The Breakdown of Skeletal Muscle in Dairy Cows During Peak Lactation

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For my grandparents

Lil & Vern

Inez & Tommy

Abstract

The decline in fertility in dairy cows is of international concern. Since 1950, milk production demands have increased while first service rates of conception have decreased. It is unclear why fertility has decreased, however current dairy management practice requires cows to be kept on a tight yearly calving schedule to ensure maximum milk production over the lifetime of the cow. The current postulate suggests that this regime places a high metabolic burden on the cows, which in turn requires breakdown of tissues such as fat and muscle to provide substrates to meet the increased energy demands of lactation.

Immediately after calving, dairy cows enter a state of negative energy balance (EB), as they cannot consume enough energy to sustain lactation. During this period of negative EB, fat is mobilised in the form of non-esterified fatty acids to help supply the body with the extra energy it needs, but fat mobilisation decreases after four weeks while cows remain in a state of negative EB for several more weeks. It is unclear whether or not muscle breakdown occurs and plays a role in the restoration of EB in lactating dairy cows. I hypothesized that the breakdown of muscle does occur in cows during peak lactation, and that it occurs to a greater extent in cows producing higher amounts of milk.

Dairy cows from three strains, NZL, NZH and OSH, representing cows with differing milk production abilities (low, intermediate and high, respectively), were studied for 12 weeks postpartum. Blood was drawn at weekly intervals and muscle biopsies taken at -1, 1, 4, 8, and 12 weeks postpartum.

Analysis of plasma revealed an increase in the abundance of troponin I-fs (a marker of muscle breakdown) over the period of study, suggesting that breakdown of skeletal muscle was occurring. Real-time polymerase chain reaction analysis showed that expression of the ubiquitin-proteasome (UbP) ligases atrogin-1 (atro1) and muscle ring finger 1 protein (murf1) increased initially, but returned to normal levels by four weeks postpartum. Concentration of mRNA of the lysosomal proteases, cathepsin B, D, H and L, did not change over the period of study. Therefore, the UbP pathway may contribute to the breakdown of muscle detected by troponin I-fs in plasma.

Proteins involved in translation initiation were examined by Western blotting. The ratio of phosphorylated over total eIF2α and 4E-BP1 remained unchanged throughout the study, indicating that the breakdown of muscle was not a result of decreased protein synthesis. However, there was a greater ratio of phosphorylated to total eIF2α in NZL cows compared with NZH and OSH, suggesting that protein synthesis was less overall in NZL cows than other strains. Measurement of myosin heavy chain composition indicated there was no change in the abundance of type I and type IIx muscle fibres and plasma myostatin levels did not change over the period of study. However, the OSH cows had less myostatin in their plasma than the NZL and NZH cows, suggesting that there may be inhibition of muscle growth occurring in this strain.

The results of this study suggest that breakdown of muscle could be important in restoring the EB in high-producing dairy cows during peak lactation. Upregulation of the UbP pathway during the first four weeks of lactation may contribute to this muscle breakdown. However, it remains unclear what processes then continue to regulate breakdown of skeletal muscle to maintain the elevated abundance of troponin I-fs in plasma from four to 12 weeks postpartum in lactating dairy cows.

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Abbreviations Used

4E-BP1	eukaryotic initiation factor 4E binding protein 1
α	alpha
APS	ammonium persulfate
atro1	muscle atrophy F-box protein 1 or atrogin 1
β	beta
bp	base pair
BCA	bicinchoninic acid
BCS	body condition score
BME	β-mercaptoethanol
BSA	bovine serum albumin
BWT	body weight
°C	degrees Celsius
Ca^{2+}	calcium
CK	creatine kinase
CO_2	carbon dioxide
Da	dalton
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	energy balance
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra acetic acid
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbant assay
fs	fast skeletal
g	grams or centrifugal force
GH	growth hormone
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HF	Holstein-Friesian
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
k	kilo (10^3)
KCl	potassium chloride
L	litre
LH	luteinising hormone
LHRH	luteinizing hormone-relaeasing hormone
HRP	horseradish peroxidase
LSB	low salt buffer
μ	micro (10^{-6})
m	metre or milli (10^{-3})
M	molar or moles per litre

MAPK	mitogen-activated protein kinase
MeOH	methanol
MgCl	magnesium chloride
MHC	myosin heavy chain
min	minute(s)
mTOR	mammalian target of rapamycin
MRFs	myogenic regulatory factors
mRNA	messenger ribonucleic acid
murf1	muscle ring-finger protein 1
n	nano (10^{-9})
NA	North American
NaCl	sodium chloride
NaOH	sodium hydroxide
NEFAs	non-esterified fatty acids
NZH	New Zealand high producer
NZL	New Zealand low producer
O_2	oxygen
OD	optical density
oligo	oligonucleotide
OSH	overseas (North American) high producer
p70S6K	S6 kinase
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	hydrogen ion concentration
PI3K	phosphoinositide 3 kinase
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
Ser	serine
ss	slow skeletal
TBS	tris buffered saline
TBST	tris buffered saline with tween 20
TEMED	N,N,N’,N’-tetra-methyl-ethylenediamine
TGF-β	transforming growth factor-β
Thimerosal	mercury-[(o-carboxyphenyl)thio]ethyl sodium salt
Thr	threonine
Tris	tris(hydroxymethyl)aminomethane
trop	troponin
Tween 20	polyoxyethylene sorbitan-monolaurate
U	units
US	United States
UbP	ubiquitin-proteasome pathway
V	volt(s)

1. Review of the Literature

1.1. Fertility in Dairy Cows

1.1.1. A Steady Decline Over Decades

There is growing concern internationally with the decline in fertility in dairy cows. The most extensive records have come from the United States (US) where, in New York state, first-service rates of conception have dropped from approximately 65% in 1951 to 40% in 1996 (Butler, 1998). The steady decline in rates of conception was not well documented initially. However, standardised documentation procedures were developed and implemented to show the greatest decline occurred after the mid-1980s (Lucy, 2001). The underlying cause of the declining fertility is unknown. Nevertheless, the trend is proposed to be related to a more focussed genetic selection for cows to produce greater yields of milk. In doing so, other important traits such as health, fertility and longevity were ignored (Weigel, 2006). Reduced fertility in dairy cows has been related to shorter oestrous cycles (the phase when an animal is sexually receptive, or in heat), shorter duration of oestrus, fewer standing events (receptive stance of a cow), as well as recurrent multiple ovulations (McDougall, 2006; Staples *et al.*, 1990; Weigel, 2006). Nevertheless, the basis of these observations could be a consequence of selection for high milk production demands (Kadokawa & Martin, 2006).

The reduced fertility seen in the dairy industry is not evident in the beef industry. Beef cows experience reduced fertility only when they are undernourished and have lost too much body weight (BWT), resulting in poor body condition (Hess *et al.*, 2005; Houghton *et al.*, 1990; Lake *et al.*, 2005; Lucy, 2003; Olson, 2005). The reproductive physiology of a postpartum cow differs to that of a heifer (in both beef and dairy cows) in that the strain of the last pregnancy and lactation render it more difficult for a postpartum cow to become pregnant. However, a beef cow with good body condition will have a conception rate similar to a heifer (Lucy, 2003). In theory, the fertility of postpartum cows should be high if they are disease-free and producing normal amounts of milk. Loss of body condition in the postpartum period may be a crucial factor in reduced fertility. Genetic selection programmes for dairy cows have exploited nutrient partitioning to increase milk yield at the expense of BWT (McDougall, 2006; Weigel, 2006). In contrast, beef cows have not been selected for production of a high milk yield and do not lose BWT or body condition during lactation (Hess *et al*., 2005; Lake *et al*., 2005; Lucy, 2003).

1.1.2. An Increased Demand for Milk Production and the Metabolic Cost to Fertility

Over the past few decades the consumer demand for milk has increased steadily and cows have been genetically selected to meet the increased demands. Several studies have shown that there is an inverse relationship between milk yield and rate of conception in cows (Dematawewa & Berger, 1998; Hansen, 2000; Laben *et al*., 1982; McDougall, 2006). Indeed, it has been suggested that the reproductive biology of today’s high milk-producing dairy cow is significantly different to that of their ancestor’s (Weigel, 2006). There is a prevalence of abnormal oestrus cycles in cows selected for high milk production, and the duration of oestrus is reduced (McDougall, 2006; Nebel & McGilliard, 1993; Butler & Smith, 1989).

Immediately after calving, milk yield increases rapidly, while the increase in compensatory feed intake is delayed (Buckley *et al*., 2003; Nebel & McGilliard, 1993). As a result, dairy cows are in a state of negative energy balance (EB) during peak lactation and body tissues, typically adipose, are mobilized to help the lactating cow meet the energy requirements of lactation (Kim & Suh, 2003; Macrae *et al.*, 2006; McDougall, 2006; Weigel, 2006). However, the dairy industry requires lactating cows to be pregnant by day 85 after calving (see section 1.1.3.) to match the length of gestation for a cow to calve and, therefore, produce milk annually.

1.1.3. The Annual Calving Schedule

A typical year in the dairy industry has a dairy cow calving once per year. The length of gestation in cows is 280 days. Cows spend approximately 305 days in lactation, and have a 60 day “dry” period (cessation of lactation) (Figure 1.1). During the “dry” period, milking is stopped gradually or abruptly, the latter being the preferred method. This enables the udder to undergo involution, to repair and regenerate secretory tissue, to give the digestive system a rest from the stress of high feed intake and to allow the cow to replenish its energy reserves (Capuco *et al*., 1997; Grum *et al*., 1996). The length and severity of negative EB varies depending on the production demands of the cow and extended periods of negative EB may affect fertility (Butler, 1998, 2000; Butler *et al*., 1981; Butler & Smith, 1989; de Vries & Veerkamp, 2000; Spicer *et al*., 1990; Staples *et al*., 1990; Veerkamp *et al*., 1994; Villa-Godoy *et al*., 1988).

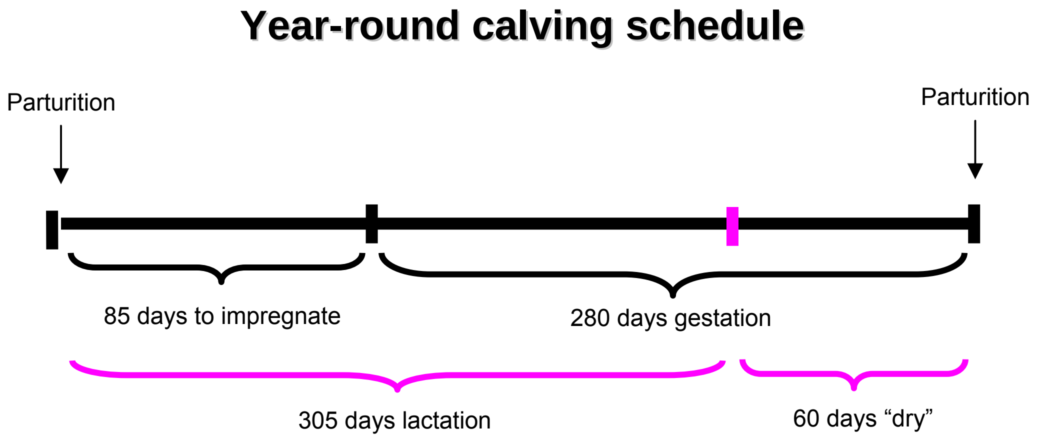

Figure 1.1: *Programme followed for year-round calving.*

The seasonal calving system used in New Zealand and Ireland is more stringent in that calving needs to be timed to coincide with peak pasture growth as well as fitting into 365 days (Buckley *et al*., 2003; McDougall, 2006). In a representative season, breeding starts on a given day in spring. In this way the majority of the herd is calving at the same time, 280 days later. Breeding programs are established to produce the highest pregnancy rate in the shortest amount of time after the start of the breeding season in order to establish a tightly regulated calving scedule for the following years (Buckley *et al*., 2003). In a country where cows are fed high energy concentrate rations and live in barns, such as the United States or Canada, it is not essential to time calving with peak pasture growth.

The interval from calving to first ovulation is a critical period for the dairy industry so that a cow conceives, ideally, at first service (Westwood *et al*., 2002). It is unclear whether or not there is a link between the interval from calving to first ovulation and energy state. Some have shown a positive relationship between EB during the first few weeks after calving and the time to first ovulation (Butler, 2000; Butler *et al*., 1981; de Vries & Veerkamp, 2000; Senatore *et al*., 1996; Staples *et al*., 1990). In contrast, other groups have not been able to relate EB to length of anoestrus (Harrison *et al*., 1990; Spicer *et al*., 1990; Villa-Godoy *et al*., 1988). Whether or not EB relates to time to first ovulation when a cow is in a state of negative EB, there is reduced secretion of luteinising hormone-releasing hormone (LHRH). As a consequence, the reduction in secretion of LHRH leads to reduced secretion of luteinising hormone (LH), the principal hormone regulating follicular development and ovulation (Lucy, 2003; Garnsworthy & Webb, 2000; Zurek *et al*. 1995). Therefore, ovarian follicles fail to reach the pre-ovulatory stage (Butler, 2000). The concentration of serum LH increases after the nadir of EB, and first ovulation follows soon after (Butler, 2000; Zurek *et al*., 1995). A likely possibility is that selection for high milk production has resulted in an increased requirement for energy to support the increased milk production which then compromises the reproductive axis, leading to a lower rate of conception, a higher rate of embryonic loss and a higher proportion of anoestrous cows (the phase when the sexual cycle rests) (Kadokawa & Martin, 2006; Weigel, 2006).

1.2. Body Condition Scoring

1.2.1. What is Body Condition Scoring?

It is crucial in dairy management to be aware of a cow's body reserve status. Before 1970, there was no simple way to measure the energy reserves or body condition of a cow (Roche *et al*., 2004). Early studies were carried out using a Beltsville open-circuit respiration chamber, or similar, in which energy consumed (feed, O_2, H_2O) as well as energy expelled (urine, faeces, CO_2), were all recorded (Eisemann *et al*., 1986; Tyrrell *et al*., 1988). It was a thorough method allowing valuable information to be obtained regarding a cow's health and condition. However, it was very tedious and time-consuming, and not ideal for large studies. Body condition scoring (BCS) was developed as a surrogate measure of energy status and is used to assess changes in the body condition of dairy cattle. It is helpful for both farm management and research. A reduction in BCS is associated with the mobilization of fat (de Vries & Veerkamp, 2000; Komaragiri *et al*., 1998), thus it is thought that BCS can be used to indicate a cow's energy state (Kim & Suh, 2003). Energy stores can vary as much as 40% in cows with the same BWT (Andrew *et al*., 1994), thus change in BWT alone cannot be used to gauge the change in adipose and lean tissue assessed using the BCS system (NRC, 2001).

There are different BCS systems used internationally. The US and Ireland use a 5-point scale, Australia uses an 8-point scale and New Zealand uses a 10-point scale. Regardless of the scale, a score on the lower end corresponds to a thin, undernourished cow, and a score on the high end reflects an obese, overnourished cow. The US and Australia assess BCS visually, while Ireland and New Zealand use palpation on the back and hind quarters in combination with visual assessment to determine BCS (Jones *et al*., 1982). Roche *et al*. (2004) examined relationships between the different BCS systems to allow studies to be compared regardless of the system in place. They found that there were strong positive linear relationships between the New Zealand 10-point BCS system and the US 5-point, the Irish 5-point and the Australian 8-point BCS scales. The correlation was much stronger when palpation and visual assessments were made, as opposed to just visual. They also found that the New Zealand pasture-based system of dairy farming results in thinner cows than the other three systems (Roche *et al*., 2004). Thus New Zealand cows are at the lower end of the BCS scale and a combination of decreased appetite and/or decreased pasture growth can reduce BCS even further.

1.2.2. How Does Body Condition Scoring Relate to Fertility?

Before 1970, it was thought that a cow's body condition was most crucial at calving. However, with the observed loss of body condition between calving and the start of breeding, the importance of good body condition has extended past calving and into lactation (Beam & Butler, 1999; Buckley *et al*., 2003). Several studies suggest that the length of time a cow remains in negative EB during lactation correlates negatively with conception (Domecq *et al*., 1997; Nebel & McGilliard, 1993; Villa-Godoy *et* *al*., 1988). Butler and Smith (1989) showed that losing as little as 0.5 to 1 point (on a 5-point scale) in BCS between calving and first service decreases the rate of conception at first service from 65% to 53%, and losing more than 1 point decreases rate of conception at first service to 17%. Genetic selection for high milk production has also resulted in a genetic trend for lower BCS throughout the period of lactation thus compounding the observed decline in fertility (Buckley *et al*., 2003).

1.3. Food Intake

Food intake is difficult to measure for an individual cow grazing at pasture (Halachmi *et al*., 2004). However, food intake can be simulated using a mathematical model that requires absolute values of feed requirements (NRC, 2001), day to day body weight fluctuations and milk yield (Halachmi *et al*., 2004). The diet of a dairy cow can be pasture-based, concentrate-based, or a combination of both. When the diet is based on high-energy concentrates, the amount of food the cow consumes is thought to be controlled via nutrient requirements. A pasture-fed cow consumes grass, which has a low digestible energy concentration and thus is digested slowly, physically limiting the intake and preventing the cow from reaching its metabolic limit (Forbes, 1970). The amount of food an animal will consume varies greatly throughout the different stages they go through. For instance, pregnant heifers have been shown to have larger appetites and eat more than non-pregnant heifers. However, during the last few weeks of pregnancy (the dry-off period) a cow’s appetite gradually declines (in both pasture and concentrate-fed cows), then increases a few days before calving and continues to increase steadily during lactation (Forbes, 1970). Using genetic correlations between milk yield and food intake, it has been calculated that high yielding Holsteins (pasture and concentrate-fed) consume less than half what is required to meet energy demands to support peak lactation. Therefore, mobilisation of tissue must be used to compensate for insufficient nutrient uptake (Pryce *et al*., 2001; Veerkamp *et al*., 1994).

1.4. Non-Esterified Fatty Acids

Triacylglycerols (glycerol esterified with three fatty acids) are a source of energy stored as adipose tissue. Non-esterified fatty acids (NEFAs) are the free fatty acids released from adipose tissue, found circulating in blood and supplying the body with energy in times of need (Stryer, 1988). During the last week before calving, there is a decrease in food intake, which causes the cow to enter into a state of negative EB. This state continues through into early lactation, as cows cannot consume as much energy as they require to sustain the high energy demands of milk production. The additional energy requirements are met by lipolysis and concentrations of NEFAs in blood typically increase a few days before calving and peak around calving, then remain higher than prepartum up until approximately 12 weeks after calving (Adewuyi *et al*., 2005; Emery *et al*., 1992; Pushpakumara *et al*., 2003). NEFAs provide several tissues, including muscle, with substrate for energy conversion, production or oxidation (Adewuyi *et al*., 2005; Haemmerle *et al.* 2002). An increase in the concentration of NEFAs in blood, along with a decrease in BCS is an indication of negative EB in postpartum cows (Adewuyi *et al*., 2005; Emery *et al*., 1992; Jaurena *et al*., 2005; Pushpakumara *et al*., 2003). There is an immediate metabolic shift from nutrient accumulation to mobilisation of fat in order to provide additional energy substrates during the period of negative EB in early and peak lactation (Adewuyi *et al*., 2005; Bauman & Currie, 1980). It is well documented that mobilisation of NEFAs from adipose tissue during and after calving is characteristic of the transition from pregnancy to lactation (Adewuyi *et al*., 2005; Buckley *et al.,* 2003; Pushpakumara *et al.*, 2003; Nebel & McGilliard, 1993; Butler & Smith, 1989). However, it is not well documented whether or not muscle is mobilised to offset the deficit in energy resulting from the inadequacy of feed intake together with mobilisation of fat.

1.5. Muscle

Skeletal muscle is the most abundant tissue in the body, making up 40-50% of body mass (Kelly *et al*., 1986). The functional integrity and quality of muscle is maintained through the dynamic balance of myogenesis, protein synthesis and degradation (Franch & Price, 2005; Guttridge, 2004; Kandarian & Jackman, 2006). This balance is controlled via several pathways which depend on inter- and intracellular signals.

1.5.1 Myogenesis

Myogenesis is the formation of muscle tissue, particularly during embryonic development. There are four main myogenic regulatory factors (MRFs); MyoD, Myf-5, myogenin and MRF4 (Sabourin & Rudnicki, 2000). MRFs belong to a superfamily of basic helix-loop-helix DNA-binding transcription factors, and are essential for the proliferation and complete development of muscle (Berkes & Tapscott, 2005). It is the MRFs that initiate the conversion of fibroblasts into myogenic cells. Postnatal growth of muscle occurs via limited fusion of new myoblasts to form myofibres (hyperplasia). Increased size of myofibres is supported by recruitment of satellite cells, which reside under the basal lamina of skeletal muscle (hypertrophy). Because satellite cells only represent 2-7% of nuclei in adult myofibres, adult muscle mass is primarily the result of hypertrophy. Therefore, it is unlikely that maintenance or breakdown of adult muscle mass is via myogenesis.

1.5.2. Muscle Atrophy

Healthy human adults typically synthesize and degrade 300 grams of protein each day. Atrophy is a change in muscle resulting from conditions of disuse, certain states of disease, fasting, and ageing. Regardless of the cause, atrophy results in a decrease in protein content, fibre size, production of force, and resistance to fatigue (Jackman & Kandarian, 2004). Various factors and signaling pathways are altered during atrophy, upsetting the balance of synthesis and degradation. Even a small change in synthesis or degradation can have a big impact on muscle mass (Franch & Price, 2005; Kandarian & Jackman, 2006). It is unclear at present whether the state of negative EB during peak lactation alters the balance of protein synthesis and degradation in skeletal muscle.

1.5.3. Protein Synthesis

Synthesis of muscle protein is mediated by several different factors, but it is principally regulated at the level of translation (Dreyer *et al*., 2008). Translation is often controlled by variations in the cellular concentration and phosphorylation states of only a few initiation factors (Rhoads, 1993). The proteins most directly involved are the eukaryotic initiation factors (eIFs), specifically eIF2α and eIF4E, and the S6 kinase (p70S6K).

The eIF2 complex (made up of eIF2α and eIF2β) binds the initiator methionyl-transfer RNA to the native 40S ribosome to form the 43S initiation complex (Figure 1.2). When the eIF2α subunit is phosphorylated, it binds very tightly to the eIF2β subunit which inhibits the catalytic activity of eIF2β, thereby downregulating synthesis (Holcik & Sonenberg, 2005; Proud, 2005; Pestova *et al.,* 2001). The eIF4 factors help bind mRNA to the 43S initiation complex to make up the 48S initiation complex. Upon phosphorylation, eIF4E associates with eIF4G allowing for the recruitment of several other initiation factors to form the eIF4F complex, initiating cap-dependent protein synthesis (Fingar *et al*., 2002; Gingras *et al*., 2001). Depending on the cellular environment, eIF4E may or may not be bound by the eIF4E binding protein 1 (4E-BP1). Unphosphorylated, 4E-BP1 remains tightly bound to eIF4E, preventing translation initiation via eIF4E.

However, phosphorylation changes the conformation of 4E-BP1 decreasing it's affinity to eIF4E, resulting in subsequent dissociation and allowing initiation of translation through initiation complex formation.

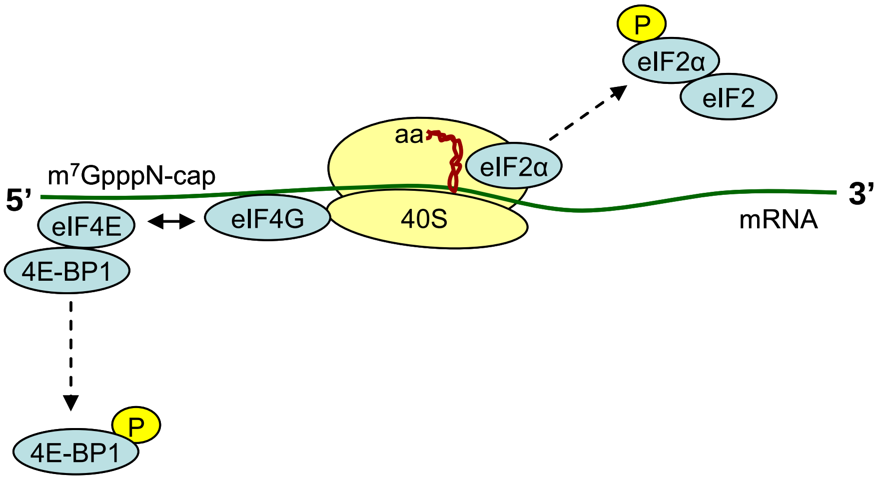

Figure 1.2: *The key regulatory components of the eukaryotic translation initiation.*

The phosphorylation of 4E-BP1 occurs in response to different extracellular stimuli encouraging translation. Phosphorylation of 4E-BP1 decreases during stress, starvation, infection and other undesirable cellular states (Gingras *et al*., 2001).

While it is known that activation of p70S6K is regulated by phosphoinositide 3 kinase (PI3K) and mammalian target of rapamycin (mTOR), and that it plays a role in initiation of translation, synthesis of protein and hypertrophy of muscle, its exact location in the signaling cascade remains unclear (Brown & Schreiber, 1996; Dreyer *et al*., 2008; Fingar *et al*., 2002; Gingras *et al*., 2001). However, it is known that phosphorylation of p70S6K correlates with hypertrophy of muscle, while a reduction in phosphorylation of p70S6K correlates with atrophy (Dreyer *et* *al*., 2008). It has yet to be determined whether or not initiation of translation is upregulated or downregulated in skeletal muscle in cows during peak lactation.

1.5.4. Muscle Degradation

1.5.4.1. The Ubiquitin-Proteasome Pathway

Atrophy of skeletal muscle occurs in pre-existing muscle fibres such that an increase in degradation or decrease in synthesis results in a faster rate of proteolysis. There are several degradation pathways that can contribute to atrophy. The Ubiquitin-Proteosome (UbP) pathway is a major contributor to atrophy of skeletal muscle. The 26S proteosome is a multi-subunit complex that covalently adds poly-ubiquitin chains to proteins (Franch & Price, 2005). Ubiquitin is a small peptide which, when polyconjugated to a protein, is used to target that protein for degradation by the proteosome. The UbP pathway has three key enzymatic components; E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. The E3 ubiquitin ligases give substrate specificity. Studies have shown that there is a significant increase in the levels of mRNA of genes encoding the components of the UbP pathway during atrophy (Cao *et al*., 2005; Dreyer *et al*., 2007; Eley & Tisdale, 2007; Glass, 2003, 2005; Szewczyk & Jacobson, 2005; Tisdale, 2005). There are three E3 ligases uniquely expressed in muscle. They are E3α-II, the muscle ring finger 1 protein (murf1) and muscle atrophy F-box protein, also referred to as atrogin 1 (atro1) (Franch & Price, 2005; Glass, 2003; Jagoe *et al*., 2002). *Murf1* and *atro1* are consistently upregulated in most muscle wasting conditions and are widely used as proxy markers of muscle wasting (Bodine *et al*., 2001). However, the UbP pathway cannot degrade intact myofibrils, and as such works in conjunction with the calcium (Ca^{2+})-dependent calpains (Koohmaraie, 1992; Solomon & Goldberg, 1996; Williams *et al*., 1999). It has not yet been determined whether or not the components of the UbP pathway are upregulated or downregulated in skeletal muscle during lactation.

1.5.4.2. The Calcium-Calpain Pathway

Calpains are Ca^{2+}-regulated, non-lysosomal, intracellular cysteine proteases that are responsible for the cleavage of specific substrates during various stages of the cell cycle (Bartoli & Richard, 2005). There are principally three calpains expressed in muscle tissue, the ubiquitous μ- and m-calpains, and calpain 3. Skeletal muscle-specific calpain 3 differs from the μ- and m-calpains in that it is quite often rapidly degraded itself, and during atrophy is downregulated (Herasse *et al*., 1999). The μ- and m-calpains are activated by μM and mM concentrations of Ca^{2+}, respectively. They have four domains, an N-terminal domain, a domain containing a distinct cysteine protease sequence, a connecting domain and a Ca^{2+}-binding domain (Bartoli & Richard, 2005; Costelli *et al*., 2005). Calpains are normally present in the cytosol in an inactive state as a dimer of an 80 kDa subunit and a 30 kDa subunit. Calpains are regulated by the indogenous protein inhibitor, calpastatin, but the precise mechanisms of this regulation are unclear (Bartoli & Richard, 2005; Costelli *et al*., 2005; Goll *et al*., 2003; Porn-Ares *et al*., 1998). However, it is known that an increase in cytosolic Ca^{2+} concentration causes the calpain 80 kDa subunit to dissociate from the 30 kDa subunit and become active. Calpains are considered key players in skeletal muscle atrophy as they initiate degradation of myofibres (Bartoli & Richard, 2005). However, Ca^{2+}-dependent calpains can perform only limited proteolysis, while the UbP pathway and the lysosomal pathway execute complete degradation, allowing the released amino acids to be recycled (Bartoli & Richard, 2005; Costelli *et al*., 2005; Koohmaraie, 1992; Solomon & Goldberg, 1996; Williams *et al*., 1999).

1.5.4.3. The Lysosomal Pathway

The lysosomal degradation pathway is another contributor to muscle breakdown. Lysosomes are membrane-bound vesicles containing hydrolysing proteins such as proteases, nucleases and phosphatases in an acidic environment (between pH 4-5). Lysosomes are responsible for the degradation of a wide range of molecules (Bechet *et al*., 2005). The endoplasmic reticulum and the Golgi are located throughout myofibers of all muscle types. They control synthesis and direction of lysosomal enzymes. Cathepsins are lysosomal proteases. Most exist as prepropeptides and are activated when exposed to the acidic lumen of lysosomes (Hasilik, 1992; Turk *et al*., 2001). There are several cathepsins involved in lysosomal degradation. The principal lysosomal proteases are cathepsin B, cathepsin H, cathepsin D and cathepsin L (Bechet *et al*., 2005; Turk *et al*., 2001). Although cathepsins are abundantly expressed, their proteolytic activity is tissue specific. Cathepsin H targets troponin T

(Bechet *et al*., 2005). Cathepsin B degrades myosin heavy chain, troponin T, troponin I and tropomyosin (Schwartz & Bird, 1977). Cathepsin L breaks down most myofibrillar proteins, excluding troponin C and tropomyosin (Matsukura *et al*., 1981). Cathepsin D degrades actin and myosin (Bird *et al*., 1981). Although cathepsin L is considered a general marker of atrophy, expression of other cathepsins can also increase depending on the cause of muscle wasting. Stimulation of the lysosomal pathway coincides with stimulation of the UbP pathway, Ca^{2+}-dependent calpains, or both during atrophy (Bechet *et al*., 2005).

1.6. Growth Factors

1.6.1. Insulin-Like Growth Factor One

Insulin-like growth factor 1 (IGF-1) is a trophic, mitogenic hormone synthesized mainly by the liver and skeletal muscle. It plays an important role in embryonic and post-natal growth, carrying out autocrine, paracrine or endocrine functions. IGF-1 is expressed in response to growth hormone (GH) stimulation and activates two principal intracellular signaling pathways, the phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway, thereby promoting protein synthesis (Favier *et al.,* 2008). The fusion of myoblasts into myotubes through IGF-1 is mediated by PI3K signaling. PI3K also mediates protein and glucose uptake, and resistance to apoptosis. The activation of MAPK is necessary for developmental IGF-1 receptor (IGF-1R)-dependent myogenesis. Cellular effects of IGF-1 are regulated by the IGF-1R, which also plays an essential role in myoblast proliferation and differentiation

(Singleton & Feldman, 2001). A connection has been made to the deactivation of the IGF-1/PI3K/Akt signaling pathway and an increase in proteolysis or an increase in the expression of proteolytic genes (Kandarian & Jackman, 2006). In conditions of atrophy, concentrations of IGF-1 mRNA decrease, subsequently decreasing the rate of protein synthesis (Favier *et al.,* 2008).

The GH/IGF-1 axis is nutritionally sensitive, and IGF-1 expression decreases during underfeeding (Jeanplong *et al.*, 2003). Concentration of IGF-1 in plasma is directly related to energy status, and IGF-1 is an essential component of ovarian follicular development, as well as stimulation of progesterone secretion from luteal cells (Patton *et al.* 2007; Butler, 2000). Increased levels of IGF-1 in plasma during the first two weeks of lactation have been linked to a higher rate of conception at first service (Patton *et al.* 2007).

1.6.2. Myostatin

Myostatin is a negative regulator of skeletal muscle growth and belongs to the transforming growth factor-beta (TGF-β) family. Myostatin inhibits activation and replication of muscle satellite cells, as well as synthesis of DNA and protein, thereby inhibiting myogenesis (McCroskery *et al*., 2003; Taylor *et al*., 2001). Myostatin is a 375-amino acid protein, highly conserved in sequence, expressed in developing and adult skeletal muscle (Gonzalez-Cadavid & Bhasin, 2004; McPherron *et al*., 1997). Myostatin is cleaved postranslationally into an N-terminal propeptide and the active component, the C-terminal myostatin protein, 110 amino acids in length. Myostatin is secreted and circulates in the blood as a dimer, complexed with the propeptide and other related proteins (Gonzalez-Cadavid & Bhasin, 2004). In the absence of myostatin (myostatin null mice), or when naturally occurring mutations are present that render the protein inactive (Belgian Blue and Piedmontese cattle), double muscling is observed, where animals have up to 50% more muscle mass caused by hyperplasia and hypertrophy (Lee & McPherron, 2001; Yang *et al*., 2001; Zhu *et al*., 2000). Myostatin has also been shown to induce muscle wasting in mice when present in excess, suggesting that myostatin plays a role in mediating cachexia (Zimmers *et al*., 2002). In support of this role, myostatin expression increases in various conditions of atrophy, such as during hindlimb unloading and in HIV-infected men (Carlson *et al*., 1999; Gonzalez-Cadavid & Bhasin, 2004; Gonzalez-Cadavid *et al*., 1998; Lalani *et al*., 2000; Ma *et al*., 2003). However, myostatin null mice are more susceptible to loss of muscle during unloading (McMahon *et al*., 2003), which suggests that myostatin does not only block myogenesis, but it may also play other roles that are important for protecting muscle against wasting. Ji *et al*. (1988) found that myostatin is expressed in the tubuloalveolar secretory lobules of porcine lactating mammary glands, and postulated that myostatin may have a role in the regulation of gestational or lactational mammary gland growth and development. It is presently unclear what role myostatin plays during peak lactation.

1.7. Markers of Muscle Wasting

1.7.1. Myosin Heavy Chains

There are two main contractile proteins responsible for muscle contraction, actin and myosin. Actin and myosin polymerize to form filaments, the thin and thick filaments, respectively. Myosin is a fundamental component of the contractile apparatus in muscle. The functional diversity of muscle fibres is due to the various isoforms of myosin (Pette & Staron, 2000). Myosin is a hexameric protein with heavy and light chains. Myosin heavy chains (MHCs) are encoded by a multi-gene family (Pette & Staron, 2000; Schiaffino & Reggiani, 1994). There are four main MHC isoforms: type I, type IIa, type IIx and type IIb. Type I is a slow twitch isoform, while the type IIs are fast twitch isoforms (Schiaffino & Reggiani, 1994). Although muscle fibres are often classed as a certain type, there is actually a continuum of hybrid fibres between the major fibre types (Ennion *et al*., 1995). MHCs are the most essential protein components found in skeletal muscle cells as they control the molecular motor and are the most abundant (Cooke, 1995). MHC isoforms are approximately 200 kDa, and they can be separated on a polyacrylamide gel so that the fraction of each isoform present in a sample of tissue may be measured (Talmadge & Roy, 1993). Separation of MHCs has played a critical role in establishing relative percentages of fibre types, which has been useful in the comprehension of what takes place during various conditions and disease states (Kohn & Myburgh, 2006). Some MHCs are muscle specific, while others are present in a variety of muscles. Muscle atrophy (brought on by various states of disuse) is usually accompanied by changes in the relative abundance of MHC isoforms (Chopard *et al*., 2001). Muscles composed of mainly fast-twitch myosin isoforms (MHC IIa, IIx and IIb, in order of increasing contraction rate) lose mass and protein faster during breakdown of protein than muscles composed mainly of slow-twitch isoforms (MHC I) (Brodsky *et al*., 2004).

1.7.2. Creatine Kinase

For many years concentrations of creatine kinase (CK) detected in blood has been used as an indication of muscle wasting (Adams *et al*., 1993; Apple *et al*., 1988). CK is present in both cardiac and skeletal muscle. CK exists as a dimer of 39 to 42 kDa and is abundant in most tissues. Muscle-CK mRNA is upregulated during atrophy (Cros *et al*., 1999), and there is a release of CK into blood after acute myocardial infarction (Apple, 1999a; Adams *et al.,* 1993). Changes in concentration of CK in blood has been noted in conditions other than acute myocardial infarction such as skeletal muscle damage or injury caused by rigorous exercise or disease-induced muscle wasting (Adams *et al*., 1993; Apple *et al*., 1997). CK is no longer the preferred marker for detecting muscle wasting after acute myocardial infarction due to its lack of specificity (it does not distinguish between cardiac and skeletal muscle), and a switch has been made to detect increased concentrations of cardiac-specific troponins to detect acute myocardial infarction (Apple, 1999a, 1999b; Jaffe *et al*., 2000). Measuring skeletal muscle-specific troponins for detecting breakdown of skeletal muscle is also becoming more common (Simpson *et al.,* 2005; Sorichter *et al*., 1997). Currently, CK remains the most sensitive marker for early detection, particularly for detecting dystrophic conditions where a compromised sarcolemma allows CK to leak into blood. Therefore high concentrations of CK are a hallmark of the pathology. However, the use of CK may not be as sensitive a marker for detecting breakdown of muscle as a skeletal muscle troponin assay would be for other types of muscle wasting conditions.

1.7.3. Troponins

In the early 1990s, clinicians began to move away from CK analyses as an indication of muscle wasting, such as after acute myocardial infarction, and troponin became a more reliable and more specific marker for the breakdown of cardiac muscle (Adams *et al*., 1993; Adams *et al*., 1996; Apple, 1999a, 1999b; Apple *et al*., 1999; Apple *et al*., 1997; Hamm *et al*., 1997; Jaffe *et al*., 2000; Katrukha *et al*., 1998; Wu *et al*., 1998). As muscle fibres degrade, troponins are released into the blood. Muscle contraction is regulated by intracellular Ca^{2+} concentrations and is a result of the thick (myosin) and thin (actin) filaments sliding back and forth (Takeda, 2005). There are three main regulatory components of the skeletal muscle thin filament, actin, tropomyosin and troponin. Actin polymerizes into helical filaments and tropomyosin (a coiled-coil dimer) associates with each strand of the actin helix such that one tropomyosin interacts with seven consecutive actin monomers (Brown & Cohen, 2005; Farah & Reinach, 1995; Filatov *et al*., 1999; Takeda, 2005). Troponin is located on the polymerized actin filaments with tropomyosin forming the muscle thin filament (Figure 1.3) (Reinach *et al*., 1997; Takeda, 2005).

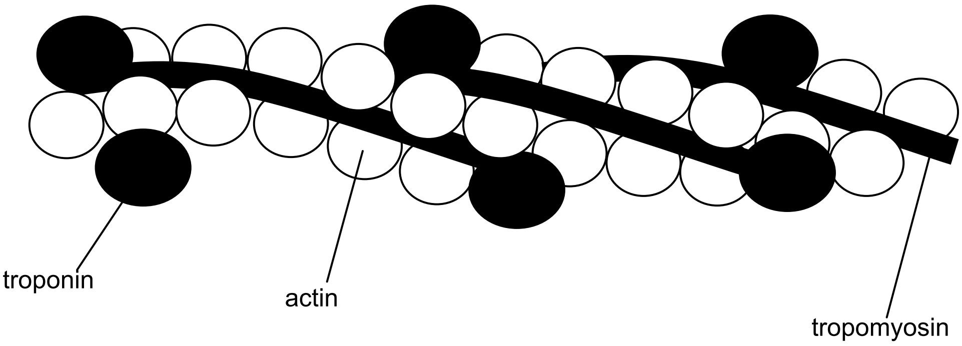

Figure 1.3: *The main regulatory components of the skeletal muscle thin filament* (Adapted from Takeda, 2005).

Troponin is an essential protein for the regulation of skeletal and cardiac muscle contraction (Takeda, 2005). It is made up of three polypeptides, each with a specific function. Troponin I is the inhibitory subunit of the protein. It inhibits the ATPase activity of actomyosin. Troponin C interacts with calcium, which then prevents troponin I inhibition of actomyosin. Troponin T allows the binding of troponin to tropomyosin (Filatov *et al.*, 1999). There are two isoforms of troponin I and T, each with a fast and slow twitch form. While it is known that contraction in skeletal and cardiac muscle is a result of Ca^{2+} binding to the troponin C subunit, the precise mechanism in which troponin regulates the interactions between myosin and actin in the thin filament with respect to muscle contraction remains unclear (Brown & Cohen, 2005; Takeda, 2005). Assay of troponin has not been used for detection of skeletal muscle breakdown to the extent that they have been used for detecting breakdown of cardiac muscle. However, assay of concentrations of troponin is gaining popularity as a marker of skeletal muscle breakdown (Simpson *et al.*, 2005; Sorichter *et al.*, 1997).

1.8. Outline of this Study

Thus far, little work has been done to determine what role muscle turnover plays in the period of time cows spend in negative EB. While it is known that the breakdown of fat (NEFAs) contributes to restoring EB initially, it is still unclear whether or not breakdown of skeletal muscle contributes to restoring EB in conjunction with breakdown of fat during lactation in dairy cows.

This study aims to:

(i) Determine whether breakdown of skeletal muscle occurs during peak lactation in cows.

(ii) Determine whether breakdown of skeletal muscle occurs in relation to the different yields of milk in different strains of dairy cows.

I hypothesize that the breakdown of muscle does occur in cows during peak lactation, and that it occurs to a greater extent in cows producing higher amounts of milk.

To asses this hypothesis, three strains of cow with different milk production abilities were investigated (see section 2.2.1.).

2. Materials and Methods

2.1. Materials

All reagents used in this research were of analytical grade or better. All water used was de-ionized and ultra-filtered in a milli-Q ultra-filtration system.

2.1.1. Antibodies

Antibodies used and their sources are listed in Table 2.1.

Table 2.1: *Antibodies*

Antibody	**Product #**	**Source**
Rabbit polyclonal troponin I slow skeletal	20645	Santa Cruz
Rabbit polyclonal troponin I fast skeletal	20644	Santa Cruz
Rabbit polyclonal troponin T slow skeletal	28269	Santa Cruz
Rabbit polyclonal troponin T fast skeletal	20643	Santa Cruz
Goat polyclonal myostatin	6884	Santa Cruz
Rabbit monoclonal 4E-BP1	9452	Cell Signaling
Rabbit polyclonal 4E-BP1	6936	Santa Cruz
Rabbit polyclonal phospho-4E-BP1	2855	Cell Signaling
Rabbit polyclonal phospho-4E-BP1	9455	Cell Signaling
Rabbit polyclonal eIF2α	11386	Santa Cruz
Rabbit polyclonal phospho-eIF2α	AB4837	Sapphire Bioscience
Rabbit polyclonal eIF4E	13963	Santa Cruz
Mouse monoclonal eIF4E	9976	Santa Cruz
Rabbit polyclonal phospho-eIF4E	12885R	Santa Cruz
Rabbit polyclonal p70S6K	9027	Santa Cruz
Rabbit polyclonal p70S6K	9202	Cell Signaling
Rabbit polyclonal phospho-p70S6K	7984R	Santa Cruz
Rabbit polyclonal phospho-p70S6K	9205	Cell Signaling
Rabbit polyclonal α-actin	A2066	Sigma Chemical Co.
Rabbit polyclonal goat	P0449	Dakocytomation
Goat polyclonal rabbit	P0448	Dakocytomation

2.1.2. Real-Time PCR Primers

Primer sequences and information are listed in Table 2.2. All primers were supplied by SIGMA Genosis.

Table 2.2: *Primers*

Gene	Direction[a]	Primer Sequence[b]	Size (b.p)[c]	Accession #
Atro1	Sense	AGCCAAGAAGAGAAAGAAAGAC	216	NM_001046155
	Antisense	GACTTTGCTATCAGCTCCAAC		
Murf1	Sense	TGACCAAGGAAAACAGCCACCAG	86	NP_001039760
	Antisense	TCACTCTTCTTCTCGTCCAGGATGG		
Cathepsin B	Sense	GGTGAATCTCCGATCCAGTTTC	165	BC_102997
	Antisense	AGTTGTGTCCAGCCTTCCAAGT		
Cathepsin D	Sense	CTGCACAAGTTCACGTCCATC	212	AB_055312
	Antisense	AAGACGACTGTGAAGCACTGCG		
Cathepsin H	Sense	AGCTGGCCGCGAACTCTTTAGA	153	NM_001034385
	Antisense	ATGTGTGGTTCCTGGCGTTGTG		
Cathepsin L	Sense	CCTTCATTCTTCCTGACTGTCC	159	NM_174032
	Antisense	ATTCTTCTCCCACACTGCTCTC		

[a] sense primers bind 5’ to 3’ and antisense primers bind 3’ to 5’, [b] sequences are written 5’ to 3’, [c] b.p = base pairs.

2.1.3. Common Solutions

Common solutions were routinely made in the lab. Common solutions and their composition are listed in Tables 2.3 and 2.4.

Table 2.3: *Common solutions*

Solution	Composition
Low Salt Buffer (LSB)	10 mM HEPES-NaOH 10 mM MgCl 5 mM KCl 0.1 mM EDTA 0.5% IGEPAL 0.5% Vanadate Made a day prior to use Add one complete protease inhibitor cocktail tablet per 50 mL immediately before use
3X Laemmli Sample Buffer	10% Glycerol 5% βME 2% SDS 62.5 mM Tris, pH 6.8 0.1% of Bromophenol Blue Store at -20 ˚C
Transfer Buffer	39 mM Glycine 48 mM Tris 20% Methanol Store at 4 ˚C
Tris buffered saline (TBS)	150 mM NaCl 20 mM Tris Adjust to pH 7.6 and store at 4 °C
Tris buffered saline with Tween 20 (TBST)	0.1% Tween 20 in TBS Store at 4 °C
Enhanced Chemiluminescence (ECL) Buffer	20 mM Tris Adjust to pH 8.6 and store at 4 ˚C
Luminol A	458.33 mg Luminol A in 5 mL DMSO
p-Coumaric acid B	840 mg p-Coumaric acid B in 3 mL DMSO

ECL Solution	50 mL ECL Buffer 120 µL Luminol A 20 µL p-Coumaric acid B 16.65 µL 30% H_2O_2

Table 2.4: *SDS-PAGE gel compositions*

Acrylamide Percentage	Composition
15%	7.5 mL 1.5 M Tris, pH 8.8 7 mL Milli-Q H_2O 15 mL 30% Acrylamide-Bis 300 µL 10% SDS 150 µL 10% APS 30 µL TEMED
12%	7.5 mL 1.5 M Tris, pH 8.8 10 mL Milli-Q H_2O 12 mL 30% Acrylamide-Bis 300 µL 10% SDS 150 µL 10% APS 30 µL TEMED
10%	7.5 mL 1.5 M Tris, pH 8.8 12 mL Milli-Q H_2O 10 mL 30% Acrylamide-Bis 300 µL 10% SDS 150 µL 10% APS 30 µL TEMED
8%	7.5 mL 1.5 M Tris, pH 8.8 14 mL Milli-Q H_2O 8 mL 30% Acrylamide-Bis 300 µL 10% SDS 150 µL 10% APS 30 µL TEMED
4%	2.5 mL 0.5 M Tris, pH 8.8 6 mL Milli-Q H_2O 1.34 mL 30% Acrylamide-Bis 100 µL 10% SDS 70 µL 10% APS 20 µL TEMED

2.1.4. Common Laboratory Chemicals and Reagents

All common chemicals and reagents used and their sources are listed in Table 2.5.

Table 2.5: *Chemicals and reagents*

Chemical or Reagent	**Source**
Bromophenol blue; chloroform; ethylenediaminetetra-acetic acid (EDTA); hydrochloric acid (HCl); hydrogen peroxide (H_2O_2); magnesium chloride (MgCl); potassium chloride (KCl); sodium chloride (NaCl); sodium hydroxide (NaOH)	BDH Chemicals Ltd.
Isopropanol; HPLC grade ethanol; methanol (MeOH)	BioLab Ltd.
30% Acrylamide-bis (N,N'-methylene-bis-acrylamide); ammonium persulfate (APS); coomassie brilliant blue R-250; glycine; sodium dodecyl sulfate (SDS); N,N,N',N'-tetra-methyl-ethylenediamine (TEMED)	Bio-Rad Laboratories
Bovine serum albumin (BSA); trizol	Invitrogen (Gibco BRL)
Acetic acid; tris(hydroxymethyl)aminomethane (Tris)	J.T.Baker
Goat Immunoglobulins; Rabbit Immunoglobulins	Med-Bio Ltd.
2-mercaptoethanol (BME); amino acid standard solution; Bicinchoninic acid (BCA); p-Coumaric acid; Copper (II) Sulfate; Diethyl pyrocarbonate (DEPC); Dimethyl Sulfoxide (DMSO); Glycerol; HEPES; IGEPAL; Luminol; Mercury-[(o-carboxyphenyl)thio]ethyl sodium salt (Thimerosal); Polyethylene Glycol (PEG); Polyoxyethylene sorbitan-monolaurate (Tween 20); Polyvinylpyrrolidone (PVP); Ponceau S; Vanadate	SIGMA Chemical Co.
Complete protease Inhibitor cocktail	Roche

2.2. Methods

2.2.1. Animal Maintenance

The animals used in this project were obtained from Dexcel farms or commercial dairy farms in New Zealand. The cows chosen milk production recorded and were purebred for more than three generations. Three strains were selected, the New Zealand 70 (NZL), New Zealand 90 (NZH), and North American 90 (OSH). NZL is a strain of NZ Holstein Friesian (HF) with high Breeding Worth (a system in which bulls and cows are ranked on their expected ability to produce profitable replacements) in the 1970s. The NZL strain represented the selection and breeding policies in NZ prior to 1970. Livestock Improvement Corporation (LIC) stored semen from that time and used it to artificially inseminate dams that represented cows in the NZ population in the 1970s selected for high milk fat yield. The NZL strain had 7% North American (NA) HF genetics. Both sires and dams used to generate this strain had a low proportion of NA genes. NZH is a strain of NZ HF with a high Breeding Worth in the 1990s. The strain represented NZ selection and breeding policies used in the 1990s (high milk production) and had 24% NA HF genetics. OSH is a strain of HF from North America, which also had a high Breeding Worth in the 1990s. NZ born cows with a high proportion of NA genetics were bred with sires of primarily NA origin to generate this strain. These sires were commonly used within NZ in the 1990s because of their high protein and milk estimated breeding value. The OSH strain had 91% NA HF genetics.

2.2.2. Sample Collection

Pregnant Holstein cows from the three strains (NZL n=12, NZH n=28, OSH n=30) were assigned to have blood samples taken at weekly intervals and a biopsy sample (up to 200 mg) taken under anaesthesia from the trapezius muscle at -1, 1, 4, 8, 12 weeks post-calving (1-12 weeks being peak lactation). A blood sample (10 mL) was obtained by jugular venepuncture into vacutainer tubes containing heparin as anticoagulant. Tubes were centrifuged at 1500 x g for 10 min to separate red blood cells and plasma, and the plasma was then harvested and stored at -80 °C for later determination of creatine kinase (CK), and for Western analysis to detect levels of troponin I-fast skeletal. Biopsy samples were taken from the trapezius muscle and snap frozen in liquid nitrogen and stored at -80 °C. This study was approved by the Dexcel Animal Ethics Committee (#**10586)**.

2.2.3. Analysis of Creatine Kinase, Troponin and Myostatin in Plasma

Plasma was stored at -80 °C until required. Plasma CK was measured in duplicate (n=9 animals per strain) using the CK NAC-activated assay (RANDOX Laboratories Ltd., Antrim UK) as per manufacturer's instructions. For determination of troponin and myostatin, two µL plasma for each time point was mixed with Laemmli loading buffer (Laemmli, 1970), boiled for 5 min, then loaded and separated in a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel under reducing conditions (12% acrylamide for troponin, 15% for myostatin). Proteins were then transferred to a BioTrace NT nitrocellulose membrane (PALL Corporation). After transfer, membranes were stained with Ponceau S to verify transfer of protein. Membranes were blocked (0.3% BSA, 1% PEG and 1% PVP, and 0.01% Thimerosal) for 2 hours at room temperature. Initially, four antibodies were titred for troponin to determine which troponin isoform was most appropriate to measure. The four antibodies titred were rabbit polyclonal troponin I fast skeletal (I-fs) (Santa Cruz 20644), I slow skeletal (I-ss) (Santa Cruz 20645), T-fs (Santa Cruz 20643) and T-ss (Santa Cruz 28269). Half-log dilutions from 1:1000 to 1:30 000 were tested with various animals. The antibodies used for plasma analysis were rabbit polyclonal troponin I-fs diluted 1:3000 (selected because it had the most distinctive band) and goat polyclonal myostatin (Santa Cruz 6884) 1:3000 diluted in blocking buffer. Membranes were incubated with primary antibody overnight at 4 ˚C on a shaking table. After overnight incubation, membranes were then washed several times in 0.02 M Tris buffered saline with 0.1% Tween 20 (TBST, pH 7.6), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (rabbit polyclonal goat (Dakocytomation P0448) or goat polyclonal rabbit (Dakocytomation P0449), diluted 1:5000 in blocking buffer) for 2 hours. Membranes were then washed several times with TBST followed by repeated washes with TBS, to rinse off any traces of Tween 20 which interferes with the peroxidase reaction. Bound HRP activity was detected with enhanced chemiluminescence for 4 min and then blots were exposed to BioMax XAR film (Eastman Kodak Company, Rochester, NY). The density of each protein band detected was determined using a densitometer (Bio-Rad

Laboratories Pty., Ltd., Auckland, New Zealand) and analysed using Quantity One software (Bio-Rad Laboratories).

To validate that concentrations of troponin are reliable markers of muscle wasting, plasma from rats that were inoculated with the AH130 tumour was separated on 12% SDS-PAGE gels as per above and the same panel of troponin antibodies was screened to determine the appropriate antibody to use and the optimal titre. Briefly, male Sprague-Dawley rats (3 months old) were injected i.p. with 100 μL of sterile saline (n=6), or 100 μL of freshly passaged ascites (n=6) from a donor rat with the AH130 tumour (Baracos *et al*., 1995). Rats were killed at 6 days and blood was obtained by cardiac puncture and the plasma harvested. Body mass was recorded and the *Tibialis anterior* muscle (a representative fast-twitch muscle known to undergo wasting during AH130-induced cancer) was excised and weighed and corrected to percent of body mass of rats on day 0. This study was approved by the Ruakura Animal Ethics Committee (#**4113)**.

2.2.4. Myosin Heavy Chains

MHCs were extracted (Seward *et al*., 2001) and between 1 and 1.5 μg of crude protein from each muscle sample was loaded and separated in a SDS-PAGE gel under reducing conditions and stained with Coomassie blue (Talmadge and Roy, 1993). The optical density of each MHC band was determined using a densitometer (Bio-Rad Laboratories) and analysed using Quantity One software (Bio-Rad Laboratories).

2.2.5. RNA Extraction and Analysis

Because not all tissue samples were available for each sample time, a representative nine were selected that comprised the most complete set available for the sample times. Frozen muscle samples (30-120 mg) were homogenized on ice in Trizol Reagent (Invitrogen) for 30 sec at 13,500 rpm using an Ultra Turrax homogenizer. Debris was removed by centrifugation for 10 min at 10,000 x g and total RNA was isolated using the Trizol protocol (Invitrogen). RNA was re-suspended in 0.1% DEPC-treated water and the final concentration determined by measuring absorbance at 260 nm. Total RNA (2.4 µg) from each sample was reverse transcribed using oligo (dT) primers and Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. The reverse transcribed (RT) reactions were then diluted 100-fold for quantification of Atrogin-1 (atro1), Muscle ring-finger protein 1 (murf1), and cathepsin B, D, H and L mRNA levels. Real-time PCR was carried out using the Roche Lightcycler 2.0 with 2.5 µL of the diluted RT reaction and 7.5 µL of the mastermix (2.0 µL Roche Faststart DNA master PLUS SYBR Green I mix, 4.0 µL water, 0.5 µL gene specific Primer 1 and 0.5 µL gene specific Primer 2). Information on the primers used can be found in Table 2.2. The reaction conditions were as follows: Denature for 5 min at 95°C, followed by 40 cycles of 95 ºC for 5 sec, 60 ºC for 10 sec and 72 ºC for 20 sec. Standard curves were generated for correlation of sample concentrations. Melt curves were examined to ensure a single cDNA species was being amplified for each gene under investigation. Products were run out on a 1% agarose gel to confirm a single species was amplified. All primers were designed using Vector NTI (Invitrogen) and made by SIGMA Genosys. Results for each sample were corrected to total single-stranded cDNA using the Quant-iT OliGreen ssDNA assay Kit as per manufacturer's instructions (Molecular Probes, Inc. OR, USA) (Lundby *et al*., 2005).

2.2.6. Protein Extraction and Analysis

Six representative animals were selected at random from each strain for analysis of total and phosphorylated p70S6K, eIF2α, eIF4E and 4E-BP1. One ml of ice cold low salt lysis buffer (10 mM Hepes, 5 mM KCl, 10 mM MgCl, pH 7.9) with 0.1 mM EDTA, 0.5% IGEPAL detergent (Sigma Chemical Co. St. Louis, MO, USA), 0.5% Vanadate and an enzyme inhibitor (Complete, Roche Diagnostics NZ Ltd., Auckland, New Zealand) was added to muscle (30-120 mg) from each animal. Samples were homogenised on ice, then centrifuged at 11,000 xg for 10 min. Supernatant was recovered, mixed with Laemmli loading buffer (Laemmli, 1970), boiled for 5 min, then stored at –20 °C until analysis. The protein concentration of the supernatant was determined using the bicinchoninic acid assay (Sigma Chemical Co.).

Twenty μg of protein from each muscle sample was loaded and separated in a SDS-PAGE gel under reducing conditions (8% acrylamide for p70S6K, 10% for eIF2α and 15% for 4E-BP1), then transferred to a BioTrace NT nitrocellulose membrane (PALL Corporation). After transfer, membranes were stained with Ponceau S to verify transfer of protein. Membranes were blocked (0.3% BSA, 1% PEG and 1% PVP, 0.01%

Thimerosal) for 2 hours, and then incubated with one of the following specific antibodies diluted in blocking buffer: rabbit monoclonal 4E-BP1 (Cell Signaling 9452) diluted 1:1000, rabbit polyclonal phospho-4E-BP1 (Cell Signaling 2855) diluted 1:5000, rabbit polyclonal eIF2α (Santa Cruz 11386) diluted 1:5000, rabbit polyclonal phospho-eIF2α (Sapphire Bioscience AB4837) diluted 1:3000, rabbit polyclonal eIF4E (Santa Cruz 13963) diluted 1:3000, rabbit polyclonal phospho-eIF4E (Santa Cruz 12885R) diluted 1:3000, rabbit polyclonal p70S6K (Cell Signaling 9202) diluted 1:3000, rabbit polyclonal phospho-p70S6K (Santa Cruz 7984R) diluted 1:3000 and rabbit polyclonal α–actin (Sigma Chemical Co.) diluted 1:5000. After overnight incubation at 4 ˚C, membranes were then washed several times in 0.02 M Tris buffered saline with 0.1% Tween 20 (TBST, pH 7.6), then incubated with HRP-conjugated secondary antibody, rabbit polyclonal goat (Dakocytomation P0449) or goat polyclonal rabbit (Dakocytomation P0448), diluted 1:5000 in blocking buffer) for 2 hours. Membranes were then washed several times with TBST followed by repeated washes with TBS, to rinse off any traces of Tween 20 which interferes with the peroxidase reaction. Bound HRP activity was detected with enhanced chemiluminescence for 4 min and then blots were exposed to BioMax XAR film (Eastman Kodak Company). The density of each protein band detected was determined using a densitometer (Bio-Rad Laboratories) and analysed using Quantity One software (Bio-Rad Laboratories).

2.2.7 Statistical Analysis

Troponin I-fs and myostatin were assessed in a representative 8 animals for each strain at -1, 1, 2, 3, 4, 8, and 12 weeks postpartum. Myosin heavy chains were assessed in a representative 6 animals for each strain at -1, 4 and 12 weeks postpartum. Atro1 and murf1 were assessed in a representative 9 animals for each strain at -1, 1, 4, 8, and 12 weeks postpartum. Cathepsin B, D and H were assessed in a representative 9 animals for each strain at -1, 1, 4 and 12 weeks postpartum. Protein analysis was carried out on a representative 6 animals for each strain at -1, 1, 4, 8 and 12 weeks postpartum. All data was analysed using a repeated-measures ANOVA. Post-hoc comparison analyses among groups were performed using a Tukey test. Data are presented as means ± standard error (s.e.m.).

3. Results

3.1. Previously Published Data on NZL, NZH and OSH cows

Milk yield, BWT, BCS and concentrations of NEFAs in plasma have been recorded previously for the three strains of cow in this study. The NZL cows exhibit a low milk yield, the NZH cows intermediate, and the OSH cows high (McDonald *et al.* 2007). In previous studies, OSH high cows have a higher BWT than the NZL and NZH cows, and they lose more BWT throughout lactation (McDonald *et al.* 2007; Chagas *et al.* 2006). OSH cows typically have a lower BCS throughout lactation than the NZL and NZH cows. Studies have shown concentrations of NEFAs in plasma increase during the first two weeks of lactation, and then steadily decrease (Guo *et al.* 2007; Roche *et al.* 2007). Concentrations of NEFAs in plasma were lower in NZL and NZH cows than in OSH cows (Roche *et al.* 2007).

3.2. Plasma Analyses

3.2.1. Creatine Kinase

Elevated concentrations of CK in plasma are often indicative of breakdown of muscle and CK detection has been used to assess skeletal muscle damage or injury caused by rigorous exercise or disease-induced muscle wasting (see section 1.7.2.). Concentrations of CK were unchanged in all strains over time, with the exception of being higher (P<0.01) in NZH cows than NZL and OSH cows at 8 weeks postpartum (Figure 3.1).

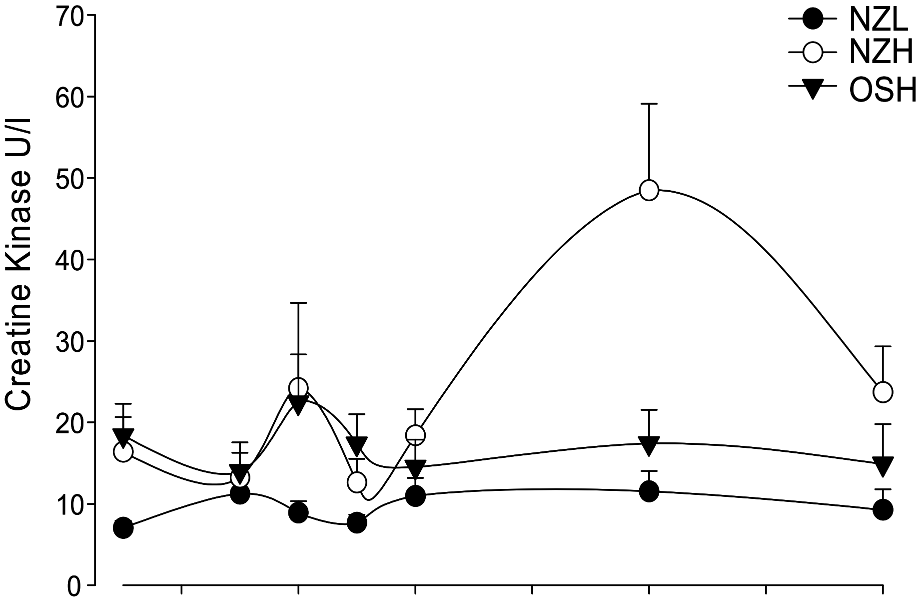

Figure 3.1: *Creatine kinase in cow plasma.* Mean (± s.e.m.) concentrations of creatine kinase in plasma of three strains of cow (NZL, NZH, OSH) (n=9 per strain) from -1 to 12 weeks of lactation.

3.2.2. Troponin

When muscle fibres undergo degradation, troponins are released into the blood. Skeletal muscle-specific troponins have recently become useful for detecting breakdown of muscle (see section 1.7.3.). Four antibodies were tested for abundance of troponin in plasma; troponin T-ss, troponin T-fs, troponin I-ss and troponin I-fs. The expected molecular size of troponin T is between 31 and 37 kDa. The expected molecular size of troponin I is 21 kDa. Of the troponin antibodies tested, only troponin I-fs consistently showed an immunoreactive band of the appropriate molecular weight (21 kDa) in cows (Figure 3.2). In rat plasma, again all four antibodies were tested and troponin I-fs had the most consistent immunoreactive band. However, the molecular size of the only immunoreactive band present was 13 kDa rather than the expected 21 kDa (Figure 3.3 A). This molecular weight band represents one of several known breakdown products (Simpson *et al*., 2005; Simpson *et al.,* 2000; McDonough *et al.,* 1999; Gao *et al.,* 1997). Therefore, troponin I-fs was used to analyse plasma concentrations in cows and rats.

Troponin I-fs was increased in plasma of cachectic rats (Figure 3.3 B) and this was associated with a decrease in mass of the *Tibialis anterior* (Figure 3.3 C). There was an overall effect of time ($P<0.05$), in which there was an increase in the abundance of troponin I-fs in plasma in all strains Figure 3.4 B), peaking at three weeks and remaining high ($P<0.05$) for the rest of the study, but no effect of peak lactation for individual strains of cow (Figure 3.4 C). Abundance of troponin I-fs was higher in NZH than OSH ($P<0.05$) and NZL ($P<0.001$) cows demonstrating a clear effect of strain (Figure 3.5).

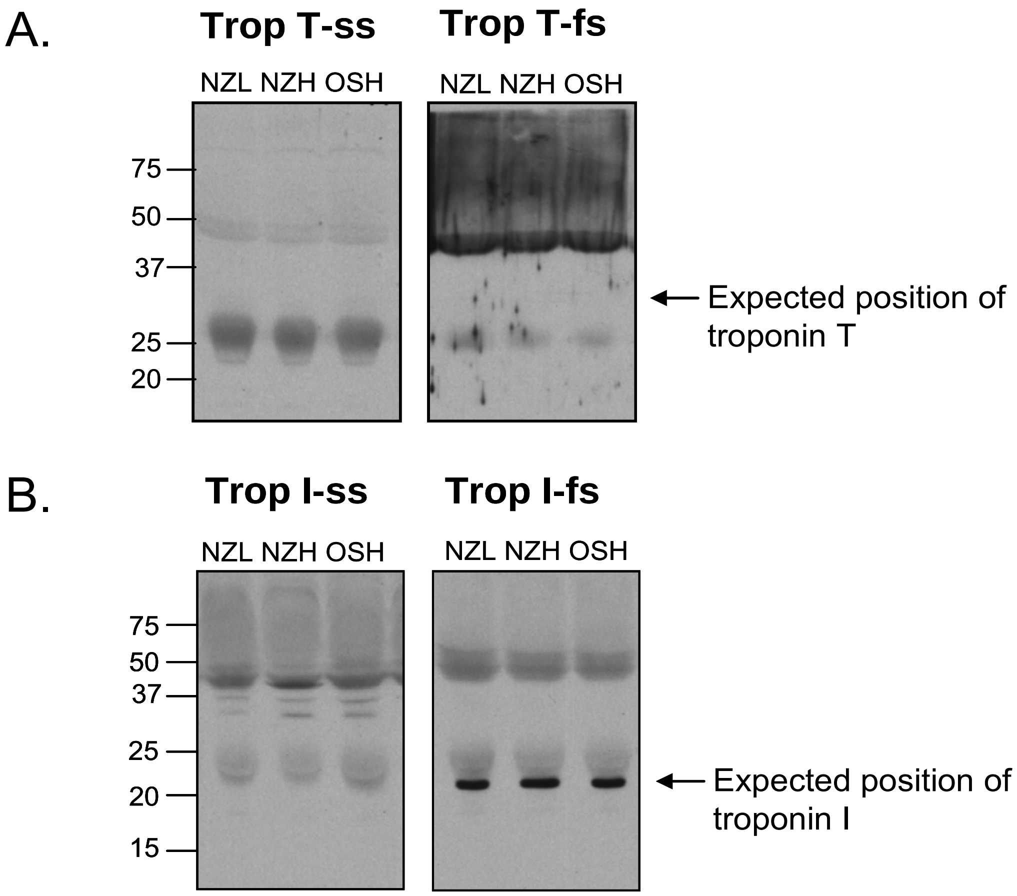

Figure 3.2: *Detecting troponin isoforms in cow plasma.* Titrations were done with dilutions from 1:1000, 1:3000 and 1:10 000 of **(A)** troponin T-ss, T-fs, and **(B)** troponin I-ss and I-fs to measure troponin in plasma. Shown is the 1:3000 titre of these antibodies to demonstrate that only troponin I-fs shows a specific immunoreactive band at 21 kDa. The expected molecular weight of troponin T is 31-37 kDa. No band was detected using either troponin T antibody.

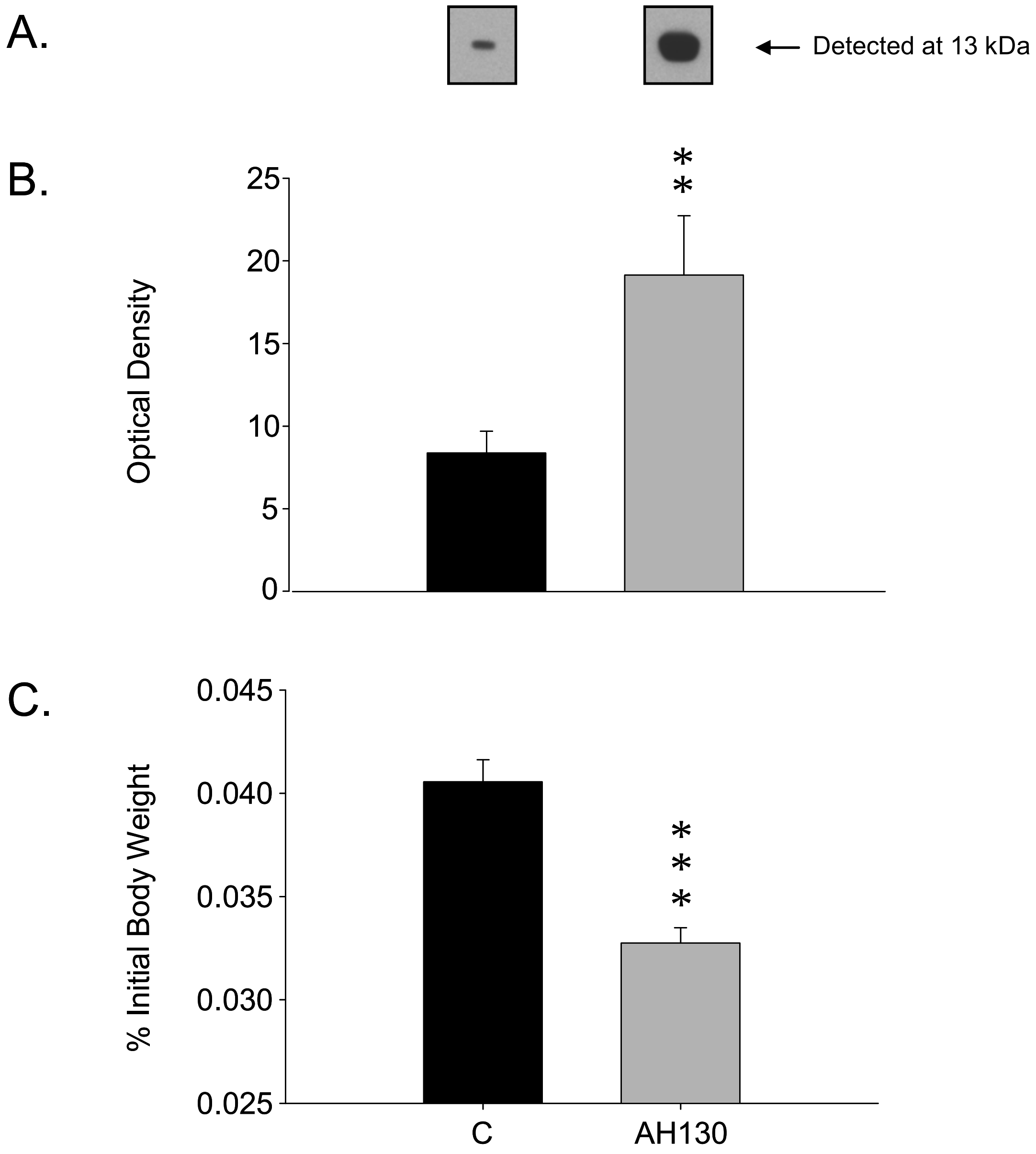

Figure 3.3: *Troponin I-fs in AH130 rats.* **(A)** Troponin I-fs detected in plasma of adult male rats subjected to vehicle (C) or AH130 tumour by Western blot. Band was detected at 13 kDa, representing one of several breakdown products of troponin I-fs.
(B) Abundance of Troponin I-fs in plasma for control rats (C) and rats at six days with tumour. **(C)** Percent loss of muscle in TA of AH130 rats compared to controls corrected to body weight at the start of the experiment (n=6). Asterisk indicates significant difference from controls (** P<0.01 , *** P<0.001).

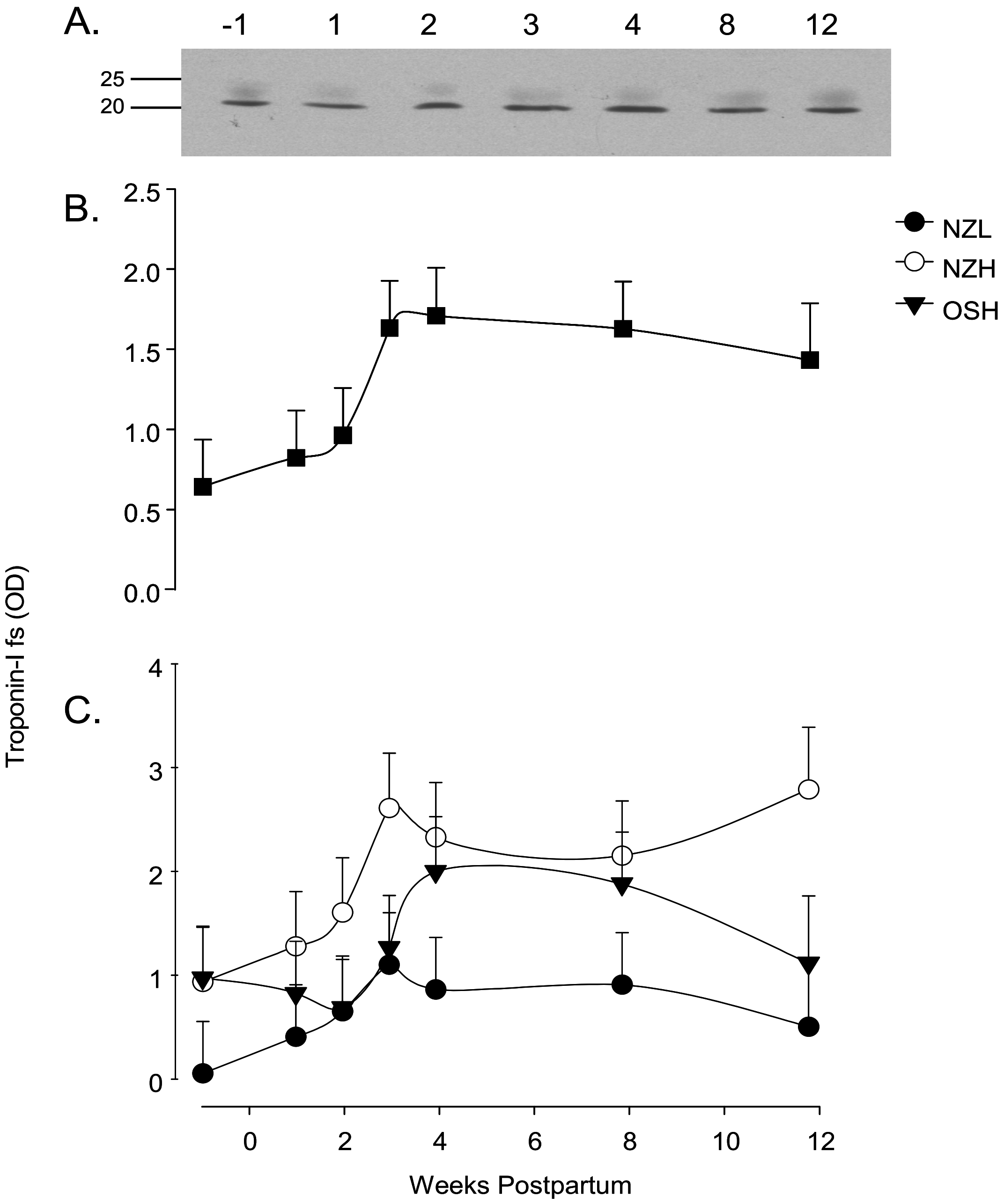

Figure 3.4: *Troponin I-fs in cow plasma.* **(A)** Single band at approximately 21 kDa representing troponin I-fs in NZH 941 from -1 to 12 weeks of lactation. **(B)** Effect of time alone (pooled mean ± s.e.m.) of troponin I-fs in plasma for all three strains (n=9 per strain) from -1 to 12 weeks of lactation. **(C)** Mean (± s.e.m.) of troponin I-fs in plasma from -1 to 12 weeks of lactation for each strain (n=9 per strain).

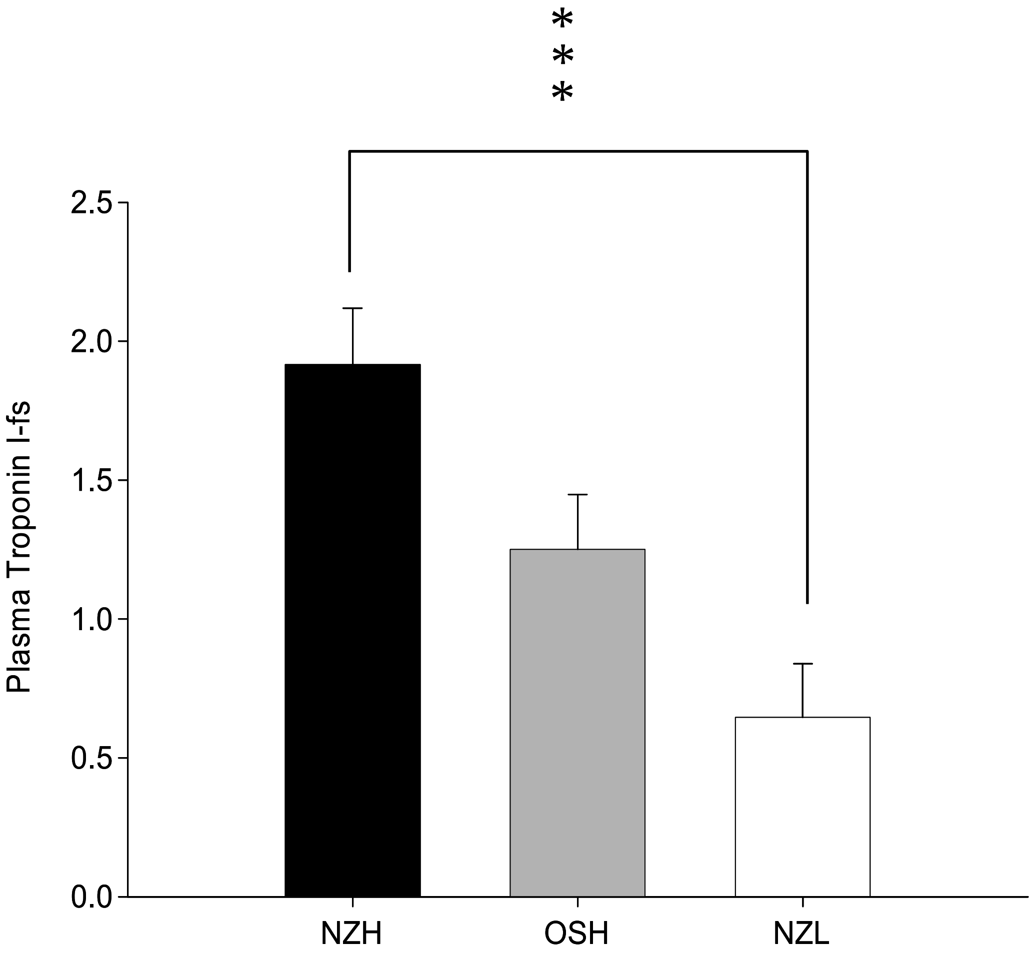

Figure 3.5: *Abundance of troponin I-fs in plasma of the three different strains of cow.* Effect of strain (pooled mean ± s.e.m., n=9 per strain) on troponin I-fs in plasma from one week before calving to 12 weeks postpartum for each strain. Asterisk indicates a significant difference among strains (***P<0.001).

3.2.3. Myostatin

Myostatin expression increases in various conditions of atrophy. An increase in the abundance of myostatin in plasma could support loss of muscle due to breakdown (see section 1.6.2.). Myostatin was detected in plasma at 26 kDa (Figure 3.6 A), which is the expected molecular weight of the mature myostatin peptide dimer. The optical density of mature myostatin did not change over the period of study for any of the three strains (Figure 3.6 B). However, there was an effect of strain ($P<0.001$), whereby OSH cows had a lower abundance of myostatin consistently detected in plasma.

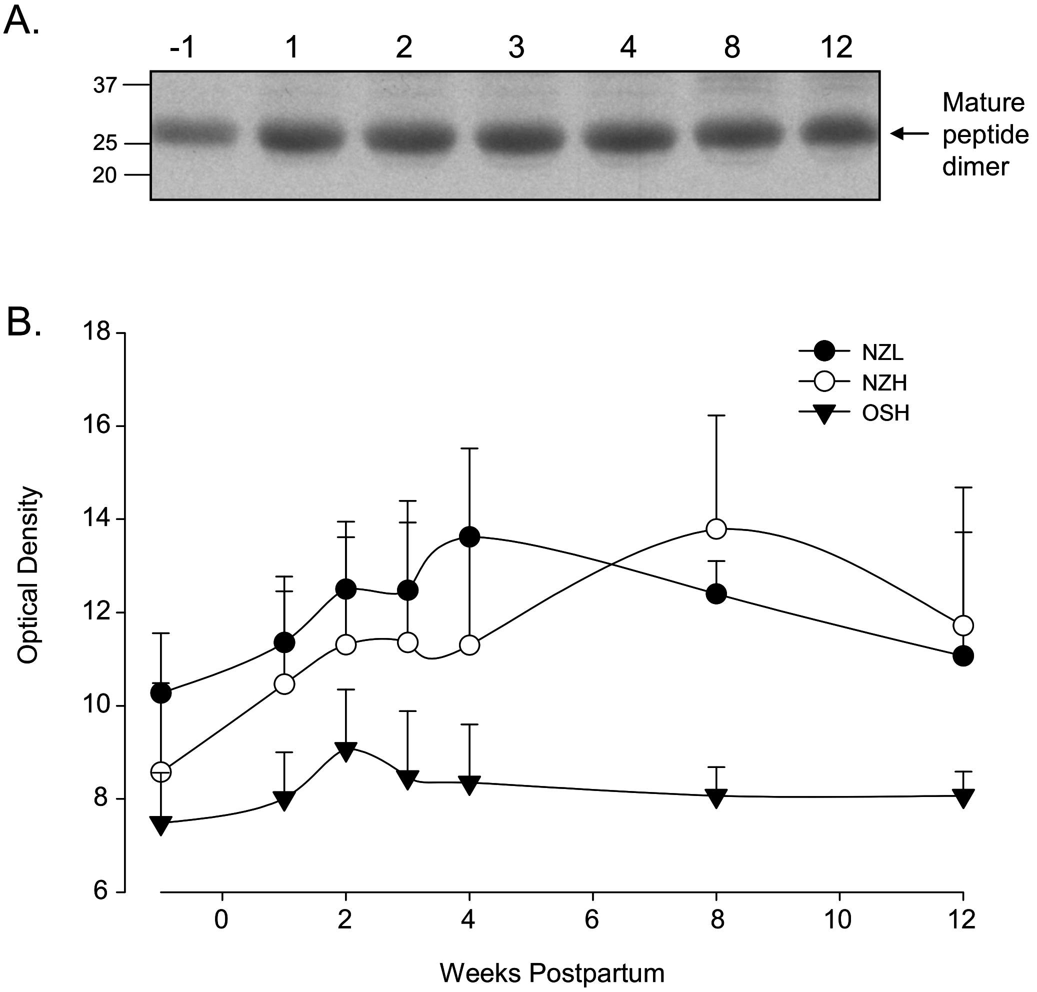

Figure 3.6: *Myostatin in cow plasma.* **(A)** A single immunoreactive band detected at 26 kDa representing mature myostatin dimer in NZH 2078 plasma samples taken at -1 to 12 weeks of lactation. **(B)** Mean (± s.e.m.) abundance of mature myostatin peptide in plasma from -1 to 12 weeks of lactation in all three strains. There is a significant effect of strain whereby OSH cows have a lower abundance of plasma myostatin ($P<0.001$).

3.3. Fibre Typing

3.3.1. Myosin Heavy Chains

The breakdown of muscle is often accompanied by changes in the abundance of MHC isoforms (see section 1.7.1.). These changes can be observed through SDS-PAGE separation of crude muscle samples. For each time point for each animal, two distinct bands could be seen in the Coomassie-stained gels (Figure 3.7 A). The upper band represents skeletal muscle fibre type IIx and the lower represents type I. There was an effect of strain whereby OSH cows had more type II than NZL and NZH cows ($P<0.05$). However, there was no overall effect of time of lactation, or effect of individual strain during peak lactation, on relative abundance of MHCs (Figure 3.7 B).

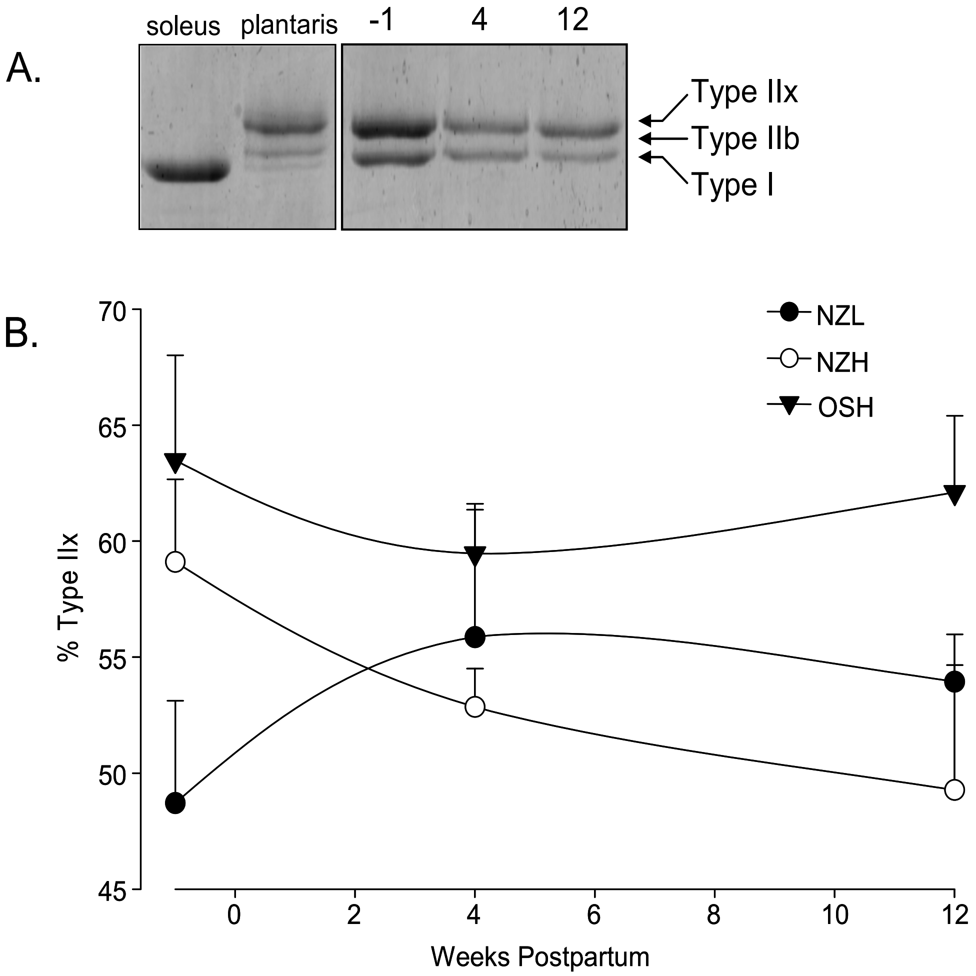

Figure 3.7: *Myosin heavy chains in cow trapezius muscle.* **(A)** MHC isoforms in trapezius muscle from NZH 9971 separated by SDS-PAGE and stained with Coomassie blue. Samples from one week before calving, and at four and 12 weeks postpartum. Soleus and plantaris samples were run for comparative purposes. **(B)** Mean (± s.e.m.) percentage of type IIx in trapezius muscle samples at -1, 4 and 12 weeks postpartum. There is a significant effect of strain whereby OSH have a higher percent of type IIx compared with either NZL or NZH ($P<0.05$).

3.4. RNA Analysis

Breakdown of muscle may be the result of an increase in protein degradation. The two key degradative pathways primarily responsible for protein degradation are the UbP pathway and the lysosomal pathway.

3.4.1. The Ubiquitin-Proteasome Pathway

The UbP ligases atro 1 and murf 1 are consistently upregulated in several conditions of muscle atrophy and are widely used as markers of muscle wasting (see section 1.5.4.1.). An increase in expression of atro 1 and murf 1 mRNA would suggest that breakdown of muscle is occurring through an increase in degradation. An effect of time was seen whereby there was a tendency for Atro1 to increase (P<0.1), then concentrations of Atro1 decreased by week four (P<0.01) and remained low for the rest of the study (Figure 3.8 A). There was no effect of strain on atrogin mRNA. A similar pattern was observed for Murf1 (Figure 3.8 B) with concentration increased from week -1 to week 1 of lactation, after which time the concentration of Murf1 mRNA decreased and remained low for the rest of the study (P<0.001).

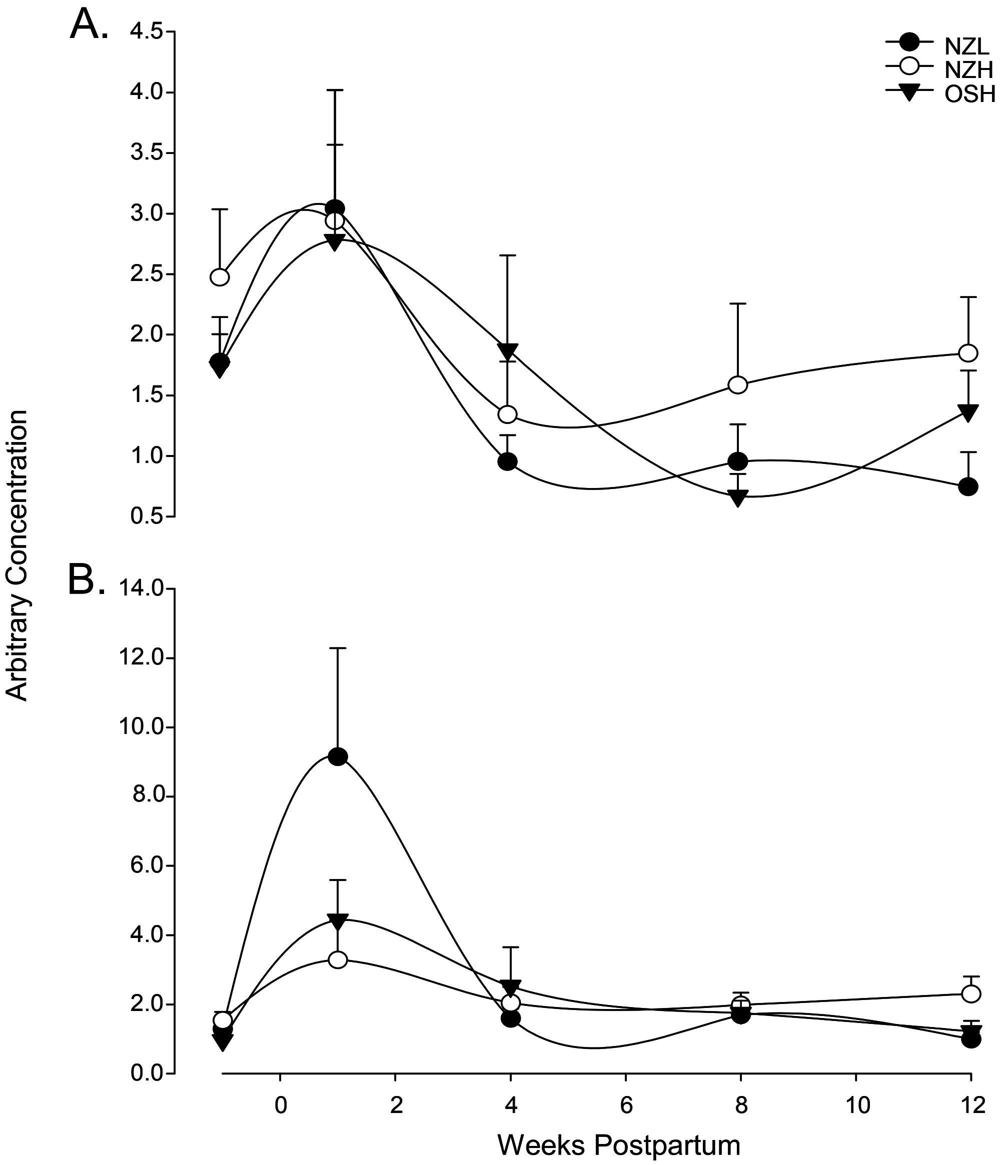

Figure 3.8: *Analysis of ubiquitin proteasome E3 ligases Atro1 and Murf1 mRNA by real-time PCR.* Mean arbitrary concentrations (± s.e.m.) of **(A)** Atrogin and **(B)** Murf1 mRNA expression in NZL, NZH and OSH from one week before calving to 12 weeks postpartum. There was an increase in expression of both genes from -1 to 1 week of lactation (at least P< 0.1).

3.4.2. The Lysosomal Pathway

Lysosomal degradation is a second pathway contributing to muscle degradation. The cathepsins are lysosomal proteases which are upregulated in conditions of muscle wasting (see section 1.5.4.3.). Cathepsin L mRNA was very low in abundance and could not be amplified to a measurable concentration (data not shown). An effect of time was seen whereby concentrations of cathepsin B mRNA decreased from -1 to 4 weeks postpartum ($P<0.01$) (Figure 3.9 A), and cathepsin D mRNA decreased from -1 to 1 weeks of lactation in NZL and NZH cows ($P<0.05$) (Figure 3.9 B). The level of cathepsin H mRNA was greater in NZH cows at 12 of lactation ($P<0.001$) (Figure 3.9 C).

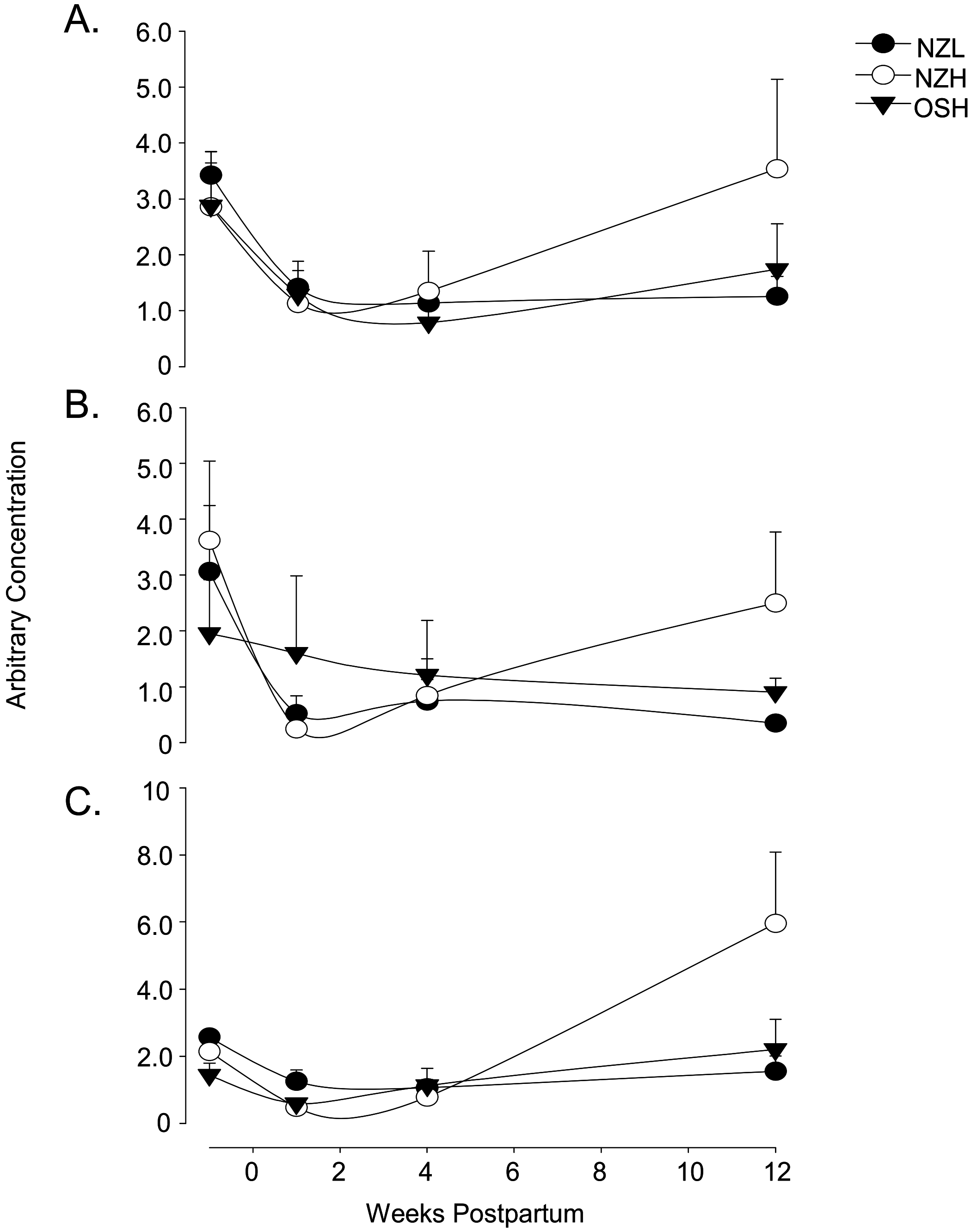

Figure 3.9: *Analysis of Cathepsin mRNA by real-time PCR.* Mean arbitrary concentrations (± s.e.m.) of **(A)** cathepsin B, **(B)** cathepsin D and **(C)** cathepsin H mRNA expression in NZL, NZH and OSH from one week before calving, 4 and 12 weeks postpartum.

3.5. Protein Analysis

Breakdown of muscle could also be the result of a decrease in protein synthesis. To determine whether or not protein synthesis was being downregulated, the components of translation initiation were investigated.

3.5.1. p70S6K

The phosphorylation of p70S6K protein correlates with an increase in protein synthesis (see section 1.5.3.). Therefore, it was expected that the ratio of phosphorylated p70S6K over total p70S6K would decrease over the period of study, representing a decrease in protein synthesis. Two antibodies were tested for total p70S6K and two for phospho-p70S6K. Out of the four tested, only one antibody for total p70S6K gave a single specific band at 70 kDa. Both antibodies for the phosphorylated state of p70S6K gave multiple immunoreactive bands at varying molecular weights (see Appendix A and B). No band was detected at the expected 70 kDa size for phosphorylated p70S6K, therefore the antibody was probably not specific for cows.

3.5.2. eIF2α

The eIF2 complex, made up of eIF2α and eIF2β, controls the association of the initiator methionyl-transfer RNA to the native 40S ribosome, forming the 43S initiator complex (see section 1.5.3.). Upon phosphorylation, eIF2α binds eIF2β very tightly and prevents the catalytic activity of eIF2β, thereby downregulating protein synthesis. It was expected that the ratio of phosphorylated over total eIF2α would increase over the period of study, in order to decrease the amount of protein synthesis taking place. One antibody was tested for total eIF2α and one for phospho-eIF2α. A distinct band at the expected molecular weight of approximately 37 kDa was seen for each antibody (Figure 3.10 A). There was no change in eIF2α total or phosphorylated for any of the three strains (Figure 3.10 B and C). The ratio of phosphorylated eIF2α over total eIF2α was greater in NZL cows ($P<0.001$) compared with NZH and OSH, but there was no effect of lactation on this ratio (Figure 3.10 D).

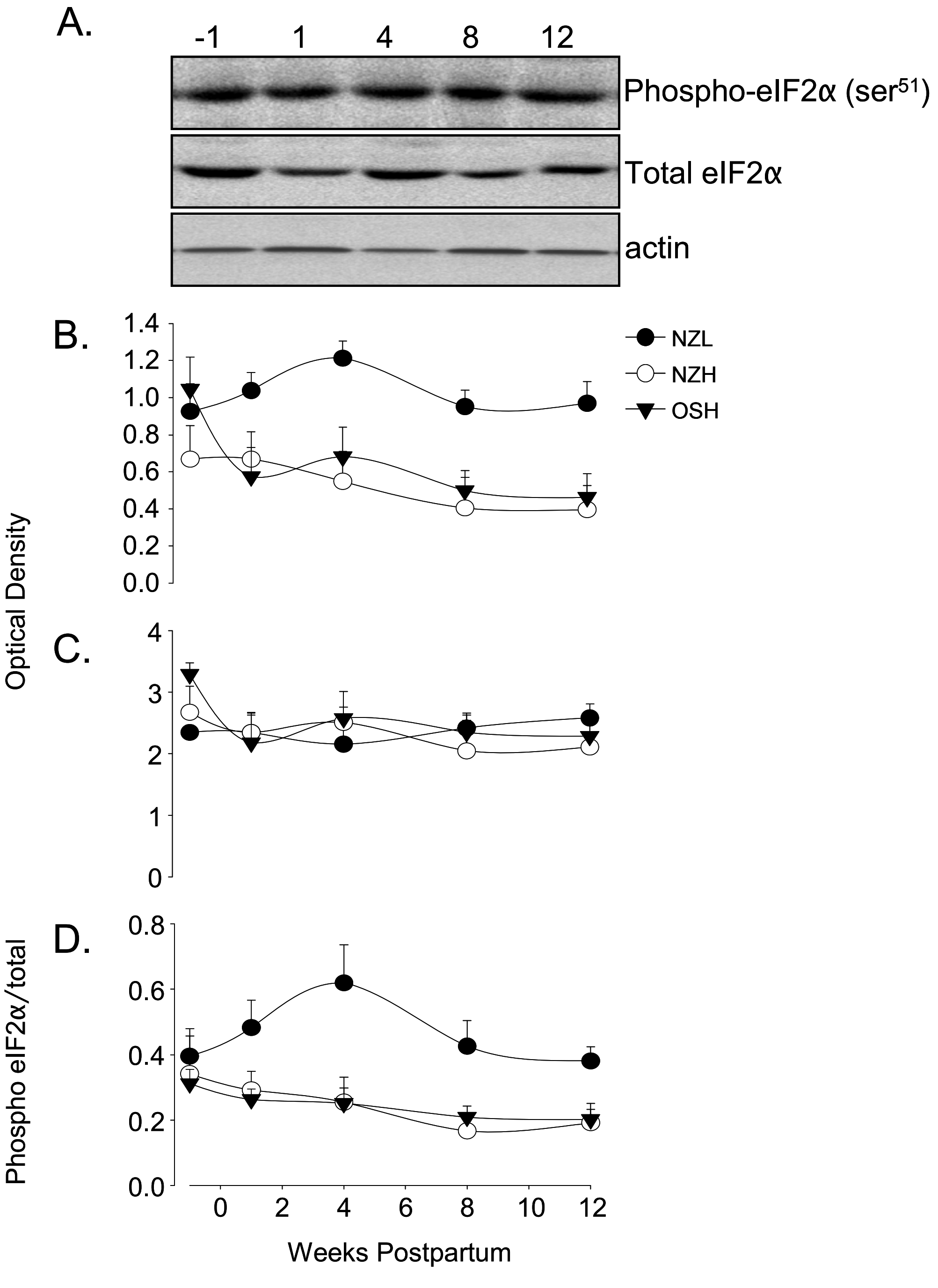

Figure 3.10: *Phosphorylated and total eIF2α in cow muscle.* **(A)** A single band representing eIF2α detected at 37 kDa the trapezius muscle of OSH 1792 at -1 to 12 weeks of lactation. **(B)** Mean (± s.e.m.) abundance of phosphorylated eIF2α in all three strains (n=6 per strain) from -1 to 12 weeks of lactation. **(C)** Mean (± s.e.m.) abundance of total eIF2α in all three strains from -1 to 12 weeks of lactation. **(D)** The ratio of phosphorylated eIF2α over total for all three strains from -1 to 12 weeks of lactation. NZL has a higher ratio than NZH and OSH cows for phosphorylated eIF2α over total eIF2α ($P<0.001$).

3.5.3. eIF4E

The eIF4 factors assist mRNA with binding to the 43S initiation complex, to form the 48S initiation complex. Phosphorylation of eIF4E allows association with eIF4G, which in turn allows recruitment of several other initiation factors, which leads to initiation of cap-dependent protein synthesis (see section 1.5.3.). It was expected that protein synthesis is downregulated. Therefore, the ratio of phosphorylated over total eIF4E was expected to decrease over the course of the study. Two antibodies were tested for total eIF4E and one for phospho-eIF4e. Both antibodies for the phosphorylated state of eIF4E gave multiple immunoreactive bands at varying molecular weights (see Appendix A). No band was detected at the expected 25 kDa size, therefore the antibody was probably not specific for cows.

3.5.4. 4E-BP1

Translation initiation via eIF4E is inhibited when eIF4E is bound to 4E-BP1. Phosphorylation of 4E-BP1 causes dissociation of the two proteins, consequently allowing the upregulation of protein synthesis via eIF4E (see section 1.5.3.). For the current study, it was expected that the ratio of phosphorylated over total 4E-BP1 would increase over time, thereby downregulating protein synthesis. Two antibodies were tested for total 4E-BP1 and two for phospho-4E-BP1. One antibody for total and one for phosphorylated gave the expected immunoreactive bands. The other two antibodies showed various non-specific bands (see Appendix B). Two bands were present at the expected molecular weight of 4E-BP1, between 15-20 kDa, for both total and phosphorylated 4E-BP1 (Figure 3.11 A). There was no change in total or phosphorylated 4E-BP1 for any of the three strains (Figure 3.11 B and C). The ratio of phosphorylated 4E-BP1 over total 4E-BP1 also did not show any change among strain or over the period of study (Figure 3.11 D).

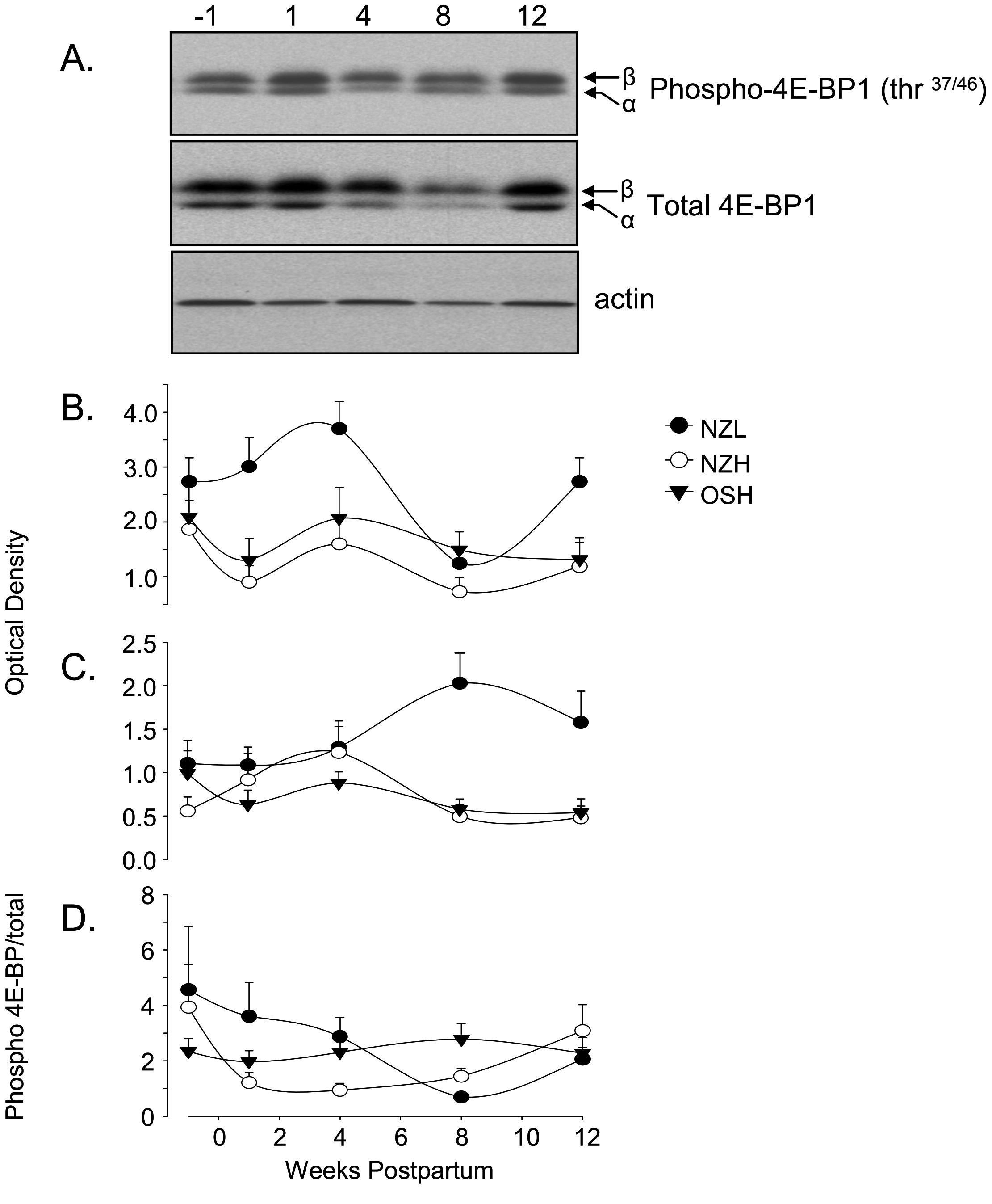

Figure 3.11: *Phosphorylated and total 4E-BP1 in cow muscle.* **(A)** A double band representing 4E-BP1 detected at approximately 12 kDa in the trapezius muscle of OSH 1982 at -1 to 12 weeks of lactation. Positions of differentially migrating forms of 4E-BP1 (α and β) are shown on the right of the blot. **(B)** Mean (± s.e.m.) abundance of phosphorylated 4E-BP1 in all three strains (n=6 per strain) from -1 to 12 weeks of lactation. **(C)** Mean (± s.e.m.) abundance of total 4E-BP1 in all three strains from -1 to 12 weeks of lactation. **(D)** The ratio of phosphorylated 4E-BP1 over total for all three strains from -1 to 12 weeks of lactation.

4. Discussion

Three different strains of cow were analysed during this study. The NZL strain represents a strain of cow from earlier than 1970, when the milk production demand was lower and milk fat content was higher. This strain had only 7% NA HF genetics. The NZH strain represents cows with high Breeding Worth from after 1990, by which time milk production demands were much higher. This strain had 24% NA HF genetics. The OSH strain represents HF cows from NA, with 91% NA genetics. These cows were born in NZ; however, both sires and dams had a high proportion of NA genetics. This strain represented the high milk-producing, high energy grain-fed cows of the NA dairy industry.

The key objective of this study was to determine if breakdown of skeletal muscle occurs in lactating dairy cows, and whether breakdown occurred to a greater extent in the OSH strain than the NZL and NZH strains.

Previous studies have shown a loss in BWT and a loss in BCS with a corresponding increase in NEFAs in HF cows fed on pasture or grains, for several weeks postpartum (Adewuyi *et al*., 2005; Emery *et al*., 1992; Pushpakumara *et al*., 2003). Currently, it is thought that breakdown of fat helps cows maintain EB during peak lactation. Increased concentrations of NEFAs in plasma are consistent with mobilisation of lipid to help offset the increased energy demand of lactation. However, concentrations of NEFAs eventually stabilise while BCS and BWT remain low. Food intake does increase, but not enough to supply the cow with the energy required to sustain lactation (Bauman & Currie, 1980; Forbes, 1970). If cows remain in a state of negative EB, other tissues must be mobilised to help meet the cow's energy requirements. It is unclear whether fat mobilisation, in conjunction with increased food intake, accounts for the restoration of energy balance. Previous studies examine the diameter of the *Longissimus dorsi* pre- and postpartum, which decrease together with a decrease in BWT and BCS (Bruckmaier *et al*., 1998; Jaurena *et al*., 2005). However, the role of muscle breakdown during peak lactation has not been determined.

Troponins have skeletal and cardiac specific isoforms. Troponin I-fs has been used for detecting breakdown of skeletal muscle after exercise-induced injury, and in soft-tissue injuries (Onuoha *et al*., 2001; Sorichter *et al*., 1997). Four skeletal muscle specific troponin isoforms were tested, and troponin I-fs gave the strongest immunoreactive signal in plasma of rats and cows.

The main finding in the current study was that the abundance of troponin I-fs in plasma increases and remains high from 3 to 12 weeks of lactation, supporting the hypothesis that there is a breakdown of skeletal muscle. Therefore, breakdown of skeletal muscle may contribute substrates to help restore EB in conjunction with lipolysis and an increase in appetite during peak lactation in cows.

The amount of troponin I-fs in cow plasma increased to over twice the normal level within 3 weeks postpartum and remained elevated, for the duration of the study. This finding suggests that breakdown of skeletal muscle is occurring during peak lactation. Furthermore, there was a significant difference in the level of plasma troponin between the NZL and NZH strains independent of lactation. Troponin was three times more abundant in the NZH cows. The OSH cows displayed an intermediate level of troponin, suggesting that breakdown of muscle was occurring during the sampling period and, collectively, these data suggest that the level of breakdown was greater in the strains selected for high milk production.

Measurement of troponins have replaced creatine kinase assays in the clinical environment to help diagnose acute myocardial infarct as they are more sensitive and tissue specific (Apple, 1999a, 1999b; Apple *et al*., 1997). Creatine kinase is expressed in cardiac and skeletal muscle. Therefore, the origin of CK detected in blood is unclear (Apple, 1999a, 1999b; Jaffe *et al*., 2000). The creatine kinase assay used in this study failed to show any consistent changes for all strains of cow over time. For this reason, the abundance of skeletal specific troponin I-fs was measured. The current study supports the view that assay of troponin I-fs is a more sensitive marker of muscle breakdown than that of CK.

In this study the mass of specific muscles could not be measured. Consequently, plasma from rats with the AH130 tumour was analysed to verify that a decrease in muscle mass correlates with an increase in plasma troponin. The rat *Tibialis anterior* obtained 6 days after injection with AH130 showed a significant decrease in muscle mass compared with the control rats injected with saline. Again troponin I-fs gave the strongest signal. However, the immunoreactive band was at 13 kDa, rather than the expected 21 kDa. This molecular weight band represents one of several known breakdown products (Simpson *et al*., 2005; Simpson *et al.,* 2000; McDonough *et al.,* 1999; Gao *et al.*, 1997). As a result, the abundance of this signal was taken to reflect the changes in the level of troponin I-fs in blood. The increased abundance of troponin I-fs in rat plasma coincided with the decrease in muscle mass observed, compared with controls. Therefore, we were confident in the use of troponin I-fs to examine the breakdown of muscle in cows during peak lactation. However, another more specific antibody will need to be used to confirm this finding.

The composition of MHCs is useful to detect the loss of specific fibre types (Pette & Staron, 2000; Tisdale, 1999). There was no significant change in the abundance of any MHC, which suggests that breakdown may not be limited to a particular fibre type. However, the pattern did suggest that there is a decrease in MHC type IIx in NZH cows from one week before calving to 12 weeks postpartum. It is also possible that other muscles undergo wasting to contribute to the increase in troponin I-fs in plasma.

Given that increased troponin I-fs in plasma suggests that breakdown of muscle occurs during peak lactation, it would be expected that expression of the components of various proteolytic pathways would increase. In several types of muscle wasting, including cachexia, HIV, sepsis, and immobilisation, mRNA of the two main E3 ligases of the UbP pathway (atro1 and murf1) consistently increase (Cao *et al*., 2005; Eley & Tisdale, 2007; Glass, 2005; Szewczyk & Jacobson, 2005). In the current study, atro1 and murf1 increased from immediately after calving to four weeks postpartum, after which the concentrations of mRNA decreased and remained low for the rest of the study. Therefore, upregulation of the UbP pathway is consistent with the breakdown of skeletal muscle during the first four weeks of lactation. However, the abundance troponin I-fs remained high throughout the study, until 12 weeks postpartum, despite the restoration of atro1 and murf1 mRNA to control levels at four weeks of lactation. Consequently, the proteins encoded by these genes are either long-lived and are functional long after transcription has ceased, or another mechanism contributes to the continued breakdown of muscle.

Cathepsins are proteases which execute degradation via the lysosomal pathway. It was expected that the cathepsin (B, D, H and L) mRNA concentration would increase during the course of this study. Cathepsin L, which is considered the general marker of atrophy, was undetectable. Cathepsin B, D and H mRNA concentrations did not increase in any strain throughout the period of the study with an isolated exception. The NZH strain showed a higher concentration of cathepsin H mRNA at week 12. However, since cathepsins B, D and H remained constant over the period of study, and cathepsin L was undetected, it is highly probably that the lysosomal pathway is not contributing significantly to the breakdown of muscle in NZL, NZH and OSH cows.

It is possible that since the UbP pathway is upregulated for the first four weeks, the lysosomal pathway is unaffected to allow for only limited degradation during this time. In disease states such as cachexia, immobilization, sepsis and HIV, significant breakdown of muscle takes place (Lecker *et al*., 1999; Tisdale, 2005). Given that lactation is not a disease-induced state, induction of large amounts of muscle degradation might not be expected, as this may be detrimental to the animal's health. Therefore, it is not unreasonable that one degradative pathway be upregulated, temporarily, while another remains constant.

The calpains, components of the Ca^{2+}-dependent degradation pathway, were not examined, as they work in conjunction with the UbP and lysosomal pathways and are responsible for only limited degradation. The calpains initiate myofibrillar degradration, while the UbP and lysosomal pathways complete the degradation (Bartoli & Richard, 2005; Costelli *et al*., 2005; Koohmaraie, 1992; Solomon & Goldberg, 1996; Williams *et al*., 1999). The early upregulation of the UbP pathway components most likely accounts for the breakdown of muscle during the first four weeks of lactation.

The phosphorylation state of the various components of translation initiation determines the rate of protein synthesis. It was expected that there would be a decrease in protein translation in lactating cows. For this to occur, the ratio of phosphorylated eIF2α over total eIF2α should increase over time such that more eIF2α is phosphorylated. The ratio of phosphorylated 4E-BP1 over total 4E-BP1, on the other hand, should decrease over time, since unphosphorylated 4E-BP1 inhibits protein synthesis. The phosphorylation of p70S6K increases the rate of synthesis. Therefore it was expected that the ratio of phosphorylated p70S6K over total p70S6K would decrease with time resulting in a decrease in translation initiation.

While the ratio of phosphorylated over total protein for p70S6K could not be assessed, the ratio of phosphorylated over total protein for

4E-BP1 was not significantly different between strains or over time. Phosphorylated over total eIF2α did not show any change over time in NZH and OSH cows. However, the ratio was greater in NZL cows, which suggests that there is less synthesis taking place in this strain, although there was no effect of lactation. Only eIF2α and 4E-BP1 could be detected reliably and these two components of the protein synthesis pathway showed no change in the relative abundance of phospho- to total protein for NZH and OSH cows. Therefore, these data suggest that the increase in abundance of troponin I-fs in plasma occurring in cows from 3 weeks to 12 weeks postpartum is not due to a decrease in protein synthesis in trapezius muscles of lactating cows.

Myostatin is a growth factor regulating myogenesis (McCroskery *et al*., 2003; Taylor *et al*., 2001). It has also been shown to play a role in muscle wasting (Zimmers *et al*., 2002). Myostatin expression increases in conditions of atrophy such as during muscle disuse and in HIV-infected men (Carlson *et al*., 1999; Gonzalez-Cadavid & Bhasin, 2004; Lalani *et al*., 2000; Ma *et al*., 2003). In the present study it was expected that there would be an increase in circulating myostatin in all strains of cow and that the levels of myostatin might be greater in those strains in which greater muscle wasting was indicated. Although there was an effect of strain, whereby the OSH cows had significantly less myostatin detected in plasma than the NZL and NZH cows, there were no significant changes in the abundance of myostatin in plasma over time in any of the three strains, suggesting that myostatin does not play a role in the breakdown of muscle during peak lactation. However, the lower abundance of myostatin in the larger OSH cows is consistent with less inhibition of post-natal growth, thereby allowing an increase in body and muscle mass compared with NZL and NZH cows.

It was expected that the NZL cows would weigh less, have less of a decrease in BCS, produce less milk and therefore have less breakdown of muscle. Several findings in this study supported that suggestion. The NZL cows did weigh less, they produced less milk and their BCS remained unchanged (Dexcel unpublished data). Troponin I-fs was least abundant in plasma of the NZL cows over the 12 weeks postpartum, suggesting that muscle breakdown was occurring to a lesser extent in the NZL cows than the NZH and OSH cows. The OSH strain was expected to weigh more, have a greater loss of BCS due to their high milk production and therefore have greater breakdown of muscle taking place. The OSH cows in this study did weigh more, had higher milk yield, and lost more body condition over the 12 weeks postpartum (Dexcel unpublished data). An intermediate abundance of troponin I-fs was seen in plasma. OSH cows had a consistently lower level of myostatin detected in plasma throughout the study, lower than that detected in the plasma of either NZL or NZH cows. Although the abundance of troponin I-fs in plasma from OSH cows was higher than that of NZL cows, none of the results indicate that the OSH cows undergo breakdown of muscle to a greater degree than the NZH cows. The NZH cows represented a hybrid of NZ and NA cows. The genetic composition of NZ cows has changed over the past 20 years due to the use of NA HF semen (Harris & Kolver, 2001). However, having only 24% NA genetics, the cows were expected to be intermediate in weight, have intermediate milk yields, and have intermediate loss of body condition. It was expected that NZH cows would have had a breakdown of muscle occurring somewhere in between NZL and OSH cows. Indeed, the NZH cows had BWTs in between those of the NZL and OSH cows. The NZH cows had higher BCS initially, but lost body condition, to a similar extent as OSH cows. Interestingly, the NZH had the highest abundance of troponin I-fs detected in plasma over the 12 weeks postpartum. This suggests that the breakdown of muscle occurs to the greatest extent in NZH cows, perhaps to provide more substrates to help restore EB.

The source of troponin I-fs remains unclear. The amount of atrophy occurring in a muscle depends on the function of the muscle. A muscle with stabilising function (i.e. the quadriceps) will undergo atrophy to a greater extent than one with an antagonist function (i.e. the hamstrings) in conditions of disuse (Kasper *et al*., 2002). Studies have shown that longissimus dorsi diameter and back fat thickness reflect changes in whole-body fat content as well as muscle mass in dairy cows (Bruckmaier *et al*., 1998; Otto *et al*., 1991; Pedron *et al*., 1993; Tamminga *et al*., 1997). Other studies use muscles involved with stabilisation or movement such as the soleus, the gastrocnemius or the quadriceps for detecting wasting (Lalani *et al*., 2000; Mancini *et al*., 1992; McMahon *et al*., 2003), and perhaps for comparative purposes any of those muscles may have provided more insight.

In conclusion, the increased abundance of troponin I-fs in plasma suggests that muscle degradation occurs in lactating cows from three to 12 weeks of lactation. Furthermore, the current study suggests that breakdown of muscle can be attributed to the UbP pathway until four weeks of lactation, and that other, as yet unidentified processes, maintain the breakdown of muscle from four to 12 weeks postpartum. The abundance of troponin I-fs in plasma is greater in the NZH and OSH cows than the NZL cows, suggesting that breakdown of muscle may occur in relation to the yields of milk in the different strains of dairy cows.

Should investigation into the breakdown of muscle in lactating cows be further pursued, several changes may increase understanding of how this degradation is being mediated. The p70S6K and eIF4E antibodies used during this study produced either non-specific signals, or no signal. Assessing a wider range of antibodies specific for these factors of translation initiation could provide the information on protein synthesis missing from the current investigation. Superior information on troponin I-fs levels could be acquired with an enzyme-linked immunosorbant assay (ELISA) for troponin I-fs. Western blots provide semi-quantitative information on proteins. However, an ELISA would be a more sensitive and rapid method of obtaining quantitative results on troponin concentrations in blood.

If this study were to be repeated, the muscle biopsied should be more carefully considered. A muscle that is commonly broken down during atrophy or one used to assess BCS would be a more suitable candidate. The study was not set up with a proper positive control. The cows were sampled at one week before calving to act as a positive control. However, it would have been preferable to also have 10 cows per strain that were not lactating for comparison with lactating cows. Unfortunately, the cost per cow, as well as a requirement for ethics approval for an animal study renders the use of 30 extra cows implausible. Therefore, the information obtained from this study may not be an accurate representation of the role muscle plays during peak lactation, although it does provide sufficient evidence to suggest that muscle degradation is involved.

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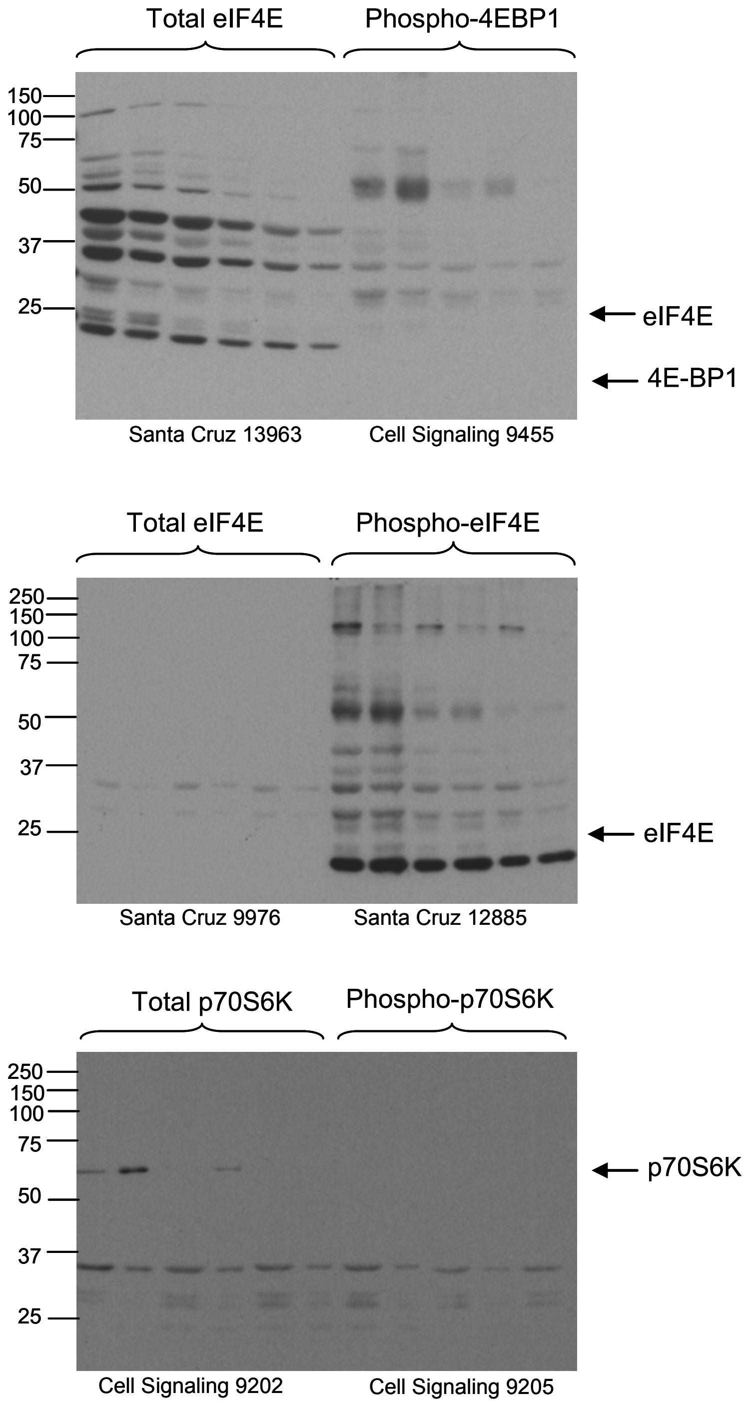
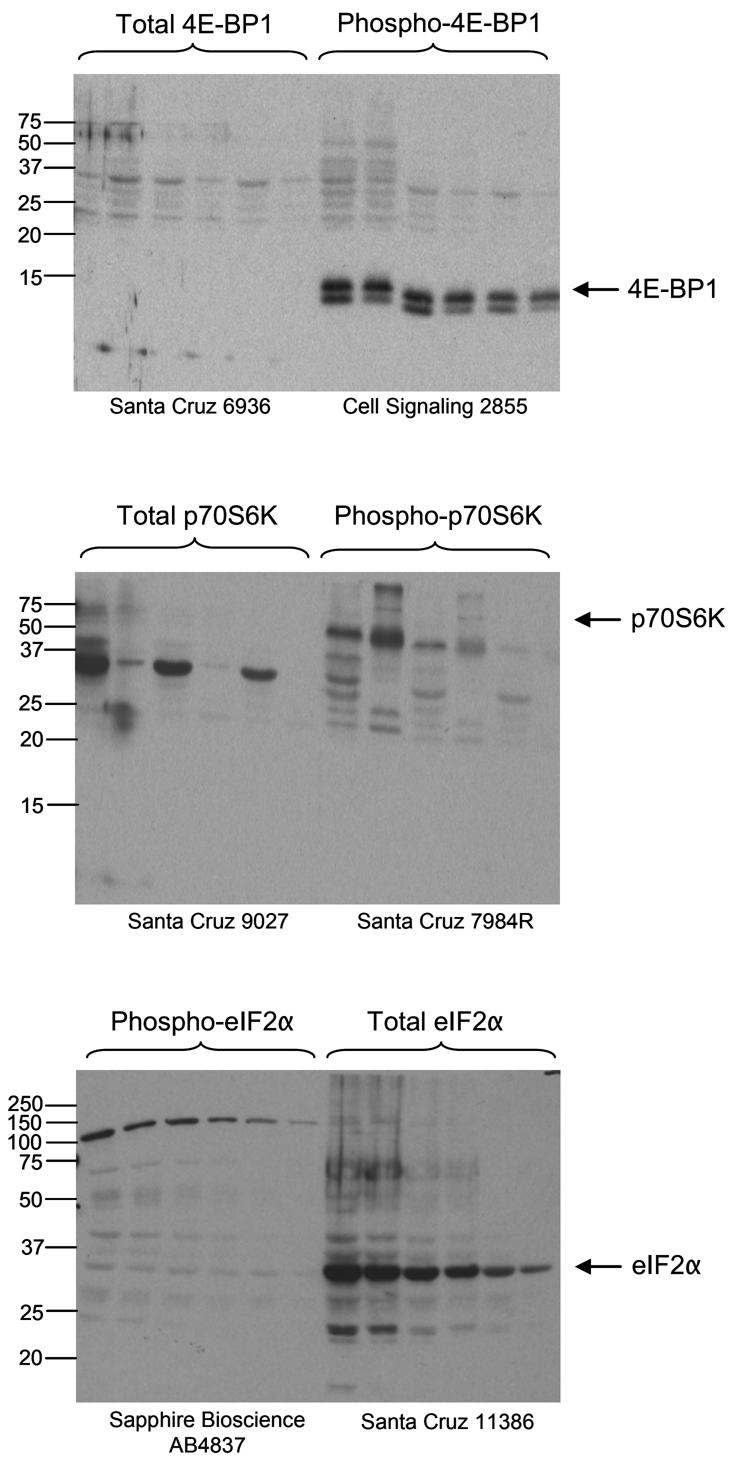


Appendix A: *Titration one of antibodies to be used for investigating the components of protein translation initiation.* 20 µg protein was loaded per well. The samples used were -1 and 4 weeks postpartum. Nitrocellulose membranes were cut into strips with sample pairs. Antibodies were diluted 1:1000, 1:3000 and 1:10 000 to check strength and specificity.



Appendix B: *Titration two of antibodies to be used for investigating the components of protein translation initiation.* 20 µg protein was loaded per well. The samples used were -1 and 4 weeks postpartum. Nitrocellulose membranes were cut into strips with sample pairs. Antibodies were diluted 1:1000, 1:3000 and 1:10 000 to check strength and specificity.