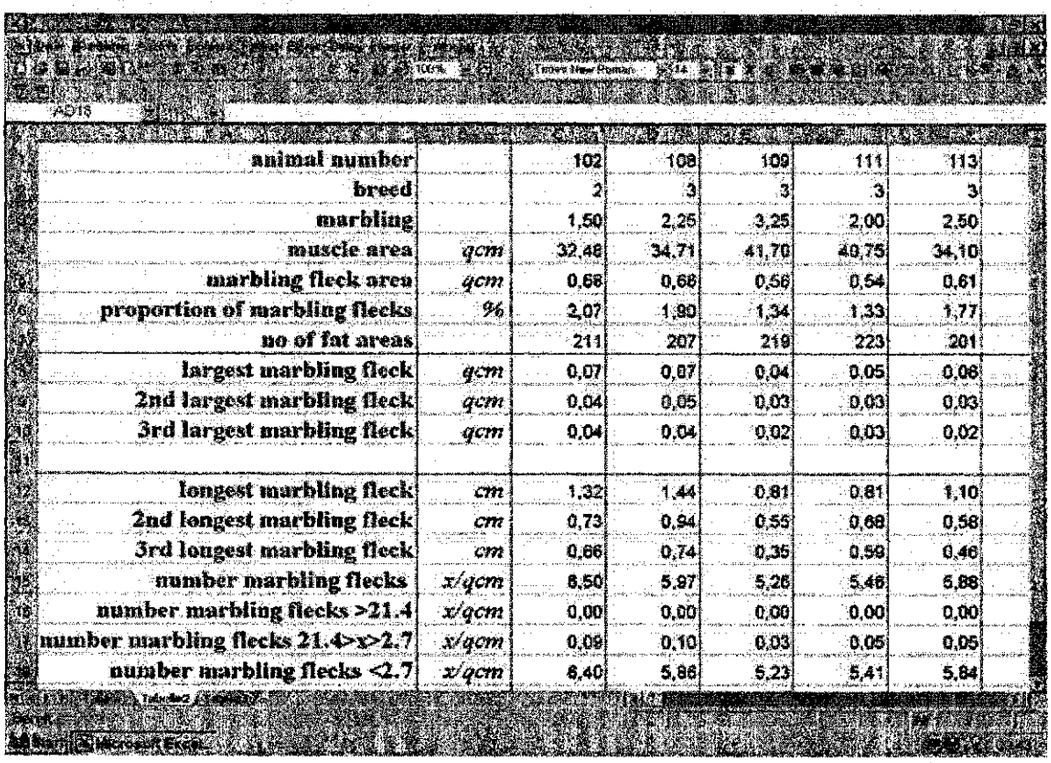
A_1 : $A \leq 2.7\ mm^2$

A_2 : 2.7 $mm^2 < A \leq 21.4\ mm^2$

Thus, marbling abundance was evaluated by using the following parameters: number of marbling flecks in both size categories expressed on the total muscle area (flecks/cm^2) and sum of marbling area in both size categories. Both parameters define two marbling densities, being the expression of count density and area density for each fleck size category, respectively.

Example of CIA Data and Concluding Comments

An example of how CIA data appears after analysis of the muscle sample is completed is shown in Figure 20. Data provided by the program can be imported into any statistical analysis software program for the analysis of any combination of i.m. fat deposition. In addition, because the data is provided in a transferable form, correlation analysis is greatly simplified. The described program was developed for the purpose of analyzing muscle tissue from any species that deposits i.m. fat in muscle tissue that can be visualized with a standard digital camera. Additional researchers who have access to the computer program have expressed interest in modifying the program to characterize the distribution of different coloured areas on the hides of cattle. As of 2003, the program had been used by Dr. Luigi Faucitano for the purpose of analyzing the deposition of i.m. fat in the muscle tissue of both cattle and swine from the Agriculture and Agri-Food Research Centre, Lennoxville, Quebec, Canada.



animal number		102	108	109	111	113
breed		2	3	3	3	3
marbling		1,50	2,25	3,25	2,00	2,50
muscle area	*qcm*	32,48	34,71	41,70	40,75	34,10
marbling fleck area	*qcm*	0,66	0,66	0,56	0,54	0,61
proportion of marbling flecks	*%*	2,07	1,90	1,34	1,33	1,77
no of fat areas		211	207	219	223	201
largest marbling fleck	*qcm*	0,07	0,07	0,04	0,05	0,06
2nd largest marbling fleck	*qcm*	0,04	0,05	0,03	0,03	0,03
3rd largest marbling fleck	*qcm*	0,04	0,04	0,02	0,03	0,02
longest marbling fleck	*cm*	1,32	1,44	0,81	0,81	1,10
2nd longest marbling fleck	*cm*	0,73	0,94	0,55	0,68	0,58
3rd longest marbling fleck	*cm*	0,66	0,74	0,35	0,59	0,46
number marbling flecks	*x/qcm*	6,50	5,97	5,26	5,46	5,88
number marbling flecks >21.4	*x/qcm*	0,00	0,00	0,00	0,00	0,00
number marbling flecks 21.4>x>2.7	*x/qcm*	0,09	0,10	0,03	0,05	0,05
number marbling flecks <2.7	*x/qcm*	6,40	5,86	5,23	5,41	5,84

Figure 20. Sample output of marbling fat characterisation data in an Excel spreadsheet obtained using computer image analysis in the ImageC program developed using Visual Basic.

APPENDIX 2

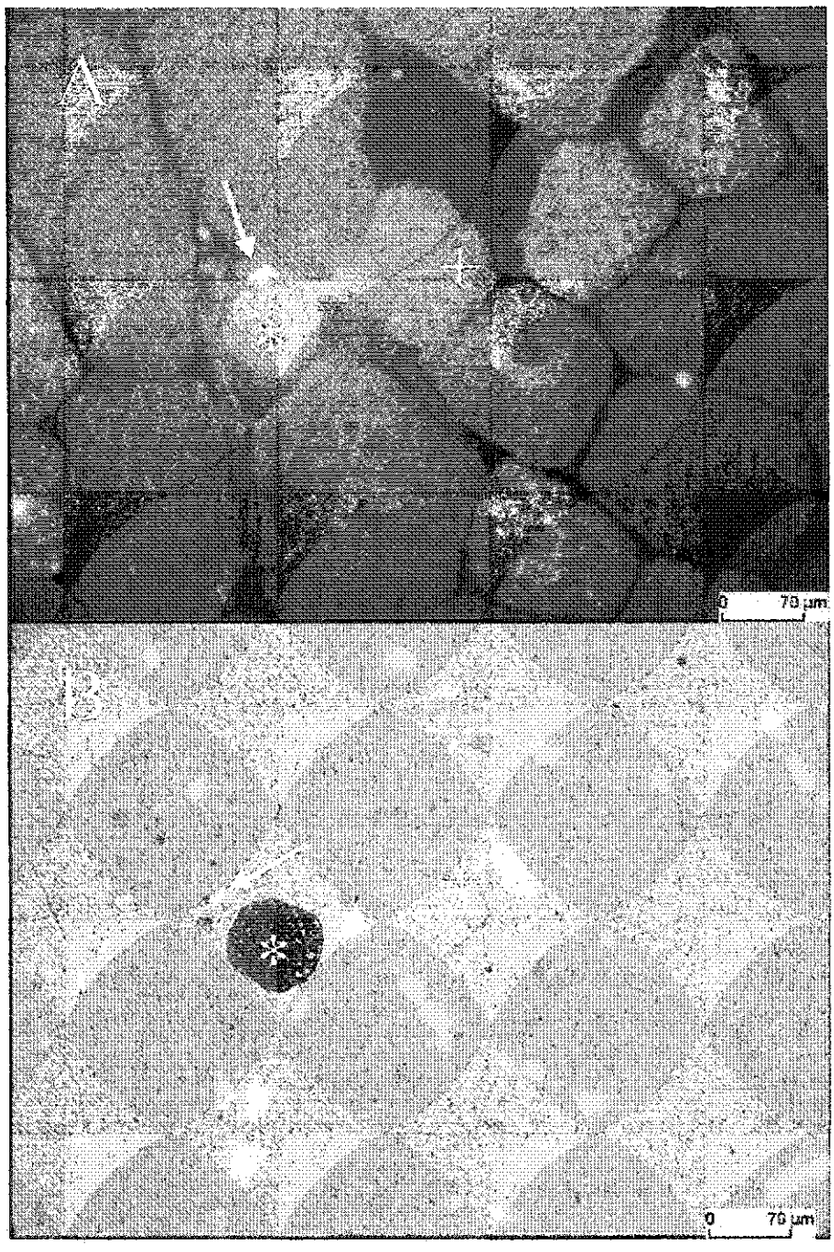


Figure 21. Immunohistochemical reaction (arrow) for pref-1 (A) and corresponding nucleic acid stain (B) in bovine *longissimus dorsi* muscle; *, Adipocyte, +, Muscle fibre containing pref-1 positive reaction. A positive reaction for pref-1 occurred in select

muscle fibres indicating the pref-1 may be expressed in a muscle fibre type specific manner.

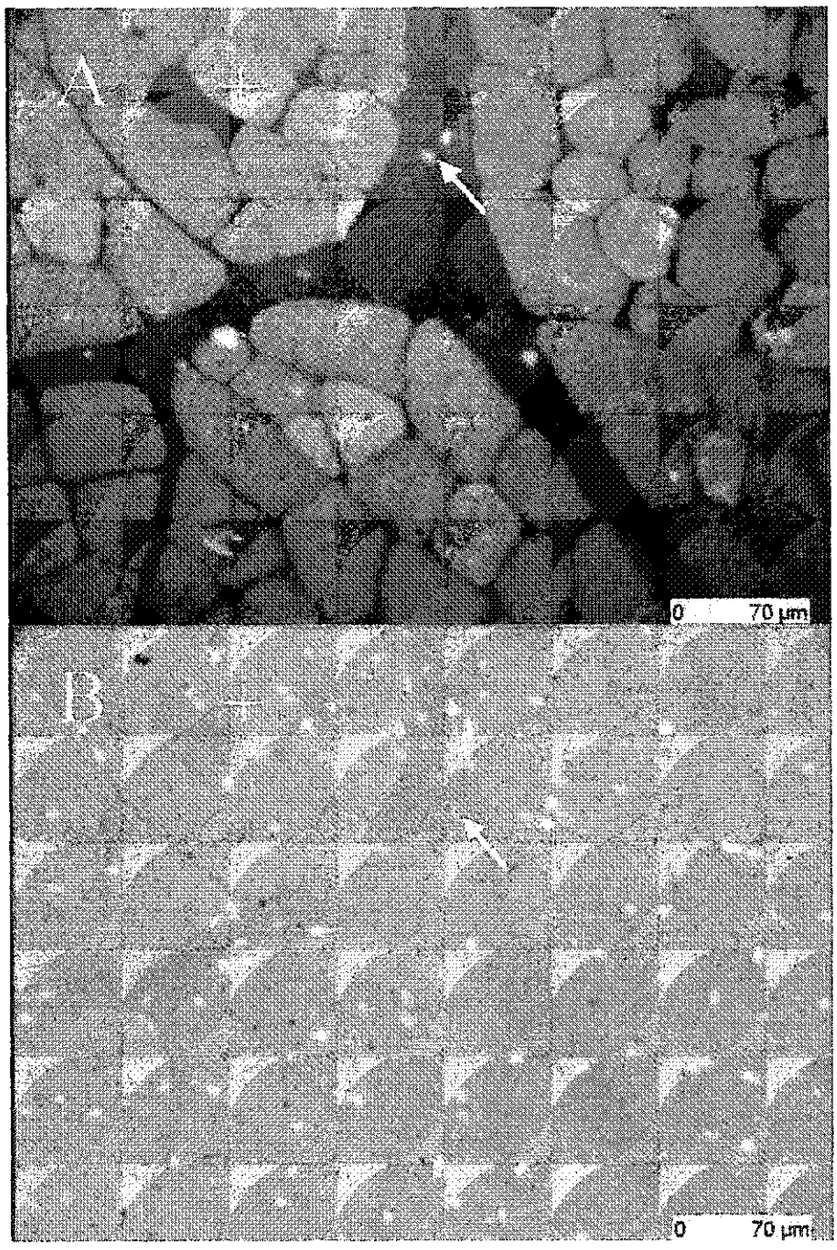


Figure 22. Immunohistochemical reaction (arrow) for pref-1 (A) and corresponding nucleic acid stain (B) in bovine *longissimus dorsi* muscle; *, Adipocyte, +, Muscle fibre containing pref-1 positive reaction. A positive reaction for pref-1 occurred in select muscle fibres indicating the pref-1 may be expressed in a muscle fibre-type specific manner.

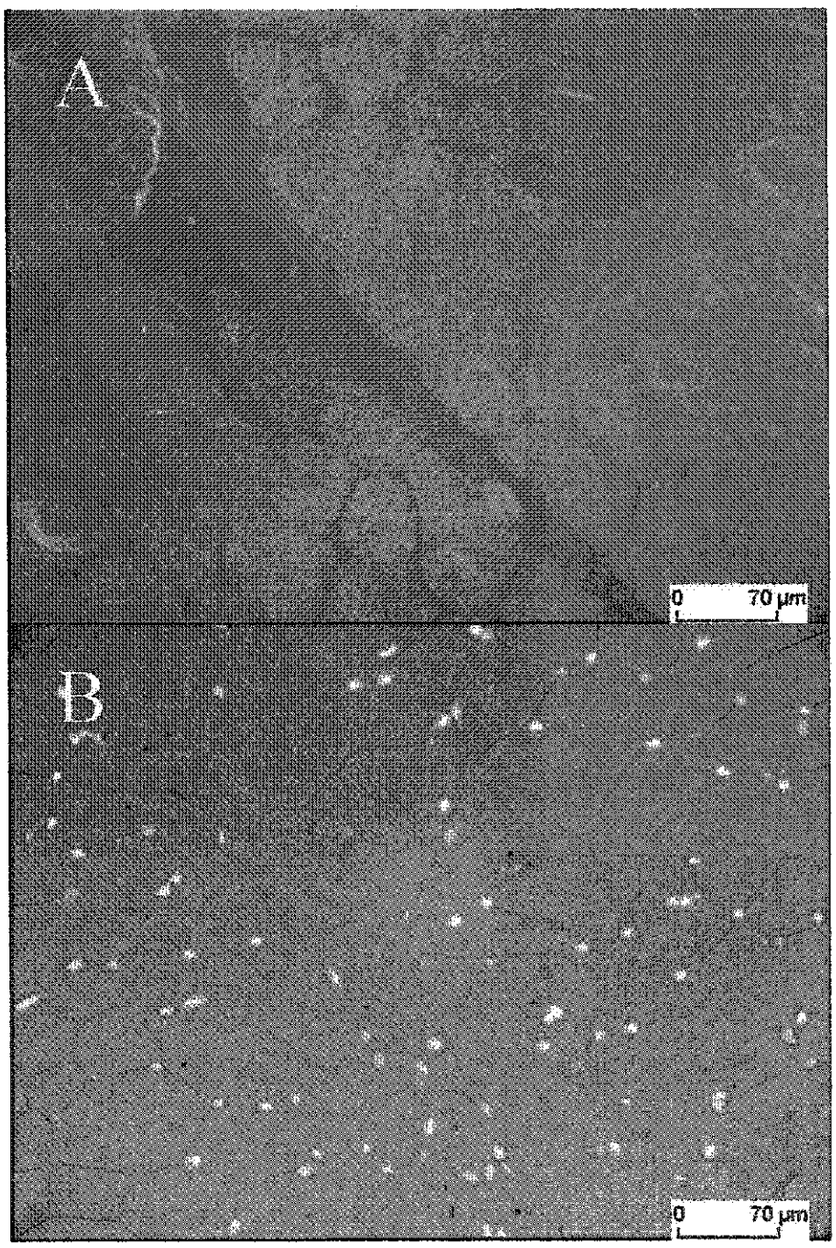


Figure 23. Control tissue for the pref-1 immunohistochemical reaction (A) and corresponding nucleic acid stain (B) in bovine *longissimus dorsi* muscle.

APPENDIX 3

ctttacctccgccggtgatcagaagcctgcgtcgtctaaattcttaagtcccctlgctta 60
gttgttcaggtttgaaagaagccacaacatacaactctaagccagagacatacaagaggg 120
acgtttccgtaaacaagtgtcattcctgaacagtcagaaattaccatggttgacacagag 180
atgccgttttggcccaccaactttgggatcagctccgtggacctttctatgatggatgac 240
cactcccatgccttgacatcaagcccttcaccaccgttgacttctccagcatttccact 300
ccgcactatgaggacattccgttcccaagagctgacccgatggttgcagattataagtat 360
gacctgaagctccaagagtaccaaagtgcaatcaaagtggagcctgtatccccaccttat 420
tattctgaaaagactcagctgtacagtaagcctcatgaagagccttccaactccctcatg 480
gccattgaatgccgggtctgtggggataaagcgtcagggttccactatggagttcatgct 540
tgtgaaggatgcaagggtttcttccggaggacaatcagattgaaacttatttacgatagg 600
tgtgatcttaactgtcggatccacaagaaaagtagaaataaatgtcagtactgtcggttt 660
cagaagtgccttgctgtggggatgtctcataatgccatcaggtttgggcgcatgccacag 720
gccgagaaggagaagctgttagcggagatctccagcgatatcgaccaactgaacccagag 780
tctgctgacctccgggccctggcaaagcatttgtatgactcgtacataaagtccttcccg 840
ctgaccaaagcaaaggcgagggcgatcttgacgggaaagacgacagacaaatcaccgttt 900
gttatctatgacatgaactccttaatgatgggagaagataaaatcaagttcaagcacatc 960
agtcccctgcaggagcccagcaaagaggtggccatccgcatcttccaggggtgtcagttt 1020
cgctccgtggaagccgtgcaggagatcacagagtacgccaagaatatccccggctttgtg 1080
aaccttgacttgaacgaccaagtaactctcctaaaatacggcgtgcacgagatcatttac 1140
acgatgctggcctccttgatgaataaggatggggtcctcatatccgagggccaaggattc 1200
atgacaagggagtttctaaagagcctgcgaaagccctttggtgactttatggagcccaag 1260
ttcgagtttgctgtgaagttcaacgcactggaattagatgacagcgacttagcaatattt 1320
atagctgtcattattctcagtggagaccgcccaggtttgctgaacgtgaagcccattgag 1380
gacatacaagacaatctgctgcaagccttggagctgcagctcaagctgaaccaccccgag 1440
tcctcccagctctttgccaagctgctccagaaaatgacagacctcagacagattgtgaca 1500
gaacacgtgcagctgttgcaagtaataaagaaaacagagacggacatgagtctccaccca 1560
ctcctacaggaaatctacaaggacttgtattagcagagaagtccgagttcactgacagca 1620
tcttccttctcccaattgcactattattttgagggaaaatctgacacctaaaaattactg 1680
tgaaagcattgaaaagaaagtttagaataat 1711

Figure 24. Nucleotide sequence for bovine PPARγ1. The forward primer (single underlined nucleotide sequence) and reverse (double underline nucleotide sequence) are noted.

cctggtcctctgtgagttattcccacctcctccaaacatttggaaacggacgtcttgact 60
cattggtgcgttcccaagttttactgccatgcatctttttctttgaacggaactggcctt 120
ttgcaagaaatagaccaaatatcggtgggagtcgtggcaaatccctgttccgtgctgtga 180
tgggtgaaactctgggagatgctcttattgacccagagagtgagcccttcgctgtcacag 240
tgtctgcaaggacctcacaagaaattaccatggttgacacagagatgccgttttggccca 300
ccaactttgggatcagctccgtggacctttctatgatggatgaccactcccatgcctttg 360
acatcaagcccttcaccaccgttgacttctccagcatttccactccgcactatgaggaca 420
ttccgttcccaagagctgacccgatggttgcagattataagtatgacctgaagctccaag 480
agtaccaaagtgcaatcaaagtggagcctgtatccccaccttattattctgaaaagactc 540
agctgtacagtaagcctcatgaagagccttccaactccctcatggccattgaatgccggg 600
tctgtggggataaagcgtcagggttccactatggagttcatgcttgtgaaggatgcaagg 660
gtttcttccggaggacaatcagattgaaacttatttacgataggtgtgatcttaactgtc 720
ggatccacaagaaaagtagaaataaatgtcagtactgtcggtttcagaagtgccttgctg 780
tggggatgtctcataatgccatcaggtttgggcgcatgccacaggccgagaaggagaagc 840
tgttagcggagatctccagcgatatcgaccaactgaacccagagtctgctgacctccggg 900
ccctggcaaagcatttgtatgactcgtacataaagtccttcccgctgaccaaagcaaagg 960
cgagggcgatcttgacgggaaagacgacagacaaatcaccgtttgttatctatgacatga 1020
actccttaatgatgggagaagataaaatcaagttcaagcacatcagtcccctgcaggagc 1080
ccagcaaagaggtggccatccgcatcttccaggggtgtcagtttcgctccgtggaagccg 1140
tgcaggagatcacagagtacgccaagaatatccccggctttgtgaaccttgacttgaacg 1200
accaagtaactctcctaaaatacggcgtgcacgagatcatttacacgatgctggcctcct 1260
tgatgaataaggatggggtcctcatatccgagggccaaggattcatgacaagggagtttc 1320
taaagagcctgcgaaagccctttggtgactttatggagcccaagttcgagtttgctgtga 1380
agttcaacgcactggaattagatgacagcgacttagcaatatttatagctgtcattattc 1440
tcagtggagaccgcccaggtttgctgaacgtgaagcccattgaggacatacaagacaatc 1500
tgctgcaagccttggagctgcagctcaagctgaaccaccccgagtcctcccagctctttg 1560
ccaagctgctccagaaaatgacagacctcagacagattgtgacagaacacgtgcagctgt 1620
tgcaagtaataaagaaaacagagacggacatgagtctccacccactcctacaggaaatct 1680
acaaggacttgtattagcagagaagtccgagttcactgacagcatcttccttctcccaat 1740
tgcactattattttgagggaaaatctgacacctaaaaattactgtgaaagcattgaaaag 1800
aaagtttagaataat 1815

Figure 25. Nucleotide sequence for bovine PPARγ2. The forward primer (single underlined nucleotide sequence) and reverse (double underline nucleotide sequence) are noted.