Nitric oxide influences muscle physiology and meat quality.

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i. Thesis abstract

Over the last 15 years, the physiological role of nitric oxide (NO) has gained prominence in biomedical literature, emerging as a regulator of many cellular processes. Importantly, the formative enzyme NO synthase (NOS) was found to have high levels of activity in skeletal muscle and the activity of the neuronal isoform of NOS was higher in muscle than in the brain. Roles for NO in the regulation of muscle contractility, energy metabolism, calcium homeostasis, and proteolysis have emerged. Since many of these processes are critical determinants of meat quality, the hypothesis of this thesis is that NO influences physiological determinants of meat quality.

To evaluate if NO influenced meat quality, inhibitors of NOS and pharmacological donors of NO were injected at concentrations of 1, 10 and 100mM into hot boned beef *Longissimus lumborum* (LL). No effect of either NOS inhibitor or NO donor was observed on tenderness, as assessed by Warner Bratzler Shear Force (WBSF). The increase in Myofibrillar Fragmentation Index (MFI) of samples injected with 1mM NOS_i and 100mM sodium nitroprussude (SNP) and combined solution of NOS inhibitors (NOS_i) was larger after 7 days post-mortem, rather than before 7 days, possibly indicating different mechanisms of meat tenderisation. Due to large increases in lipid oxidation with the NO donor SNP it was concluded the concentrations of SNP used were supraphysiological and did not represent effects mediated by enzymatic NO production. It was concluded that the minimal influence of NOS_i on meat tenderness, were most likely due to low levels of NOS activity post-mortem.

The effects of NO on muscle sarcoplasmic reticulum (SR) Ca^{++} metabolism were investigated since increases in cytosolic Ca^{++} concentrations have been hypothesised to initiate proteolysis in muscle and meat by enzymatic or non-enzymatic means. The NO donor diethylamine NONOate (NONO) increased the maximal rate of Ca^{++} uptake in ovine sarcoplasmic reticulum (SR). It is unlikely that this resulted in higher clearance of Ca^{++} from the cytosol, since NONO also decreased the Ca^{++} sensitivity of Ca^{++} uptake. These data indicate while the maximal rate of Ca^{++} removal from the cytosol was higher with NONO, initiation of uptake required higher Ca^{++} concentrations. It is likely that this resulted in less Ca^{++} uptake into the SR lumen and higher cytosolic Ca^{++} concentrations. It was concluded that this was a potential mechanism to initiate muscle proteolysis.

Systemic infusion of the specific NOS inhibitor L-arginine methyl ester hydrochloride (L-NAME) inhibited systemic NOS activity. Inhibition of NOS then resulted in an initial increase, followed by a decrease relative to control in arterio-venous difference (AVD) of glucose concentrations across the ovine hind-limb, indicating increased hind-limb glucose uptake. Since arterial insulin concentrations were unaffected by L-NAME infusion, it was concluded that NOS inhibition may increase glucose uptake independent of hind-limb insulin sensitivity. Increased venous nonesterified fatty acid (NEFA) concentrations were also observed following L-NAME infusion, indicating increased lipolysis. From this, it was concluded that NO is involved in muscle carbohydrate and fat metabolism.

The involvement of NO in muscle carbohydrate metabolism was then investigated following intravenous injection of L-NAME into lambs 135 minutes pre-slaughter.

Increases in glycogenolysis and glycolysis were observed post-slaughter in *Longissimus thoracis et lumborum* (LTL) and *Semimembranosus* (SM) muscles following L-NAME infusion, indicating that NO inhibits muscle glycogenolysis and glycolysis. Infusion of L-NAME reduced shear-force, and thus improved tenderness in the LTL muscle by approximately 12%, indicating that NO inhibits muscle tenderisation.

These experiments demonstrate that NO influences physiological processes in ovine muscle pre-slaughter, post-slaughter muscle metabolism and meat quality.

ii. Acknowledgements

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iii. List of abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AVD	Arterio-venous difference
BH_4	Tetrahydrobiopterin
$[Ca]_{0.5}$	Calcium concentration at half maximal ATPase activity
CAM	Calmodulin
CANP	Calcium activated neutral protease
cGMP	Cyclic guanosine monophosphate
GPT	Glutamic pyruvic dehydrogenase
DFD	Dark firm dry
GLUT4	Skeletal muscle specific glucose transporter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GS	Glycogen synthase
HSL	Hormone sensitive lipase
LDH	Lactate dehydrogenase
L-NAME	N^G-nitro-L-arginine methyl ester hydrochloride
L-NOARG	N_ω-nitro-L-arginine
LPL	Lipoprotein lipase
LTL	*Longissimus thoracis et lumborum*
MFI	Myofibrillar fragmentation index
NO	Nitric oxide
NO_x	NO_3/NO_4 or other higher oxide of NO
NO^+	Nitrosonium anion

NO^-	Nitroxyl anion
NAD	Nicotine adenine dinucleotide
n_H	Hill co-efficient
NEFA	Non-esterified fatty acid
NONO	Class of NO donor drug
NOS	Nitric oxide synthase
eNOS	Endothelial type NOS
iNOS	Inducible type NOS
nNOS	Neuronal type NOS
O_2^-	Superoxide
OH	Hydroxyl radical
$ONOO^-$	Peroxynitrite
RyR	Ryanodine receptor
PSE	Pale soft exudative
PCA	Perchloric acid
SR	Sarcoplasmic reticulum
SERCA	Sarcoplasmic/ endoplasmic reticulum Ca^{++} ATPase
SM	*Semimembranosus*
SNP	Sodium nitroprusside
SNAP	S-nitroso-N-acetylpenicillamine
WBSF	Warner-Bratzler shear force

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v. Publications arising from thesis

Refereed journal

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Cottrell, J. J., Warner, R. D., Dunshea, F. R., Thomson, B. and Trout, G. R. (2000). Arginine diffuses freely through hot boned beef *M. Longissimus lumborum*. *Asian-Australasian Journal of Animal Science,* **13 Supplement**; B:131.

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Chapter 1: Introduction

1.1 General introduction

Within Australia, programs have been developed to guarantee meat quality to consumers. Part of this process has involved intensive research into genetic, environmental and processing factors that determine meat quality. However, despite the best controls, there will always be variation in meat produced, due to the large biological and environmental variation encountered by meat producing animals. Improved understanding of the problems of meat production has led to solutions improving current practice and reducing variation in meat quality.

The following is a review of muscle and its components that are important to meat quality. The review will present basic muscle anatomy, biochemistry and physiology, since it is the principal component of meat. Then, the biochemical changes that occur in muscle during the conversion of muscle to meat, and how these processes are influenced by pre-slaughter factors will be discussed. The review will then focus on the physiological roles of NO in skeletal muscle and other tissues, its mechanisms of action and the influence of NO on the biochemical pathways that are important, or potentially important for meat production.

1.2 Overview of muscle anatomy and physiology

1.2.1 Overview of muscle anatomy

There are three types of muscle- skeletal, smooth and cardiac muscle, comprising the musculo-skeletal and vascular systems and cardiac tissue respectively. All three types

possess adaptations for their anatomical and physiological roles. The most prevalent muscle tissue, skeletal muscle performs four important functions; providing force for movement, maintenance of posture, stabilising joints and generating heat (Marieb, 1992a). The circulatory system supplies blood via arteries and capillaries made of smooth muscle, which is responsible for delivery of nutrients and removal of waste products (via the venous circulation) from tissues and cardiac muscle provides force for blood circulation.

While muscle fibres are the primary constituent of meat and therefore a prime factor determining meat quality (Judge et al., 1989), skeletal muscle contains other cell types which influence meat quality. These include smooth muscle, adipose and connective tissues. Adipose tissue consists of adipocytes, which are specialised cells for storing energy in the form of triacylglycerol (TAG). The framework for the physical structure of skeletal muscle is provided by connective tissue, produced by fibroblastic cells, which arrange muscle, vasculature and adipose tissues.

1.2.2 Muscle fibre structure

1.2.2.1 Contractile structure

Muscle fibres are elongate, rod shaped cells that vary greatly in diameter from 10 to 100μM within a single muscle (Hedrick et al., 1994). Muscle fibres are highly specialised cells, containing contractile structural proteins for the generation of muscular force. Force is generated within myofibrils, which consist of overlapping bands known as the "A" band, since it is anisotropic and polarises visible light, and the isotropic, non-polarising "I" band (Figure 1.1). The I band, which appears pale under the microscope and the A band, which appears dark, have a dark and light

midline interruption known as the "Z" line and "H" (helle, or bright) zone respectively. The area between the Z lines is known as the sarcomere, and is the contractile unit of the myofibril. The region bisecting the Z lines is the "M" or middle line (Stromer et al., 1974).

The two contractile filaments are the "thin", actin containing filament and "thick" myosin containing filament. In the sliding filament model proposed by Huxley, (1957), muscular force is generated by attachment of contractile filaments followed by force production then detachment of filaments. Myosin is a rod shaped protein containing a globular "head", which both binds to actin and hydrolyses ATP. The thick filament consists of myosin polymers, aligned in a staggered helical structure with the tails together and heads exposed. The myosin molecules reverse alignment at the M line resulting in a "bare zone" consisting entirely of tails (Alberts et al., 1994). The thin filament consists of a multiple actin molecules in a α-helix. Interspersed within the α-helix is tropomyosin and bound to tropomyosin is the troponin complex (Tn).

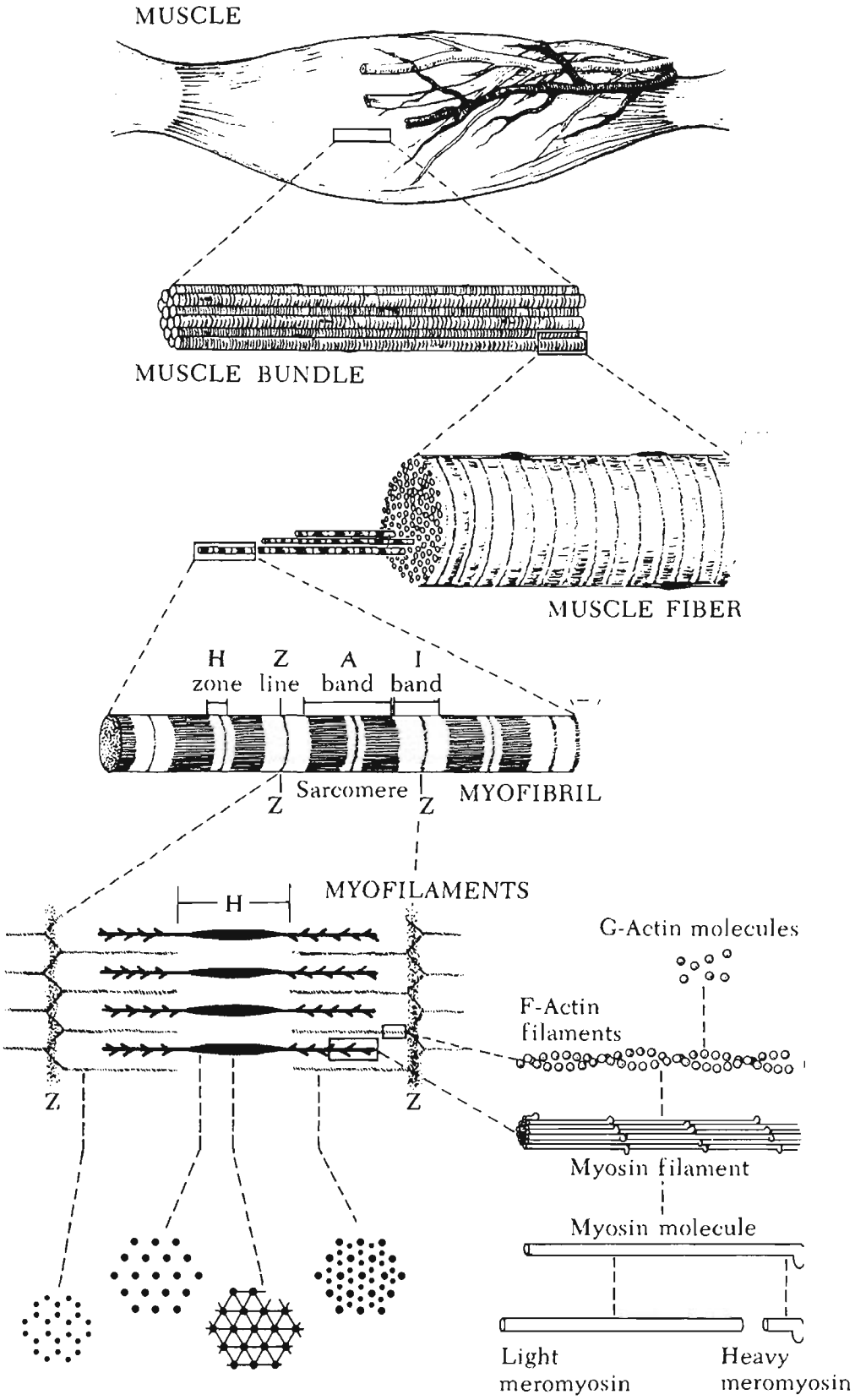


Figure 1.1: Gross anatomy of the muscle fibre (Bloom and Fawcett, 1969, as cited in Judge et al. 1989).

1.2.2.2 Cytoskeletal structure of the myofibril

Muscle fibres display a high level of internal structural organisation, as evidenced by their highly conserved hexagonal cross section (Figure 1.2). This level of organisation is provided by the cytoskeleton, which consists of a molecular lattice of structural proteins. For the purpose of this review, the cytoskeleton comprises of three groups of proteins, varying in their cellular location, physical attributes and specific roles. These include the contractile filaments and associated proteins, intermediate filaments that span from the Z-line region to the costameres and the cell membrane skeleton (Robson et al., 1997).

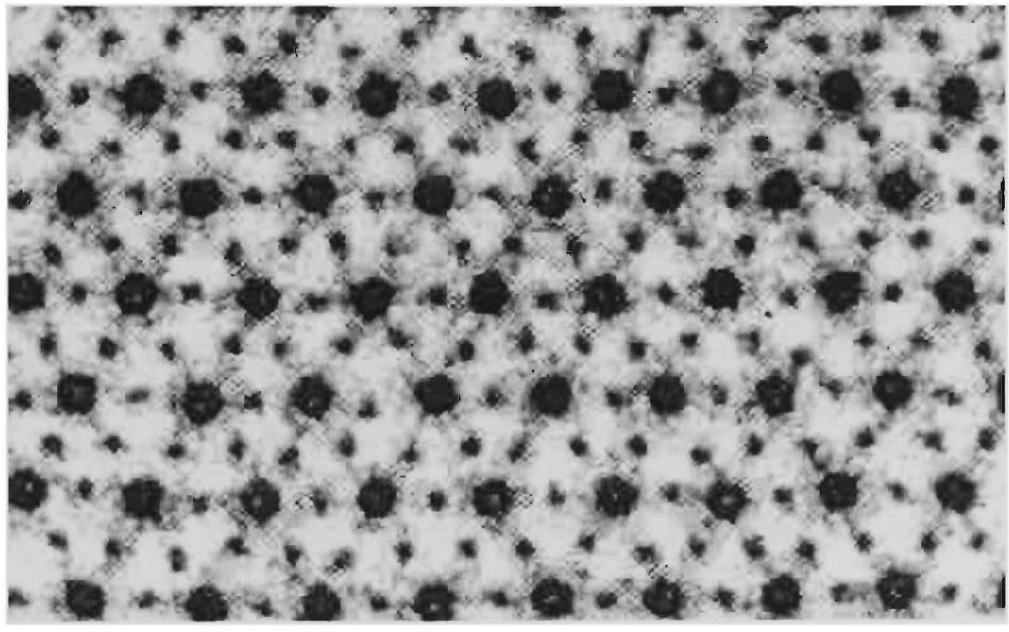


Figure 1.2: A molecular lattice of proteins ensures a highly conserved arrangement of myofibrils as evidenced by their hexagonal cross section (Alberts et al., 1994).

Associated with thin (actin containing) and thick (myosin containing) filaments are the giant proteins titin and nebulin. Titin is the largest protein yet described, having a molecular weight (MW) of 3,700,000 (Alberts et al., 1994). Titin spans longitudinally

from the M line (Mid line of the sarcomere) to the Z disc accounting for approximately 10% of the skeletal muscle cytoskeleton (Wang, 1996) and is thought to align the thick filament within the sarcomere and elasticity and prevent sarcomere over stretching (Robson et al., 1997). Nebulin is associated with the thin filaments and has a MW of 773,000 (Robson et al., 1997). Nebulin accounts for approximately 3% of cytoskeletal protein content (Robson et al., 1997). Studies of human nebulin found that it is primarily comprised of 185 blocks of a repeating 35 amino acid chain. Each repeating module binds to one actin monomer (Labeit and Kolmerer, 1995). It is proposed that nebulin serves as a "molecular ruler" for regulating the length of the thin filaments during myofibrillogenesis (Wang, 1996).

Intermediate filaments span from the Z-lines to the cell membrane skeleton and are important for the organisation of myofibrils within the muscle fibre. Filamin and desmin are associated with the Z-lines of the myofibril where they link with other myofibrils and organelles such as nuclei and mitochondria (Granger and Lazarides, 1978, Judge et al., 1989, Milner et al., 1996). The cell membrane skeleton organises cell membranes via a network of proteins such as spectrin and actin filaments anchored to trans-membrane proteins. The trans-membrane proteins form junctional complexes, which serve structural roles, but also position cellular receptors, channels and enzymes within the sarcolemma (Alberts et al., 1994).

1.2.3 Muscle fibre biochemistry

1.2.3.1 Excitation contraction coupling

Excitation contraction coupling is initiated by an action potential in an excitatory neuron, causing acetylcholine release into the synaptic cleft (Marieb, 1992a). Ligand

binding of acetylcholine to receptors in the sarcolemma cause depolarisation of the sarcolemma membrane, which in turn triggers contraction via the sarcotubular system. The sarcotubular system is a specialisation of the sarcolemma and endoplasmic reticulum in skeletal muscle to form two specialised membrane enclosed vesicles. The transverse, or T-tubule system is a series of invaginations in the sarcolemma, extending towards the Z-line of the myofibril (Stromer et al., 1974, Bendall, 1969). The structure in which each transverse tubule co-localises with two sarcoplasmic reticulum (SR) vesicles to form a "triad" (Franzini-Armstrong, 1980, Dauber et al., 2000). The SR in turn surround each myofibril (Stromer et al., 1974). Depolarisation of the sarcolemma is propagated down the T-tubule, triggering conformational change in the L-type voltage gated Ca^{++} channel, situated adjacent to the SR in the T-tubule membrane (Ji et al., 1999). The L-type Ca^{++} channel stimulates rapid calcium release from the adjacent SR bound Ca^{++} channel, the ryanodine receptor (RyR) via an unknown mechanism (Kasai et al., 1999). Opening of RyR's in response to membrane depolarisation releases Ca^{++} into the cytoplasm, increasing cytosolic Ca^{++} (Rios et al., 1991) from approximately 0.1 to 10μM (Bagshaw, 1993) and is a precursor to contraction (Hasselbach and Oetliker, 1983).

The efflux of calcium from the SR causes conformational changes in the troponin/ tropomyosin regulatory complex. Tropomyosin is a long rod shaped protein located in the cleft of the α helix of actin (Phillips et al., 1986) adjacent to the troponin complex. The Troponin complex (Tn) consists of three proteins, Tn- T (tropomyosin binding), Tn- C (Calcium binding) and Tn- I (inhibitory). Binding of Tn-T to tropomyosin is thought to position the troponin complex along the actin filament (Alberts et al., 1994). Tn-I binds to actin and forms a complex with Tn-T and

tropomyosin to inhibit myosin binding. Binding of myosin to actin is inhibited by tropomyosin, which occupies the myosin-binding site. An increase in cytosolic calcium concentrations after calcium efflux from the SR during excitation contraction coupling results in binding of calcium to Tn-C and a conformational change, releasing tropomyosin from the myosin binding site (Alberts et al., 1994). This allows tropomyosin to slide into the cleft of actin's α-helix, exposing the myosin binding site and enabling cross bridge formation (Tobacman and Butters, 2000) (Figure 1.3).

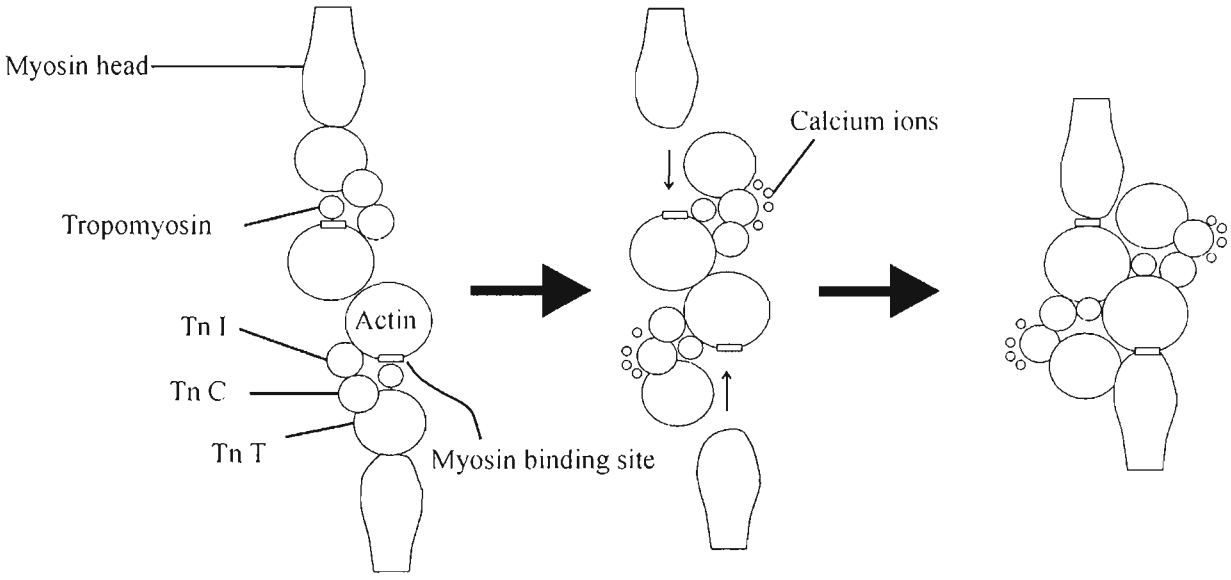


Figure 1.3: Binding of Ca^{++} to Tn-C initiates conformation changes in the troponin/ tropomyosin complex allowing the myosin head to bind to actin (Anon, 2002).

Prior to contraction, muscle is in a "rigor" state, during which the myosin heads form cross-bridges with actin filaments (Figure 1.4A). Rigor is the state responsible for rigor mortis, but in physiological states, rigor is short lived. Contraction is initiated when the cross bridges are broken following binding of adenosine triphosphate (ATP) to the myosin head while in the rigor state (Figure 1.4B). Hydrolysis of the ATP

molecule results in a conformational change of the myosin head so that it moves the thin filament towards the M line. Weak binding of myosin to actin at the new binding site results in the release of P_i from ATP hydrolysis (Figure 1.4C), resulting in tight binding between actin and myosin and a conformational change in the myosin head back to its original state of rigor, releasing ADP in the process. The conformational change during ADP release is the force generating component of the cycle (Alberts et al., 1994) (Figures 1.4D and 1.4E).

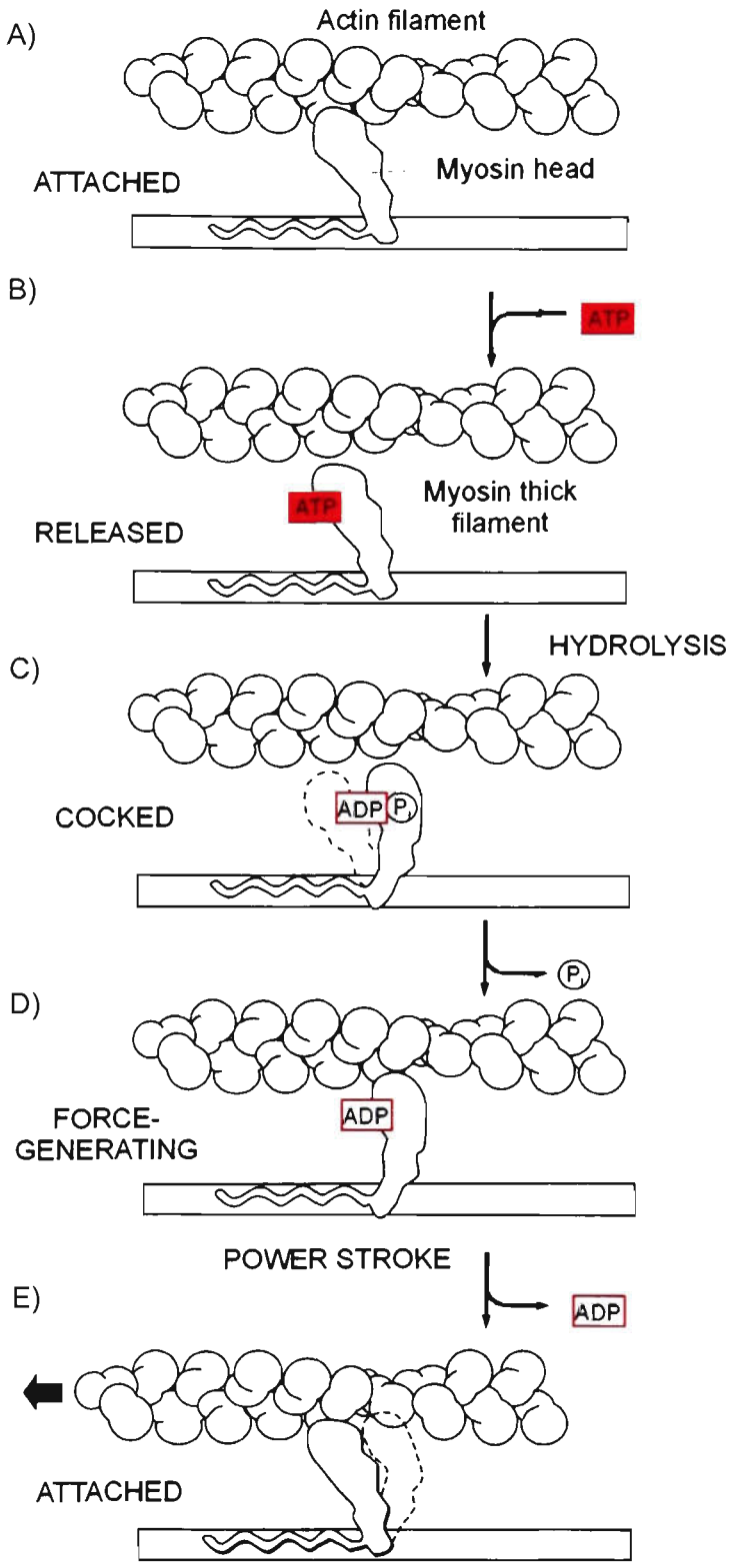


Figure 1.4: Interactions between myosin and actin filaments during muscular contraction (Alberts et al., 1994).

1.2.3.2 Types of muscle fibres

Muscle fibres differ markedly in their physical and biochemical properties and nomenclature exists, grouping fibres of similar physical and biochemical properties into "fibre types". Padykula and Herman, (1955) differentiated between two muscle fibre types which were termed type I and type II by their myofibrillar ATPase activity. Ogata and Mori (1964) subsequently identified succinate dehydrogenase and cyclooxygenase variability between fibre types. Further sub-typing of type II fibres into IIa and IIb was achieved by measuring actomyosin ATPase after pre-incubation of myofibrils in acid and alkali (Guth and Samaha, 1969). Other fibre sub-types have been identified in human trunk and limb muscles (Staron and Hikida, 1992), but for this review, only types I, IIa and IIb will be considered.

1.2.3.3 Metabolic characterisation of fibres

Fibre types differ in shortening velocities, type I fibres being slower than fast twitch type II fibres (Buchthal and Schmalbruch, 1970, Larsson and Moss, 1993). Type I fibres, also known as slow twitch oxidative fibres, are fatigue resistant and able to maintain sustained slow contractions. Type I fibres therefore posses many functional adaptations to sustain prolonged contraction. The contractile proteins are less sensitive to calcium in type I than type II fibres (Stephenson and Williams, 1981) partly due to lower abundance of Tn-C (Collins, 1991) and also Tn-T (Ogut et al., 1999). Uptake of intracellular calcium into the SR after contraction by sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) is also much slower in type I and than type II fibres (Wu and Lytton, 1993), helping sustain longer contractions.

Mitochondria are the site of cellular oxidative phosphorylation and occupy approximately 6%, 4.5% and 2.3% of muscle fibre volume respectively in oxidative types I, IIa and IIb (Howald et al., 1985). Type IIb fibres, also known as fast twitch glycolytic, are quickly fatiguing fibres. Intermediate between types I and IIb fibres are type IIa fast twitching, oxidative, glycolytic (FOG) fibres (Ouali et al., 1989). Type II fibres possess higher Na/K pump density (Ruff, 1992) and approximately 20% more pumps than type I fibres (Everts and Clausen, 1992), enabling greater charge movement in type II fibres (Dulhunty and Gage, 1983). Expression of L-type Ca^{++} channels are 3-5 fold greater in type II fibres compared to type I (Delbono and Meissner, 1996, Renganathan et al., 1998). It is not clear whether there is differential expression of RyR's in the sarcoplasmic reticulum between fibre types (Flucher et al., 1999). Type II fibres have higher levels of SERCA than type I (Everts et al., 1989, Leberer and Pette, 1986, Salviati et al., 1982) allowing more rapid removal of calcium from the cytosol. Different isoforms of SERCA have been identified in slow and fast twitch fibres, SERCA-1 and SERCA-2 respectively (Lytton et al., 1992).

Muscle fibre types also vary in their metabolic properties, concentration and content of substrates for provision of energy for contraction. Oxidative type I and IIa fibres have higher triglyceride content, while glycolytic IIb fibres have a higher glycogen content (Essen et al., 1975). Different isoforms of lactate dehydrogenase (Leberer and Pette, 1984), AMP deaminase (Morisaki and Holmes, 1993), succinate dehydrogenase and cyclooxygenase (Ogata and Mori, 1964) can be found expressed in different fibre types. There are also differences in expression of myofibrillar proteins. Long (T1) and short (T2) titin isoforms have been isolated from slow and fast twitch muscle

respectively, where they have been correlated to resting muscle tension (Wang et al., 1991).

Expression of a mix of different muscle fibre types conveys flexibility to force production, allowing muscles to respond to their diverse roles, ranging from chewing, maintenance of posture, tasks requiring fine motor control and repeated powerful contractions (Figure 1.5). It is the heterogeneity of biochemical and physical properties of muscle fibres that allow skeletal muscles to fill these diverse roles (Bottinelli and Reggiani, 2000).

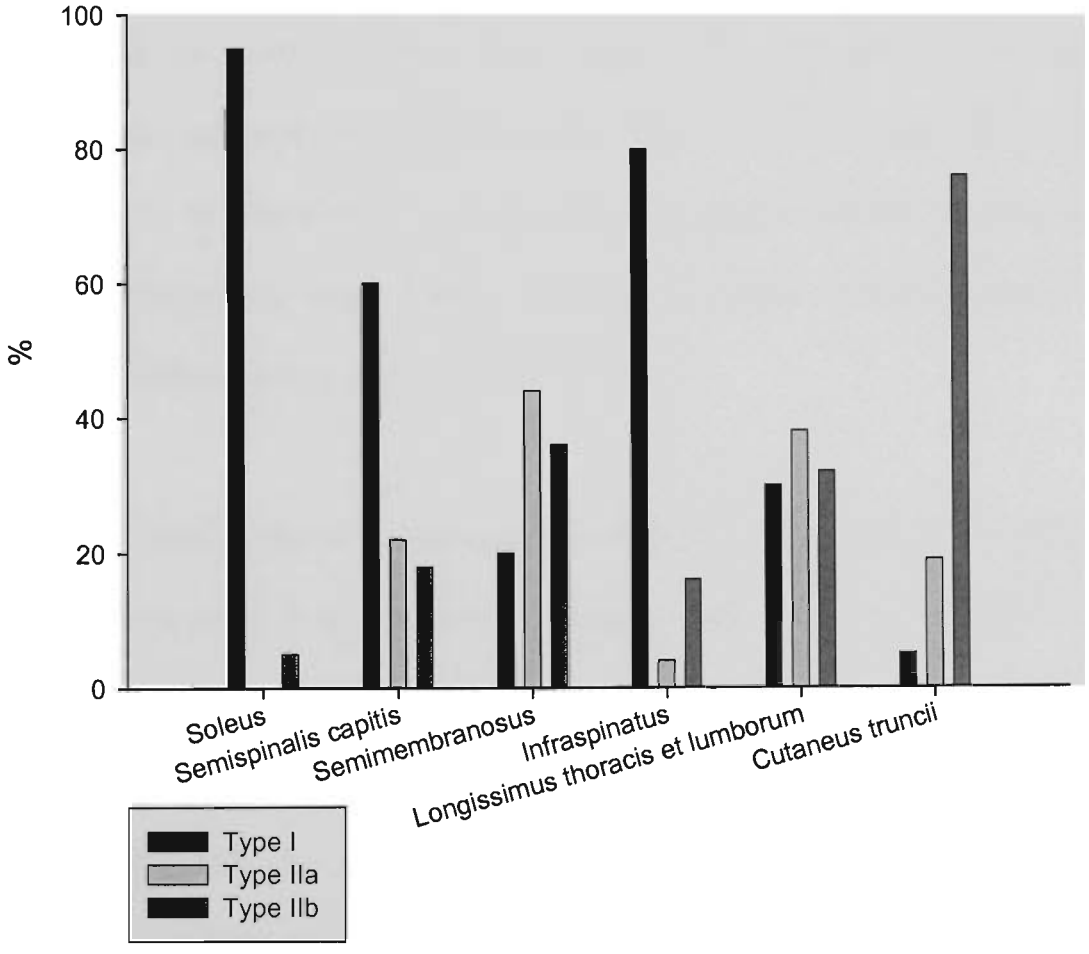


Figure 1.5: Relative proportions of Types I, IIa and IIb fibres in bovine skeletal muscles (Totland and Kryvi, 1991).

1.2.4 Connective tissue of skeletal muscle

Just as muscle fibres have an intracellular latticework of proteins organising their structure, muscle fibres are grouped into fascicles and into distinct muscles by connective tissue. Individual muscle fibres are surrounded by connective tissue called endomysium (Figure 1.6), which provides support for capillaries and nerve endings. Muscle fibres are then grouped into bundles called fascicles by the perimysium and into muscles by the epimysium.

Connective tissues contain two major types of fibres, the prime constituents of which are collagen and elastin (Pearson and Young, 1989, Judge et al., 1989). Collagen fibres are far more prevalent than elastin fibres and are major constituents of epimysium, perimysium and endomysium (Pearson and Young, 1989). The protein collagen is so prevalent, it is estimated to compose 20-30% of total mammalian protein (Bailey and Light, 1989). Conversely, elastin is only present in muscle in trace amounts (Pearson and Young, 1989).

Of the three layers of connective tissue found in muscle, the epimysium and perimysium form fibrous connective tissues (Judge et al., 1989). These tissues are high in collagen content, and preserve the coherent structure of the muscle. By forming a fibrous network, these tissues are able to reinforce the muscle providing structural rigidity and preventing over-extension of the myofibrils. The structure of these tissues also enables a framework for movement, ensuring that the force of contraction is efficiently transferred to the bones. The epimysium and perimysium are contiguous with the muscle tendons and bones and provide support for muscle

vasculature. The endomysium is a non-fibrous sheath that surrounds each myofibril, serving as a basement membrane (Bailey and Light, 1989).

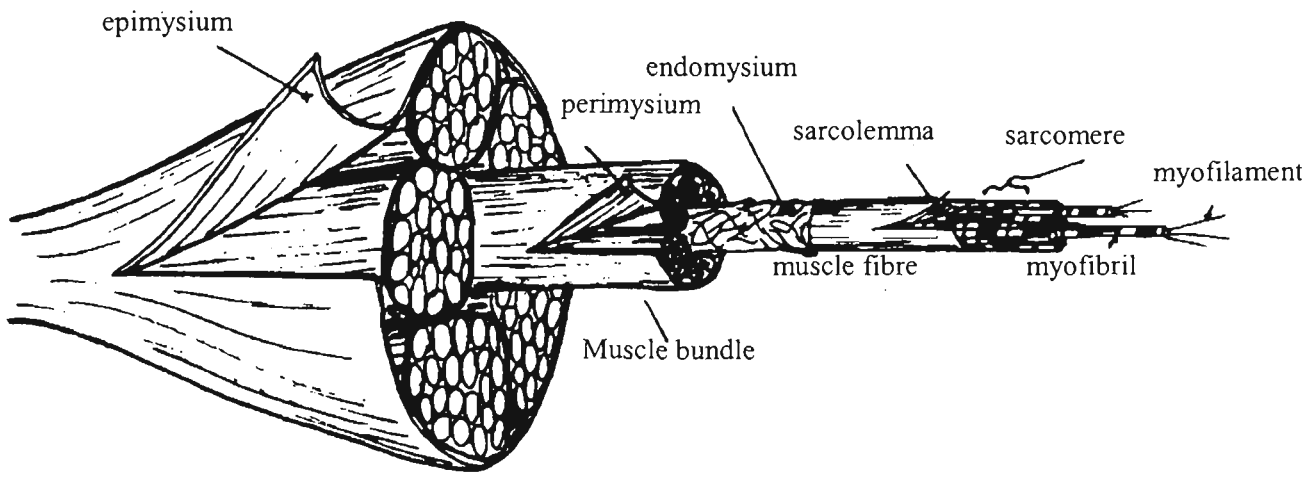


Figure 1.6: Anatomy of connective tissues in skeletal muscle (Judge et al., 1989).

1.3 Muscle energy metabolism

Energy substrates available to skeletal muscle are provided via uptake of nutrients absorbed from the intestines and released from the liver, or from catabolism of endogenous stores. Substrates for energy metabolism in skeletal muscle include volatile fatty acids, glucose, long chain fatty acids, ketones and amino acids. Muscle glucose can be stored as glycogen (glycogenesis) or metabolised via glycolysis and the citric acid cycle (also known as the tricarboxylic acid cycle, or TCA cycle). An overview of major metabolic pathways is presented in Figure 1.7.

1.3.1 Muscle glucose uptake, storage and metabolism

Skeletal muscle glucose uptake is predominantly augmented by the hormone insulin (Weekes, 1991) or muscle contraction. Glucose uptake into skeletal muscle is effected on a cellular level by glucose transporters. While both insulin and contraction result in translocation of insulin-sensitive glucose transporters (GLUT4) to the sarcolemma (Goodyear et al., 1992, Thorell et al., 1999), the underlying biochemical mechanism of translocation varies between the two stimuli (Lund et al., 1995). Re-location of glucose transporters need not be the rate-limiting step of glucose uptake as regulation may occur downstream of receptor relocation (Weekes, 1991). Transported glucose molecules are rapidly phosphorylated by hexokinase to glucose 6-phosphate to prevent loss from the cell since glucose 6-phosphate is incompatible with cellular glucose transporters (Moran et al., 1994a). Unless glucose 6-phosphate is required for glycolysis it is converted to glycogen (glycogenesis) by glycogen synthase (GS), a process stimulated by insulin (Halse et al., 2001). Glycogen stores are mobilised (glycogenolysis) by the enzyme glycogen

phosphorylase producing glucose-6-phosphate which is metabolised via glycolysis and the citric acid cycle (Moran et al., 1994a).

Glycolysis consists of 10-enzyme catalysed steps, summarised in Table 1.1; the net reaction of glycolysis is presented below (Equation 1.1). The final product of glycolysis is pyruvate, which can have two metabolic fates, oxidative decarboxylation to form Acetyl-CoA and enter the citric acid cycle, or anaerobic reduction to lactate. The citric acid cycle further oxidises the acetate moiety or acetyl-CoA, reducing the oxidising agents $NAD^{\oplus}$ and ubiquinone (Q). Production of lactate from pyruvate by lactate dehydrogenase is a metabolic end-point. Lactate produced by skeletal muscle is transported via the bloodstream to the liver to be converted to pyruvate by hepatic lactate dehydrogenase (Moran et al., 1994a). Accumulation of muscle lactate results in metabolic acidosis and low muscle pH.

Equation 1.1: $Glucose + 2ATP + 2NAD^{\oplus} + 2P_i \rightarrow 2Pyruvate + 2ATP + 2NADH + 2H^{\oplus} + 2H_2O$

Table 1.1: Enzyme catalysed reactions of glycolysis (Moran et al., 1994b)

Reaction		Enzyme
1.	Glucose + ATP $\rightarrow$ Glucose 6-phosphate + ADP + $H^{\oplus}$	Hexokinase, glucokinase
2.	Glucose 6-phosphate $\leftrightarrow$ Fructose-6-phosphate	Glucose-6-phosphate isomerase
3.	Fructose 6-phosphate + ATP $\rightarrow$ Fructose 1,6-*bis*phosphate + ADP + $H^{\oplus}$	Phosphofructkinase-1
4.	Fructose 1,6-*bis*phosphate $\leftrightarrow$ Dihydroxyacetone phosphate + Glyceraldehyde 3-phosphate	Aldolase
5.	Dihydroxyacetone phosphate $\rightarrow$ Glyceraldehyde 3-phosphate	Triose phosphate isomerase
6.	Glyceraldehyde 3-phosphate + $NAD^{\oplus}$ + P_i $\rightarrow$ 1,3-*Bis*phosphoglycerate + NADH + $H^{\oplus}$	Glyceraldehyde-3-phosphate dehydrogenase
7.	1,3-*Bis*phosphoglycerate +ADP $\rightarrow$ 3-Phosphoglycerate + ATP	Phosphoglycerate kinase
8.	3-Phosphoglycerate $\rightarrow$ 2-Phosphoglycerate	Phosphoglycerate mutase
9.	2-Phosphoglycerate $\rightarrow$ Phosphoenolpyruvate + H_2O	Enolase
10.	Phosphoenolpyruvate + ADP + $H^{\oplus}$ $\rightarrow$ Pyruvate + ATP	Pyruvate kinase

1.3.2 Muscle fat storage and metabolism

Ruminants differ from monogastrics in that the majority of ingested carbohydrate is broken down into the volatile fatty acids (VFA's), principally acetic, propionic and butyric acids. Ruminant skeletal muscle has a high dependency on VFA's as an energy source, utilising acetic and butyric acids (converted to β-hydroxybutyric acid in the rumen wall) (McDonald et al., 1995). Acetate is the major product of carbohydrate metabolism in ruminants. Acetate is utilised as an energy substrate following conversion to acetyl CoA and entry to the citric acid cycle (M^c Donald et al., 1995). Propionic acid is converted at the rumen wall to propionyl CoA, which is converted to oxaloacetate in the liver as a substrate for hepatic gluconeogenesis (Moran et al., 1994a, M^c Donald et al., 1995). The more metabolically active pools of fats are represented by nonesterified fatty acids (NEFA) and triacylglycerol (TAG). Fats stored within adipocytes as triacylglycerol (TAG) can be esterified from NEFA (Pethick and Dunshea, 1993). Fat mobilisation in adipose tissue is reflected in hydrolysis of TAG via hormone sensitive lipase (HSL) to form glycerol and NEFA (Dunshea et al., 1990). Triacylglycerol may also be transported in the bloodstream and lymphatic system bound to carrier proteins (lipoproteins). Uptake of circulating TAG is preceded by hydrolysis by lipoprotein lipase (LPL) at the endothelial wall (Pethick and Dunshea, 1993, Hocquette et al., 1998).

1.3.3 Muscle amino acid metabolism

Utilisation of amino acids for energy metabolism in skeletal muscle is provided by uptake of amino acids from the blood stream or degradation of endogenous proteins to amino acids and subsequent conversion to pyruvate, acetyl-CoA or citric acid cycle

intermediates. These substrates can subsequently be used for the provision of glucose via muscle gluconeogenesis (refer to section 1.3.4) or converted to alanine and transported to the liver for hepatic gluconeogenesis (Moran et al., 1994a).

1.3.4 Gluconeogenesis

Mechanisms exist to synthesise glucose from non-carbohydrate precursors (gluconeogenesis). Gluconeogenesis is the pathway whereby pyruvate is converted to glucose in reverse to glycolysis. Many of the intermediates between glycolysis and gluconeogenesis are shared, as some of the reactions are common to both pathways. Enzymatic reactions unique to gluconeogenesis are required to bypass the highly exergonic reactions catalysed by pyruvate kinase, phosphofructokinase-1 and hexokinase (Moran et al., 1994a). The net equation for gluconeogenesis is supplied by Equation 1.2.

Equation 1.2:

$$2\ \text{Pyruvate} + 2\ \text{NADH} + 4\ \text{ATP} + 2\ \text{GTP} + 6\ H_2O + 2\ H^{\oplus} \rightarrow \text{Glucose} + 2\ NAD^{\oplus} + 4\ \text{ATP} + 2\ \text{GDP} + 6\ P_i$$

In addition to allowing repletion of glucose, hepatic gluconeogenesis allows generation of glucose from non-glucose stores. The principal gluconeogenic substrate in the ruminant is propionic acid, absorbed from the rumen. Repletion of muscle lactate to glucose can occur via the Cori cycle, in which lactate from muscle is transported in the bloodstream to the liver and re-supplied to the muscle following gluconeogenic conversion to glucose. Conversion of amino and fatty acids to

pyruvate or citric acid cycle intermediates can also supply glucose via gluconeogenesis (Moran et al., 1994a, M^{c} Donald et al., 1995).

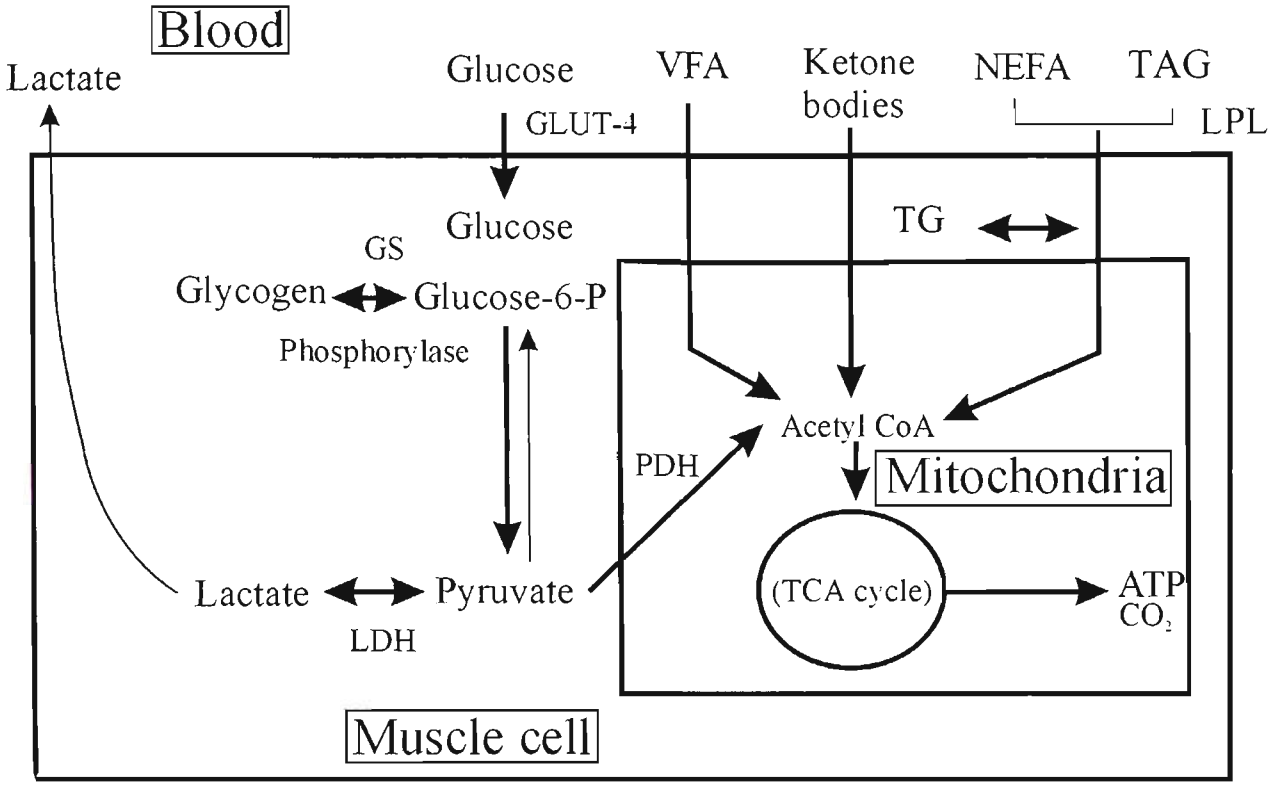


Figure 1.7: Summary of skeletal muscle metabolism, adapted from Hocquette et al. (1998).

1.4 Proteolytic systems of muscle fibres and protein turnover

The following section discusses the three main proteolytic pathways in skeletal muscle in context of their normal function, their roles in meat quality are later discussed in section 1.5.2.2. The metabolic turnover of protein in muscle fibres is the process whereby protein is continually degraded by proteolytic enzymes and replaced by protein synthesis (Schoenheimer and Rittenberg, 1940). The bulk of extracellular protein is degraded by cathepsic proteases in the lysosomes (Lowell et al., 1986). However, myofibrils are too large to be transported to the lysosomes and must first be disassembled (Goll et al., 1992). Degradation of the Z-line and myofibrillar disassembly is performed by the Ca^{++} sensitive protease calpain, of which there are two principal isoforms. Proteolysis of proteins by calpain results in their hydrolysis to peptide fragments, which are broken down into amino acids by the proteasome (Goll et al., 1989).

1.4.1 Endolysosomal proteolysis

The endolysosomal system is responsible for non-specific degradation of large amounts of endocytosed extracellular (Funato et al., 1997, Turk et al., 2000) and intracellular proteins transported by a carrier mediated mechanism (Cuervo and Dice, 1996). Proteins are "sorted" and packaged within primary endosomes which mature to endosome carrier vesicles and finally late endosomes (Murphy, 1991) (Figure 1.7). Targeted degradation of cellular proteins occurs via chaperone mediated autophagy, during which labelled proteins are complexed to surface endosomal proteins and transported into the lumen (Cuervo and Dice, 1996, Agarraberes and Dice, 2001).

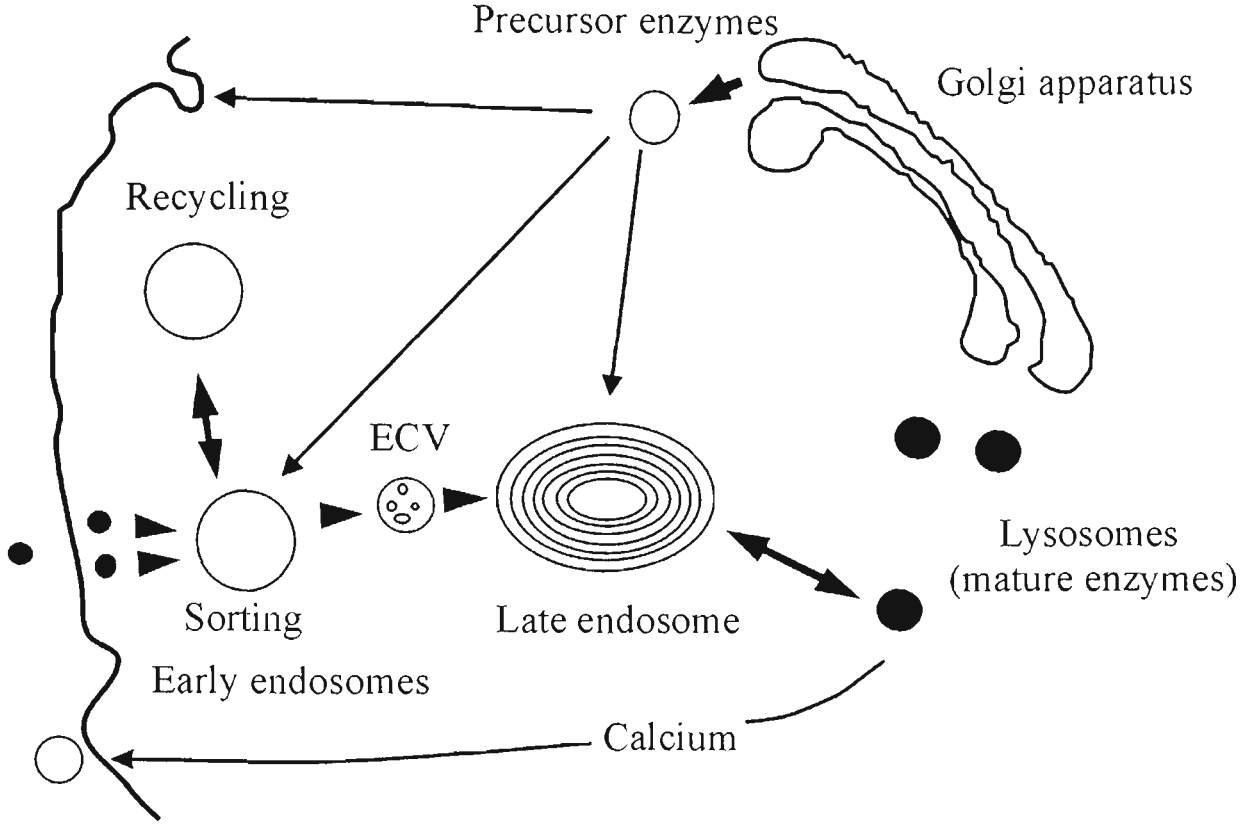


Figure 1.8: Maturation of endosomes and formation of endosomal/lysosomal hybrid in the endosomal/ lysosomal pathway (Pillay et al., 2002).

The luminal pH is lowered by H^+-ATPases to 6.3-6.5 for an early endosome (Fuchs et al., 1989, Cain et al., 1989) to 5.0-5.5 for a late endosome (Killisch et al., 1992). The terminal vesicle of the endolysosomal system is the lysosome (Kornfeld and Mellman, 1989), which is a highly acidic organelle. The pH of the lysosome can reach as low as 3.8 in intact cells (Lloyd and Masopn, 1996). While only containing 20% of the total protease pool, late endosomes are the main site of proteolysis (Tjelle et al., 1996, Bright et al., 1997). The lysosomes, which contain the bulk of the pool of proteolytic enzymes only account for 20% of proteolysis, leading to the hypothesis that lysosomes are a store for proteases (Tjelle et al., 1996). Fusion of the late endosome and the lysosome forms a hybrid organelle with the properties of both (Thilo et al.,

1995, Bright et al., 1997, Luzio et al., 2000). Rather than being a terminal organelle as often thought, the lysosomal membrane and proteolytic enzymes can be re-formed into a lysosome (Bright et al., 1997, Mullock et al., 1998).

The principal lysosomal proteolytic enzymes are the cathepsins of which there are 11 human isoforms (Turk et al., 2000), the principal muscle isoforms are B, D, H and L (Schwartz and Bird, 1977, Okitani et al., 1980, Okitani et al., 1981). Synthesised as zymogens, procathepsins undergo processing by the proteolytic (Nishimura et al., 1988, Rowan et al., 1992) or pH dependant autocatalytic removal (Cuozzo et al., 1995) of a propeptide to form the mature enzyme. Activity of the mature enzyme is regulated by lysosomal pH as cathepsins require an acidic, reducing environment for optimal activity (Turk et al., 2000). Further regulation of cathepsins is offered by cystatin, a specific cathepsin inhibitor (Turk and Bode, 1991).

Despite the contribution of the endolysosomal pathway to protein degradation and the observation of myofibrillar proteins in lysosomes (Gerard and Schneider, 1979) and detection of cathepsins outside lysosomes (Turk et al., 1993, Wang et al., 1998), current evidence does not support a significant contribution of cathepsins and the endolysosomal pathway to direct proteolysis of myofibrillar proteins (Reeves et al., 1981, Lowell et al., 1986, Furuno et al., 1990).

1.4.2 The ATP-Ubiquitin-Proteasome pathway

Protein degradation by the ubiquitin system is a two step process. Multiple ubiquitin molecules are covalently attached to target proteins, then tagged proteins are degraded by the 26S proteasome (Ciechanover, 1998). Conjugation of ubiquitin to proteins

targeted for degradation relies on the ubiquitin carrier (E_2) and conjugating enzymes (E_3) being catalysed by the ubiquitin activating enzyme (E_1) (Hershko et al., 1983).

The 26S proteasome is generated by the ATP dependant association between two 19S regulatory complexes and a 20S catalytic core (Brooks et al., 2000, Ciechanover, 1998). The 19S regulatory complexes contain approximately 20 different subunits, 6 of which are ATP dependant (Brooks et al., 2000). The 19S regulatory complexes serve as a port of entry to the 20S catalytic core, as well as providing regulatory functions for the selective degradation of ubiquitin tagged substrates (Ciechanover, 1998). The 20S core possesses multiple proteolytic sites, is ATP-independent and capable of ubiquitin-independent proteolysis (Hough et al., 1987). Solomon and Goldberg (1996) observed that while the proteasomal degradation pathway was primarily responsible for muscle protein degradation, dissociation of myofibrillar proteins was the limiting step.

1.4.3 Proteolysis by Calpains

While the proteasome and endolysosomal system perform the bulk of cellular protein degradation, they cannot initiate degradation of the myofibrillar proteins, which constitute 50-60% of skeletal muscle protein (Goll et al., 1992). Specific cleavages initiating the disassembly of myofibrils to myofilaments, capable of degradation by the proteasome and lysosomes are initiated by the Ca^{++} dependant cysteine protease calpain (Canonico and Bird, 1970, Busch et al., 1972, Goll et al., 1989, Croall and DeMartino, 1991).

Busch et al. (1972) observed the Ca^{++} dependant degradation of the Z-lines in rabbit skeletal muscle, without damaging other components of the myofibril. The properties of this enzyme, calcium activated neutral protease (CANP), later called calpain, were further characterised by Dayton et al. (1976b). It was hypothesised by Suzuki et al. (1981) and later confirmed (Dayton, 1982) that there are high and low calcium requiring isoforms of CANP in skeletal muscle. Dayton et al. (1981) discovered two isoforms of CANP in porcine skeletal muscle, requiring 5μM and 0.1mM Ca^{++} respectively for optimal activity (μ- and mCANP respectively). From this, they hypothesised that μCANP was active under physiological conditions, while mCANP was an inactive "storage form". Suzuki et al. (1981) observed that autolysis of mCANP resulted in a significant increase in its calcium sensitivity, leading them to conclude μCANP was a product of the high-calcium requiring protease. Dayton (1982) later showed that autolysis of the 30kDa subunit was common to both isoforms, discounting the theory of Suzuki and co-workers.

Regulation of endogenous regulation of calpain isoforms remained a perplexing question since both isoforms are inactive at resting cytoplasmic free Ca^{++} concentrations *in vitro*. Autolysis of the 30kDa subunit and subsequent activation of calpain required abnormally high Ca^{++} concentrations (Suzuki et al., 1981, Coolican et al., 1986, Cong et al., 1989, Baki et al., 1996), while the high-calcium requiring isoform (m-calpain) required supraphysiological Ca^{++} concentrations. Tompa et al. (1996) observed that the activities of low- and high calcium requiring calpain (μ- and m-calpain) at micromolar Ca^{++} concentrations were higher than the sum of their individual activities. This indicated a cascade where μ-calpain regulated the activity of m-calpain by cleavage of the 30kDa subunit. However, the autolysis of the 30kDa

subunit results in the formation of an active, but unstable enzyme (Elce et al., 1997), while continued autolysis of the 80kDa subunit deactivates the active enzyme (Coolican et al., 1986).

Calpain activity is also regulated by its specific endogenous inhibitor calpastatin (Waxman and Krebs, 1978, Hattori and Takahashi, 1982). The interaction between calpain and calpastatin is complex, as calpastatin abundance appears to far outweigh calpain activity. Each calpastatin molecule has four internal repeats, each able to inhibit calpain (Emori et al., 1987, Maki et al., 1987, Emori et al., 1988). Concentrations of calpastatin in bovine cardiac muscle are sufficient to provide 10-fold excess of inhibitory capacity over calpain activity (Waxman and Krebs, 1978, Otsuka and Goll, 1987). The calpain-calpastatin interaction is Ca^{++} dependant (Nishimura and Goll, 1991), with binding of calpastatin occurring below calcium concentrations required for calpain activity (Otsuka and Goll, 1987, Kapprell and Goll, 1989). Clearly some form of regulation of this system exists. It has been observed that of the three subdomains of each calpain inhibitory site, only one subdomain is inhibitory of calpain, the other two subdomains stimulate calpain activity (Tompa et al., 2002). From this Tompa et al. (2002) hypothesised an interaction with other proteases to remove the inhibitory subdomains of calpastatin resulting in stimulation of calpain activity.

Further to the identification of μ- and m-calpain, a 94kDa skeletal muscle-specific isoform has recently been identified (p94) (Kinbara et al., 1998). The p94 isoform is particularly unstable and has an *in vitro* half-life of 30min (Sorimachi et al., 1993). While it is found in the myofibrillar fraction (Kimura et al., 1984, Kinbara et al.,

1998) and can bind to titin, p94 is Ca^{++} and calpastatin-independent and its physiological role is not clear (Kinbara et al., 1998).

1.5 Pathophysiology and the conversion of muscle to meat

1.5.1 Pathophysiology of skeletal muscle

While an animal may be considered deceased following exsanguination, the line defining when muscle becomes meat is blurred. The time that skeletal muscle remains metabolically active post mortem varies between species, but may be in excess of 15 hours in non-electrically stimulated cattle carcasses (Bendall, 1978, George et al., 1980). Following slaughter there is a sequence of biochemical events, culminating in rigor mortis.

Ischaemia is defined as a low oxygen state usually due to obstruction of the arterial blood supply or inadequate blood flow leading to hypoxia in the tissue. Muscle metabolism continues post-exsanguination, with finite oxygen and energy stores. The resulting ischaemic environment results in a shift from oxidative metabolism to glycolysis (Lipton, 1999). Metabolic by-products such as lactic acid and H^+ accumulate from muscle glycogenolysis, resulting in a falling muscle pH (Tarrant and Mothersill, 1977, Lipton, 1999). In the absence of continued nutrient supply from the blood stream, metabolic exhaustion occurs, resulting in depleted ATP (Lipton, 1999). This inhibits ATP-dependent membrane-bound ion pumps and subsequent loss of ion gradients, increasing intracellular calcium and sodium. Efflux of Ca^{++} down concentration gradients from the SR to the cytosol results in indiscriminate activation of Ca^{++} dependent processes, including proteases such as calpain (Dransfield, 1994) and lipases (Cotran et al., 1989). Sodium influx increases osmotic pressure within the cell and can result in lysis (Cotran et al., 1989) and it has been demonstrated that osmotic pressure in beef *sterenomandibularis* and *psoas* muscles increases post-rigor (Winger and Pope, 1980-81). Breakdown of structural proteins, membranes and

increased osmotic pressure can result in a loss of cellular compartmentalisation and eventually leads to cell death (Cotran et al., 1989, Lipton, 1999).

1.5.2 Conversion of muscle to meat and factors affecting meat quality

Rigor mortis is often referred to as the line that defines the conversion of muscle to meat. Rigor mortis occurs when there is insufficient ATP to break actin/myosin cross-bridges and maintain a relaxed state (Judge et al., 1989). In ovine and bovine species this is typically between 6 and 12 hours post-mortem (Goll et al., 1964). Development of rigor mortis coincides with peak tension of the muscles in the carcass (Devine et al., 1999). Post-rigor, the muscle tension declines and the texture becomes softer in a process known as ageing (Goll et al., 1964). The rate and extent of meat ageing is a product of biochemical and processing factors including muscle pH, enzymatic tenderisation, sarcomere length and fibre type.

1.5.2.1 Post–mortem muscle pH decline and ultimate pH

As previously discussed, muscle pH fall post-mortem is driven by glycolysis, which is influenced by a number of biochemical, environmental and processing factors. Muscle glycogen is the substrate and therefore limiting factor for post-mortem muscle glycolysis. Depleted muscle glycogen at slaughter results in reduced post-mortem glycolysis, less meat acidification and a high ultimate pH (Lister, 1988, Pethick et al., 1995). Symptomatic of high ultimate pH meat is its dark colouration, it is due to this property that dark, high ultimate pH meat is often referred to as "dark cutting". Muscle glycogen concentrations are heavily influenced by the level of nutrition, but there are also significant environmental factors (Pethick et al., 1995). It has been demonstrated that stress (Lawrie, 1958, Mc Veigh et al., 1982), adrenaline (Lacourt

and Tarrant, 1985, Hocquette et al., 1998) and exercise (Apple et al., 1994, Pethick and Rowe, 1996) all reduce muscle glycogen, resulting in meat of high ultimate pH (refer to sections 1.6.1 and 1.6.2).

While more common in pork, "pale soft exudative" or PSE meat has also been reported in beef (Tarrant and Mothersill, 1977). Pale soft exudative meat is a product of excessively fast carcass pH fall at a high carcass temperature due to elevated glycolysis and lactate accumulation, usually due to acute pre-slaughter stress (Briskey and Wismer-Pedersen, 1961, Boles et al., 1994). Pre-slaughter stress in pigs can cause extreme metabolic activity and acidosis, resulting in PSE. Pale, soft exudative characteristics in meat are the result of excessive denaturation of muscle proteins, decreasing their ability to bind water (Hultin, 1985). Processing factors such as electrical stimulation of the carcass can also induce PSE meat due to over-stimulation of post-mortem glycolysis, resulting in abnormally fast rates of pH fall (Hammelman et al., 2003).

The ultimate pH of meat has significant effects on tenderness and other quality traits (Bouton et al., 1971, Bouton et al., 1982). Investigations into the effect of ultimate pH on meat tenderness have revealed a curvilinear relationship in many, but not all studies. Meat of an intermediate ultimate pH of approximately 6 has reduced tenderness when compared to meat of low (≈5.6) or high (>6.3) ultimate pH in bovine and ovine carcasses (Yu and Lee, 1986, Watanabe et al., 1996, Purchas and Yan, 1997, Purchas et al., 1999). Purchas (1990) proposed that part of the effect of pH on tenderness was due to changes in sarcomere length. However, in later work this was not observed (Purchas and Yan, 1997, Purchas et al., 1999). Yu and Lee (1986)

observed significant changes in the degradation patterns of meat with low, intermediate and high ultimate pH and proposed that muscle pH heavily influenced enzymatic tenderisation of meat.

The rate of post-mortem glycolysis, pH decline and rigor onset is affected by the rate of temperature decline (Marsh, 1954). Bendall (1978) demonstrated that variation in intra-carcass cooling had a significant impact on rates of post-mortem glycolysis. Modifications in temperature and pH in turn affect the rate of proteolysis and hence meat tenderness (Koohmaraie et al., 1986, Dransfield, 1992).

1.5.2.2 Enzymatic tenderisation of meat

Post-mortem degradation of Z-lines (Henderson et al., 1970, Hattori and Takahashi, 1979) was shown to be a Ca^{++} dependant process (Davey and Gilbert, 1967), ultimately leading to the discovery of calpains (Busch et al., 1972). Since then the involvement of calpains in meat tenderisation has been demonstrated in numerous experiments and according to Koohmaraie (1994), calpain is the principal proteolytic enzyme for tenderising meat. However, while calpain may be the principal tenderising enzyme it is not solely responsible for meat tenderness (Koohmaraie, 1994, Alarcon-Rojo and Dransfield, 1995).

While dissolution of lysosomes occurs post-mortem (Moeller et al., 1977, Chambers and Bowers, 1993) and lysosomal enzymes are present outside the lysosomes (Turk et al., 1993, Wang et al., 1998) and can perform extra-lysosomal proteolysis (Authier et al., 1995), the involvement of cathepsins in meat quality remains controversial. Cathepsins have been observed to degrade myofibrillar proteins (Schwartz and Bird,

1977, Gerard and Schneider, 1979) and tenderise meat (O'Halloran et al., 1997). This is despite other experiments concluding that myofibrillar proteins are poor substrates for cathepsins (Dayton et al., 1976a, Lowell et al., 1986). While cathepsins display low levels of activity at cytosolic pH, it is possible that cathepsin activity is increased by acidic conditions post-slaughter (Ouali, 1990). Lysosomal dissolution is pH sensitive, increasing in magnitude as pH falls (Etherington, 1984), but conserved in high ultimate pH meat (Ertbjerg et al., 1999).

Once ATP is depleted, active compartmentalisation of Ca^{++} is lost (Mickelson, 1983). There are substantial differences in the literature for free calcium concentrations in resting muscle and rigor, largely dependent on the methodologies used (Hopkins and Thompson, 2002). It is accepted that cytosolic, or "free" calcium concentrations increase after death from approximately 10μM to as much as 500μM, stimulating calpain activity (Dransfield, 1993). The stimulatory effect of Ca^{++} on meat tenderness has been demonstrated by substantial inhibition of muscle proteolysis after sequestration of calcium with EGTA (Busch et al., 1972, Uytterhaegen et al., 1994). Calpain activity *in vitro* is optimal around a neutral pH, but significant activity still exists as low as pH 6.5 (Dayton et al., 1976b). However, most meat tenderisation occurs at pH values lower than this (Bouton et al., 1971). Cottin et al. (1981) observed that inhibition of calpain with calpastatin was attenuated with lower pH ranges found in meat. This may be in part due to the high rate of degradation of calpastatin post-mortem, coupled with favourable ionic conditions for calpain activation (Geesink and Koohmaraie, 1999).

1.5.2.3 Sarcomere length

Sarcomere shortening during rigor mortis was first observed by Bendall (1951). Later, a link between sarcomere length and meat quality was established when it was observed that rapid chilling increased both sarcomere shortening and beef toughness (Locker, 1960, Locker and Hagyard, 1963). This observation was not limited to 'cold shortening', as 'heat-shortening has also been observed. While 'cold-shortening' typically occurs when muscle temperature falls below 15-16°C pre-rigor (Hedrick et al., 1994), carcasses or muscles stored between 32 and 49°C post-mortem also exhibited sarcomere shortening (Davey and Gilbert, 1973, Bowling et al., 1978, Lee and Ashmore, 1985, Devine et al., 1999). The mechanism of shortening under the different imposed environmental temperatures is thought to be due to leakage of calcium from the sarcoplasmic reticulum (Davey and Gilbert, 1973, Cornforth et al., 1980).

1.5.2.4 Muscle fibre type

As reviewed in sections 1.2.2.4 and 1.2.2.5, different muscle fibres possess vastly different physical, contractile and metabolic properties. It has been proposed that this is critical in the development of meat quality (Geay and Picard, 1995). Muscle fibre type has been linked to meat quality, particularly in the areas of tenderness, pH and colour (Ashmore, 1974, Klont et al., 1998). Ouali et al. (1989) proposed that variation in tenderness is largely accounted for by differences in muscle fibre type. The rate of tenderisation has been demonstrated to be faster in white (type II) than red (type I) muscle fibres (Ouali, 1990). Three separate mechanisms have been proposed for this: 1) different levels of proteases and inhibitors, 2) sensitivity of muscle proteins to proteolysis and 3) osmotic pressure (Ouali, 1990, Monin and Ouali, 1987).

Patterns of glycogen depletion have been shown to differ between muscle fibre types (Tarrant and Mothersill, 1977, Lacourt and Tarrant, 1985). In one experiment increased proportions of type I muscle fibres increased redness in bovine muscles (Hunt and Hedrick, 1977). Alternatively, it has been proposed that the higher glycolytic rates on high type II fibres is a pre-disposing factor for dark cutting in a muscle, since it is more likely that these muscles deplete their glycogen stores pre-slaughter (Talmant and Monin, 1986). Predisposition to dark cutting has obvious impacts on meat colour, but importantly, colour is also influenced by concentrations of the colour pigment myoglobin and its oxidation status. Higher mitochondrial content in type I fibres allows greater scavenging of diffused O_2, limiting its reactions with myoglobin and reducing the depth of the bright red oxymyoglobin layer (Monin and Ouali, 1987). According to Hood (1980), the higher oxidative state of type II fibres accounts for almost half the variation in colour stability in packaged beef.

1.6 Physiological responses to stress and effects on meat quality

The term 'stress' is frequently used, but poorly understood. Stress has been defined as a non-specific response of the body to any demand. In terms of physiology, stress is an unpleasant situation that elicits a response from the hypothalamus (Marieb, 1992a) or adaptations to attempt to re-establish physical and psychological homeostasis (Cook et al., 1996). The "response" from the hypothalamus will vary according to the size of the stimulus. Whether the stressor is chronic or acute will also affect the response. The response in the animal to acute and chronic stressors is different. An acute stress is short term, and is principally mediated through nerve impulses generated in the hypothalamus causing the release of catecholamines from the adrenal medulla via the neurotransmitter acetylcholine (Figure 1.9). Chronic

stress is mediated through the prolonged release of glucocorticoids and mineralcorticoids from the adrenal medulla and by an endocrine release of adrenocorticotropic hormone (ACTH) (Marieb, 1992a).

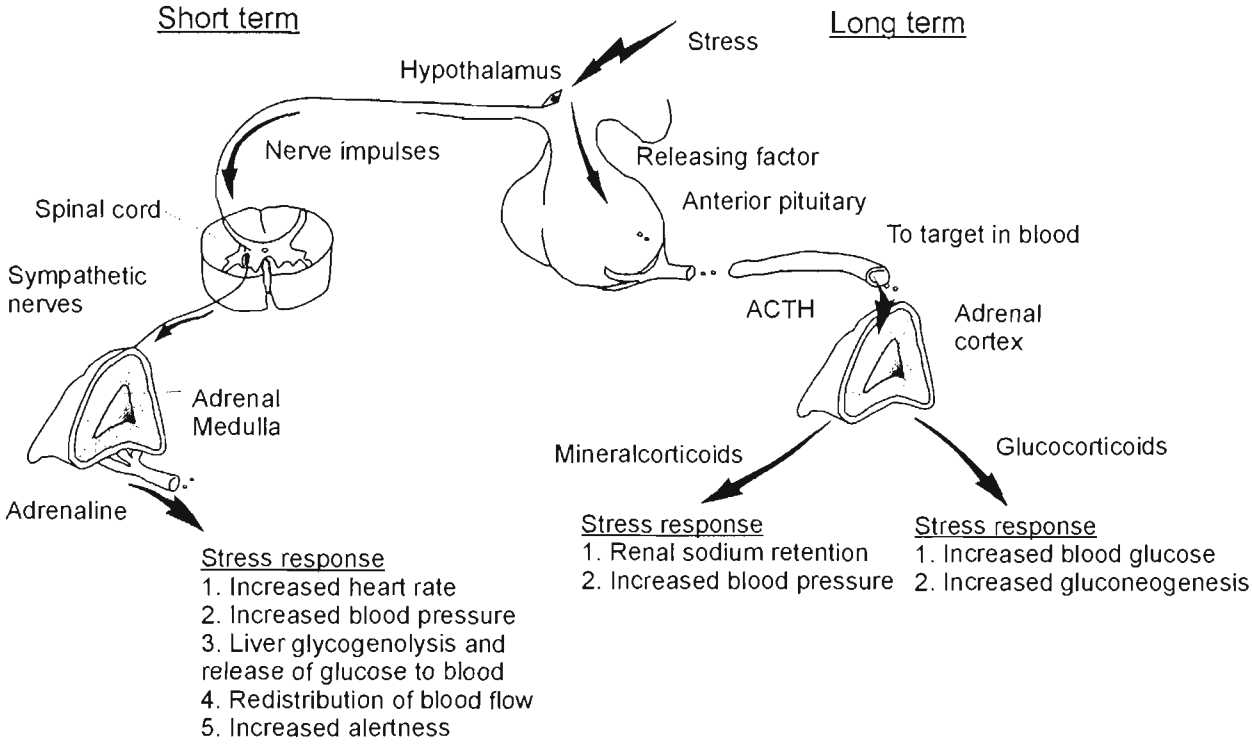


Figure 1.9: Mediation of short and long term stress responses (Marieb, 1992b).

1.6.1 Psychological and physical stress of animals that influences meat quality

Fear is a common stressor experienced amongst meat animals during the marketing and slaughter process. Due to unfamiliar events and environment, animals are frightened in many ways. Physical and psychological stressors are frequently linked, including responses to 'negative handling' (Shorthose and Wythes, 1988) and mixing of unfamiliar animals pre-slaughter (Monahan Raj et al., 1992, Daly et al., 1996). Dantzer and Mormede (1983) observed increases in the levels of plasma ACTH in pigs when exposed to a new environment, or when given electrical shocks.

Mixing of unfamiliar animals leads to a variety of stress behavioural responses. This includes exertion (mounting and fighting) and fear (Tarrant, 1989, Monahan Raj et al., 1992, Daly et al., 1996). Mc Veigh et al. (1982) demonstrated that mixing unfamiliar groups of cattle led to increases in rectal temperature, heart rate, serum NEFA and creatine kinase while muscle glycogen fell to 41% of pre-stress levels. Poorly designed facilities increase stress in animals by several mechanisms. This includes increased pre-slaughter handling (due to increased coercion from stock handlers) and injury of animals on poorly placed fittings (Tarrant, 1989). Stress can also arise due to a poorly designed facility (Webster, 1983, Tarrant, 1989). For example, housing animals in cubicles that do not allow animals enough space to move, results in chronic discomfort (Webster, 1983) and concrete floors can predispose animals to hoof injuries (Webster, 1981). Animal responses to these conditions can result in damage of skins and hides, bruising of the carcass and increased risk of dark cutting carcasses.

Transportation and relocation of livestock from farm to sale yard or abattoir is a significant source of stress (Hutcheson and Cole, 1986) and fatigue (Shorthose and Wythes, 1988). Responses to transportation-induced livestock stress include elevated levels of cortisol, glucose and packed cell volume (Tarrant et al., 1992, Schaefer et al., 1997) dehydration, weight loss, glycogen depletion and bruising (Wythes et al., 1981).

1.6.2 Effects of stress on meat quality

Depending on the type of stress, physiological responses to stress often influence meat quality. An extreme case of adverse stress response occurring in pigs is "porcine

stress syndrome", during which an extreme catecholamine response to stress can result in fatal metabolic acidosis (Webster, 1983). The resultant accelerated pH fall post-mortem, resulting in PSE meat, is further outlined in section 1.5.2.1. The opposite extreme is the occurrence of DFD, due to glycogen depletion and reduced post-mortem pH fall, also discussed in section 1.5.2.1. Development of PSE and DFD represent what could be termed classical models of the effects of stress on meat quality, whereby stress influences pH fall, meat colour and water holding capacity via pre-slaughter glycogen concentrations and post-mortem glycolysis.

1.7 The physiological roles of nitric oxide in skeletal muscle and regulation of meat quality determinants

1.7.1 Overview

Nitric oxide is synthesised by a family of enzymes known as the nitric oxide synthases (NOS), which are analogous to cytochrome P-450 reductase (Bredt et al., 1991). The NOS family contains three isoforms, nomenclature of the three isoforms follows the tissues they were initially isolated from; endothelial NOS (eNOS) (Janssens et al., 1992, Lamas et al., 1992), macrophage or inducible NOS (iNOS) (Stuehr et al., 1989) and neuronal NOS (nNOS) (Wu et al., 1994a). Nakane et al. (1993) identified nNOS as the most prevalent isoform in skeletal muscle, and its expression in muscle was greater than in the brain. One year later, Kobzik et al. (1994) published a comprehensive study on the effects and mechanisms of NO on muscle contraction and many studies have since followed. Nitric oxide has since been demonstrated to play a crucial role in skeletal muscle, emerging as a model of nitric oxide function and redox-related signalling in biology (Stamler and Meissner, 2001).

1.7.2 Nitric oxide synthases

The three distinct isoforms of NOS have been isolated from neuronal, macrophage and endothelial cells are increasingly being referred to as NOS I (nNOS), NOS II (iNOS) and NOS III (eNOS) in recognition of the wide tissue distribution of each isoform. Nitric oxide synthases were classified as constitutive (calcium activated) or inducible in their activity, but this has proved unreliable as a means of classification since each isoform may be regulated dynamically (Stamler and Meissner, 2001) and iNOS can be expressed constitutively (Guo et al., 1995, Park et al., 1996). However,

typically nNOS and eNOS activities are calcium dependant (Michel and Feron, 1997), and while iNOS activity is not influenced by calcium, it does contain a calmodulin binding domain (Bredt and Snyder, 1994).

Expression, activity and distribution of NOS in muscle vary between species and muscles. For example Kobzik et al. (1994) observed considerably higher content of nNOS in type II fibres than in type I rat skeletal muscle while Tews et al. (1997) observed nNOS in both fibre types in rat facial muscles. All three NOS isoforms are expressed in skeletal muscle tissue (Stamler and Meissner, 2001), but vary in their sub-cellular and organelle distribution. Neuronal NOS is expressed in surface membranes (Nakane et al., 1993), where it is associated with membrane bound dystrophin complexes (Chang et al., 1996), sarcoplasmic reticulum (Xu et al., 1999), neuromuscular junctions (Frandsen et al., 1996, Kobzik et al., 1994), myotendinous junctions and costameres (Chang et al., 1996). The diverse expression of nNOS in skeletal muscle underlines its importance in skeletal muscle physiology.

Expression of eNOS and iNOS is less prevalent than nNOS in skeletal muscle. Endothelial NOS is expressed in the caveolae of endothelial and skeletal muscle cells where it is thought to be associated with signal transduction (Segal et al., 1999, Garcia-Cardena et al., 1997). Expression of eNOS in mitochondria is thought to regulate oxidative enzymes (Kobzik et al., 1995). The role of iNOS in skeletal muscle is not clear, as it is normally expressed in low levels (Park et al., 1996). However, the expression of iNOS in human skeletal muscle increases during congestive heart failure (Riede et al., 1998).

Biosynthesis of NO is tightly regulated, since NO is a free radical and cannot be stored (Bredt and Snyder, 1994). All NOS isoforms oxidise the terminal guanido nitrogen of L-arginine in a two-step process that consumes five electrons and yields stoichiometric amounts of NO and citrulline. The reaction requires the co-substrates O_2 and NADPH and co-factors heme (Xie et al., 1996, Abu-Soud et al., 1994), tetrahydrobiopterin (BH_4) (Tayeh and Marletta, 1989), flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN) (Zhang et al., 2001) and calmodulin (CAM) (Stevens-Truss et al., 1997, Abu-Soud and Stuehr, 1993, Figure 1.10).

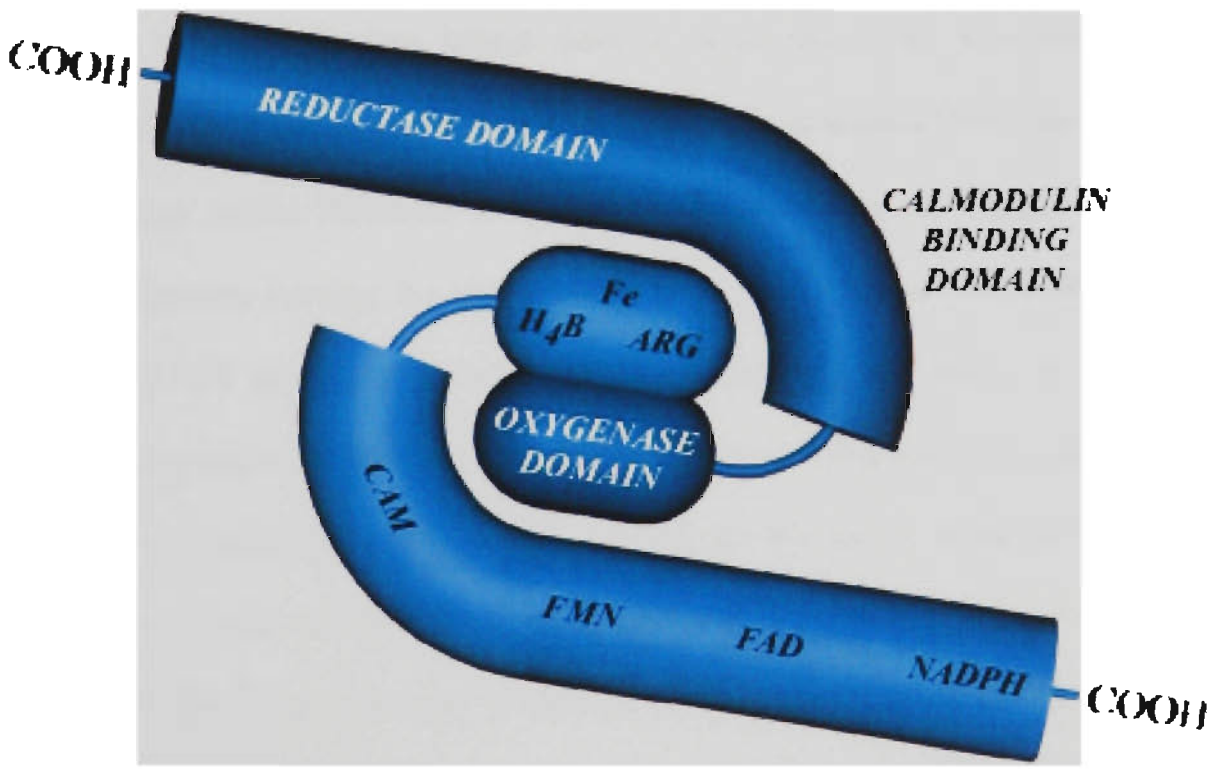


Figure 1.10: Structure and binding domains of co-factors on NOS dimer (Anon, 1999)

NOS monomers catalyse marginal NO production and a heme prosthetic group is a pre-requisite of dimerisation and binding of BH_4 and arginine (Klatt et al., 1996), which are essential to NOS activity (Tayeh and Marletta, 1989). The presence of NADPH reduces the flavins, but not the prosthetic heme group, which is initiated by

binding of calmodulin in response to increasing intracellular calcium. Binding of calmodulin results in transfer from the one electron reduction of NADPH to the prosthetic heme (Abu-Soud and Stuehr, 1993), resulting in reduced heme binding O_2 and conversion of L-arginine to N-hydroxyarginine (Marletta, 1993). In the second step of the reaction the second atom of the O_2 molecule is released as water, again reducing the heme and oxidising N-hydroxyarginine to form NO, citrulline and water (Nedospasov, 1998).

1.7.3 Biochemistry of NO and related oxides

NO is a generic term for the free radical form of nitric monoxide, sometimes represented as $NO^{\bullet}$. Nitrogen monoxide is a free radical with an unpaired electron in its $2p$-π antibonding orbital (Marletta et al., 1990). Oxidation of NO results in removal of this electron, forming the nitrosonium cation (NO^{+}) (Lee et al., 1990), while reduction of $NO^{\bullet}$ forms the nitroxyl anion (NO^{-}) (Murphy and Sies, 1991). Changes in the redox state of NO stem primarily from reactions with transition metal complexes and diatomic oxygen (Stamler et al., 1992c), but NO can be quenched by other free radicals (Szalai and Brudvig, 1996, Lepoivre et al., 1994). Nitric oxide differs from other signalling molecules as it does not propagate signals by ligand binding, but rather by covalent binding with redox sensitive biomolecules. The reactivity of NO with different biomolecules are characteristically different with the different redox states of NO and are a product of the surrounding redox milieu (Stamler, 1994, Figure 1.11).

As mentioned previously, $NO^{\bullet}$ reacts with transition metal centres and diatomic oxygen. Transition metal centres are found on a variety of metalloproteins, including heme and non-heme complexes. This can include hemoproteins such as myoglobin, guanylate cyclase and cytochrome c (Cleeter et al., 1994), iron-sulfur centres in aconitase (Hausladen and Fridovich, 1994), or zinc finger proteins (Kroncke et al., 2002). The ratio of uptake and release of $NO^{\bullet}$ by Fe(II)-hemoglobin is five to six fold greater than that of O_2 (Doyle and Hoekstra, 1981) and unlike carbon monoxide or O_2, $NO^{\bullet}$ can bind to Fe(III) porphyrins (Sharma et al., 1983). Oxidation of $NO^{\bullet}$ to NO^{+} and reduction of Fe(III) to Fe(II) and subsequent attack by ambient nucleophiles results in release of NO^{+} via an NO^{+}/Fe(II) complex (Wade and Castro, 1990). By this mechanism transition metal nitrosyls formally donate NO^{+} within biological tissue (Stamler, 1994).

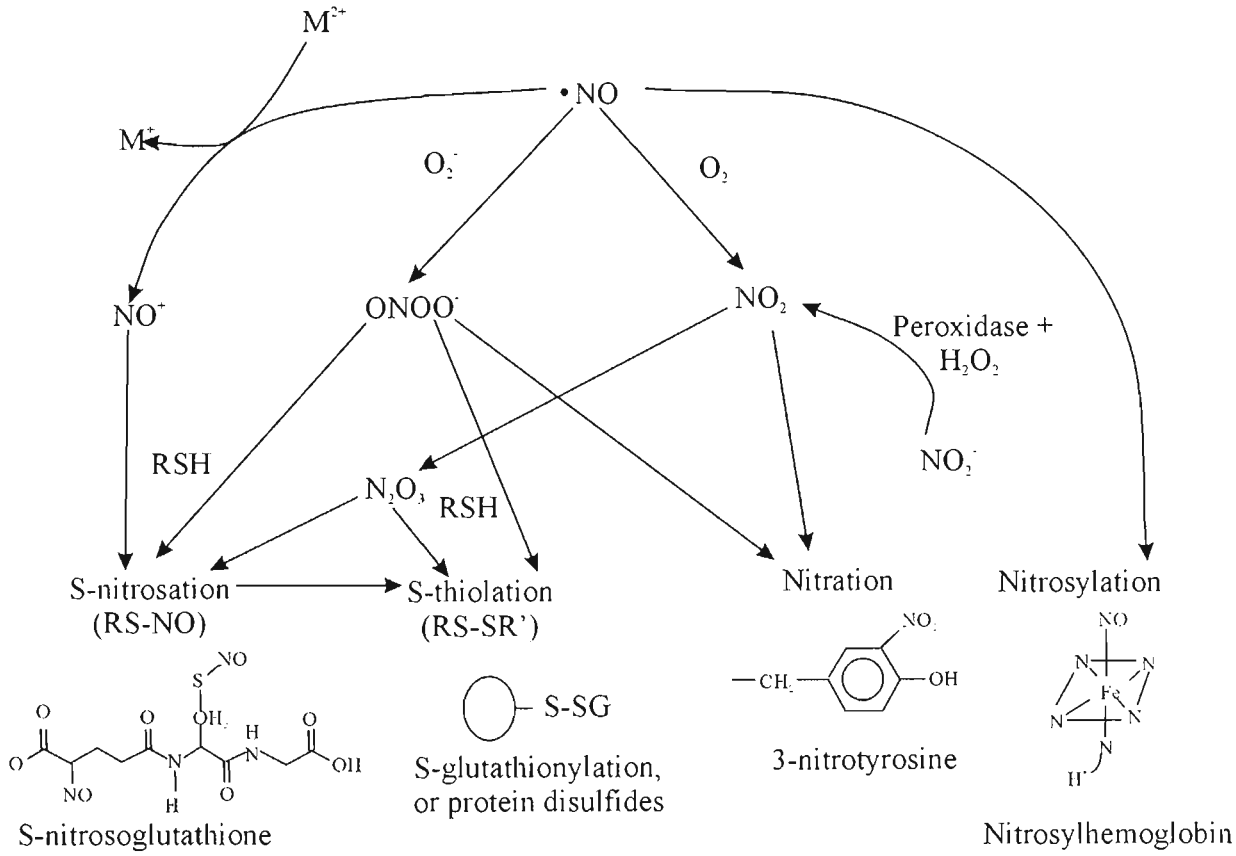


Figure 1.11: Mechanisms of NO-dependent modifications of biological molecules, metal complexes (M) and oxygen species (Patel et al., 2000).

Loss of one electron from the $2p$-π of $NO^{\bullet}$ confers electrophilicity to NO^{+} increasing its addition and substitution reactions with nucleophilic –S, -N, -O and –C centres in organic molecules (Simon et al., 1996). However, under physiological conditions S-nitrosation of sulfur with NO^{+} (RS-NO) is preferred (Wink et al., 1991, Stamler et al., 1992b, Stamler et al., 1992c). Due to the propensity of RS-NO to undergo heterolytic transfer of NO^{+}, rather than homolytic release of $NO^{\bullet}$, the circuit of nitrosation can be extended to S-thiolation (RS-SR) (Stamler, 1994).

Reactions of $NO^{\bullet}$ with diatomic oxygen in the form of superoxide (O_2^{-}) and oxygen (O_2) yield peroxynitrite ($ONOO^{-}$) and NO^{-}. Peroxynitrite is not a free radical as the unpaired electrons from superoxide and nitric oxide have combined to form a new chemical bond (Equation 1.3, Beckman and Koppenol, 1996). While $ONOO^{-}$ is not a free radical, it is a potent initiator of lipid (Radi et al., 1991, Brannan and Decker, 2001) and protein oxidation (Sharpe and Cooper, 1998, Alayash et al., 1998). The high cytotoxicity of $ONOO^{-}$ is due in part to its exceptional stability and long half life, purified $ONOO^{-}$ is stable for days in alkaline solution (King et al., 1992). Peroxynitrite exists in *cis* formation, but protonation (which occurs readily under physiological conditions) to peroxynitrous acid (ONOOH) results in isomerisation towards *trans* configuration which lengthens the O-O bond enabling the terminal oxygen to attack the nitrogen (Tsai et al., 1994). In this excited state peroxynitrite can react like a hydroxyl (•OH) plus nitrogen dioxide radicals ($\bullet NO_2$) (Beckman et al., 1990, Tsai et al., 1994, Beckman and Koppenol, 1996, Figure 1.12). Peroxynitrite can cause S-nitrosation, S-thiolation (sulfhydryl oxidation) and nitration in biological

tissues (Patel et al., 2000) and large quantities of $ONOO^-$ are produced by iNOS in macrophages to destroy invading bacteria (Xia and Zweier, 1997).

Equation 1.3: $^-$:O-O• + •NO→ $^-$:O-O-N-O

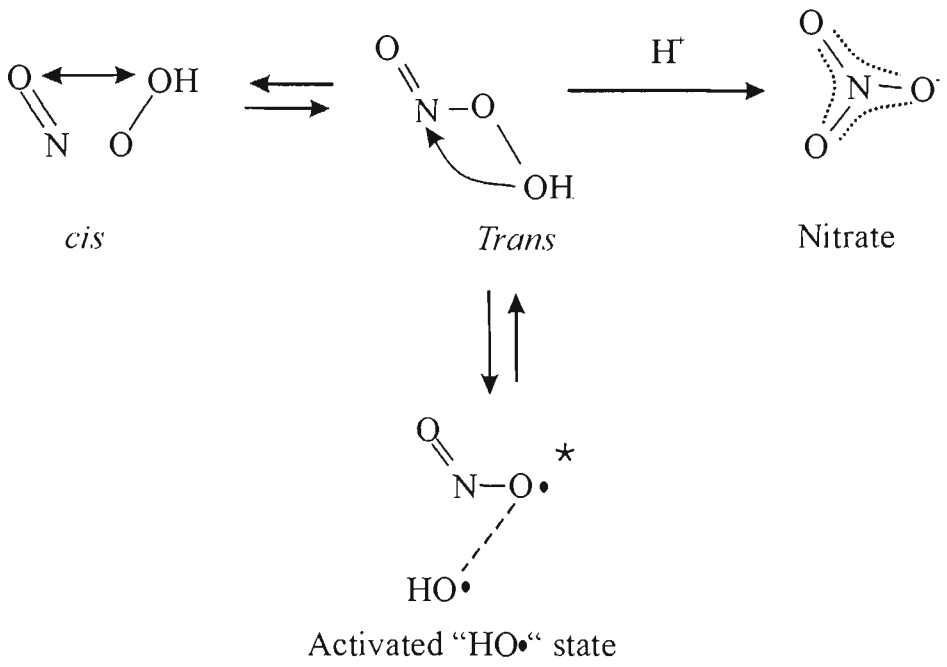


Figure 1.12: Different stereoisomers of peroxynitrite found in biological tissues (Beckman and Koppenol, 1996). Lengthening of the terminal –OH bond in the *trans* confirmation results in peroxynitrite acting like two free radicals.

The nitroxyl anion, formed by reaction of $NO^•$ with O_2 will rapidly react with other $NO^•$ molecules to higher oxides of NO, N_2O and $•NO_2$ (NO_x) (Seddon et al., 1973). The nitroxyl anion may also undergo reversible addition to thiols, resulting in sulfhydryl oxidation (Doyle et al., 1988) and S-nitrosothiols are believed to be a minor product of reactions between NO^- and disulfides. Analogous to $NO^•$, NO^- can also reduce Fe(III) to Fe(II) heme (Doyle et al., 1988, Wink et al., 1991).

1.7.4 Metabolic fate of NO and related oxides in skeletal muscle

Nitric oxide may mediate its effects indirectly via cyclic guanosine monophosphate (cGMP) or directly via oxidation. The heme containing enzyme guanylate cyclase is particularly sensitive to oxidation by NO, increasing cGMP synthesis and propagating the stimulus within the cell (Schmidt et al., 1993). The direct effects of NO depend on the concentration of NO and the surrounding environment. Hart and Dulhunty (2000) observed that the cardiac RyR activity was increased by low concentrations and inhibited by high concentrations of a NO donor. This led to the hypothesis that the effects of NO on contraction are mediated by its concentration. Certainly in the brain, feedback loops with NO and the N-methyl-D-aspartate (NMDA) receptors have been observed (Manzoni et al., 1992). The NMDA receptor activity stimulates NOS activity (Garthwaite et al., 1989), increasing NO concentration. Since the NMDA receptor both stimulates NOS and is inhibited by NO, a feedback mechanism was proposed (Manzoni et al., 1992).

The fate of NO is linked to its surrounding redox environment. For example diffusion of NO synthesised in the endothelium is limited, since NO is rapidly scavenged by serum proteins (Stamler et al., 1992a, Jourd'heuil et al., 2000). While this may appear limiting, restriction of NO to near its site of synthesis ensures that it is able to perform highly specific and localised signalling functions. Dynamic regulation of NO also occurs via the presence of other free radicals. Depending on the type of free radicals present, they could scavenge NO, propagate into other species or even potentiate the effects of NO. For example, peroxynitrite has been observed to induce smooth relaxation and increase cGMP 50 to 1000 fold higher than NO (Liu et al., 1994, Wu et

al., 1994b, Tarpey et al., 1995), yet in other experiments peroxynitrite has been observed to be highly cytotoxic.

1.7.5 Activity of NOS in skeletal muscle

Muscle NOS activity has been quantified by the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline and depending on species, resting skeletal muscle NOS activity generally varies between 2 and 25 pmol.min^{-1}.mg^{-1}, averaging at about 10 pmol.min^{-1}.mg^{-1} (Stamler, 1994). Nitric oxide synthase activity in isolated skeletal muscles is increased during contraction by various electrical stimulation protocols (Kobzik et al., 1994, Reiser et al., 1997, Stamler and Meissner, 2001) and exercise (Roberts et al., 1999). Increases in higher oxides of NO (NO_x) in human plasma have been correlated to both acute bouts of exercise and increases in physical fitness over time (Jungersten et al., 1997). The interplay between NO and muscle function has been demonstrated on a gross scale in numerous experiments. Expression of nNOS and eNOS is increased during exercise training in humans and rats (Balon and Nadler, 1997, Tatchum-Talom et al., 2000). Removal of weight bearing for 10 days reduced nNOS expression in the rat hind-limb, but returned to control levels upon resumption of weight bearing. Inhibition of NOS activity with arginine analogues reduced muscle weight gain and sarcomere addition after atrophy in rats (Koh and Tidball, 1999).

1.7.6 Roles of NO in skeletal muscle and implications for meat quality

The use of NO to improve meat quality is not new as reduction of nitrates and nitrites to yield NO has traditionally been used for curing meat, increasing shelf life and improving colour (Walters and Taylor, 1964, Cornforth, 1996). However, it is only

recently that a link between NO and fresh meat quality, specifically the tenderisation process, has been implicated (Cook et al., 1998). In the study by Cook et al. (1998), NO donors and inhibitors of NOS were injected into hot boned (still metabolically active) meat, improving and reducing meat tenderness respectively (Figure 1.13). However, while a link exists between NO and fresh meat quality, the mechanism by which NO could affect meat quality remains unclear. Following is a brief review of physiological processes important to meat quality that are influenced by NO.

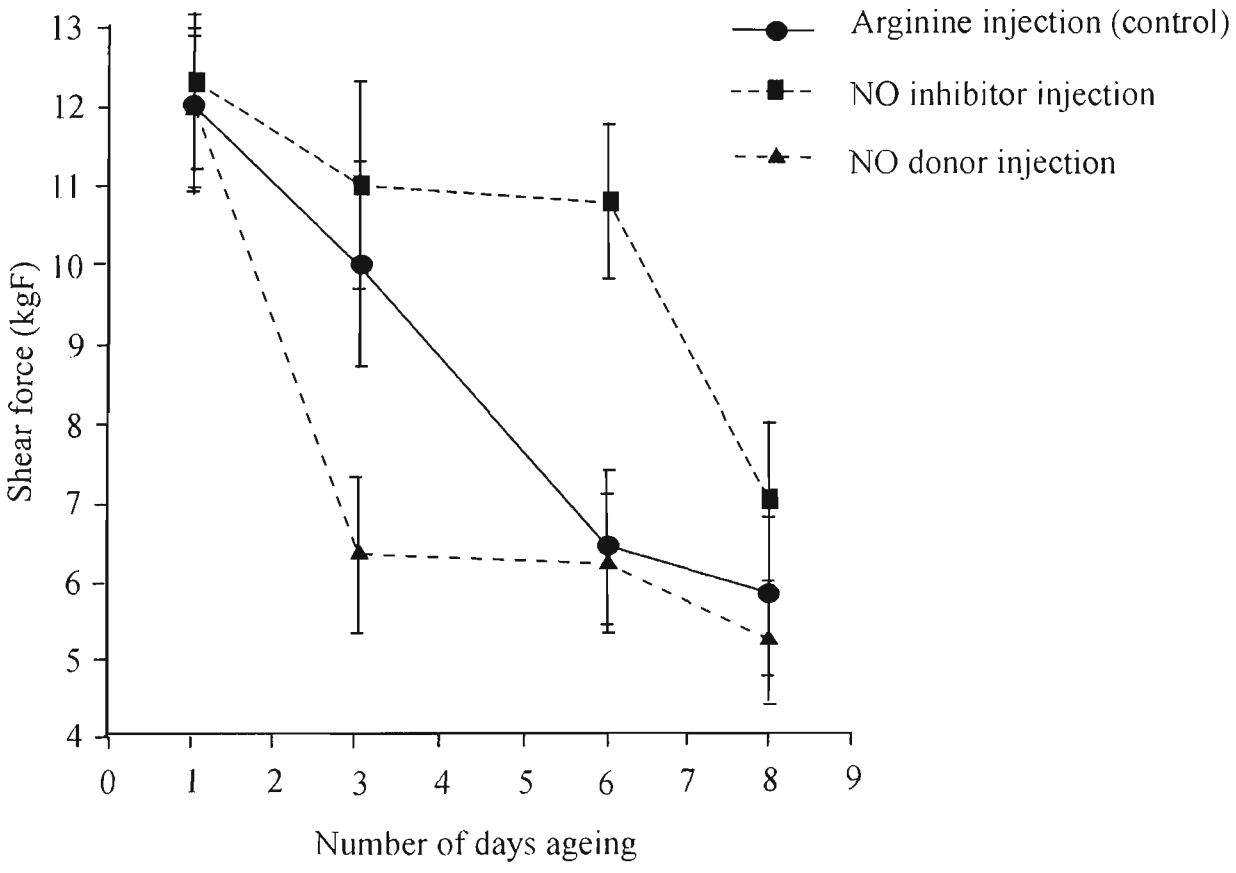


Figure 1.13: Changes in shear force with injection of 100mM NO and NOS inhibitor cocktails or arginine into hot boned beef *Longissimus lumborum* (Cook et al., 1998).

1.7.6.1 Regulation of cellular calcium homeostasis and muscular fatigue

Whilst involvement of NO in regulation of RyR and sarcoplasmic/ endoplasmic reticulum ATPase (SERCA) has been demonstrated in many experiments, results have

been conflicting, suggesting a complex relationship (Meszaros et al., 1996, Stoyanovsky et al., 1997, Zahradnikova et al., 1997, Xu et al., 1998). The effect of NO on the RyR is dependent on the concentration of NO. For example, S-Nitrosylation of RyR by NO was agonistic at low, but antagonistic at high NO concentrations, respectively (Hart and Dulhunty, 2000). As with the RyR, NO has also been observed to increase both activation and inhibition of calcium uptake by SERCA (Ishii et al., 1998, Trepakova et al., 1999, Cohen et al., 1999, Adachi et al., 2001). However, it should be noted that this effect is not restricted to NO, SERCA inhibition by other oxidants has been observed (Scherer and Deamer, 1986, Trimm et al., 1986, Zhang et al., 1999). As reactive nitrogen and oxygen species are increased during exercise, it has been hypothesised that inhibition of calcium release forms a component of muscular fatigue (Reid, 1998). Increasing muscle calcium concentrations *in vivo* and post-mortem impacts on meat quality via stimulating calpain activity (Dransfield, 1993, Koohmaraie, 1994), non-enzymatic weakening of Z-disks (Takahashi, 1992, Takahashi et al., 1987), involvement in cold-shortening (Davey and Gilbert, 1973, Cornforth et al., 1980) and the effect of malignant hypothermia on pork quality (Fujii et al., 1991).

1.7.6.2 Muscle metabolism

Nitric oxide is known to influence many metabolic pathways in muscle including glycolysis (Dimmeler et al., 1992, Zhang and Snyder, 1992, Mohr et al., 1994, Depre et al., 1998), mitochondrial respiratory chain (Cleeter et al., 1994, Brown, 2000, Clementi et al., 1998), glycogenolysis (Borgs et al., 1996) and ATP recycling (Gross et al., 1996, Kaasik et al., 1999, Konorev et al., 2000). Nitric oxide mediates vasodilation (Ignarro et al., 1987, Palmer et al., 1987) and therefore nutrient delivery

to muscle. Nitric oxide is also involved in propagating the effects of some hormones. In particular, insulin increases cGMP levels in cultured smooth muscle cells (Kahn et al., 2000) and insulin resistance has been observed with inhibition of NOS (Sadri and Lautt, 1999). Furthermore, NO increases glucose uptake, possibly by translocation of glucose transporters (Etgen et al., 1997). This indicates that NO could have a significant impact on meat quality via regulation of muscle metabolism.

1.7.6.3 Oxidative regulation of proteolysis

Oxidative damage to proteins is an adjunct of aerobic metabolism and removal of oxidatively damaged proteins by proteolytic enzymes is integral to normal cell cycling (Mehlhase and Grune, 2002). However, the proteolytic enzymes themselves are in turn regulated by reactive nitrogen and oxygen species. While proteasome and cathepsin activities are sensitive to oxidation (Strack et al., 1996, Obin et al., 1998, Percival et al., 1999), it is the interactions with calpain that may prove most important for meat quality. This can occur indirectly as oxidative stress can increase cellular calcium and increase calpain activity resulting in cytoskeletal damage (Miyoshi et al., 1996, Mehlhase and Grune, 2002). As with other physiological systems, the effects of oxidation on calpain activity are concentration dependent. Pronzato et al. (1993) observed that low levels of carbon tetrachloride (CCl_4^-) induced injury stimulated calpain activity, while high doses of CCl_4^- inhibited calpain activity.

Alternatively, inhibition of calpain can occur via direct interactions. Calpain contains cysteine (Sorimachi et al., 1997), making it susceptible to S-nitrosation by NO. Reversible inhibition of calpain by NO donors has been observed in separate studies (Michetti et al., 1995, Koh and Tidball, 2000, Rackoff et al., 2001). However, in what

may constitute a cell signalling pathway, NOS isoforms are sensitive to degradation by proteasome (Musial and Eissa, 2001) and calpain (Walker et al., 2001).

1.7.6.4 Lipid and myoglobin oxidation

Oxidation of lipids results in rancidity and associated off colours and brown colour, reducing shelf life and quality of meat and meat products (Gray et al., 1996). It has been proposed that NO may initiate lipid peroxidation through its ability to react with superoxide to form peroxynitrite (Brannan et al., 2001, Brannan and Decker, 2001). As discussed in section 1.7.3, peroxynitrite is a potent initiator of lipid peroxidation. Nitric oxide also reacts with the heme pigment myoglobin, as evidenced by the pink colour of cured meats that occurs through the formation of nitrosomyoglobin (Hultin, 1985, Cornforth, 1996). However, it is not known whether endogenous concentrations of NO in muscle post-slaughter are sufficiently high to significantly alter lipid peroxidation or meat colour.

1.8 Types of pharmacological donors of NO and inhibitors of NOS

Pharmacological compounds are used to experimentally increase or decrease the effects of free NO in a biological system. The following is a brief review of some commonly used NO donors and NOS inhibitors. As discussed in section 1.7.3 the term "nitric oxide" is used to loosely describe the many redox states of NO and higher oxides (NO_x). Similarly, different NO donors can be used to release NO in different redox states to mimic various physiological events.

A commonly used NO donor is the metal-NO complex sodium nitroprusside (SNP), which undergoes reductive activation to yield NO^+, as occurs in biological tissues

following reactions between NO and Fe (Schulz, 1984, Bates et al., 1991). The reaction between endogenous thiol and NO can also be mimicked using S-nitrosothiol donors such as S-*nitroso*-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione. In physiological buffers, S-nitrosothiols undergo relatively rapid homolytic fission of the S-N bond to produce disulfide and NO (Nguyen et al., 1992, Feelisch, 1998, Al-Sa'doni and Ferro, 2000). Donors such as molsidomine are prodrugs and require enzymic activation to NO producing sydnonamines, which can be used to replicate the effects of peroxynitrite by production of NO and O_2^- (Bergstrand et al., 1984, Rosenkranz et al., 1996, Noack and Feelisch, 1989). Other donors such as the nucleophile/ NO adducts diazeniumdiolates and NONOates release NO by spontaneous dissolution in aqueous media (Maragos et al., 1991, Hrabie et al., 1993, Keefer, 1998). Alternatively guanylate cyclase agonists and antagonists, cGMP and phosphodiesterase inhibitors can be used to manipulate NO and cGMP dependent signalling cascades.

Alternatively, endogenous enzymatic NO production can be antagonised with arginine analogues. Arginine analogues are not suicide substrates as their inhibition of NOS can be overcome with an excess of arginine. Rather, arginine analogues are competitive inhibitors of NOS (Klatt et al., 1996, Griffith and Gross, 1996). There are advantages and disadvantages in using both NO donors and NOS inhibitors to study effects of NO in biological systems. Delivery of NO donors often lacks specificity inherit to physiological NO production, while NOS inhibitors do not mimic NO production, but rather remove the influence of NO on the system measured

1.9 Conclusions

Meat quality is dependent on a multitude of factors. Some of these are the level of stress pre-slaughter and interaction with post-slaughter processing factors. While much is already known about how these factors affect meat quality, little is known about the physiological regulation of some of these processes. Nitric oxide offers new insights into the regulation of these processes since it interacts with many of the physiological stimuli that determine meat quality.

While NO has been demonstrated to affect meat quality (Cook et al., 1998), many questions remain unanswered. Cook et al. (1998) investigated the effects of single concentrations of NO and NOS inhibitors on meat quality. Since the effect of NO is frequently concentration dependent, the effects of differing concentrations of NO donors and inhibitors need to be examined, particularly if the concentrations used by Cook et al. (1998) may not represent enzymatic NO production.

The mechanisms by which NO may affect meat quality are unclear. It has been demonstrated that NO plays an important role in calcium homeostasis. This may influence tenderisation of meat by enzymatic means, increasing calpain activity, or non-enzymatic weakening of Z-disks. However, NO has been demonstrated to directly inhibit the activity of calpain, suggesting it may be part of a complex system whereby NO participates in activation or inhibition of calpain activity.

The rates of post-mortem pH fall and muscle metabolism are an important factor in determining meat quality. Nitric oxide has a regulatory role in muscle metabolism, including roles in muscle glucose uptake and glycolysis. Since NOS is part of a

second messaging system, relaying signals from hormones to intracellular targets, NO may play a role in nutrient uptake in muscle. Under certain conditions, NO has also been demonstrated to play a role in pathophysiological events leading to cell death. The ramification of this on meat quality has not been investigated. It is highly likely that NO is involved in the stress response in livestock due to its involvement in muscle contraction and potentiation of hormonal signals.

1.10 Aims of thesis

1. To determine whether NO donors and inhibitors injected into hot boned muscles influence tenderness, pH and oxidation.

2. To determine whether injection of NO donors and inhibitors into hot boned muscles provides a suitable model to study the effects of NO on meat quality.

3. To determine if NO influences sequestration of calcium ions into the SR via the RyR's and SERCA's as a potential mechanism of activating endogenous calpains.

4. To quantify the metabolic effects of systemic NOS inhibition with L-arginine methyl ester hydrochloride (L-NAME) on whole body and hind-limb (predominantly skeletal muscle) metabolism.

5. To determine the temporal pattern of metabolic response to L-NAME infusion.

6. To determine whether pre-slaughter inhibition of NOS with L-NAME influences meat quality.

7. To determine whether L-NAME influences the effect of exercise on muscle metabolism and meat quality.

Chapter 2: Measurement of arginine diffusion through hot boned beef *m. Longissimus lumborum.*

2.1 Introduction

The molecule nitric oxide (NO) has a diverse range of physiologically significant functions in skeletal muscle. Cook et al. (1998) demonstrated that NO plays a role in meat tenderness, finding that hot boned beef tenderness was improved following injection of pharmacological donors of NO and reduced after injection of NOS inhibitors.

Inhibition of NOS is routinely performed with structural analogues of L-arginine. Arginine analogues (AA) inhibit NO production by competing with endogenous L-arginine as substrates for NO production by NOS. Injection of L-arginine or AA's into meat to study the effects of NO on meat quality poses methodological difficulties since L-arginine is a highly basic amino acid (Moran et al., 1994a). Therefore injection of L-arginine or AA's may increase muscle pH or buffer post-mortem pH fall, confounding measurements on meat quality. Delivery of AA into muscle fibres of hot boned muscle poses obstacles since there is no longer an active circulatory system and metabolic activity is reduced. Beef *Longissimus lumborum* (LL) has variable rates of glycolysis, pH fall and water holding capacity, depending on proximity to the meat surface (Tarrant and Mothersill, 1977). Therefore, to maximise inhibition of NOS in hot boned beef LL, injected L-arginine or AA's will need to diffuse away from their injection sites. If diffusion of AA's from the site of injection is limited, inhibition of NOS will be localised around the site of injection, resulting in increased variability of the hot boned muscle. It is expected that this would increase

the error around any measurements of meat quality, particularly those that require small tissue samples. Finally, it is important that concentrations of injected compounds lost in the meat purge are insignificant, indicating retention within the tissue.

2.2 Aim

To determine if injection of 10% w/w 0.1M arginine in a 2cm by 1cm matrix is a suitable model for studying the effects of arginine analogues on hot boned beef LL meat quality.

2.3 Hypotheses

1. Injection of 10% w/w 0.1M L-arginine solution will not slow pH fall in hot boned LL.
2. Injected ^{3}H-L-arginine will diffuse away from the site of injection within hot boned beef LL tissue as determined by the decrease in the coefficient of variation (%CV) of tissue sub-samples.
3. The level of injected ^{3}H-L-arginine lost from hot boned LL in purge loss will be insignificant.

2.4 Methodology

2.4.1 Sample collection and preparation

Seven beef LL muscles, each weighing approximately 2-3kg and 40-60cm long were hot boned from carcasses derived from male Hereford or Hereford cross cattle (hot

carcass weight 191-244 kg) from a local commercial abattoir at approximately 25 minutes post-slaughter. One LL was collected on each visit to the abattoir and each loin was transported approximately 20 minutes in an insulating container to the meat laboratory. The large size of the first LL obtained enabled it to be halved and performed two replicates of the experiment on the muscle. Each LL was trimmed of dorsal adipose tissue and cut into five steaks (transverse to the carcass mid line) ~4cm thick and approximately 10cm of remaining LL kept for determination of pH. Each portion was randomly allocated to one of five sampling time-points; 0, 3, 6, 9, or 24 hours post-injection, then injected with a solution of L-arginine (0.1 M), ^{3}H-L-arginine (^{3}H-L-arg, 0.1μCi/mL) in .09% NaCl (saline) to 10%w/w. The injection solutions and samples were stored at room temperature (22-25°C) before injection and the steaks were stored in plastic bags at 2°C post-injection. Injection was performed approximately one hour post-slaughter, using a single syringe and 18-guage needle across a 1cm by 2cm matrix approximately 1cm deep through the epimysium. The matrix was every 1cm transverse and every 2cm along the midline (Figure 2.1).

2.4.2 Measurement of muscle pH

Muscle pH was measured using portable pH meter with temperature probe (model 6009, Jenco Electronic Ltd, USA) and polypropylene spear type gel electrode (Ionode IJ421, Australia). The pH of the injection solution was 10.6 and control steaks for pH determination were not injected.

2.4.3 Liquid scintillation counting

Before quantification of ^{3}H-L-arg at the aforementioned time-points, steaks were trimmed of external surfaces before cutting the remaining sample into six

approximately equal pieces. Sub-samples (2g) were taken and homogenised with an ultra-turrax dispersing head (Ultra-turrax T25B, IKA Works, Malaysia) in 10mL of 10% TCA for 2 minutes and then centrifuged at 10,000g for 15 minutes. The individual 2g sub-samples were considerably smaller than the distance between injection sites to enable accurate determination of arginine diffusion. Disintegrations per minute (DPM) of meat samples was determined using a Wallac 1410 liquid scintillation counter (LSC) with 2mL of supernatant added to 13mL of scintillation fluid.

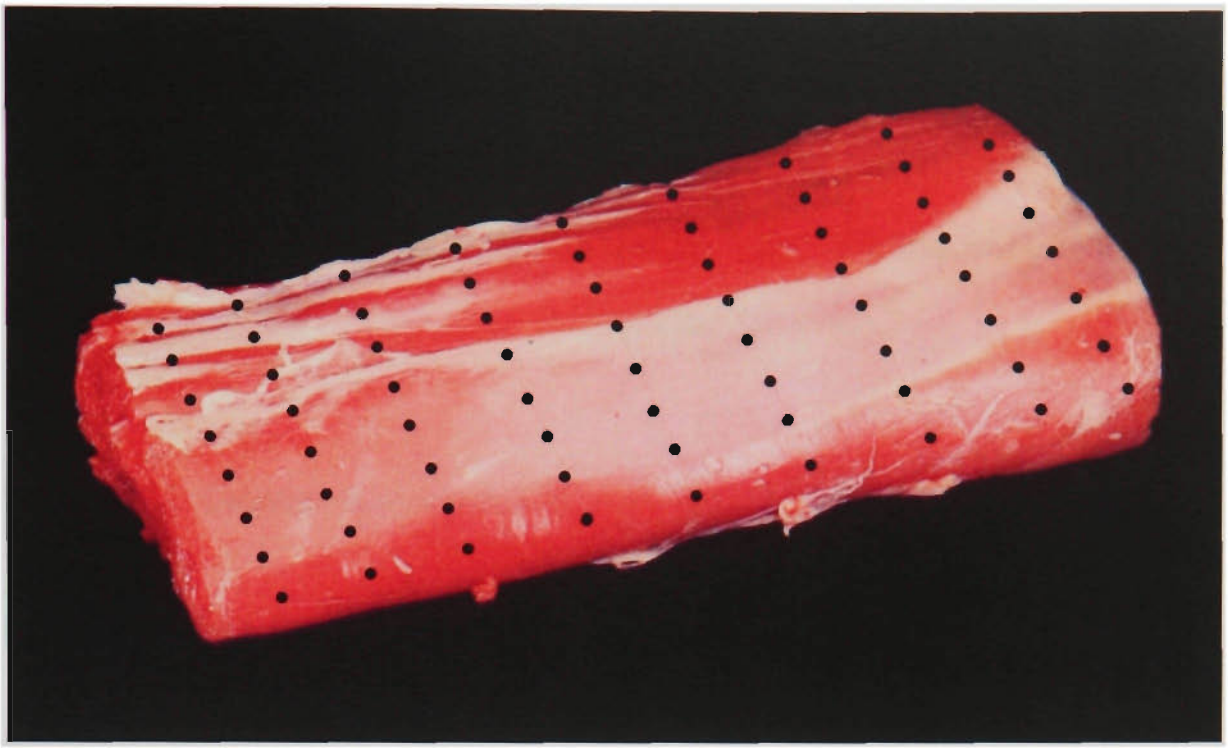


Figure 2.1: Representation of injection sites across beef LL muscles. Injections were performed every 2cm along the length and 1cm across the width of the loin. Figure representation only, not to scale and individual steaks, not the whole loin was injected. Injection sites represented by black dots.

The arginine retention was determined as the ratio of ^{3}H-L-arg injected (per gram) (Equation 2.1) to the ^{3}H-L-arg counted (Equation 2.2) in DPM per gram (detected:injected ratio) at each time point. Arginine diffusion was measured by calculating the percent co-efficient of variation (%CV) of each steak at each time (Equation 2.3).

Equation 2.1: Calculation of DPM of injected ^{3}H-L-Arginine:

$$= \text{DPM (stock) mL} * \frac{((\text{post-inj. wt (g)} - \text{pre-inj. wt (g)}) \times 1000\text{mL})/\ \text{wt 1L stock (g)}}{\text{post-injection wt (g)}}$$

Equation 2.2: Calculation of detected/injected ratio:

$$= \frac{\text{DPM detected} \times (\text{TCA added (mL)} + (\text{Wt sample (g)} \times \%\ H_2O}{\text{Sample wt (g)} + \text{sample volume (mL)}}$$

Equation 2.3: Calculation of the %CV.

$$= \frac{\text{St deviation actual/injected} \times 100\%}{\text{Average actual/injected}}$$

2.4.2 Statistical analyses

All statistical analyses were performed using Genstat 5.41 (Payne et al., 1993). Post mortem pH fall was tested by a paired two tailed t-test on the slope of the simple linear regressions of the control and injected meat samples respective pH curves. The change in arginine retention and diffusion with respect to time were tested by ANOVA's on the natural logarithm of the raw data. The quadratic regression on the log scale was then back transformed and graphed.

2.5 Results

Figure 2.2 displays the adjusted means for muscle pH of the control and arginine injected steaks at 0, 3, 6, 9 and 24 hours post-injection. Muscle pH fell from initial values during injection of approximately 6.5, to approximately 5.6 at 24 hours post-mortem. The rate of pH fall was unaffected (P=.73) by arginine injection.

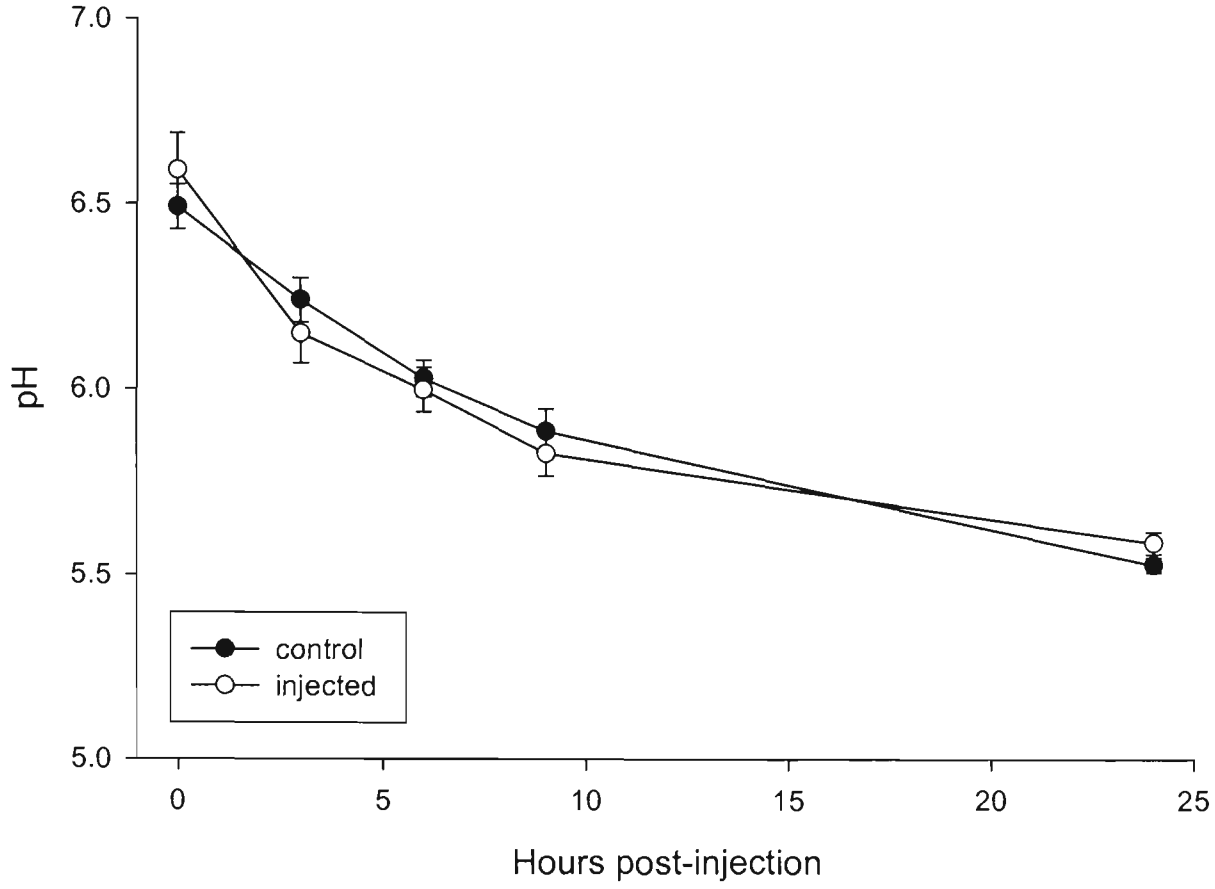


Figure 2.2: Effect of treatment (control v. 0.1mL-arginine) on the rate of pH fall post-injection.

Figure 2.3 shows the degree of arginine diffusion in hot boned LL muscles post-mortem, depicted as the decrease of the back transformed mean %CV. The log_{10} transformed values of the %C.V. at 0, 3, 6, 9 and 24 hours were 1.72, 1.64, 1.66, 1.51 and 1.53 ± .0721 respectively. The %CV decreased (P=.036) with time with maximum diffusion occurring between 6 and 9 hours post-injection.

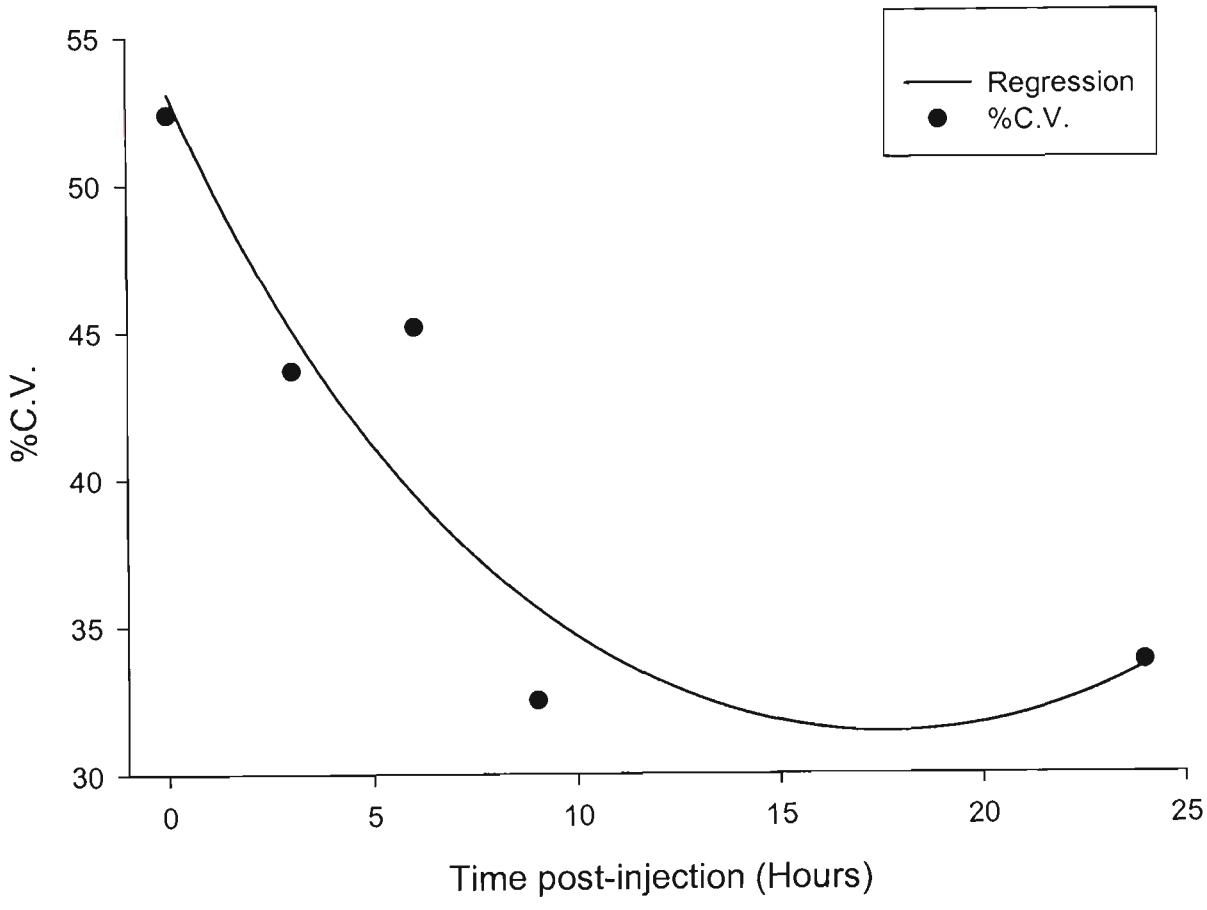


Figure 2.3: Degree of arginine diffusion in hot boned LL muscles post mortem. Depicted as the decrease of the back transformed mean %CV.

Arginine retention as depicted by the detected:injected DPM per gram ratio in Figure 2.4 decreased up to 6 hours post-injection, after which time loss of ^{3}H-L-arginine in the hot boned beef LL was minimal. The reduction in the detected:injected ratio was approximately 18% and was not statistically significant (P=.13) from zero.

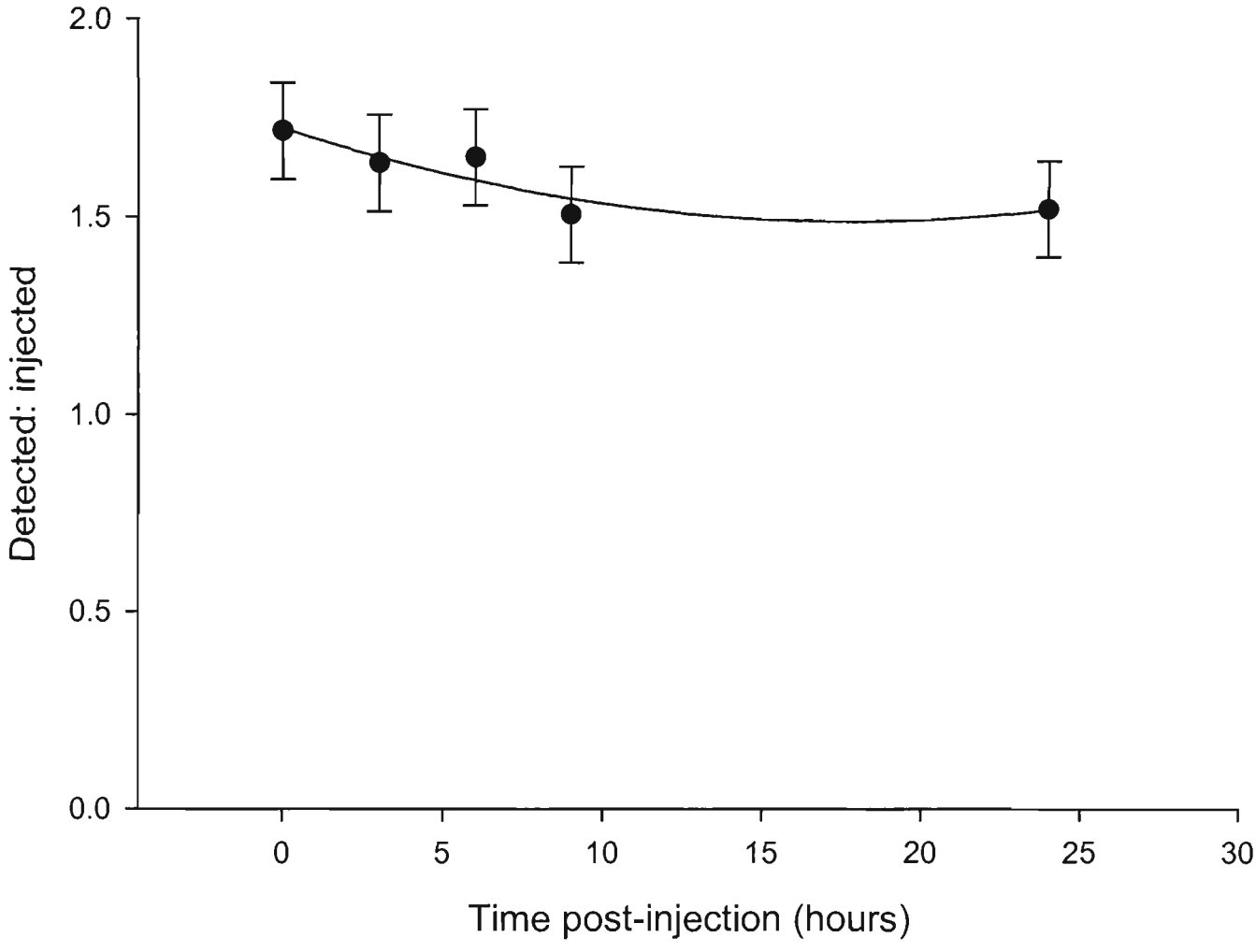


Figure 2.4: Retention of ^{3}H-L-arginine with time in injected hot boned LL muscle samples assessed by the ratio of the DPM of ^{3}H-L-arginie detected in the meat to the DPM of ^{3}H-L-arginine injected.

2.6 Discussion

Post-mortem pH fall in meat is a result of the accumulation of metabolic by-products in the muscle tissue. Differences in muscle glycogen levels at slaughter and in glycolytic rates post-slaughter can affect the rate and extent of muscle pH fall, which can significantly affect meat tenderness and quality (Pethick et al., 1995, Watanabe et al., 1996, Tarrant, 1989). Since arginine is a basic amino acid and the 0.1M L-arginine injection solution had a pH of 10.6, it was possible that the pH fall could be artificially changed by use of the injection solution 10%w/w. This in turn could affect meat quality, particularly tenderness (Marsh et al., 1980-81). However, these data support hypothesis 1, showing that steaks injected 10%w/w with 0.1M L-arginine were not significantly different in their pH fall to non-injected steaks. Therefore, injection of arginine or arginine based solutions up to 0.1M in concentration and 10% w/w is not likely to slow pH fall of hot boned beef LL.

Injection of solutions into meat samples to modulate meat tenderness is not new. Injection of calcium chloride ($CaCl_2$) into pre and post-rigor muscle (Koohmaraie and Shackelford, 1991, Clare et al., 1997) and lactic acid (Berge et al., 2001) has been successful in improving tenderness. Calcium chloride and lactic acids have smaller molecular weights (111 and 90.08 respectively) than arginine (MW 174.2) and due to their smaller size should diffuse more readily through muscle tissue. It is possible that diffusion of amino acids through muscle tissue may be restricted, making it important to determine the level of diffusion of amino acids in hot boned muscles. This experiment measured diffusion with radioisotopes rather than dyes to simulate the diffusion of arginine or similar molecules rather than chemically dissimilar dyes. Diffusion of L-arginine was determined by the decline of the %CV between six sub-

samples randomly taken from each steak over time. Arginine did diffuse from the site of injection over the period 0 and 24 hours post-injection, with maximum diffusion occurring between 6 and 9 hours post-injection, supporting hypothesis 2. If AA's are to be used to inhibit NOS activity in further experiments, they will need to diffuse away from the site of injection, or the result will be a series of localised effects. Many measurements of meat quality require small amounts of tissue and thus poor diffusion will lead to increased sample variability and an artificially increased error. Therefore, to decrease the error around meat quality measurements, injected arginine, or AA's, should diffuse away from injection sites. These data indicate that arginine does diffuse from its site of infusion, to parts of the LL not directly injected with L-arginine.

Arginine retention within the hot boned beef LL's was measured by the ratio of detected ^{3}H-L-arginine to injected ^{3}H-L-arginine. The decrease of the ratio from 1.313 to 1.083 was not statistically significant, supporting hypothesis 3, but represented a loss of approximately 18% of the injected arginine. The ratio of arginine injected/measured was larger than 1 indicating that arginine does not diffuse well in hot boned LL. However it is likely that this was due to the method of injection, since injections were not performed on the outer edges of the sample, but rather 1cm into the steak. The outer edges were trimmed and discarded before determination of DPM, since they are exposed to drip loss (containing ^{3}H-L-arginine) from the sample, but the ratio is calculated on the weight of the whole steak. Therefore the high ratio reflected the tendency for injecting into the centre of the steak to increase retention of the injection solution.

2.7 Conclusion

The data from this experiment shows that injection of arginine 0.1M 10%w/w across a 2cm by 1cm matrix does not change hot boned beef LL pH fall. Injected arginine diffuses away from the injection site without significant levels being lost in purge. Therefore, the model meets the criteria selected for studying the effect of NOS inhibition with AA's on meat quality.

Chapter 3: The influence of different concentrations of a donor and inhibitor of nitric oxide on hot-boned beef Longissimus lumborum meat quality.

3.1 Introduction

In addition to inhibiting bacterial growth on meat, nitrites also stabilise meat colours via the formation of nitric oxide (NO), which reacts with heme pigments in meat to produce the pink colour of cured meat products after heating (Lindsay, 1985). However, studies on the effects of NO on fresh meat quality have received little attention. Nitric oxide influences multiple physiological pathways in muscle that are important to meat quality, including glycolysis and calcium homeostasis. In one of the few studies to investigate the role of NO in post-slaughter muscle metabolism, Cook et al. (1998) soaked hot-boned beef *Longissimus lumborum* (LL) in solutions of pharmacological donors of NO and inhibitors of nitric oxide synthase (NOS) for approximately 2 hours post-slaughter. Tenderisation during ageing post-mortem was accelerated with NO donors and inhibited with inhibitors of NOS, suggesting that NO can affect beef tenderisation.

NO is a small, gaseous, locally acting, lipophilic molecule, able to diffuse within and between cells (Lancaster, 1997). Nitric oxide is enzymatically synthesised from cleavage of the guanidino group from L-arginine by NOS. There are three isoforms of NOS, the nomenclature of the different isoforms follows the tissues they were originally isolated from. The neuronal isoform (nNOS) is the most prevalent isoform

in skeletal muscle (Kobzik et al., 1994) where it is found in higher levels than in the brain (Nakane et al., 1993).

Nitric oxide is also a free radical, and reacts with intracellular thiol, iron-sulfur clusters and heme targets present in many enzymes, receptors and ion channels, enabling NO to regulate many cellular processes (Stamler, 1994). While NO is a free radical, it possesses low reactivity with lipid membranes and has a limited effect on lipid peroxidation under physiological conditions (Beckman and Koppenol, 1996). Background levels of NO in muscle are in the low picomolar range (Roberts et al., 1999), and are likely rapidly scavenged by myoglobin (Eich et al., 1996) and other cellular anti-oxidants (Wakulich and Tepperman, 1997). Nitric oxide itself may act as an antioxidant (Kroncke et al., 1997), but under conditions of oxidative stress, NO can propagate with other free radicals to form peroxynitrite ($ONOO^-$), the degradation products of which are highly reactive with lipid membranes (Beckman et al., 1990, Radi et al., 1991).

The modulation of tenderness observed by Cook et al. (1998) may be a product of multiple factors influenced by NO. Nitric oxide influences calcium homeostasis through ryanodine receptors (Meszaros et al., 1996, Suko et al., 1999, Eu et al., 1999) in the sarcoplasmic reticulum (SR). It has been hypothesised that intracellular calcium can tenderise meat via direct degradation of the myofibrillar structure by Z-disk weakening (Takahashi et al., 1987, Takahashi, 1992) or by calcium dependant proteases such as the calpain proteolytic system (Dransfield, 1993). Direct (Michetti et al., 1995) and indirect (Koh and Tidball, 2000, Rackoff et al., 2001) inhibition of calpain activity with NO has been observed experimentally. Nitric oxide clearly plays

a substantial role in muscle physiology and in the production of cured meat products, but the effects of NO and NOS activity on fresh meat quality is poorly understood.

3.2 Aims

To determine if injection of the NO donor sodium nitroprusside (SNP) and a NOS inhibitor cocktail (NOS_i) of 90% L N^G-N-nitro-L-arginine methyl ester hydrochloride (L-NAME) and 10% N-nitro-L-arginine (L-NOARG) on meat quality and to establish whether a dose-response relationship exists between 0, 1, 10 and 100mM SNP and L-NAME/ L-NOARG.

3.3 Hypotheses

1. Neither SNP nor NOS_i will change pH fall in the *Longissimus lumborum* (LL) post-slaughter.
2. SNP will increase tenderness in hot-boned beef LL.
3. NOS_i will increase toughness in hot-boned beef LL.
4. SNP will increase oxidation in beef LL.
5. NOS_i will not affect oxidation or colour.

3.4 Materials and Methods

3.4.1 Experimental design

Forty-two LL muscles from Hereford or Hereford cross cattle between 191 and 244 kg hot carcass weight were hot-boned approximately 25 minutes post-slaughter at a local abattoir over 5 separate visits. Before hot-boning, carcasses were electrically

stimulated (180V) with a rigidity probe during hide removal for approximately 5 seconds, 10-15 minutes post-slaughter. Muscles were transported to the laboratory and trimmed of surface fat, overlying muscles and connective tissue and cut into steaks as described in section 2.4.1. Five steaks were cut from each LL and randomly allocated to an ageing period of 1 day (15cm thick), 2, 4, 7 or 14 days (4cm thick).

3.4.2 Injection of SNP and NOS_i

Each LL was randomly allocated to one of seven treatments: control- saline (0.9% NaCl), saline plus 1, 10 or 100mM sodium nitroprusside (SNP) (cat. S-0501 Sigma Aldrich, USA,) or saline plus 1, 10 or 100mM NOS inhibitors (NOS_i). The inhibitor consisted of 90% L N^G-N-nitro-L-arginine methyl ester hydrochloride (cat. 80210 L-NAME, Cayman Chemical Company, USA) and 10% N_ω-nitro-L-arginine (cat. N-5501L-NOARG, Sigma Aldrich, USA) molar equivalent for each concentration. The treatment solutions were injected 10% w/w, approximately 1 to 2 hours post-slaughter using the same injection pattern outlined in section 2.4.1. Steaks used for analyses after 1-day were vacuum packed and stored at 2°C until required.

3.4.3 Muscle pH

Post-injection pH fall was measured on the steak used for analysis at 1 day post-mortem. The steak was kept in a plastic bag at 2°C and pH measured as described in section 2.4.2 at 30mins, 2, 3, 4, 5, 6, 9 and 24 (pH_u) hours post-injection. After the final pH reading, the end of the sample was trimmed and a 2cm thick steak cut, placed on a foam tray with plastic over-wrap and displayed under constant fluorescent light at 2°C for 14 days.

3.4.4 Surface colour

At 24 hrs and 14 days post-slaughter, the pH and colour after a 20 min bloom at 2°C (CR 200 Minolta Chromameter, Minolta, Japan) were measured. Colour measurements were taken in the CIE - L^*, a^*, b^* system (where L* measures relative lightness, a* relative redness and b* relative yellowness), with the average of three measurements taken across the same cross section of muscle avoiding areas of connective tissue or intramuscular fat. The chromameter was calibrated on a red tile (Y=15.6, x=.446, y=.313) before measuring colour. The measuring head had D_{65} lighting with a 2° standard observer and 8 mm aperture.

3.4.5 TBARS assay

Lipid oxidation was measured by the TBA (2-thiobarbituric acid) assay based on a modification of the method described by Witte et al. (1970). Modifications to the assay included use of an ULTRA-TURRAX rather than a blender, homogenisation of 10g of muscle, rather than 20g and centrifugation of homogenised muscle at 4000 x g for 10 minutes before filtration. Meat samples were taken from the centre of the muscle 24 hours post-mortem and after 14 days from displayed samples and immediately processed. Samples were diced and homogenised on ice in 50 mL chilled distilled water for 1 minute with an ultra turrax dispersing head at approx 19,000rpm. A further 50 mL of 20% TCA (trichloroacetic acid) and 2M H_3PO_4 (orthophosphoric acid) was added and the sample homogenised for a further 15 sec. Forty mL of the homogenate was centrifuged at 4,000 x g and –1°C for 20 minutes and the supernatant filtered through Whatman N°3 filter paper. Four mL of filtered

supernatant and 4mL of 5mM TBA were added to 10mL polypropylene tubes and incubated in the dark for 15 hours at room temperature (approx. 22°C), with a standard curve constructed of TEP (1,1,3,3-tetraethoxypropane) standards (Sinnhuber and Yu, 1958). Absorbance (533nm) of TBA reactive substances (TBARS) were determined spectrophotometrically (U-2000, Hitachi Ltd, Tokyo, Japan)

3.4.6 Warner Bratzler Shear Force

Sub-samples (150g) for Warner-Bratzler shear force (WBSF) were removed from samples at 1, 2, 4, 7 and 14 days post-slaughter, placed in a plastic bag, suspended in a hot water bath and cooked at 80°C for 1 hour. Samples were then cooled for 30 minutes in running water, blotted dry and stored at 2°C overnight. The following day, meat samples were cut into strips approximately 5cm long, oriented parallel to the grain of the muscle fibres with a $1cm^2$ cross section and WBSF measured with a texture analyser (model 4465, Instron, USA) fitted with an inverted V-blade and cross head speed of 300mm/min perpendicular to fibre orientation (Bouton et al., 1971). Shear force values represent the mean of a minimum of six readings (shears) on separate muscle strips per sample.

3.4.7 Myofibrillar fragmentation index

Myofibrillar fragmentation index (MFI) was measured on muscle samples (approx. 20g) wrapped in aluminium foil and flash frozen in liquid nitrogen before storage at –70°C until analysis. Myofibrillar fragmentation index was measured at time-points coinciding with WBSF measurements by the method described by Culler et al. (1978). Samples were thawed and 2g duplicates weighed and homogenised in 20mL ice cold

MFI buffer (MB) (100mM KCl, 10mM KH_2PO_4, 10mM K_2HPO_4, 1mM ethylene glycol-bis (β-aminoethyl ether)- N,N,N',N'- tetraacetic acid (EGTA), 1mM $MgCl_2$, 1mM NaN_3) with an dispersing head (Ultra-turrax T25B, IKA Works, Malaysia) for 30 seconds while being maintained at ice cold temperatures. Homogenates were centrifuged at 1000-x g for 15 minutes at 2°C, the supernatant was then discarded while the pellet and fat cap (if present) were retained. The pellet and fat cap were washed by suspension in 20 mL MB using a plastic spatula and centrifuged as before. The supernatant and fat cap were then discarded and the pellet containing the myofibrils re-suspended in 20mL MB. Connective tissue was removed by pouring the washed and resuspended pellet through 4-ply gauze. The tube was rinsed with a further 10mL of MB and re-poured through the gauze.

Protein concentration in the sample was determined by the Biuret method (Gornall et al., 1949) in duplicate by adding 3mL of Biuret reagent (1.5g $CuSO_4$, 6g NaK tartate, 30g NaOH in 1L dH_2O) to 250μL of suspension and 750μL of MB. Standards between 0 and 1mg/mL Bovine Serum Albumin (BSA) were prepared from 4mg/mL stock solution. Stock solution BSA concentration was calculated from Equation 3.1 before conducting the assay. Samples and standards were incubated at room temperature in absence of light for 30 minutes and A_{540} read spectrophotometrically. Duplicate suspensions were diluted to 0.5mg/protein in MB, vortexed and A_{540} on duplicates of the original sample was read (4 readings/sample). MFI was calculated as the mean of A_{540}*200, MFI values are unitless.

Equation 3.1: Determination of BSA concentration by its ultraviolet absorbance (Wilson and Walker, 2000).

$$\text{BSA concentration (mg/mL)} = \frac{(A_{280} - (A_{320} * 1.7)}{0.66}$$

3.4.8 Statistical analyses

The experiment comprised of injection of saline and 1, 10 and 100mM of NOS_i and SNP. Control (saline) injections represented a 0mM concentration of NOS_i and SNP respectively and the effects of SNP or NOS_i concentration were analysed relative to 0mM. The effects of SNP and NOS_i relative to each other were not analysed. The effect of time and concentration and the interaction between time and concentration were analysed within SNP or NOS_i using an ANOVA with blocks placed on each LL within each kill. Where an effect of concentration was observed, linear and quadratic regressions were used to describe the type of dose-response relationship between 0,1, 10 and 100mM concentrations each of SNP or NOS_i. All statistical analyses were performed using Genstat version 5.4.1 (Payne et al., 1993) and all errors are presented as the standard error of the difference (SED). Data from the TBARS assay were not normally distributed and ANOVAs were performed on log transformed means, values are presented as transformed means, except for Table 3.1, where back transformed means are also included.

3.5 Results

Muscle pH decreased with time post-slaughter (P<.001) from approximately 6.40 at 30 min post-injection to an ultimate (pH_u) of 5.61 at 24 hours post injection. The LL pH post-mortem was unaffected by SNP (5.97, 5.94, 5.98 and 6.02 ± .102 for 0, 1, 10 and 100mM, P=.90) or NOS_i (5.97, 5.96, 5.95 and 5.99 ± .078, P=.96) injections and no interaction between concentration and time was observed for either SNP (P=.60) or NOS_i (P=.53, Figure 3.1A, 3.1B).

The LL WBSF decreased between 1 and 14 days post-mortem from approximately 11 kg/cm^2 to 5.5 kg/cm^2 (P<.001). No effect of SNP (7.97, 8.54, 9.77 and 8.81 ± 1.175, P=.50) or NOS_i (7.97, 8.67, 8.10 and 8.57 ± 1.251. P=.93) on LL WBSF values were observed. Likewise, the interaction with time post-mortem and injection of SNP (P=.73) or NOS_i (P=.93) was not significant (Figure 3.2A, 3.2B). The MFI increased with time post-mortem from approximately 50 to 80 (P<.001 for SNP and NOS_i). An inverse relationship between MFI values and SNP concentration was observed (70.7, 67.7, 58.7 and 50.1 ± 6.47, P=.019), and was found to be linear (P=.005). No interaction between SNP and time was observed (P=.61, Figure 3.3A). Overall, injection of NOS_i did not affect MFI values (70.7, 60.7, 64.9 and 57 ± 8.90, P=.47), but an interaction between NOS_i and time was observed (P=.049). This interaction was neither linear (P=.61), nor quadratic (P=.104), reflecting reduced myofibrillar fragmentation at the 1mM and 100mM NOS_i concentrations compared to 0 and 10mM NOS_i concentrations at 7 days of ageing (Figure 3.3B).

Increases in TBARS concentrations between 1 and 14 days post-mortem were observed with both SNP and NOS_i (P<.001 for both Table 3.1). There was a

quadratic relationship between SNP concentration and TBARS concentration during ageing, with TBARS being increased with 1 and 10mM SNP compared to control and 100mM SNP (1.25, 2.35, 2.34, and 1.55 ± .185, P<.001). The increase in TBARS concentration over time was significant for 0, 1mM and 10mM SNP, but not for 100mM SNP (P=.006; Table 3.1). There was no overall effect of NOS_i concentration on TBARS (1.25, 1.20, 1.25 and 1.59 ± .235, P=.35), nor was there an interaction between NOS_i concentration and time during the ageing period (P=.62; Table 3.1)

The effects of NOS_i and SNP on the surface lightness (L*), redness (a*), yellow (b*) and TBA values of the injected LL's are presented in Table 3.2. With the exception of a tendency for an increase in b* values with time with SNP (P<.10), surface L*, a* and b* values were unaffected by time post-mortem in SNP and NOS_i injected samples (P>.10). While no overall effect of SNP concentration on L* was observed (33.7, 33.7, 33.4 and 31.2 ± 1.43, P=.33), there was a tendency for a linear reduction in L* values with increasing SNP concentration after 1 day of ageing, indicating darker meat in samples injected with 100mM SNP (P=.054). An effect of SNP concentration was observed on a* (19.2, 19.1, 18.5 and 14.7 ± .91, P<.001). This relationship was found to be linear (P<.001) such that a* values decreased with increasing SNP concentration, indicating reduced redness of the meat. Surface b* values were unaffected by SNP injection (P=.61). No effects of NOS_i concentration on L* (33.7, 34.8, 35.6 and 33.1 ± 1.26, P=.23), a* (19.2, 18.4, 17.7 and 17.5 ± .81, P=.19) and b* values (9.18, 8.73, 8.63 and 7.96 ±.71, P=.41) were observed and no interactions between time and concentration for SNP and NOS_i injections were observed (P>.10).

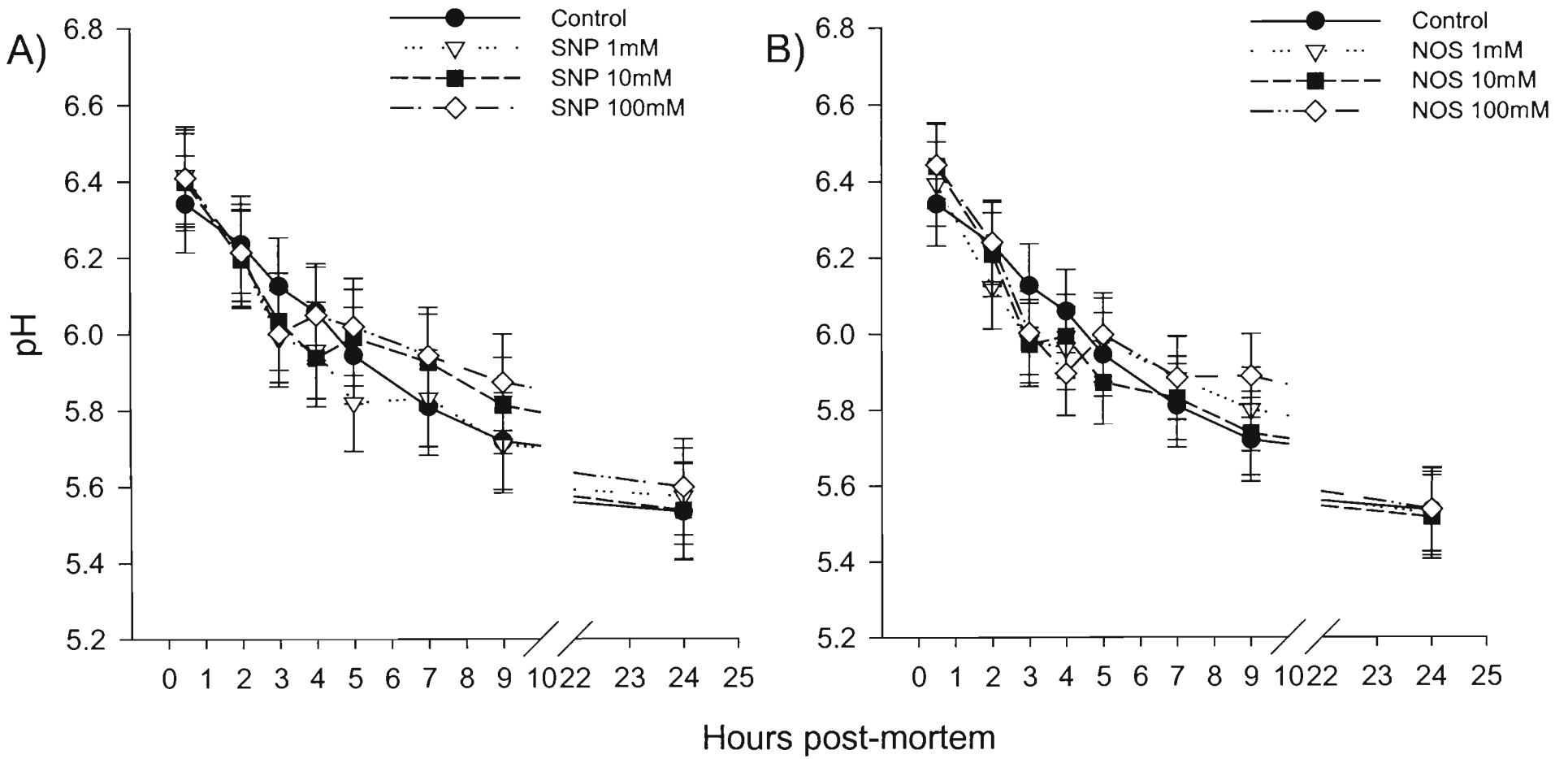


Figure 3.1: The reduction in hot-boned beef LL pH post-injection of muscles injected with 0, 1, 10 and 100mM A) SNP and B) NOS_i. A) P= .90 and .60 for the effects of SNP concentration and the interaction between SNP and time respectively. B) P= .96 and .53 for the effect of NOS_i concentration and interaction between NOS_i and time respectively.

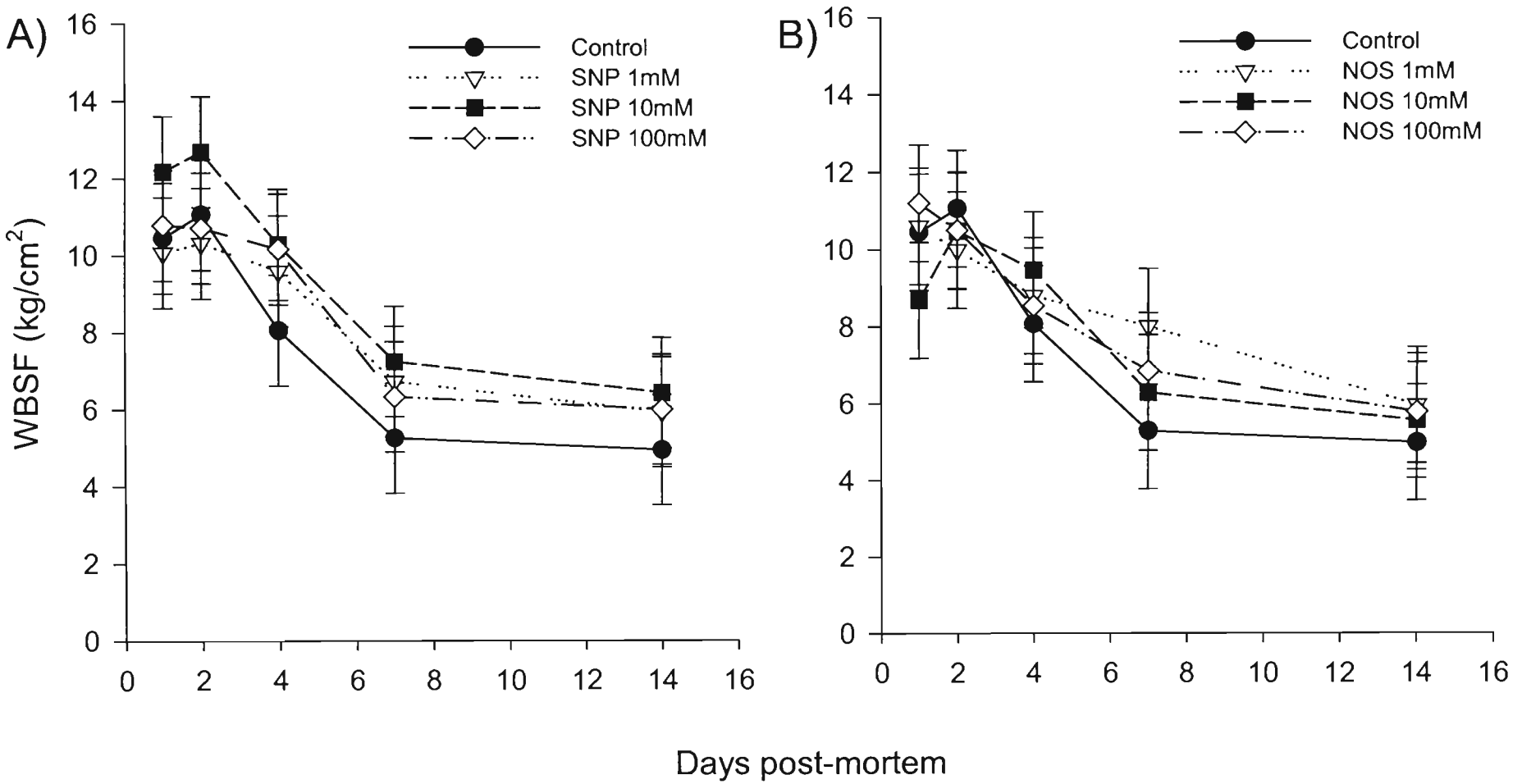


Figure 3.2: The reduction in hot-boned beef LL WBSF of muscles injected with 0, 1, 10 and 100mM A) SNP and B) NOS_i. A) P= .50 and .73 for the effects of SNP concentration and the interaction between SNP and time respectively. B) P= .93 and .93 for the effect of NOS_i and interaction between NOS_i concentration and time respectively.

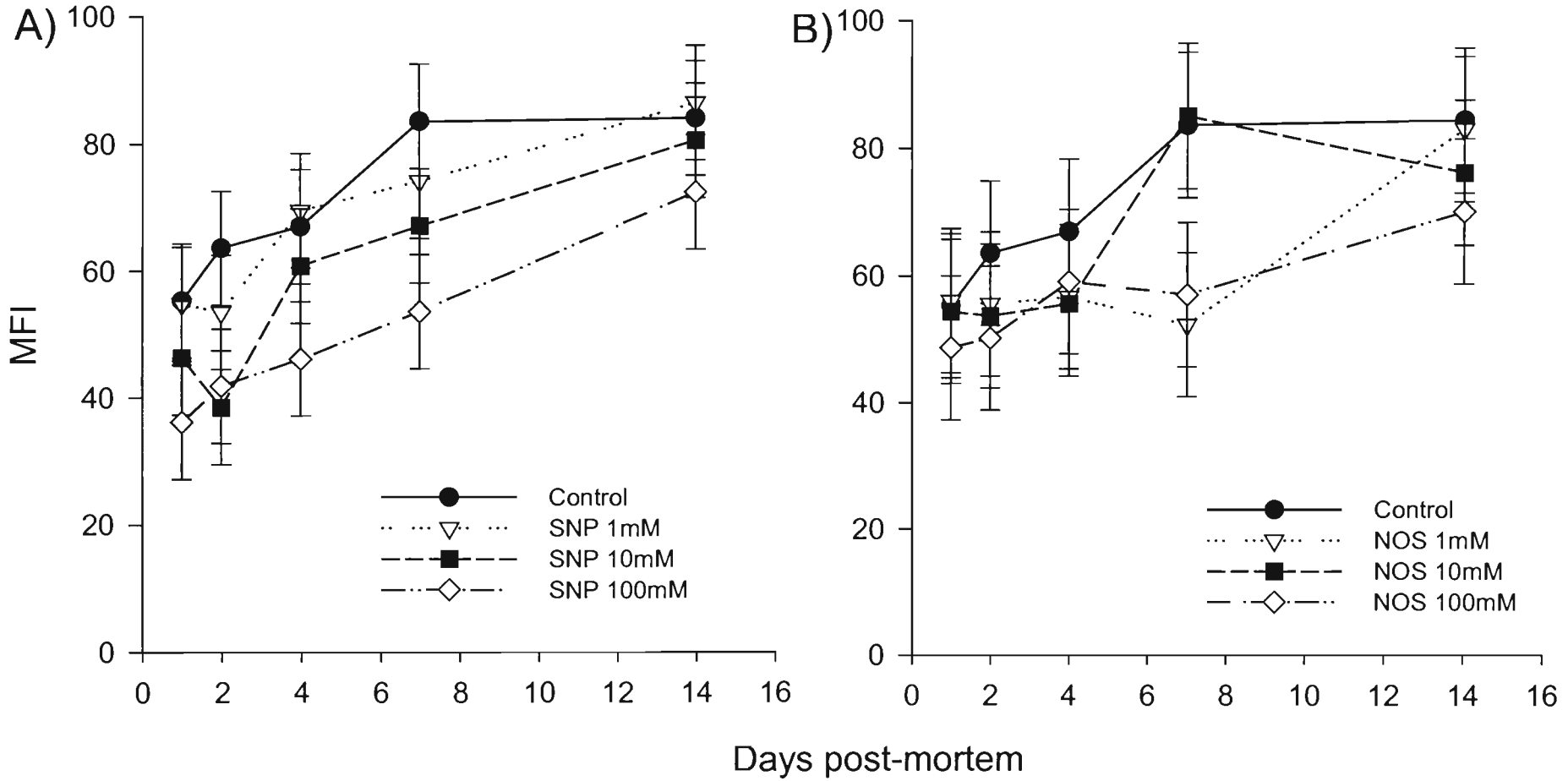


Figure 3.3: The reduction in hot-boned beef LL MFI of muscles injected with 0, 1, 10 and 100mM A) SNP and B) NOS_i. A) P= .019 and .61 for the effects of SNP concentration and the interaction between SNP and time respectively. B) P= .47 and .049 for the effect of NOS_i and interaction between NOS_i concentration and time respectively.

Table 3.1: The effects of time (1 and 14 days) and concentration (0, 1, 10 and 100 mM) of injected SNP and NOS_i on hot boned beef TBARS.

Trt.	Time (Day)	Concentration				SED	Significance		
		0[2]	1	10	100		Time	Conc.	Time*Conc.
SNP	1	.78	1.81	1.91	1.50	.225	<.001	<.001	0.006
	(mg/kg)	(6.0)	(64.7)	(80.7)	(4.5)				
	14	1.72	2.89	2.77	1.60				
	(mg/kg)	(52.5)	(772.7)	(588.8)	(5.0)				
NOS_i	1	.78	.92	.86	1.23	.279	<.001	0.35	0.62
	(mg/kg)	(6.0)	(8.4)	(7.2)	(16.9)				
	14	1.72	1.47	1.65	1.95				
	(mg/kg)	(52.5)	(29.4)	(44.3)	(88.3)				

[1]Log_{10} transformed values presented on top line, back-transformed means presented in parentheses (mg/kg) (SED values not back-transformed).

[2]0mM concentrations are common between SNP and NOS_i treatments.

Table 3.2: The effects of time (1 and 14 days) and concentration (0, 1, 10 and 100 mM) of injected SNP and NOS_i on hot boned beef colour (L*, a*, b*).

			Concentration					Significance		
	Trt.	Time (Day)	0[1]	1	10	100	SED	Time	Conc.	Time*Conc.
L	SNP	1	34.5	33.2	32.5	30.6	1.71	.33	.27	.33
		14	33.0	34.2	34.3	31.9				
	NOS_i	1	34.5	34.3	35.3	32.6	1.54	.65	.23	.46
		14	33.0	35.3	35.9	33.6				
a	SNP	1	18.4	20.2	18.8	14.4	1.48	.81	<.001	.44
		14	19.9	18.0	18.2	14.9				
	NOS_i	1	18.4	18.9	18.3	17.6	1.09	.69	.19	.28
		14	19.9	17.9	17.1	17.4				
b	SNP	1	8.3	9.8	9.7	8.7	.93	.066	.61	.44
		14	10.1	9.6	10.3	10.4				
	NOS_i	1	8.3	9.0	8.5	8.0	.99	.43	.41	.43
		14	10.1	8.5	8.8	7.9				

[1]0mM concentrations are common between SNP and NOS_i

3.6 Discussion

Post-mortem LL pH fall was not affected by either of the injected solutions, supporting hypothesis 1. The results from this experiment conform with the results from Chapter 2 that showed arginine and presumably arginine analogues, would not significantly affect pH. These data indicate that any changes in WBSF or MFI caused by injection treatments are not due to pH dependant mechanisms. Watanabe et al. (1996) and Yu and Lee (1986) observed a curvilinear relationship between meat tenderness and pH suggesting that tenderisation from cellular proteases is pH dependant (Hwang and Thompson, 2001, Watanabe et al., 1996).

Injection of NOS_i or SNP had little effect on WBSF. However, the MFI of 1mM NOS_i and 100mM NOS_i and SNP injections at 7 days post-mortem were significantly lower than control values, indicating that less proteolytic degradation of myofibrillar structure had occurred over the first 7 days of ageing in these treatments (Culler et al., 1978). By 14 days post-mortem, MFI at all concentrations of NOS_i and SNP were not different from controls, indicating that the degree of myofibril fragmentation that occurred during the last seven days of ageing was greater in the 1mM and 100mM NOS_i and 100mM SNP treatments. Although it is difficult to speculate on the roles of proteolytic systems that are responsible for this, the μ- and m-calpains and the cathepsins retain little activity after 7 days post-mortem (Boehm et al., 1998). Therefore it is likely that tenderisation beyond 7 days is largely due to an alternate mechanism than enzymatic tenderisation. These data do not support hypotheses 2 and 3, that SNP and NOS_i will increase tenderness and toughness respectively as observed by Cook et al. (1998).

Nitric oxide synthase does not appear to be rapidly degraded post-mortem as it retains its activity *in vitro* for at least 24h when extracted from pork muscle post-mortem (Brannan and Decker, 2002). This occurs despite isoforms of NOS being sensitive to degradation by calpain (Laine and de Montellano, 1998, Walker et al., 2001). While the structure of NOS appears to be conserved post-mortem, it is unlikely that NOS would remain active in hot-boned muscles, where there is low availability of substrates, cofactors, oxygen, coupled with the inhibitory effect of reduced pH and temperature (Venturini et al., 1999). The activity of NOS *in vivo* is tightly regulated, requiring O_2 and NADPH as substrates and FAD, FMN, BH_4, heme and calmodulin as cofactors (Reid, 1998) and pH dependent (Anderson and Meyer, 2000). Reductions in pH values below 7 uncouple NADPH oxidation by NOS, inhibiting the enzyme (Gorren et al., 1998) and it is likely that pH reductions in hot boned muscles will also have an inhibitory effect on NOS activity. Brannan et al. (2001) postulated that activity of NOS assayed in meat was most likely an artefact of the assay itself, since the assay provides optimal conditions. Due to the unfavourable conditions for NOS activity found in meat, it is likely that NOS activity in bovine muscle post-slaughter is limited. This may also explain the failure of the NOS_i injection to affect WBSF as observed by Cook et al. (1998), since the substrate inhibitors used will only inhibit an active enzyme.

Levels of oxidation measured by TBA, were significantly increased by addition of 1 and 10 mM w/w injections of SNP, but not 100mM SNP or NOS_i. The increases in oxidation of 1and 10mM SNP support hypotheses 4 and 5, that SNP would increase oxidation, while NOS would not influence oxidation. Since NO is a weak oxidant, the high levels of oxidation observed with the 1 and 10mM concentrations most likely

represent a large excess of NO, or formation of peroxynitrite. Peroxynitrite is formed from NO and superoxide (O_2^-) (Beckman et al., 1990), both of which are endogenously produced in skeletal muscle. Brannan and Decker (2001) demonstrated that peroxynitrite increases TBARS in post-mortem trout skeletal muscle and the pro-oxidant effect of peroxynitrite on lipid peroxidation is widely reported. Radi et al. (1991) also reported that peroxidation of lipids by peroxynitrite increases with decreasing pH. Loss of the pro-oxidant effect of SNP with 100mM 10% w/w injections may be due to peroxynitrite induced loss of myoglobin pro-oxidant activity (Jourd'heuil et al., 1998), or cyanide (CN^-) production as a by-product of nitroprusside breakdown (Schulz, 1984). Cyanide competitively inhibits oxidative phosphorylation (Beasley and Glass, 1998) and therefore is likely to inhibit superoxide and peroxynitrite concentrations.

Low concentrations of SNP did not appear to influence meat colour, while 100mM SNP reduced L* and a* values. The darker, less red meat was particularly evident after cooking, particularly near the site of injection (data not shown), indicating that SNP decomposed rapidly after injection, effectively poisoning the surrounding meat. The dark colouration may reflect the formation of covalent ferric myoglobin complexes, metmyoglobin and cyanmetmyoglobin which are brown in colour (Francis, 1985), providing supporting evidence for cyanide release from SNP breakdown.

Increased oxidation with 1 and 10 mM SNP may be due to lower concentrations of CN^- than with 100mM SNP. If this is so, 1 or 10mM concentrations are less likely to be influenced by CN^-. These data indicate a confounding effect when SNP was

injected, particularly at 100mM 10% w/w, most likely due to release of CN^- from degradation of the nitroprusside moiety of SNP, simultaneous with free NO release.

3.7 Conclusion

These data indicate that NOS activity is minimal post slaughter. Synthesis of NO by NOS is dependent on O_2 and NADH, which are in short supply in ischaemic muscle. Since NOS_i had only a minor effect on meat quality it appears that NOS must only be active briefly post-slaughter. Therefore, addition of a substrate inhibitor would be unlikely to alter NOS activity or meat quality in hot-boned muscle. Inhibition of NOS may be more effective if injected at an earlier time post-slaughter or alternatively *in vivo*.

Injection of SNP had little effect on the meat tenderness as shown by WBSF and MFI, with only the highest dose of 100mM having an effect on MFI at 7 days. Increased oxidation in response to SNP injection was attributed to supraphysiological concentrations of NO released from SNP at the concentrations used. However, at 100mM SNP the observed decreased oxidation was most likely due to cyanosis, demonstrating reactions with degradation by-products from SNP other than NO with muscle proteins post-mortem. In the absence of differential effects of SNP and NOS_i, it appears that alterations in meat quality with SNP were due to supraphysiological concentrations and therefore not representative of physiological conditions.

Chapter 4: Nitric oxide alters the rate and sensitivity of sarcoplasmic reticulum calcium uptake.

4.1 Introduction

During cell death there is a loss of Ca^{++} concentration gradients across the sarcoplasmic reticulum (SR) membrane, resulting in increased cytosolic concentrations of Ca^{++} (Cotran et al., 1989, Lipton, 1999). Increasing cytosolic Ca^{++} concentrations catalyse muscle proteolysis in a process integral to meat tenderisation (Koohmaraie, 1994, Dransfield, 1994). The Ca^{++} dependence of meat tenderisation has been demonstrated experimentally by inhibition of muscle proteolysis upon injection of the Ca^{++} chelator EGTA (Busch et al., 1972, Uytterhaegen et al., 1994). Recently, Hopkins and Thompson (2002) found that post-mortem free [Ca^{++}] and levels of meat proteolysis were highly correlated. Clearly, factors regulating cytosolic [Ca^{++}] in skeletal muscle are of critical importance to meat tenderness, an important facet of meat quality.

Calcium sensitive muscle proteolysis can occur by enzymatic or non-enzymatic means. The endogenous protease calpain is hypothesised to be the principal protease involved in meat tenderisation (Dransfield, 1993, Koohmaraie, 1994). Calcium is a pre-requisite for calpain activity, such that nomenclature of the principal calpain isoforms in skeletal muscle follows their calcium sensitivity (Dayton, 1982). Furthermore, Ca^{++} has been reported to non-enzymatically degrade Z-disks which is associated with improving meat tenderness (Takahashi et al., 1987, Takahashi, 1992).

The biomolecule nitric oxide (NO) has been demonstrated to influence muscle contraction in numerous species, partly due to the regulatory effect of NO on SERCA and RyR (Reid, 1998, Stamler and Meissner, 2001). Both excitation and inhibition of sarcoplasmic/ endoplasmic reticulum ATPase (SERCA) and ryanodine receptor (RyR) by NO have been observed, depending on the concentration and redox chemistry of NO, concentrations of NO scavenging molecules and methodology used (Suko et al., 1999, Hart and Dulhunty, 2000, Stamler and Meissner, 2001). The mechanism by which NO regulates Ca^{++} transport can occur by indirect and direct means. Nitric oxide increases cyclic guanosine monophosphate (cGMP) production via interaction with the heme moiety of the enzyme guanosine cyclase (Murad et al., 1990, Schmidt et al., 1993). Since cGMP is a second messenger of NO, its effects mirror the direct effects of NO on SERCA and the RyR (Weisbrod et al., 1998, Belia et al., 1998, Adachi et al., 2001). Both SERCA and RyR are rich in redox sensitive cysteine residues, which are sensitive to nitrosation and oxidation (Scherer and Deamer, 1986, Trimm et al., 1986, Xu et al., 1997, Eu et al., 1999). Cysteine oxidation by NO or reactive oxygen intermediates (ROI's) influence the function of SERCA and RyR (Viner et al., 2000, Stamler and Meissner, 2001, Xu et al., 1997). In particular, Sun et al. (2001) demonstrated that separate cysteine residues within the RyR were adapted to mediate nitrosative and oxidative stimuli.

The activity of NO synthase (NOS) in skeletal muscle is increased during exercise (Jungersten et al., 1997, Roberts et al., 1999, Tatchum-Talom et al., 2000). Accordingly, various electrical stimulation protocols applied to muscles have been used to experimentally increase skeletal muscle NO production (Balon and Nadler, 1994, Kobzik et al., 1994, Tidball et al., 1998). Since electrical stimulation (ES) is

routinely applied to ovine and bovine carcasses following commercial slaughter, and electrical stimulation increases Ca^{++} efflux from the SR (Jeacocke, 1982), the effects of NO and ES on SR Ca^{++} homeostasis may be linked.

4.2 Aims

This experiment aims to investigate the effects of different redox states of NO (NO^+ and NO•) and ES on the uptake, efficiency and release of SR Ca^{++} in muscle necropsies.

4.3 Hypotheses

1. Pre-incubation of purified SR with the NO donors DEA NONO and SNP will influence SR Ca^{++} uptake.
2. Pre-incubation of purified SR with the NO donors DEA NONO and SNP will influence release of Ca^{++} from the SR.
3. Electrical stimulation applied to the carcass will reduce the activity of the SR ATPase and therefore reduce the rate of SR Ca uptake.
4. Electrical stimulation applied to the carcass will increase the rate of Ca^{++} leakage from the SR due to increased membrane damage.

4.4 Methods

4.4.1 Sample collection and electrical stimulation:

A total of 9 lambs (24.4 ± 1.9 kg hot carcass weight) were slaughtered on three separate days and muscle necropsies (approx 10g) collected from the LTL at the 13th

thoracic vertebra approximately 10 minutes post-mortem following control (none), medium (300V, 14Hz) or high (700V, 14Hz) voltage electrical stimulation applied for 1 minute approximately 5 minutes post-mortem. Stimulation was applied via two electrically joined multipoint electrodes, one in the lateral musculature of each hind leg and one multipoint electrode inserted in the dorsal aspect of the neck, on the dressed carcass. The stimulation current comprised of a unipolar rectangular waveform providing 146.5 mA with a frequency of 13.5 Hz and pulse width of 8 msec.

Necropsies were immediately homogenised and crude SR preparations were isolated as described by Martinosi et al. (1968). Muscle necropsies (approx 10g) devoid of visible fat and connective tissue were homogenised at room temperature in 9 volumes of sucrose buffer (0.3M sucrose, 20mM MOPS, pH 7.0) with four 15 second bursts at high speed (22,000 rpm) with a Waring commercial blender (Warings Laboratory and Science, Model 38BL41, Connecticut, USA) and placed on ice. The homogenate was centrifuged at 10,000 x g at 4°C for 30 min and filtered through glass wool before the filtrate was then centrifuged at 40,000 x g at 4°C for 60 minutes and the pellet re-suspended in 1.5mL of sucrose buffer and frozen in aliquots of approximately .5mL. Samples were then frozen in liquid nitrogen and stored at –80°C until analysis.

4.4.2 Pre-incubation of SR membranes

Ten µL of either diethylamine NONOate (NONO, Cayman Chemical Company, cat 82100) or sodium nitroprusside dihydrate (SNP, Sigma Aldrich Pty Ltd, cat S-0501) (NONO and SNP stocks were 500µM) were added to 40µL of purified SR membranes in a 0.5mL microcentrifuge tube immediately before sample incubation. The final

concentration of NO donor was 100μM and all dilutions were made in homogenising buffer. The final concentration of 100μM for NONO and SNP was determined from the experiments of Lipton et al. (1993), Kobzik et al. (1994) and Viner et al. (2000) using the same NO donors where final concentrations between 100 and 500μM were used.

4.4.3 Measurement of SR Ca^{++} uptake and release

The rate of SR Ca^{++} transport was determined as described by Kargacin et al. (1988). Incubated SR samples (20μL) were pipetted into 4mL quartz cuvettes containing 3mL phosphate buffer (100mM KCl, 20mM Histidine, 25mM $KHPO_4$, 5mM $MgCl_2$, 5mM ATP, 5mM NaN_3, 15mM $CaCl_2.2H_2O$ and 2.5μM fura-2 sodium salt (Sigma Aldrich Pty. Ltd., USA, pH 7.0). The phosphate buffer was pre-warmed to 25°C in a water bath before addition of SR. Phosphate was added to the buffer to serve as an intra-vesicular Ca^{++} precipitating anion. Free [Ca^{++}] was determined fluorometrically by the change in emission at 510nm after excitation at 340 and 380nm (LS50B Luminescence spectrophotometer, Perkin Elmer Limited, USA). The ratio of excitation at 340 and 380nM (R) was achieved by the addition of a fast filter attachment to the spectrofluorimeter while operated in ratio mode. Assays were conducted in duplicate at 37°C with a magnetic stirrer attachment underneath the cuvette and magnetic flea placed inside the cuvette to prevent sedimentation of the SR during the assay.

The linear rates for Ca^{++} uptake and the rate of leakage/ release of Ca^{++} from the SR after pharmacological inhibition of Ca^{++} uptake by SERCA with the specific inhibitor

thapsigargin (Lytton et al., 1991) were calculated from the raw data (Figure 4.1). Due to inherent noise in the 340/380 v. time curve data were smoothed by a moving average calculation using smoothing windows containing 27-31 data points. The maximum rate of Ca^{++} uptake velocity (V_{max}) and calcium concentration at half-maximal activity ($[Ca^{++}]_{0.5}$) were calculated from the smoothed $[Ca^{2+}]$ v. time curves. These values were then fitted to the Hill equation with a curve fitting program and the kinetics of uptake determined from the fit. From the Hill equation, the Hill co-efficient (n_H), describing the Ca^{++} dependence of uptake was determined (Kargacin et al., 1988). All raw data collection, smoothing and calculations were conducted using the built in functions of FLWINLAB v. 3.0 software (Perkin Elmer Limited, USA).

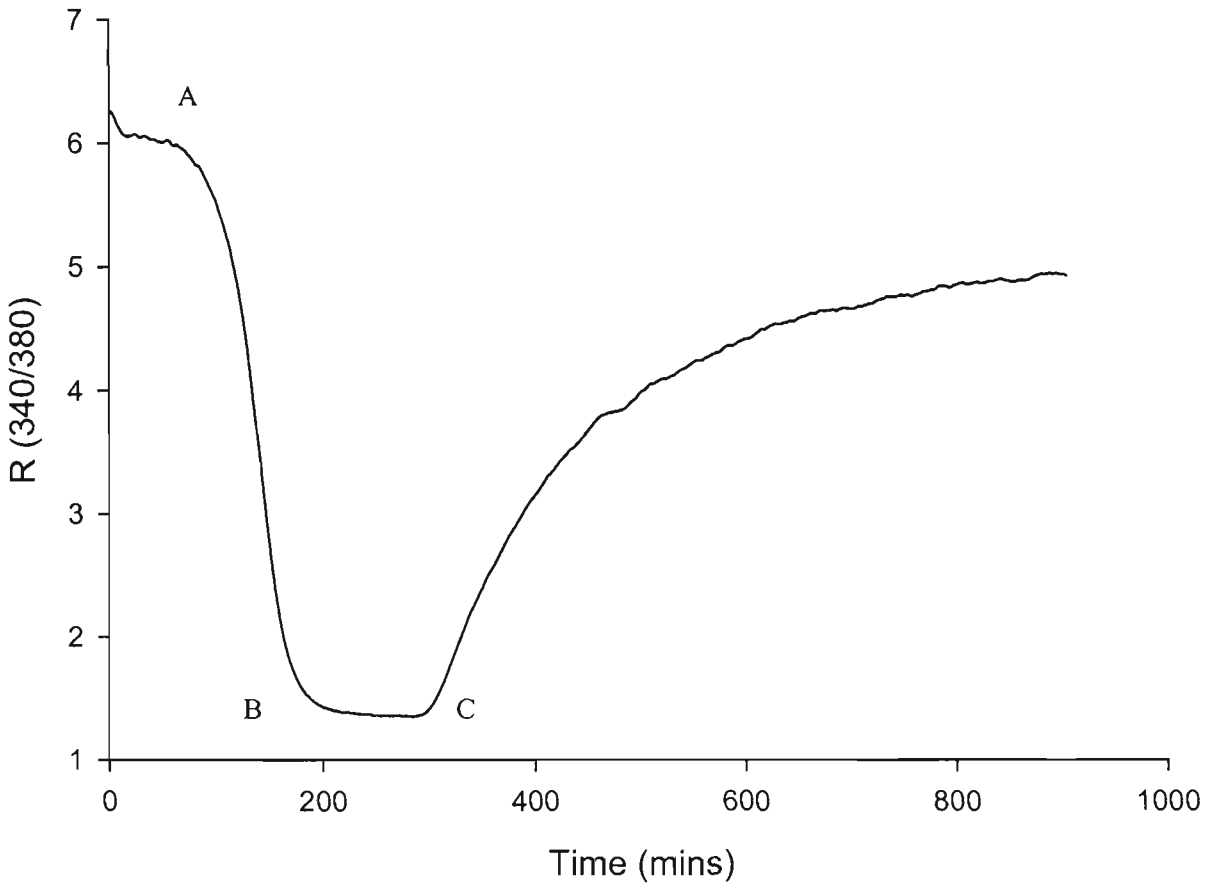
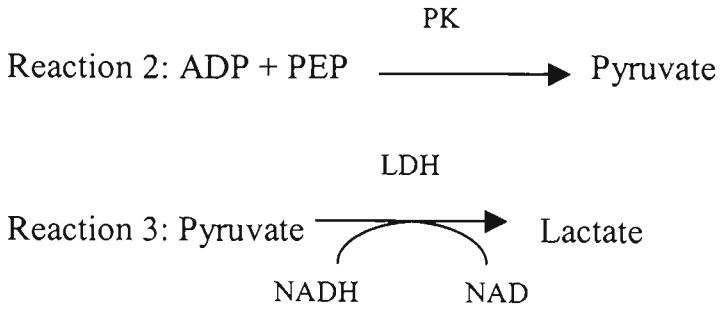


Figure 4.1: Sample trace of Ca^{++} uptake and release induced by inhibition of Ca^{++} uptake via pharmacological blockade of SERCA with thapsigargin in crude lamb LTL SR preparation. A) Addition of SR to assay buffer and subsequent uptake of Ca^{++} from buffer. B) Limit of Ca^{++} uptake. C) Addition of thapsigargin and release of Ca^{++} sequestered in SR. $[Ca^{++}]_{0.5}$ is approximately half way between A and B. The V_{max} is the fastest rate between A and B.

4.4.4 Measurement of SR Ca^{++} ATPase activity

The ATPase activity of pre-incubated crude SR preparations were determined fluorometrically in duplicate as described by Takahashi and Putnam (1979). Crude SR preparations (20µL) were added to 3mL of ATPase buffer containing 100mM KCl, 20mM Histidine, 5mM $MgCl_2$, 1mM EGTA, 5mM NaN_3, 70µM NADH 50mM Na_2ATP, 25mM Phosphoenolpyruvate (PEP), 5mM b-Nicotinamide adenine dinucleotide phosphate (NADPH), 214 U/mL coupled pyruvate kinase/ lactate dehydrogenase solution (PK/LDH) (cat. 109100, Roche Diagnostics, Switzerland), pH 7.0. The PK/LDH solution was dialysed in 100mM KCl and 20mM MOPS to remove interfering preservatives before use in assays. Activity of the ATPase was determined by NADH oxidation in a coupled enzyme system (Reactions 1-3). The assay was conducted using the same equipment and conditions described in section 4.4.3. Concentrations of ATP were recorded using FLWINLAB v. 3.0. Two modes of ATPase activity have been characterised in the SR, Ca^{++} independent ATPase (Mg ATPase) and Ca^{++} dependant ATPase, which is stimulated by micromolar concentrations of Ca^{++} and is tightly coupled to Ca^{++} transport (Flaherty et al., 1975, Berman, 1982, Figure 4.2). Calcium ATPase activity was stimulated by addition of 20µL of 200mM $CaCl_2$ to the reaction mixture. All data acquisition and calculations were performed using FLWINLAB v. 3.0 software. The Ca^{++} ATPase activity was determined by subtracting the Mg^{++} ATPase (nmoles/ mg/ min SR) from the total ATPase (nmol/mg/min) (Figure 4.2). The coupling ratio was determined by dividing V_{max} (nmol/ mg/ min SR) by Ca^{++} ATPase activity (nmoles/ mg/ min).

Reaction 1: $ATP \xrightarrow{SERCA} ADP + P_i$



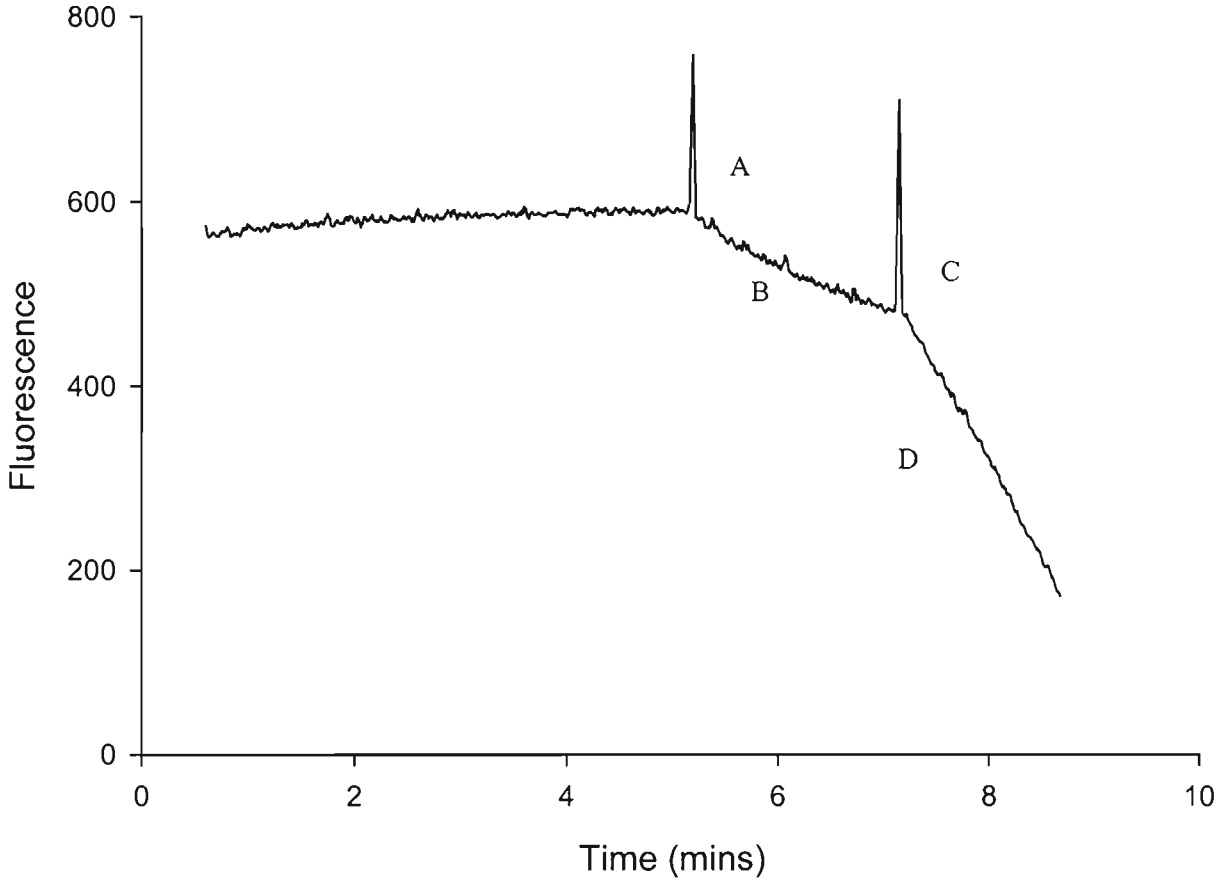


Figure 4.2: Sample trace of Mg^{++} and Ca^{++} ATPase activity in crude lamb LD SR preparation. A) Addition of crude SR preparation to assay buffer B) Basal Mg^{++} ATPase activity C) Addition of $CaCl_2$ D) Total ATPase activity.

4.4.5 Statistical analyses

The main effects of NO donor (control, NONO and SNP), electrical stimulation (none, medium, high) and their interaction were analysed by an analysis of variance

(ANOVA) with blocks on lamb and day of slaughter. All errors were calculated as the standard error of the differences of the means (SED) and all statistical analyses performed in Genstat 5.41 (Payne et al., 1993).

4.5 Results

A significant effect of NO donor on the linear and maximal (V_{max}) rates of Ca^{++} uptake was observed. Increased Ca^{++} uptake by SERCA was observed after pre-incubation with NONO ($P<.05$ and $P<.01$ for linear and V_{max} Ca^{++} uptake, respectively) but not SNP (Table 4.1). There were no effects of electrical stimulation or interactions between NO donor and electrical stimulation on linear and maximal (V_{max}) rates of Ca^{++} uptake ($P<.10$). While the ATPase activity in purified SR preparations was unaffected by NO donors or electrical stimulation ($P>.10$), an interaction between the two was observed ($P<.05$). An interaction between electrical stimulation and NO donor on ATPase activity was observed, indicating that the activity of control and NONO was reduced by high voltage electrical stimulation (.42, .42, .46 and .50, .46, .44 and .40, .40, .48 ± .050 nmol/mg/min for control, NONO and SNP after low, medium and high voltage stimulation, $P<.05$). The significant increase in V_{max}, independent of ATPase activity increased the coupling ratio, indicating that NO improved efficiency of Ca^{++} transport by SERCA ($P<.05$, Table 1). Electrical stimulation did not affect the coupling ratio and an interaction between NO donors and electrical stimulation was not observed ($P>.10$ for both).

An effect of NO donor was observed on $[Ca^{++}]_{0.5}$ of the SERCA. Higher concentrations of Ca^{++} were present at half-maximal activity with NONO, but not SNP ($P<.05$, Table 1). A tendency for a lower Hill co-efficient (n_H) with NONO

incubation compared to control was observed (P=0.06), reflecting negative cooperativity of Ca^{++} binding to the two binding sites of the SERCA. This indicates reduced affinity of Ca^{++} binding of the SERCA incubated with NONO compared to control. No effect of electrical stimulation, or interaction between NO donor and electrical stimulation were observed on $[Ca^{++}]_{0.5}$ or n_H.

No effect of NO donor was observed on the rate of Ca^{++} release after thapsigargin induced inhibition of SERCA (P>.10) (Table 4.1). The rate of Ca^{++} release after thapsigargin induced inhibition of SERCA was increased by high voltage electrical stimulation (P<.05). No interactions between NO donor and electrical stimulation were observed for the rates of Ca^{++} release.

Table 4.1: Effect of incubation of the NO donors DEA NONOate and SNP coupling ratio on the sarcoplasmic/ endoplasmic reticulum ATPase (SERCA) and ryanodine receptor purified lamb SR membranes from carcasses with no (control), medium and high voltage electrical stimulation.

	Donor (D)				Voltage (V)				P-value		
	None	NONO	SNP	SED	control	medium	high	SED	Donor	Voltage	D x V
Uptake (μmol/mg/min)	105	134	102	12.3	127	110	104	16.5	.042	.42	.32
Vmax (nmol/mg/min)	.41	.47	.38	.022	.47	.42	.37	.065	.004	.40	.26
Ca++ ATPase activity (nmol/mg/min)	.44	.43	.46	.017	.43	.47	.43	.044	.191	.63	.040
Coupling ratio (V_{max}/ATPase)	.97	1.12	.84	.081	1.10	.91	.93	.244	.015	.72	.36
$[Ca^{++}]_{0.5}$ (nmol/mg/min)	.79	.83	.76	.022	.80	.77	.81	.029	.013	.50	.92
Hill co-efficient (n_H) (nmol/mg/min)	2.35	2.11	2.28	.093	2.15	2.29	2.36	.263	.060	.73	.38
Ca^{++} Release (μmol/mg/min)	6.65	7.32	6.80	.408	4.12	6.65	9.99	1.336	.27	.029	.21

4.6 Discussion

The main findings of this experiment were that the NO donor NONO increased the linear and maximal rates of Ca^{++} uptake *in vitro*. The increase in the rate of Ca^{++} uptake with NONO was not associated with an increase in the SR ATPase activity, thus resulting in an increased coupling ratio. While Ca^{++} uptake was faster with NONO, higher concentrations of Ca^{++} were required to stimulate SERCA, as evidenced by increased $[Ca^{++}]_{0.5}$. It is possible that the reduced Ca^{++} sensitivity with NONO was due to decreased calcium binding cooperativity as the n_H tended to be reduced by NONO.

Nitric oxide has been shown to increase the rate of Ca^{++} sequestration into the SR in rabbit smooth muscle (Cohen et al., 1999) and human platelets (Trepakova et al., 1999). The mechanism of a NO mediated increase in Ca^{++} uptake remains perplexing, as the increased uptake was independent of a concomitant increase in ATPase activity. This is not elucidated by the studies of Cohen et al. (1999) or (Trepakova et al., 1999), as the energetics of Ca^{++} transport could not be calculated since ATPase activity was not measured in their experiments. The SERCA has two calcium binding sites facing the cytosol (Inesi et al., 1980). Transportation of two moles of Ca^{++} requires one mole of ATP, giving the SERCA a maximum theoretical coupling ratio of 2 (Worsfold and Peter, 1970, Inesi et al., 1980, Hasselbach and Oetliker, 1983). However, coupling ratios of 2 do not occur experimentally due to processes such as back inhibition of luminal Ca^{++} (Ikemoto, 1975), activity of ATP regeneration systems (Inesi and de Meis, 1989) and calcium leakage via the membrane or "slippage" of the Ca^{++} pump (Berman, 2001). Coupling ratios of 1 in this experiment probably reflect damage to the SR from slaughter, purification and freezing processes. Therefore, it is possible

that the increase in the coupling ratio observed in this experiment represents a reversal of damage due to slaughter, purification and/or freezing, rather than an improvement in SERCA function above pre-slaughter rates.

It is probable that the effect of NONO in this experiment is mediated by nitrosylation of SERCA thiol residues. Skeletal muscle guanylate cyclase activity is low compared to other tissues (Stamler and Meissner, 2001) and guanylate cyclase is expressed in the sarcolemma (Sulakhe and Sulakhe, 1976, Levine et al., 1979) and, therefore, will not be present in the purified SR. Since SERCA monomers contain 22 free cysteine residues, they are particularly sensitive to oxidation (Murphy, 1976, Thorley-Lawson and Green, 1977). A detailed study by Viner et al. (2000) into SERCA cysteine oxidation found that nitrosylation of SERCA thiol by NO was highly specific. After finding that oxidation of cysteine residues did not attenuate ATPase activity, Viner et al. (2000) hypothesised that nitrosylation had a protective effect against reactive oxygen intermediates (ROI's) on SERCA function. Therefore, the increase in the coupling ratio observed after pre-incubation with NONO may represent a reversal of oxidative damage by ROI's post-mortem.

While the half-lives of the NO donors SNP and NONO at 25°C are similar (Diodati et al., 1993), their mechanism of donation and redox state of the NO they formally donate are not. Sodium nitroprusside is a metal NO complex, that requires reductive activation to yield NO^+ (Bates et al., 1991, Feelisch, 1998). This differs from NONO which liberates $NO^\bullet$ spontaneously in aqueous media (Keefer, 1998). The different redox states confer different reactivities of NO donated by SNP and NONO with other biomolecules (Feelisch, 1998). Reactions with nucleophilic targets such as thiols are

preferred by NO^+ (Wink et al., 1991, Stamler et al., 1992b, Stamler et al., 1992c), while $NO^\bullet$ prefers reactions with metal complexes and O_2 (Stamler et al., 1992c) and reactions with thiols are limited under physiological conditions (Gloldstein and Czapski, 1996). Therefore, the biochemical reactions favoured by NO are largely determined by its redox state. While NO liberated from SNP may be able to freely react with endogenous thiol groups, $NO^\bullet$ donated from NONO will need prior oxidation of $NO^\bullet$ to NO^+ by reactions with endogenous metal complexes (Stamler, 1994). In addition, the effects mediated by NONO may be concentration dependant since 1 mole of NONO yields 1.5 moles of NO (Hrabie et al., 1993), while SNP yields equimolar concentrations of NO (Schulz, 1984).

The SERCA has two Ca^{++} binding sites, one with low Ca^{++} affinity, and one with high Ca^{++} affinity (Chevallier and Butow, 1971) with the stoichiometric ratio of Ca^{++} binding to catalytic sites of 2:1 (Inesi et al., 1980). Binding of Ca^{++} to the high affinity sites activates the SERCA for cation transport (Meissner et al., 1973, Ikemoto, 1975) by a conformational change in protein structure allowing phosphorylation (Yamamoto and Tonomura, 1967, Martonosi, 1969) and exposure of the low affinity Ca^{++} binding site. In what is described as a cooperative process, occupation of the low affinity Ca^{++} binding site is dependent on binding at the high affinity site.

The increase in Ca^{++} uptake with NONO was independent of an increase in n_H, which would indicate improved cooperativity of Ca^{++} binding to the SERCA Ca^{++} affinity sites. This indicates that faster rates of Ca^{++} uptake observed after pre-incubation with NONO were independent of improved Ca^{++} binding. Since the increased rate of Ca^{++} uptake into the SR lumen was independent of increased ATPase activity or calcium

binding, the increased rate of Ca^{++} transport may be due to increased recruitment of SERCA's.

Release of Ca^{++} from the SR following thapsigargin-induced inhibition of SERCA was not affected by either NO donor. The principal site of Ca^{++} 'release' from the SR is via RyR opening, but Ca^{++} can exit the SR via membrane leakage, or reversal of the SERCA, albeit at significantly lower rates than RyR opening (Inesi, 1985). Reversal of the SERCA has only been induced experimentally and is not expected to contribute to Ca^{++} release in this experiment. Since opening of RyR's results in rapid release of Ca^{++}, it appears that neither NONO, SNP nor electrical stimulation increased opening of RyR channels. Hart and Dulhunty (2000), observed that RyR's need to be active before their activity was inhibited by NO. Since the opening of RyR channels was not stimulated in this experiment, the effect of NO mediated inhibition of RyR has not been observed, only that NO does not result in increased RyR opening. For the inhibitory effect of NO to be observed, a non-fluorescing RyR agonist would need to be used in conjunction with the NO pre-incubation. Silver nitrate is frequently used for this task, but was not used during this experiment due to the similarity of action between silver nitrate and NO, which both oxidise the RyR (Abramson et al., 1983).

Numerous other experiments have observed activation and inhibition of RyR by NO. Meszaros et al. (1996) observed that the nitrosothiol S-nitroso-n-acetylpenicillamine increased SR Ca^{++} uptake due to lower rates of concurrent RyR mediated Ca^{++} release in rabbit heavy SR vesicles. This effect was removed by addition of L-NAME, a substrate inhibitor of NOS, demonstrating that concentrations of NO synthesised endogenously are sufficient to inhibit SR Ca^{++} release. Suko et al. (1999) reported

both activation and inhibition of RyR with pharmacological donors of NO. Furthermore, Suko et al. (1999) observed that oxidation of cysteine residues by NO was highly specific. This was later supported by Sun et al. (2001), who found specific, separate residues within the RyR were adapted to mediate nitrosative and oxidative responses.

Electrical stimulation significantly increased thapsigargin-induced Ca^{++} release. Since the Ca^{++} release in this experiment was most likely through leakage through the SR membrane, rather than opening of the RyR, it appears that electrical stimulation increases SR "leakiness", probably due to increased permeability or damage to the SR membrane or SERCA pumps. Electrical stimulation has been observed to increase Ca^{++} leakage from the SR in one experiment (Jeacocke, 1982) and a reduction in SR ATPase activity in electrically stimulated ovine LD muscle was observed in a different experiment (Tume, 1979). The high calcium concentration gradient between the SR lumen and cytosol is continually maintained by the SERCA. Reduced ATPase activity would result in reduced Ca^{++} uptake and hence contribute to higher rates of Ca^{++} leakage.

The effect of NO on calcium homeostasis and in turn meat quality is difficult to interpret. It is likely that the reduced sensitivity of the SERCA would shift towards increasing cytosolic Ca^{++} concentrations. Oxidative stress in cells has been observed to increase $[Ca^{++}]$ and the Ca^{++} dependant protease calpain (Miyoshi et al., 1996, Mehlhase and Grune, 2002) and calpains are postulated to be the principal meat tenderising enzyme (Dransfield, 1993, Koohmaraie, 1994). Furthermore, Ca^{++} can also weaken Z-disks by ionic interactions, tenderising meat (Takahashi et al., 1987,

Takahashi, 1992). However, increasing cytosolic [Ca^{++}] pre-rigor can also result in muscle shortening and meat toughening (Davey and Gilbert, 1974, Cornforth et al., 1980). While it can not be concluded from these data whether NO causes a rise in myofibrillar [Ca^{++}] sufficient to cause sarcomere shortening, the likelihood of NO causing sarcomere shortening does not appear to be high since NO did not induce RyR opening. Opening of RyR's would rapidly increase Ca^{++} efflux from the SR, resulting in a higher [Ca^{++}] in the muscle fibre closer to slaughter when the muscle has a greater capacity to shorten. However, it should be noted that the conditions of the assay do not necessarily replicate conditions in muscle post-mortem. Assays were conducted under stable, reproducible environments, while muscle experiences variable and falling ATP, O_2 and pH levels, all which are critical to NO production, SERCA and RyR activity. For example, it is expected that increased NO induced Ca^{++} uptake is probably not as important as rising cytosolic Ca^{++} concentrations since in post-mortem muscle SERCA activity will be reduced by depleted ATP stores.

4.7 Conclusion

The results of this experiment support the hypothesis 1, since pre-incubation of purified SR membranes with DEA NONO increased the rate of calcium intake into the SR lumen. However, it is likely that the onset of Ca^{++} uptake is delayed as evidenced by the increased $[Ca^{++}]_{0.5}$. It is possible that endogenous release of NO in physiological systems will increase cytosolic Ca^{++} concentrations due to reduced Ca^{++} mediated Ca^{++} uptake. The mechanism of increased calcium uptake is perplexing since an increase in Ca^{++} binding (n_H) was not observed. This may be due to increased recruitment of SERCA's by an unknown mechanism. Hypothesis 2 was not supported by this experiment, since Ca^{++} release was not affected by NONO or SNP,

however this may be in part to the methodologies used and a different result may be observed on rates of stimulated Ca^{++} release. Hypothesis 3 was not supported by the data since electrical stimulation of carcasses did not appear to reduce the activity of the SR Ca^{++} uptake or RyR functionality. However electrical stimulation did increase rates of Ca^{++} release. The slow rate of release is was likely to be due to disruption of the SR membrane and not activation of the RyR, supporting hypothesis 4. From these data it is concluded that endogenous release of NO in skeletal muscle may increase cytosolic Ca^{++} concentrations, a precursor for increased Ca^{++} mediated meat tenderisation.

Chapter 5: Inhibition of endogenous nitric oxide production influences ovine hind-limb energy metabolism by a mechanism independent of insulin concentrations.

5.1 Introduction

The importance of muscle metabolism to meat quality is highlighted by the development of dark cutting (DC) and dark firm dry (DFD) meat, which occurs due to reduced conversion of glycogen to lactate post-slaughter (Lawrie, 1958, Pethick et al., 1995). Depletion of muscle glycogen can occur by endocrine responses to stress including adrenaline release or increased physical activity (Lacourt and Tarrant, 1985). Since many physiological pathways in skeletal muscle are influenced by NO, including muscle contraction and metabolism, it is hypothesised that the response to stress is partly mediated by nitric oxide (NO). Muscle contraction is inhibited by pharmacological donors of NO with the involvement of endogenous NO being demonstrated following an increase in muscle force upon inhibition of endogenous NO production (Kobzik et al., 1994, King-Vanvlack et al., 1995). Other experiments have shown that skeletal muscle NOS activity and expression is increased by exercise (Roberts et al., 1999, Tatchum-Talom et al., 2000). Due to the close relationship between muscle contraction and NOS activity, it is likely that increases in muscle fibre NOS activity are associated with the contractile response to stress.

Nitric oxide has been demonstrated to have multiple regulatory roles in muscle metabolism. Young et al. (1997) observed that SNP increased rat *soleus* glucose

uptake and lactate production, while inhibiting rates of insulin-stimulated glycogenesis. Other experiments conducted in rats *in vivo* have shown increases and decreases in muscle glucose uptake respectively using SNP and NOS antagonists (Balon and Nadler, 1997). For example, insulin-stimulated glucose uptake is partly mediated by vasodilation and increased tissue perfusion. Inhibition of the vasodilatory response to insulin with inhibitors of NOS, which is expressed in the endothelium, result in insulin resistance due to reductions in tissue perfusion (Baron et al., 1995, Sadri and Lautt, 1999). Alternatively, NOS may influence muscle metabolism via endogenous synthesis of NO within the muscle fibre. This has been demonstrated in hepatic and endothelial mitochondria, where NO influences mitochondrial metabolism, principally via inhibition of cytochrome c oxidase (Giulivi et al., 1998, Giulivi, 1998, Clementi et al., 1999).

5.2 Aims

The aims of this experiment are to investigate the effect of intravenous infusion of the NOS inhibitor L-arginine methyl ester hydrochloride (L-NAME) on NO synthesis and hind-limb metabolism prior to investigations into the involvement of NO during stress and subsequent meat quality.

5.3 Hypotheses

1. Infusion of 30mg/kg L-NAME will reduce plasma NO_x concentrations.
2. L-NAME will act as an insulin antagonist, decreasing glucose uptake.
3. L-NAME will increase NEFA concentrations.

5.4 Material and Methods

5.4.1 Animals and Surgery

All procedures were approved by the Victorian Institute of Animal Science Animal Ethics Committee. Eight Border Leicester x Merino lambs 50-55kg live weight were housed in individual metabolism crates. Lambs were fed *ad libitum* on pelleted feed (Table 5.1) dispensed at three-hourly intervals via an auto-feeder with supplementation of lucerne hay. Catheters were placed in the jugular vein, medial saphenous artery and lateral saphenous vein (Oddy et al., 1987, Mc Donagh et al., 1999) 2-3 days pre-infusion. The jugular vein was catheterised in the conscious animal with a 12 gauge "Dwellcath" (cat. 351-365 Sutherland Medical, Australia)

Table 5.1: Nutritional analysis of pelleted feed ration expressed as % of dry matter.

Metabolisable energy	12MJ/ME/kg
Min crude protein	19%
Min crude protein from natural sources	16.3%
Non protein nitrogen (or urea or crude biuret) equiv. To crude protein	2.7%
Urea	1.0%
Min crude fat	2.0%
Max crude fibre	16.0%
Max added salt (NaCl)	1.0%
Calcium (Ca)	1.0%
Phosphorous (P)	0.5%
Max Fluoride	0.02%
Min vitamin A	6000IU/kg
Min vitamin D3	500IU/kg
Vitamin E	26mg/kg

(Sutherland Medical, Australia), while hind-limb catheterisation was performed under halothane (Rhone Merieux, Australia) gas anesthesia (approximately 3% halothane, .5 L/min air) after intravenous administration of 10-15 mg/kg thiobarb (thiopentone sodium, Durax Pty Ltd, Australia). Catheters were introduced to the lateral saphenous vein and the tips placed approximately 40 cm (measured from the insertion point to the pin bone) in the deep femoral vein. Arterial blood was collected from the abdominal aorta via the lateral saphenous artery with an insertion distance of approximately 20 cm (Teleni and Annison, 1986). All lambs were given approximately 15mg/kg Engemycin (Oxytetracycline, Intervet Australia Pty Ltd, Australia) by intramuscular injection upon completion of surgery. Catheter material was 1.50mm outer diameter x 1.00mm inner diameter polyethylene tubing (Dural Plastics and Engineering Pty Ltd, Australia).

5.4.2 L-NAME challenge and blood sampling

Saline or 30 mg/kg of L-arginine methyl ester hydrochloride (L-NAME, Cayman Chemical Company) in saline was administered in a 10 mL bolus via the indwelling jugular catheter. The concentration of 30mg/kg was determined from information supplied by Griffith and Lilbourne (1996) and from the results of a brief trial where concentrations of L-NAME between 10 mg/kg and 30mg/kg were infused and effects on plasma glucose and lactate concentrations analysed (data not presented). Blood samples (approx. 3 mL) were simultaneously removed from the hind-limb arterial and venous catheters at -60, -30, -15, 0, 15, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 minutes relative to the infusion. Haematocrit % was determined by removal of a small sample of whole arterial blood by capillary action into a 75 μL heparinised haematocrit tube (Hirschman Laborgerate, Germany) and centrifugation for 5 minutes

(Haematokrit, Hettich Zentrifugen, Germany). The remaining blood was dispensed into a heparinised blood tube with separating gel (cat. 46.9924.197 Sarstedt Australia Pty Ltd) and centrifuged for 10 minutes at 4000g (JB-40, Beckman, USA) before the plasma was removed and separated into aliquots and frozen at –20°C until analyses. Each lamb was used for each treatment (control and L-NAME) by bleeding on two separate experimental days separated by a 3-day "washout" period. Patency of indwelling catheters was maintained between experimental days by daily flushing with heparinised saline solution (50,000 U /L, David Bull Laboratories, Australia). On experimental days, the catheters were flushed with 12.5g/L K_2EDTA/.9% NaCl after each blood sample to prevent clot formation as heparin increases the activity of lipoprotein lipase (Gartner and Vahouny, 1966, Olivecrona and Egelrud, 1974). Infusions were randomised for each animal and day (refer to statistical design section below).

5.4.3 Biochemical analysis of plasma

5.4.3.1 Plasma NO_x assay

Plasma NO_x concentrations were determined using an enzymatic kit as per the manufacturers instructions (Cat 7810001, Cayman Chemical Co., USA). Approximately 400μL of plasma was ultrafiltered using centrifugal filter units with 10,000 molecular weight cut offs (Ultrafree MC, Millipore, USA). Samples were centrifuged for approximately 5 hours at 2°C and 6500 x g with a microcentrifuge (EBA 12, Hettich Zentrifugen, Germany). Ultrafiltered plasma was frozen and stored at –20°C until analysis. Before the assay, assay buffer (AB) was re-constituted with 100mL deionised H_2O. The AB was then used to re-constitute nitrate reductase and enzyme co-factors (1.2mL AB) and nitrate standard stock (1mL AB). The nitrate

standard was further diluted 10-fold to 200μM to form a fresh working standard. Plasma samples (60μL) and nitrate standards were pipetted in duplicate into microtitre plates (Nalgene, NUNC). Assay buffer (20μL) and 10μL of enzyme co-factors and nitrate reductase were added to microtitre plate wells, covered with an adhesive strip and incubated at a room temperature of approximately 22°C for 3 hours. The adhesive strip was then removed and 50μL of Greiss reagents R1 and R2 added to each well. The reaction was incubated at room temperature for 10 minutes and the A_{540} of the chromophore formed between NO_2^- and Greiss reagent measured using a microtitre plate measured (Titretek Multiscan, Lab Systems Finland).

5.4.3.2 Plasma glucose assay

Plasma glucose concentrations were measured using a commercially available kit as per the manufacturers instructions (cat 510-A, Sigma-Aldrich, USA). Prior to use, the contents of the *o*-Dianisidine vial was re-constituted with 20mL deionised H_2O and enzyme capsules were re-constituted with 100mL of dH_2O. Glucose reagent was made by adding 1.6mL *o*-Dianisidine to the re-constituted enzyme capsule. Plasma (4μL) and glucose reagent (300μL) and a glucose standard were dispensed into microtitre plates using a diluter (Microlab 1000, Hamilton, USA). Microtitre plates were incubated at 37°C for 30 minutes and A_{450} of a chromophore measured as previously outlined (section 5.4.3.1).

5.4.3.3 Plasma lactate assay

Plasma lactate concentrations were measured using a commercially available kit as per the manufacturers instructions (cat 735-10, Sigma-Aldrich, USA). Plasma (3μL), lactate standard and lactate reagent (300μL) were dispensed to microtitre plates as

outlined in section 5.2.3.2. The A_{540} of a chromophore was measured following a 10 minute incubation period at room temperature (approximately 22°C, section 5.4.3.1).

5.4.3.4 Plasma NEFA assay

Plasma nonesterified fatty acids (NEFA) were determined using kits supplied by Wako, USA (NEFA C, cat 279-75401), modified to conduct extra assays by a five-fold dilution of all diluents A and B (contents unknown) in 25mM phosphate buffer (pH 7.8) (Dunshea and King, 1995). Plasma (5μL) and diluent A (95μL) were dispensed into microtitre plate wells using a Hamilton diluter as per section 5.2.3.2 and incubated at room temperature (approx 22°C) for 120min. Diluent B (200μL) was then added to all wells and incubated for 60min and the concentration of a chromophore determined at A_{540}.

5.4.3.7 Plasma TAG assay

Plasma triacylglycerol (TAG) concentrations were determined using infinity triglycerides reagent (cat 343-25P, Sigma-Aldrich Pty Ltd). Plasma (3μL) and infinity triglyceride reagent were added to cuvettes and incubated for 5 minutes at 37°C using a COBAS MIRA S auto-analyser (Roche, Switzerland). Concentrations of TAG were determined by changes in absorbance at 520nm relative to a triglyceride standard.

5.4.3.6 Plasma Urea assay

Plasma urea concentrations were determined using a commercially available kit as per the manufacturers instructions (cat 640-B, Sigma Diagnostics, USA). Prior to assay, vials containing urease buffer reagent were re-constituted with 30mL deionised H_2O.

Plasma or standard (10μL) was added to 500μL of urease buffer reagent in 10mL polypropylene tubes and incubated in a 37°C water bath for 30 minutes. The following were then added in order: phenol nitroprusside solution (1mL), alkaline hypochlorite solution (1mL) and deionised H_2O (5mL). Tubes were then returned to the water bath for 30 minutes and A_{570} of a chromophore measured using a spectrophotometer with peristaltic pump "sipper" attachment with a 4 sec. draw time (U-2000, Hitachi Ltd, Japan).

5.4.3.7 Plasma insulin assay

Arterial plasma from each lamb was pooled within baseline (-60 to 0 min), acute (15 to 120 min) and semi-acute (150-360 min) phases and insulin concentrations determined using a commercially available kit (cat. PI-12K, Linco Research Inc., USA). All volumes were halved from the manufacturers guidelines to allow extra assays to be conducted from each kit. Recombinant human insulin standards or samples (50μL) and 150μL of assay buffer and 50μL of porcine bound I^{125}-insulin antibody were pipetted into borosilicate test tubes. Tubes were vortexed and incubated overnight at 4°C before 500μL of cold precipitating reagent was added to all tubes. Tubes were vortexed again and incubated for 20min at 4°C, then centriguged at 3000 x g for 15 minutes at 4°C. The supernatant was decanted and bound I^{125}-insulin concentrations in the pellet determined with a gamma counter (1277 Gamma Master, LKB Wallac, Finland).

5.4.4 Statistical analyses

Lambs (n=8) were given a bolus of saline (control) or L-NAME on two separate days in a balanced randomised block design. The pre-infusion samples (baseline) were

averaged to obtain the baseline mean between –60 and 0 min relative to infusion. Data from the response period (15 to 360 minutes post-infusion) were analysed using analysis of variance (ANOVA), using the baseline mean as a co-variate and blocks on day of infusion and individual lambs. From these analyses, the effects of time, treatment (control v. L-NAME) and interaction between time and treatment were obtained. A subsequent analysis of arterial and venous data was completed as above, but including the baseline mean (covariate) in the response period to obtain an initial time point (0 min) for graphical representation. An exception was arterial insulin, which was pooled within baseline (-60 to 0 min), acute (15 to 120 min) and semi-acute (150 to 360 min) phases relative to infusion. These data were analysed as above with the acute and semi-acute phases as the response periods. All errors were calculated as the standard error of the differences of the means (SED) and all statistical analyses performed in Genstat 5.41 (VSN International Ltd, UK) (Payne et al., 1993).

5.5 Results

Arterial NO_x concentrations were decreased by L-NAME treatment (19.0 v. 16.7 ± .94μM for control and L-NAME respectively, $P<.05$, Figure 5.1A), while venous NO_x concentrations were unaffected (19.7 v. 18.1 ± 1.60, $P>.10$, Figure 5.1B). Despite reductions in arterial NO_x concentrations not being reflected in venous plasma, no effect of treatment on hind-limb AVD was observed (.62 v. –1.50 ± 3.016, $P>.10$, Figure 5.1C). No effects of time or interaction between treatment and time were observed on arterial or venous NO_x concentrations ($P>.10$). The hind-limb AVD of NO_x initially decreased with time following L-NAME or saline infusion, then returned

to pre-infusion concentration differences. No interaction between treatment and time was observed on hind-limb NO_x AVD. Arterial (3.5 v. 3.6 ± .19 mmol/L, P=.39) and venous (3.3 v. 3.4 ± .16 mmol/L, P=.55) plasma glucose concentrations and glucose AVD (.19 v. .27 ± .065 mmol/L, P=.20) (Figures 5.2A, 5.2B) were unaffected by L-NAME treatment. The interaction between L- NAME treatment and time was not significant.

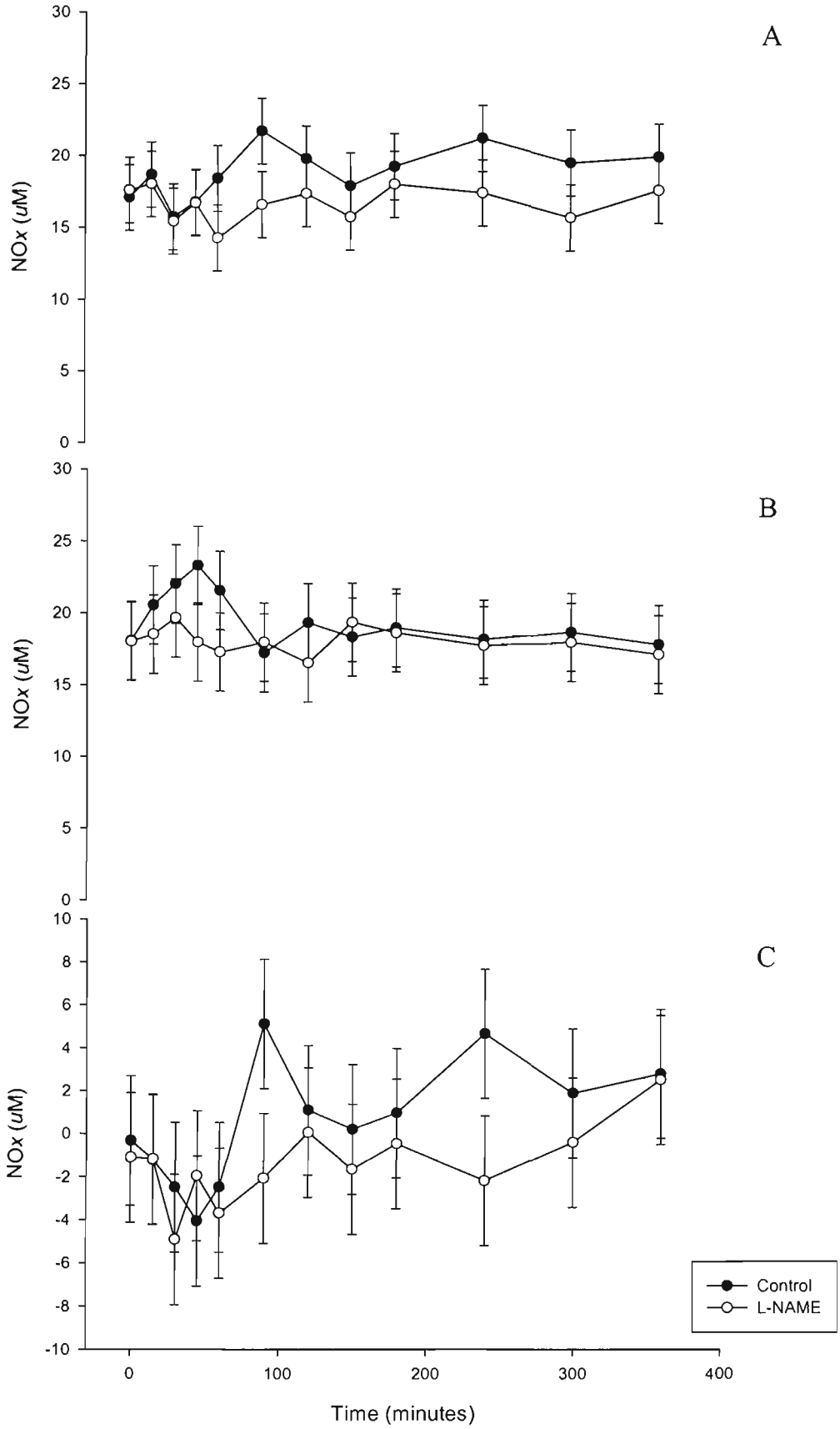


Figure 5.1: Responses in A) arterial and B) venous plasma NO_x or C) AVD after control or 30mg/kg L-NAME infusions. A) P=.20, .049, and .82 ± .256 for time, treatment, and time * treatment, respectively B) P=.54, .37, and .71 C) P= .026, .19, and .51.

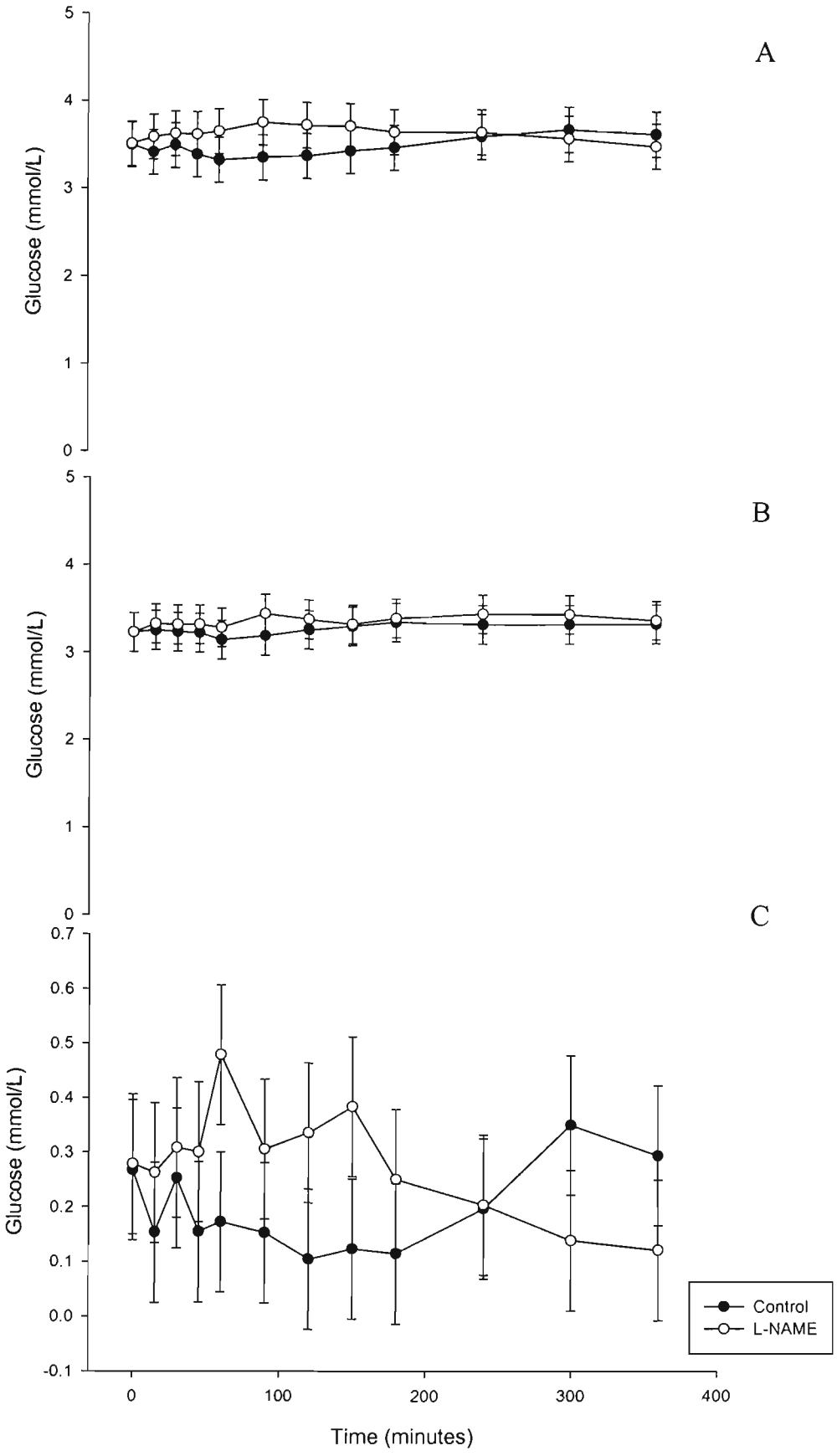


Figure 5.2: Responses in A) arterial, B) venous plasma glucose or C) AVD after control or 30mg/kg L-NAME infusions. A) P=.98, .39, and .54 for time, treatment, and time * treatment, respectively B) P=.88, .55, and .99 C) P= .90, .20, and .038.

for arterial (P=.54) or venous plasma glucose concentrations (P=.99). An interaction (P=.038) between L-NAME treatment and time was observed, such that the glucose AVD was initially increased in the L-NAME infused lambs (up to 180 min), while control concentrations decreased in the corresponding period. The divergence between control and L-NAME responses diminished or was even reversed beyond 240 mins (Figure 5.2C). Arterial plasma insulin concentration was unaffected by L-NAME treatment (25.3 v. 27.8 ± 3.62 mU/L, P=.85), time (P=.85) and no interaction between treatment and time were observed (P=.37).

Arterial (.66 v. .66 ± .101 mmol/L, P=.99), venous (.60 v. .62 ± .100 mmol/L, P=.83) and AVD plasma lactate concentrations (.024 v. .088 ± .0487 mmol/L, P=.24, Figure 5.3) were unaffected by L-NAME treatment. While there was no effect of time on either arterial plasma lactate concentrations (P=.65) or lactate AVD (P=.38), venous plasma lactate tended (P=.060) to increase with time. A short-term increase in arterial lactate concentrations was observed with L-NAME treatment approximately 30 minutes post-infusion, followed by a return to control concentrations (P=.072). Venous (P=.41) and AVD (P=.11) plasma lactate concentrations were unaffected by the acute increase in arterial concentrations.

While arterial plasma NEFA concentrations were unaffected by L-NAME treatment (288 v. 273 ± 19.0 μmol/L, P=.42), increased venous plasma NEFA (222 v. 272 ± 13.2 μmol/L, P=.007) and NEFA AVD concentrations (79.4 v. −13.3 ± 31.5 μmol/L, P=.018) were observed following L-NAME treatment (Figure 5.4). No effects of time on arterial (P=.67) and venous (P=.86) plasma or AVD NEFA concentrations (P=.75)

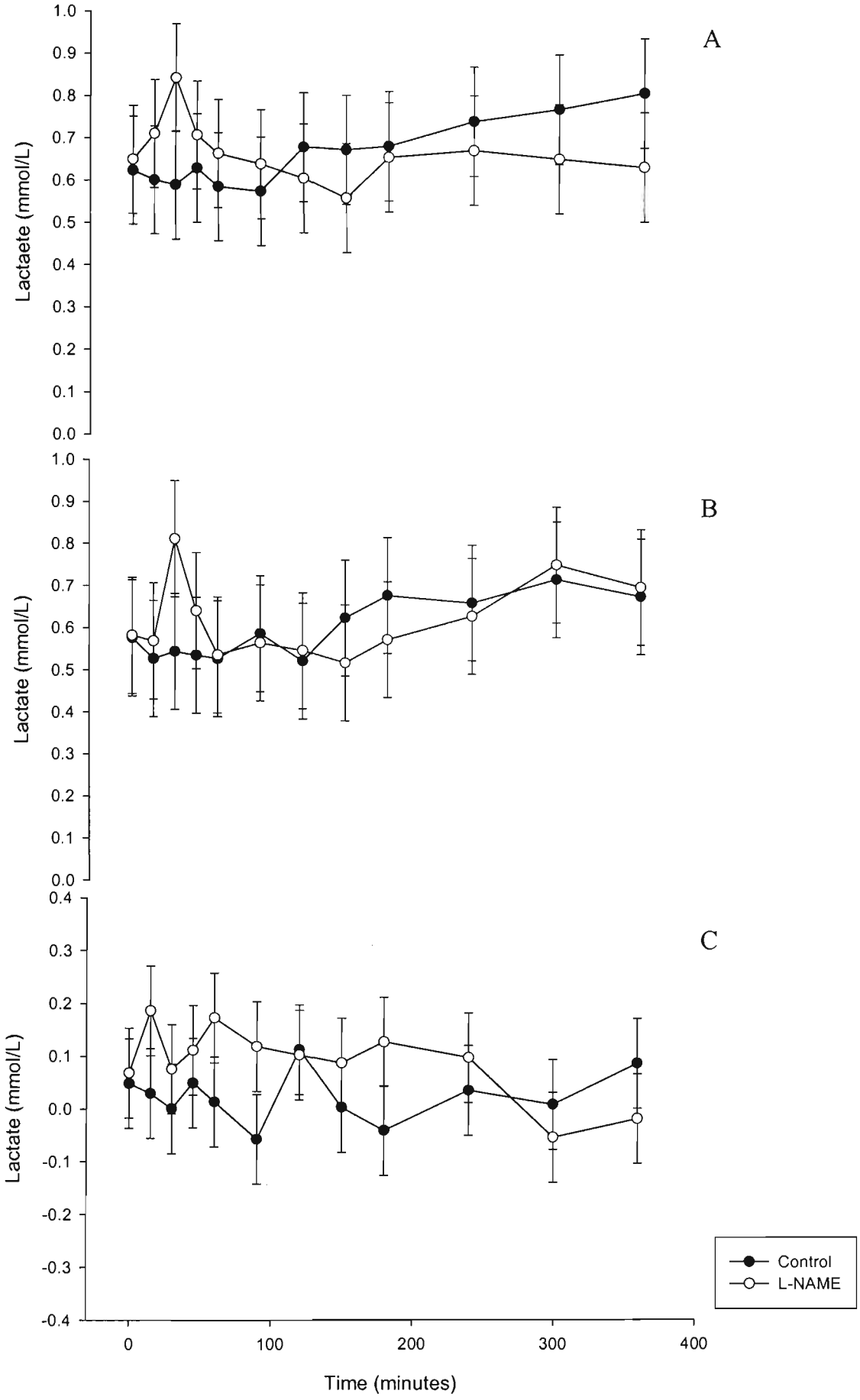


Figure 5.3: Responses in A) arterial, B) venous plasma lactate or C) AVD after control or 30mg/kg L-NAME infusions. A) P= .65, .99 and .072 for time, treatment and time * treatment respectively B) P= .060, .83, and .41 C) P= .38, .24, and .11.

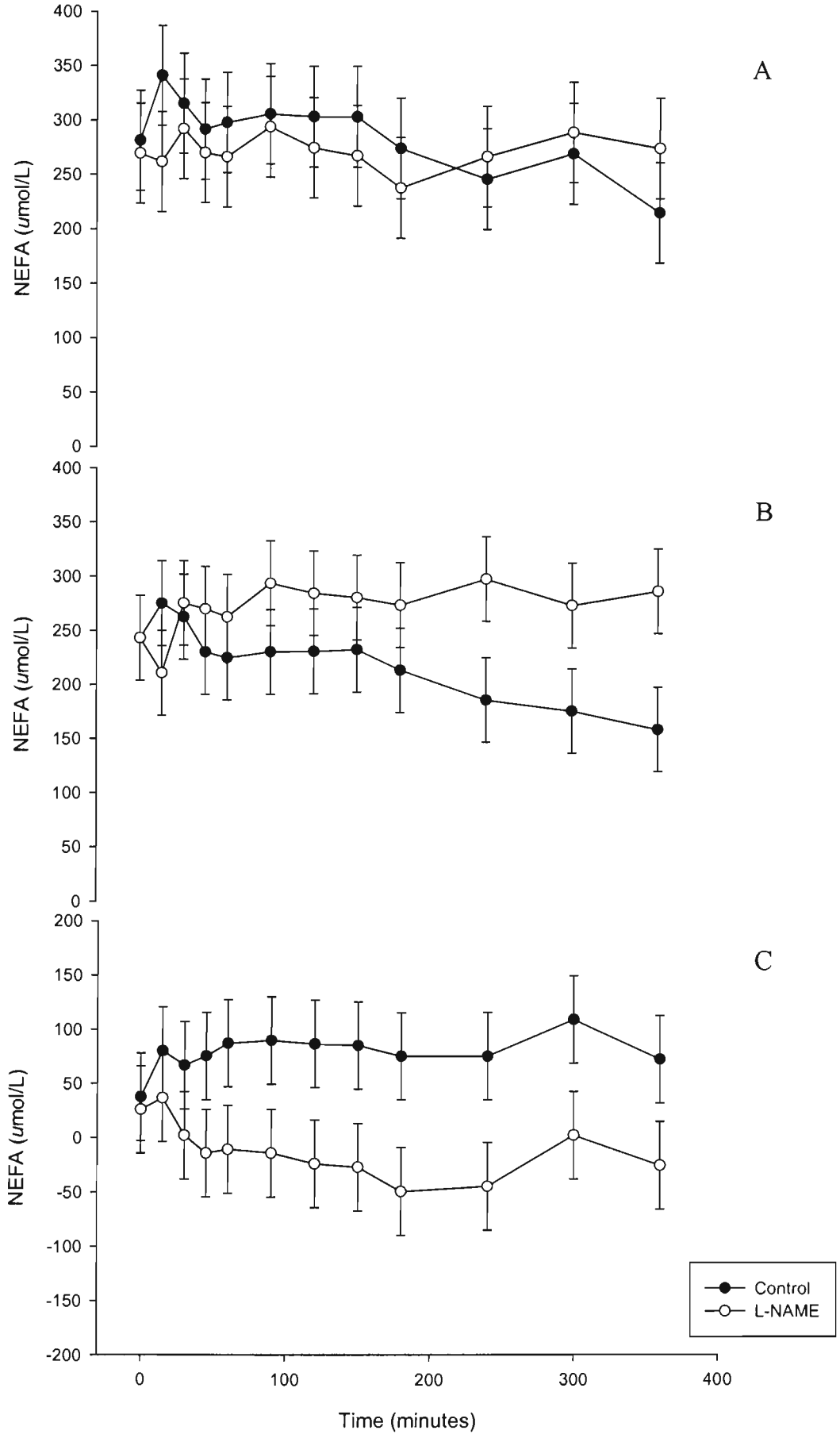


Figure 5.4: Responses in A) arterial, B) venous plasma NEFA or C) AVD after control or 30mg/kg L-NAME infusions. A) P= .67, .42 and .73 for time, treatment and time * treatment respectively B) P= .86, .007 and .063 C) P= .75, .018, and .91.

Venous NEFA concentrations tended to initially increase, then plateau following L-NAME treatment, whereas control plasma NEFA concentrations declined with time (Figure 5.4B). The interaction between L-NAME treatment and time was not significant for arterial (P=.73) or NEFA AVD concentrations (P=.91).

Overall, arterial (156 v. 142 ± 20.8 μmol/L, P=.58) and venous (154 v. 140 ± 20.5 μmol/L, P=.50) plasma TAG concentrations were unaffected by L-NAME treatment (Figure 5.5A, 5.5B). An acute increase in plasma TAG concentration was observed approximately 15 minutes following L-NAME treatment (P<.001), after which concentrations returned to control levels. The acute increase in arterial TAG concentrations with L-NAME treatment was reflected in venous TAG concentrations (P<.05, Figure 5.5B). No effect of L-NAME treatment (-1 v. 2.9 ± 3.17 μmol/L, P=.23) or time (P= .38) were observed on plasma TAG AVD concentrations (Figure 5.5C). The interaction between time and treatment for the AVD of TAG approached significance (P=.088). It is possible that the interaction with time after L-NAME was a reflection of a slight inversion of responses.

Arterial plasma urea concentrations were reduced by L-NAME treatment (7.7 v. 7.3 ± .15 mmol/L, P=.011, Figure 5.6A). While overall venous urea concentrations were unaffected by L-NAME treatment (7.8 v. 7.6 ± .19, P=.35), venous urea concentrations tended to decrease with time following L-NAME infusion (P=.099). There were no effects of L-NAME treatment on AVD concentrations of plasma urea (-.10 v. -.31 ± .188 mmol/L, P=.28). With the

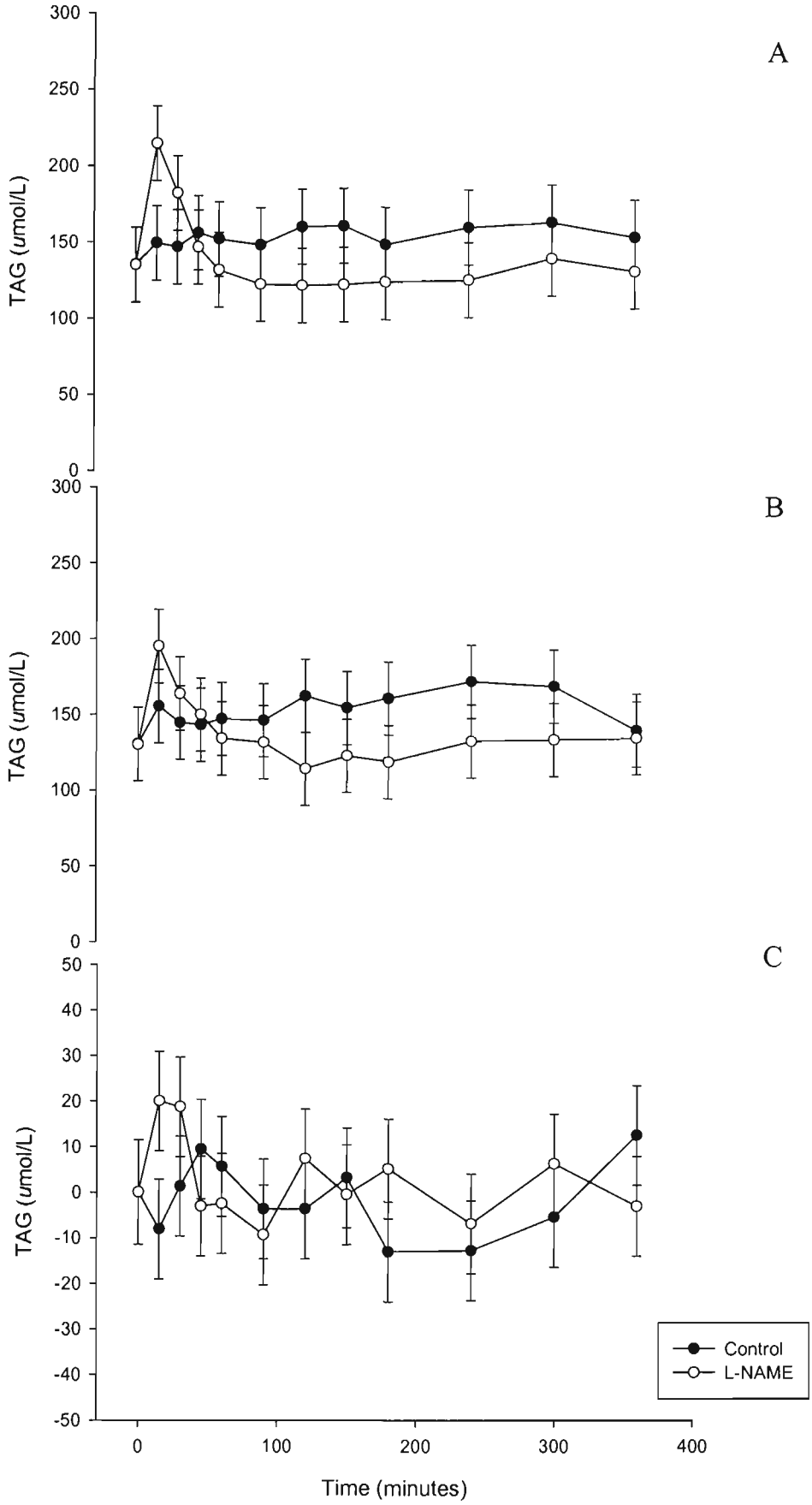


Figure 5.5: Responses in A) arterial, B) venous plasma TAG or C) AVD after control or 30mg/kg L-NAME infusions. A) P<.001, .58 and <.001 for time, treatment and time*treatment respectively B) P= .003, .50 and <.001 C) P= .38, .23 and .088.

exception of arterial urea concentrations, which tended to reduce with time (P=.097), no effect of time, or interaction between time and L-NAME treatment was observed (P>.10).

Arterial plasma haematocrit was not affected by L-NAME (27.4 v. 27.9 ± .79, P=.55) but did tend to increase with time after infusion (.21%/h, P=.10).

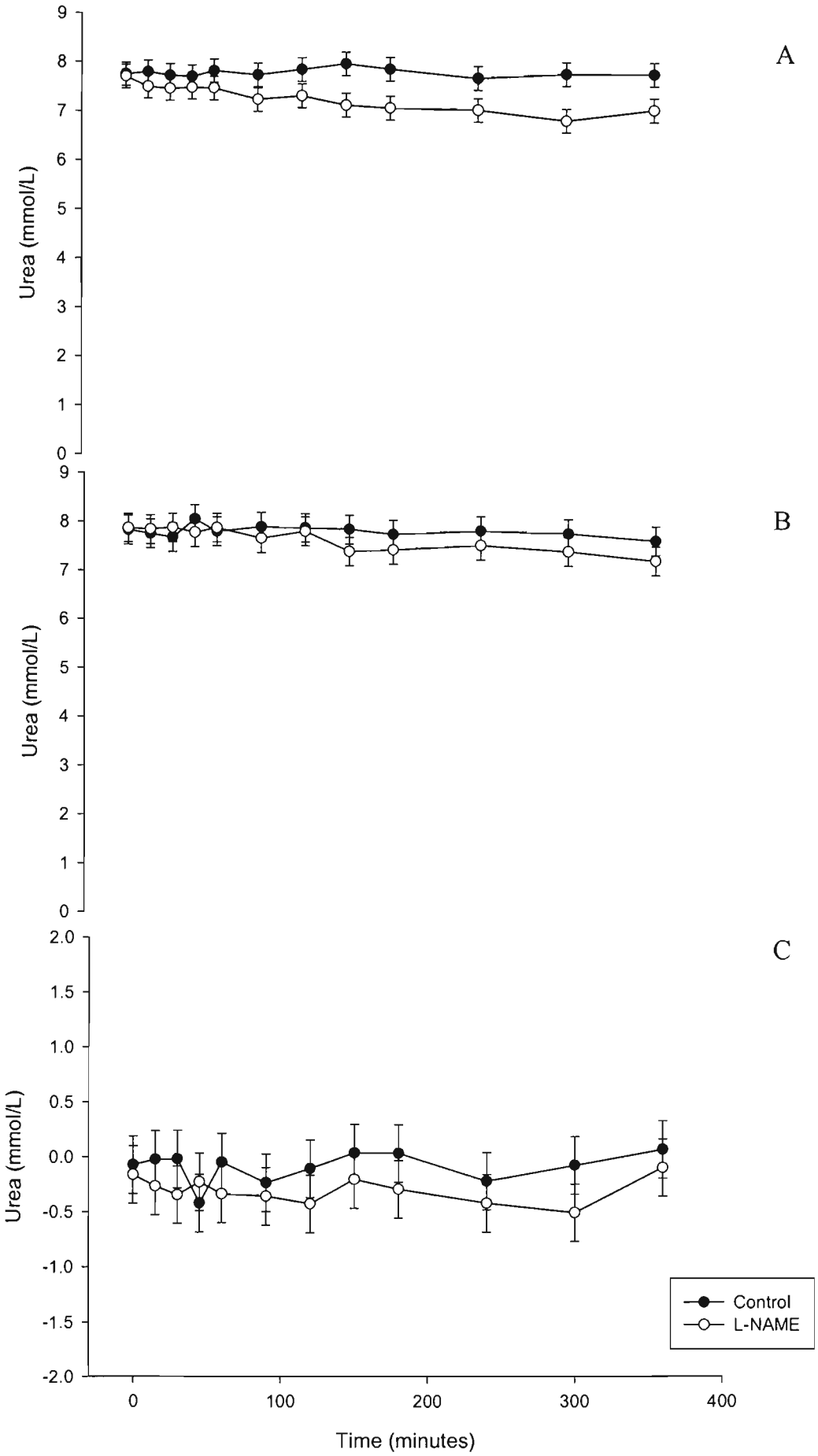


Figure 5.6: Responses in A) arterial, B) venous plasma urea or C) AVD after control or 30mg/kg L-NAME infusions. A) P= .097, .011 and .099 for time, treatment and time*treatment respectively B) P= .14, .35 and .58 C) P= .54, .28 and .85.

5.6 Discussion

Infusion of 30mg/kg L-NAME inhibited systemic NO synthesis, as demonstrated by the decrease in arterial NO*x* concentrations. This supported the first hypothesis of the experiment. Inhibition of NOS with L-NAME influenced both hind-limb and systemic metabolism. The short-term increase in hind-limb glucose AVD indicated increased hind-limb glucose uptake. Venous plasma NEFA concentrations were increased by NOS inhibition, suggesting an increase in lipolysis and/or a decrease in hind-limb NEFA utilisation. Increases in glucose uptake and lipolysis were not influenced by insulin concentrations, which were unaffected by NOS inhibition. Changes in systemic metabolism included decreased urea concentrations, most likely due to altered hepatic amino acid catabolism and/or gluconeogenesis.

Inhibition of NO synthesis through infusion of L-NAME caused an acute increase in glucose AVD across the hind-limb. Increased muscle glucose uptake after infusion of arginine analogues is not unique. For example, Butler et al. (1998) observed increased glucose uptake and leg blood flow with the arginine analogue N^G-monomethyl-L-arginine (L-NMMA) in human calf muscle. Also, Balon et al. (1999) observed that chronic oral ingestion of L-NAME for 14 days by rats increased muscle glucose uptake after an insulin challenge, but not under basal conditions. In the same animals, a decreased insulin response to oral glucose challenges was observed, indicating that while chronic NOS inhibition increased peripheral insulin responsiveness, it inhibited insulin production. Lajoix et al. (2001) found that pancreatic ß-cells have nNOS localised in insulin secretory granules and insulin release was stimulated (rather than inhibited) by inhibition of NOS with L-NAME,

while inhibition was observed with SNP. Therefore, the apparent increase in hind-limb glucose uptake in the present study may be a function of increased insulin sensitivity, rather than increased insulin concentrations. This supports hypothesis 2, that L-NAME will act as an insulin agonist.

The present data contrast with other experiments that found inhibition of NOS with L-NMMA decreased rat muscle glucose uptake (Baron et al., 1995, Balon and Nadler, 1997, Baron et al., 2000). Also, Young et al. (1997) found that NO donors increased insulin- or contraction-mediated glucose uptake. They proposed that muscle glucose uptake was increased due to elevated muscle blood flow mediated by insulin or pharmacological vasodilators. According to Baron et al. (1995, 1996) as much as 30% of insulin-dependant glucose uptake can be attributed to increased muscle blood flow. However, Bradley et al. (1999) observed that L-NMMA reduced leg glucose AVD in the exercising human independently of blood flow. In a similar study, Higaki et al. (2001) showed that L-NMMA did not change contraction-induced glucose uptake, whereas SNP increased glucose uptake. From this, Higaki et al. (2001) hypothesised that NO increases glucose uptake in skeletal muscle by a mechanism independent of insulin or contraction. Although our data indicate that there was an increase in hind-limb glucose uptake with NOS inhibition (rather than a NO donor), both experiments indicate the involvement of NO in regulating limb glucose uptake is independent of insulin concentrations.

While some of these findings may appear contradictory, one must be careful when making direct comparisons between different NOS inhibitors. For example, the arginine analogue L-NAME is a more specific and longer-acting inhibitor of nNOS

than L-NMMA (Klatt et al., 1996). Studies by Baron et al. (1995, 1996) and (Bursztyn et al., 1997) investigated haemodynamic effects of insulin on glucose uptake, mediated by eNOS during L-NMMA induced vasoconstriction. The choice of L-NAME for the present experiment reflects the different aim, to measure metabolic changes in skeletal muscle after perturbation of metabolic homeostasis by inhibition of nNOS, the most prevalent skeletal muscle isoform of NOS (Nakane et al., 1993, Kobzik et al., 1994).

As discussed previously, an acute increase in hind-limb glucose uptake may be due to increased non-insulin concentration-dependent glucose uptake by skeletal muscle or increased insulin sensitivity rather than to altered haemodynamics. In the sheep, approximately 80% of whole body glucose utilisation is non-insulin-dependent (Petterson et al., 1993) and even in tissues that are reliant on insulin, such as skeletal muscle, there is a component of glucose uptake that is not insulin-dependent. This component of glucose utilisation would presumably increase when glycemia is increased during L-NAME treatment (Gottesman et al., 1983). In addition, expression of glucose transporters on the surface membrane of muscle cells are NO-sensitive. Etgen et al. (1997) observed increased glucose uptake due to increased surface expression of GLUT-4 glucose receptors in isolated rat *epitrochlearis* muscles pre-incubated with SNP for 90 minutes. While this observation runs counter to our data, it does demonstrate that regulation of glucose uptake can be influenced on the cellular level by NO and that experiments in live animals can yield vastly different results than in isolated muscles.

Neither arterial nor venous plasma lactate concentrations, nor hind-limb lactate AVD were significantly affected by infusion of 30 mg/kg L-NAME, indicating that NOS inhibition did not affect the rate of glycolysis in resting, *ad-libitum* fed lambs. Likewise, Licker et al. (1998) observed that concentrations up to 100mg/kg L-NAME had no influence on circulating lactate concentrations in anaesthetised pigs. However, Mills et al. (1999) reported that infusion of a 20mg/kg L-NAME significantly increased plasma lactate in exercising horses. From this, it does not appear that NO influences lactate production in resting tissues, but rather NO may influence lactate production when glycolysis is stimulated.

Plasma NEFA concentrations were significantly increased by NOS inhibition, particularly after 180 minutes post-infusion. Plasma NEFA are derived from lipolysis of triglycerides in either adipose tissue or from hydrolysis of circulating triglycerides (Pethick and Dunshea, 1993). While the surgical hind-limb preparation is used primarily as a model for skeletal muscle metabolism (Dunshea et al., 1995), the hind-limb of sheep also contains, depending on age, breed and nutrition, between 5% (Oddy et al., 1984) and 30% (Ulyatt and Barton, 1963) adipose tissue. The mechanism initiating increases in venous NEFA concentrations is unlikely to involve plasma TAG hydrolysis since plasma TAG concentrations were not reduced by NOS inhibition. Therefore, it is likely that increased venous NEFA concentrations are due to increased adipose or skeletal muscle TAG hydrolysis or reduced NEFA utilisation.

Chronic feeding of rats with an arginine analogue has been observed to inhibit the activity of the rate-limiting enzyme of fatty acid oxidation, carnitine palmitoyltransferase, while fatty acid synthesis was unaffected. This indicated that

increases in circulating triglyceride concentrations associated with NOS inhibition were due to reduced fatty acid oxidation (Khedara et al., 1999). Conversely, Picard et al. (2001) observed that reduced fatty acid oxidation during endotoxin-induced hypertriglyceridemia was due to inhibition of lipoprotein lipase (LPL) activity in rat skeletal muscle by NO overproduction. In a demonstration that lipolysis is redox-sensitive, lipolysis in white adipose tissue was increased and decreased respectively by pharmacological donors of NO yielding the nitrosonium cation (NO^+) and $NO^\bullet$ (Gaudiot et al., 1998). Other experiments conducted *in vivo* have linked NO to lipolysis, Fruhbeck and Gomez-Ambrosi (2001) observed elevated levels of plasma NO_x during leptin-stimulated lipolysis. It is not known whether NOS inhibition resulted in substantial LPL inhibition in this experiment, nor whether LPL inhibition alone could result in the acute increase in arterial TAG observed or the apparent reduction in NEFA utilisation. Likewise the involvement of HSL cannot be excluded, particularly due to the NO mediated effects on insulin sensitivity and tissue perfusion reported elsewhere.

5.7 Conclusions

These data indicate that NO infusion of 30mg/kg L-NAME is sufficient to inhibit endogenous production of NO in lambs, supporting hypothesis 1. The results of NOS inhibition with L-NAME indicate that NO is involved in regulating carbohydrate and fat metabolism since NOS inhibition caused an acute increase in hind limb glucose uptake and a sustained decrease in NEFA uptake in rested, fed lambs. These data did not support hypothesis 2, since L-NAME increased insulin sensitivity, rather than act as an insulin antagonist. The decrease in NEFA uptake is due to either increased adipose tissue lipolysis, a decrease in adipose re-esterification, or a decrease in the

uptake of pre-formed fatty acids liberated after hydrolysis of circulating triglycerides. Therefore, these data did not support hypothesis 3, since L-NAME did not increase lipogenesis. This was observed without alteration in lactate formation, indicating that in resting animals, NO is not involved in glucose oxidation. Nitric oxide may mediate its metabolic effects by a variety of mechanisms including metabolic changes at a cellular level including non-insulin dependent glucose uptake, via endocrine hormones other than insulin or by changing hind-limb blood flow. From these data, it is evident that NO is involved in carbohydrate metabolism and therefore may provide mechanisms to buffer against pre-slaughter glycogen depletion.

Chapter 6: Inhibition of nitric oxide synthase pre-slaughter increases post-mortem glycolysis and improves tenderness in ovine muscles.

6.1 Introduction

Nitric oxide (NO) has emerged as an important regulator of skeletal muscle homeostasis, where it interacts with many physiological pathways influencing meat quality. Nitric oxide may prove a useful agent for improving meat quality as it influences many systems important to meat quality. This potentially includes protecting against "dark cutting", which is normally a result of stress induced glycogen depletion pre-slaughter (Lawrie, 1958). Glycogen depletion may be due to the ability of NO to influence muscle glucose uptake (Balon et al., 1999, Etgen et al., 1997), glycogenolysis (Borgs et al., 1996, Jaffrey et al., 2001) and glycolysis (Mohr et al., 1996). Furthermore NO has been observed to inhibit calpain activity (Michetti et al., 1995, Koh and Tidball, 2000) and may therefore influence muscle protein turnover and inhibit meat tenderisation. Nitric oxide also influences calcium homeostasis via direct and indirect effects on the sarcoplasmic/ endoplasmic reticulum ATPase (SERCA) and ryanodine receptors (RyR) (Meszaros et al., 1996, Ishii et al., 1998, Eu et al., 1999). Levels of cytosolic calcium can have a significant impact on meat quality via activation of proteases (Suzuki et al., 1981, Dayton, 1982), direct weakening of Z-discs (Takahashi et al., 1987, Takahashi, 1992) or, depending on conditions, sarcomere shortening (Joseph, 1996, Van Moeseke et al., 2001).

While injection of inhibitors of nitric oxide synthase (NOS) into hot-boned beef has been observed to reduce tenderness in one study (Cook et al., 1998), it has been hypothesised that NOS activity post-slaughter is limited (Brannan et al., 2001, Brannan and Decker, 2002, Chapter 3). Biosynthesis of NO is tightly regulated, and NOS requires many co-factors as well as O_2 and NADPH as co-substrates for activity (Bredt and Snyder, 1994). It is unlikely that NOS remains active for very long post slaughter. Since inhibition of endogenous NOS has only been attempted in hot boned muscles (Cook et al., 1998), where activity is likely to be attenuated (Brannan et al., 2001, Brannan and Decker, 2002, Chapter 3), the real effect of endogenous NO on meat quality remains untested.

6.2 Aims

The aims of this experiment were to investigate the effect of endogenous NO on meat quality by increasing and decreasing muscle NOS activity with exercise (Jungersten et al., 1997, Roberts et al., 1999) and pharmacological inhibitors, respectively.

6.3 Hypotheses

1. L-NAME will increase glycolysis in LTL and SM muscles post-slaughter, reducing glycogen and increasing lactate concentrations respectively.
2. L-NAME will increase WBSF in LTL and SM muscles.
3. L-NAME will decrease sarcomere length in LTL and SM muscles.
4. Exercise will increase LTL and SM glycolysis, evidenced by decreased glycogen and increased lactate concentrations respectively.

5. L-NAME will influence the effects of exercise on plasma glucose and lactate and muscle glycogen and lactate metabolism.

6.4 Methodology

6.4.1 Animals and catheterisation

Forty Border Leicester/ Merino cross lambs, approximately six months old, ranging between 33.5 and 51.0 kg live weight were housed in individual pens with visual and audial contact for 10-14 days pre-slaughter and acclimatised to handlers, diet and environment. During the acclimatisation period, lambs had access to lucerne chaff, lamb cubes (Barastoc Stockfeeds, Table 5.1) and water *ad libitum*. Lambs were subjectively assessed twice daily for their levels of aversion to stock handlers (low, medium or high) within each group (4 groups of 10 lambs). At one day pre-slaughter, an indwelling catheter (12G) was inserted intravenously (i.v.) into the jugular vein and animals were deprived of food approximately 12 hours pre-slaughter, with *ad libitum* access to water as per normal slaughter practices. Catheter patency was maintained by flushing with 12.5g/L K_2EDTA in saline (0.9% NaCl).

6.4.2 Experimental treatments and blood sampling

In a 2 x 2 factorial design, lambs were allocated to the treatments 0 (saline) v. 30 mg/kg L-NAME and 0 v. 15min exercise. Treatments were balanced equally across the whole experiment and level of aversion to the stock handler index (low, medium or high). On the day of slaughter, lambs were infused with either saline or L-NAME (30 mg/kg in saline) injected as a bolus (10mL) i.v. via the jugular catheter 135 minutes pre-slaughter, as per Chapter 5. Venous blood samples (5mL) were removed

–90, -60, -30, 0, 30, 60, 90 and 120 minutes relative to infusion. After the 120min post-infusion blood sample, all lambs were walked approximately 150m from holding pens to abattoir. The exercise regime was conducted in a diversionary paddock, where individual lambs were exercised for 15 minutes by a stock handler. Lambs isolated in the diversionary paddock all avoided the stock handler and behaviour included sprinting and occasional running into fences. All exercised lambs displayed heavy panting and increasingly reduced avoidance over the 15 minute exercise period, indicating muscular fatigue. The stock handler minimised lambs running into fences by following the lambs rather than chasing them. Exercise has been observed to increase NOS activity in human skeletal muscle (Roberts et al., 1999), where it inhibits muscular contraction (Marechal and Gailly, 1999) and is hypothesised to be involved in the development of muscular fatigue (Stamler and Meissner, 2001). Therefore, muscular fatigue was used as a behavioural end-point of the exercise regime and not an exercise bout with pre-defined timing. This enabled the level of exercise to be controlled with the individual lambs disposition and relative level of fitness. It is highlighted that exercise does not specifically activate NOS signalling cascades as exercise also involves a significant endocrine (eg. adrenergic) response. Non-exercised sheep were allowed 15 minutes to move from the sheep shed to abattoir accompanied by a flock of 5 trained sheep. Minimal coercion was given and potential stressors minimised. One final blood sample was removed approximately 135 minutes post-infusion while the lamb was restrained in the pilot abattoir V-restrainer, immediately before slaughter.

6.4.3 Slaughter and measurements

6.4.3.1 Slaughter and carcass dressing

Lambs were restrained in a V-restrainer and electrically stunned with a dual point electrode placed to the head, exsanguinated and killed via cervical dislocation. Carcasses were trimmed according to the specifications of AUS-MEAT (Anon, 1992) and chilled at 2°C and ~86% humidity overnight.

6.4.3.2 Muscle pH

The post-mortem pH and temperature was measured at the 13th thoracic vertebra in the LTL and at a depth of approximately 5cm in the SM at .5, 1, 2, 3, 4, 5, 6 and 24 hours post-slaughter. Measures were obtained using equipment outlined in section 2.4.2. Muscle temperature at pH 6, defined as the rigor temperature was calculated as per Equation 6.1 (Hwang and Thompson, 2001). The linear rate of pH fall between 30 min and 6 hours was calculated as the slope (dpH/dTime)

Equation 6.1: Rigor temperature = T_n- ((pH_n-6)/ (pH_n-pH_{n-1})*(T_n-T_{n-1}))

Where:

T_n = Temp immediately before pH 6

T_{n-1} = Temp immediately after pH 6

pH_n = pH immediately before pH 6

pH_{n-1} = pH immediately after pH 6

6.4.3.3 Warner-Bratzler shear force and cook loss

Approximately 24 hours post-slaughter, the LTL and SM were removed from one side of the carcass (randomly selected). Each muscle was halved and randomly allocated to 1 and 3 days ageing. The sample for 1 day of ageing was trimmed to 80g (exact

weight recorded) and cooked as outlined in section 3.4.6, while the 3 day sample was vacuum packed in plastic bags and stored at 2°C. The aged samples were then cooked as per 1 day samples. Meat samples were blotted dry after cooking and the percent difference between the cooked weight and initial weight calculated as cook loss.

6.4.3.4 Total water content, purge and drip loss

Samples of approximately 20g were taken from the LL at 24hrs post-slaughter and allocated for measurement of total water content, muscle purge and drip loss. Samples for total water content were accurately weighed and dried in an oven at 100°C for 24 hours. The total water content of the muscle was then calculated as the percent of the dried weight relative to the initial weight.

Samples for muscle purge were blotted of excess moisture and accurately weighed prior to vacuum packaging. Following storage at 2°C for 48hrs, samples were removed from vacuum bags, blotted of excess moisture and weighed. Muscle purge was measured as the percent difference in weight of vacuum-packed samples stored at 2°C between day 1 and day 3.

Drip loss was measured by suspending an accurately weighed 20 g meat sample in a plastic jar for 24 hours at 2°C (Rasmussen and Andersson, 1996). Following this, the meat sample was then re-weighed and the percent difference recorded as drip loss.

6.4.3.5 Surface colour (L,a*,b*)*

Surface colour L* (Lightness), a* (Redness) and b* (yellow) were measured as per section 3.4.4 at 24 hours and 3 days post-mortem.

6.4.3.6 Sarcomere length

Sarcomere length was determined using a helium-neon laser diffraction unit (custom built by the University of New England, Armidale). Muscle samples were cut parallel with the fibres in 4cm x 1cm^2 strips, frozen in liquid nitrogen and stored at –20°C. A thin slice of frozen muscle was placed between two microscope slides, thawed and squeezed flat before measuring the distance of light band diffraction in the muscle samples. Sarcomere length was then calculated as per Equation 6.1 (Ruddick and Richards, 1975).

Equation 6.1: Sarcomere length (mm) = 0.635/sin(arctan(d/75))

6.4.4.7 Plasma glucose and lactate

Plasma glucose and lactate were determined as outlined in sections 5.4.3.2 and 5.4.3.3.

6.4.3.8 Muscle glycogen

Muscle necropsies were removed from the LTL at 2 minutes, 6 and 24 hours post-slaughter and from the SM at 2 and 30 minutes, 1, 2, 4, 6 and 24 hours post-slaughter. Necropsies (200mg) devoid of visible fat or connective tissue were weighed and immediately homogenised in 1mL 3.6% perchloric acid (PCA) with 0.5mm glass beads using a mini bead-beater (Biospec Products Inc., USA) at 5500rpm for 2min. Homogenates were centrifuged at 13,500 x g for 5 minutes (EBA 12, Hettich Zentrifugen, Germany), the supernatant decanted, frozen and stored at –20°C until analysis.

Muscle glycogen in the supernate (50μL) was hydrolysed to glucosyl units from acid extracted supernatant by amyloglucosidase (Boehringer Mannheim, Germany cat 737160) diluted in acetate buffer (500μL) for 90 min at 37°C (Chan and Exton, 1976). Acetate buffer consisted of 40mM sodium acetate (.82g), pH adjusted to 4.8 with glacial acetic acid, 150U/mL amyloglucosidase was added fresh before assay. Glucosyl units were then measured using a modification of the plasma glucose assay described in section 5.2.3.2. The concentration of glucosyl units is equivalent to glycogen and is referred to as glycogen from here on. Hydrolysed glycogen in acetate buffer (80μL) was added to 1mL glucose reagent in a polypropylene test tube and incubated in a shaking water bath (model SWB20, Ratek Instruments Pty, Ltd, Australia) for 30min at 37°C. Glucose concentrations were then measured by changes in A_{450} (U-2000, Hitachi Ltd, Japan) relative to glucose standards.

6.4.3.9 Muscle lactate

Muscle lactate was determined from the same supernate used for measuring glycogen concentrations (section 6.4.3.6). Before the assay 43 U/mL lactate dehydrogenase (LDH, cat 107069, Boehringer Mannheim, Germany) and 1.77 U/mL glutamic pyruvic transaminase (GPT, cat 737127, Boehringer Mannheim, Germany) were added to aminopropanol buffer (200mM 2-amino-2-methylpropanolol, 40mM sodium glutamate and 100mM NAD^+). Supernate (20μL), dH_2O (80μL) and aminopropanolol buffer containing LDH and GPT (900μL) were added to polypropylene test tubes and incubated at 25°C for 60 minutes in a shaking water bath (model SWB20, Ratek Instruments Pty, Ltd, Australia). During the incubation period, background absorbance of supernate (20μL), and aminopropanolol buffer (excluding

NAD^+, LDH and GPT) was measured at 340nm (U-2000, Hitachi Ltd, Japan). The difference in A_{340} after production of NADH from NAD by LDH and background absorbance was used to determine lactate concentrations in the wet weight of the muscle (Noll, 1985, Passonneau and Lowry, 1993)

6.4.3.10 Detection of calpain autolysis by western blotting

Muscle necropsies were removed 5 min, 6 and 24 hours post-mortem, wrapped in aluminium foil, flash frozen in liquid nitrogen and stored at –80°C until analysis. Muscle samples (150mg) were homogenised in 1.5mL of buffer (40mM KCl, 40mM KH_2PO_4, 40mM K_2HPO_4, 1mM EGTA, 1mM $MgCl_2.6H_2O$, 1mM NaN_3, pH 7.0) using a Mini Bead-Beater (Biospec Products Inc, USA) with 0.5mm zirconia-silica beads for 3 minutes. Protein concentration was determined using the Biuret method outlined in section 3.4.7. The homogenate was diluted to 2 mg/mL in loading buffer (8 M urea, 2 M thiourea, 75 mM DTT, 3% SDS, .05% bromophenol blue, 50 mM Tris, pH 6.8) and heated in a dry block heater (model DB3D, Thermoline, Australia) at 100°C for 1 minute immediately before addition to gel. Fifteen micrograms of protein per lane and a single lane of pre-stained molecular weight standard (cat 161-0305, BIORAD laboratories, USA) were added to gels (resolving gel: 8% acrylamide, .5% *Bis*-acrylamide, pH 9.3, 4% stacking gel). Electrophoresis of proteins was performed with a 40 mA current at room temperature using a Mini-Protean II cell and Power Pac 300 (BIORAD laboratories, USA).

Upon completion of SDS-PAGE gels were immediately placed on a .2 μ PVDF membrane (cat. 162-0184, BIORAD, USA), previously activated in 100% methanol and rinsed in deionised H_2O. The activated membrane and gel were returned to the

Mini-Protein Cell and protein transfer was achieved using a 400 mA current at 4 °C for 4 hours. The gel was then removed and the membrane rinsed with dH_2O and blocked in 5% milk powder/ TBST (200 mM NaCl, .05% Tween-20, 50 mM Tris.HCl, pH 7.5) for one hour. The solution was discarded and the membrane washed twice (10 min each) with TBST solution. The membrane was incubated with either anti-μ-calpain domain III (1:2000 dilution) or anti-m-calpain domain III (1:500 dilution) mouse monoclonal antibodies (cat. SA-257 and SA-255 respectively, BIOMOL Research laboratories Inc. USA) for 4 hours on a rocking platform (Vibrax-VCR, IKA Labortechnik, Germany). Following two more rinses in TBST, the membrane was returned to the rocking platform for 2 hours with (1:7500 dilution) an alkaline phosphatase linked anti-mouse IgG 2° antibody (cat. S372B, Promega, USA). The membrane was then washed twice with TBS (no Tween) and moistened with chemiluminescent alkaline phosphate substrate (cat. 2041677, Roche Diagnostics, Switzerland). Chemiluminescence was detected using a CCD camera at –20°C for 30 minutes (LAS 3000, Fujifilm medical systems, USA).

6.4.5 Statistical analyses

Four slaughters, consisting of 10 animals each, were conducted on separate days in a 2 x 2 factorial with a balanced randomised block design. The effects of L-NAME (L, 0 v. 30 mg/kg), exercise (E, no v. yes) and time (T) were tested for significance with an analysis of variance (ANOVA) using Genstat v5.41 (Payne et al., 1993). Data were blocked for the slaughter day (n=4) and qualitative assessment of the lamb's level of aversion, obtained during the familiarisation period (low, medium and high). Interactions between L-NAME, exercise and time were analysed for plasma glucose and lactate and muscle glycogen and lactate, where a number of sequential

observations were made. A freezer malfunction resulted in loss of half of the blood samples taken immediately pre-slaughter (n=20). These data were excluded and remaining data analysed with intra-block ANOVA's and regression procedures used to predict means when they were not supplied. Data from drip and purge loss measures was not normally distributed and was analysed following a $\log_{10}$ transformation. Data was then back-transformed and presented as geometric means. All errors are presented as the SED.

6.5 Results

Plasma glucose concentrations before exercise were unaffected by L-NAME infusion (3.1 v. 3.0 mmol/L ± .04 for 0 v. 30 mg/kg L-NAME, P=.56). While plasma glucose concentrations gradually declined prior to exercise (P=.007, Figure 6.1A), there was no interaction between L-NAME and time (P=.57). Plasma lactate concentrations before exercise were unaffected by L-NAME (.44 v. .45 ± .016 mmol/L, P=.40, Figure 6.1B) or time (P=.34). Exercise increased plasma glucose concentrations 2-fold (P<.001) and lactate concentrations 6-fold (P<.001) respectively (Table 6.1). Plasma glucose concentrations after exercise were reduced in lambs infused with L-NAME (P<.01), while post-exercise lactate concentrations were unaffected by L-NAME (P>.10). Plasma lactate concentrations tended to be reduced by L-NAME in non-exercised lambs (P<.10).

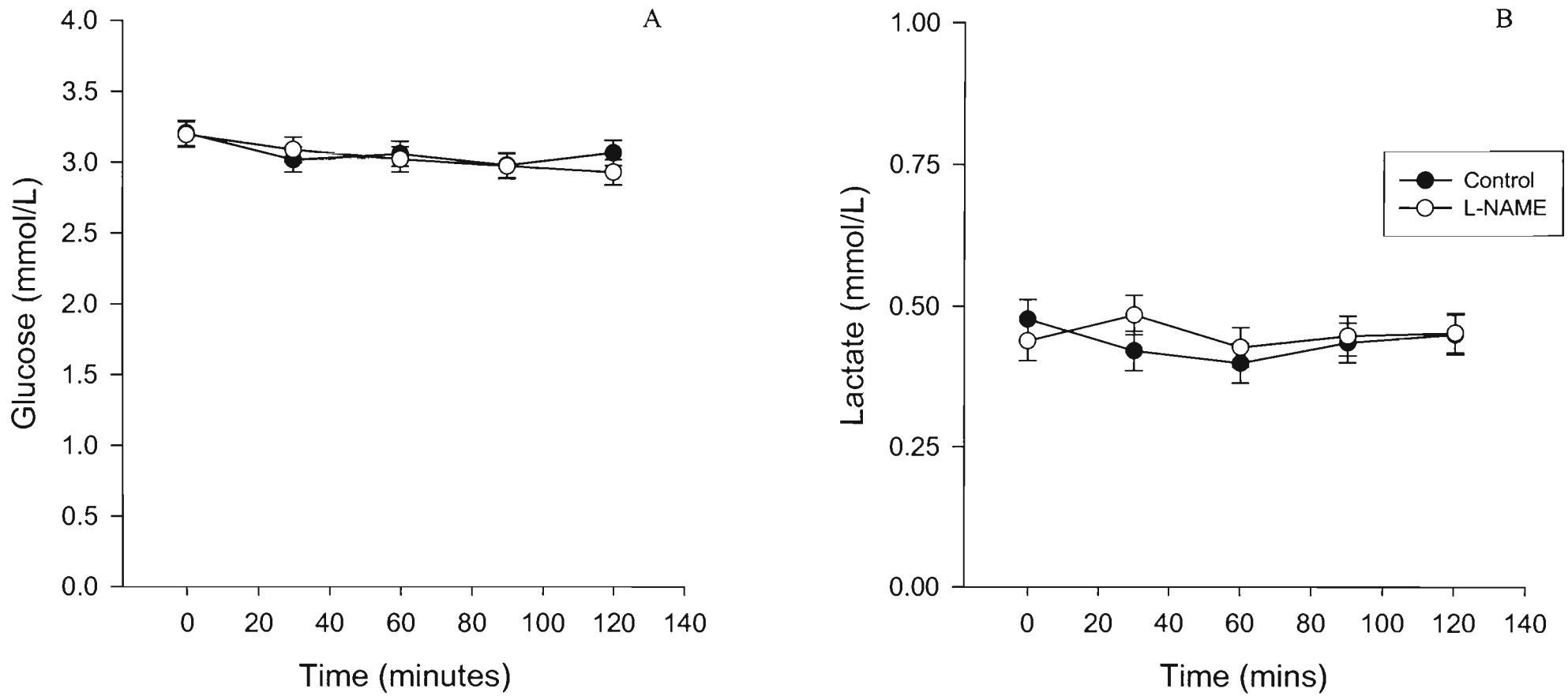


Figure 6.1: Infusion of L-NAME did not affect plasma concentrations of glucose (A) or lactate (B). Sample concentrations between –90 and 0 minutes pre-infusion are presented as an average. P=.57 and .34. for the interaction between L x T for Figures A and B respectively.

Table 6.1: Plasma glucose and lactate concentrations immediately pre-slaughter after L-NAME (0 v. 30 mg/kg) and exercise (no v. yes) treatments.

Exercise (E)	No		Yes				Significance		
L-NAME (L)	0	30	0	30	SED	n	L	E	L x E
Glucose (mmol/L)	3.7	3.0	9.4	6.4	.56-.67	20	.003	<.001	.017
Lactate (mmol/L)	3.7	1.3	13.8	14.2	.74-.84	20	.13	<.001	.058

Muscle glycogen concentrations were reduced by L-NAME infusion in SM (38.2 v. 35.0 ± 1.42 μmol/g for 0 v. 30 mg/kg L-NAME, P=.025), but not LTL muscles (30.8 v. 29.1 ± 2.55, P=.52). Exercise halved glycogen concentrations in SM (41.7 v. 18.2 ± 2.55 for no v. yes exercise, P<.001) and LTL muscles (51.3 v. 21.9 ± 1.42, P<.001). Infusion of L-NAME reduced glycogen concentrations in non-exercised SM (P<.001, Figure 6.2A) and LTL (Figure 6.3A, P<.01) muscles. Concentrations of glycogen in both the SM and LTL muscles fell with time between slaughter and 24 hours post-mortem (P<.001 for SM and LTL), but no interactions between L-NAME or exercise and time (P>.10 for LTL and SM respectively) or L-NAME, exercise and time were observed (P>.10 for SM and LTL respectively, Figures 6.2A and 6.3A).

Infusion of L-NAME increased lactate concentrations in the SM (16.5 v. 17.9 ± .53 μmoles/g for 0 v. 30 mg/kg L-NAME, P=.009) while no main effect of L-NAME was observed in the LTL (25.9 v. 25.6 ± 1.30 P=.86). Exercise increased lactate concentrations in the SM (15.0 v. 19.3 ± .53 for no v. yes exercise, P<.001) and to a lesser degree in the LTL (24.6 v. 26.8 ± 1.3, P=.094). Infusion of L-NAME increased lactate concentrations in exercised SM (P<.001, Figure 6.2B) and LTL muscles (P=.018, Figure 6.3B). Concentrations of lactate in both the SM and LTL muscles increased with time between slaughter and 24 hours post-mortem (P<.001 for SM and LTL), but no interactions between L-NAME or exercise and time (P>.10) or L-NAME, exercise and time were observed (P>.10).

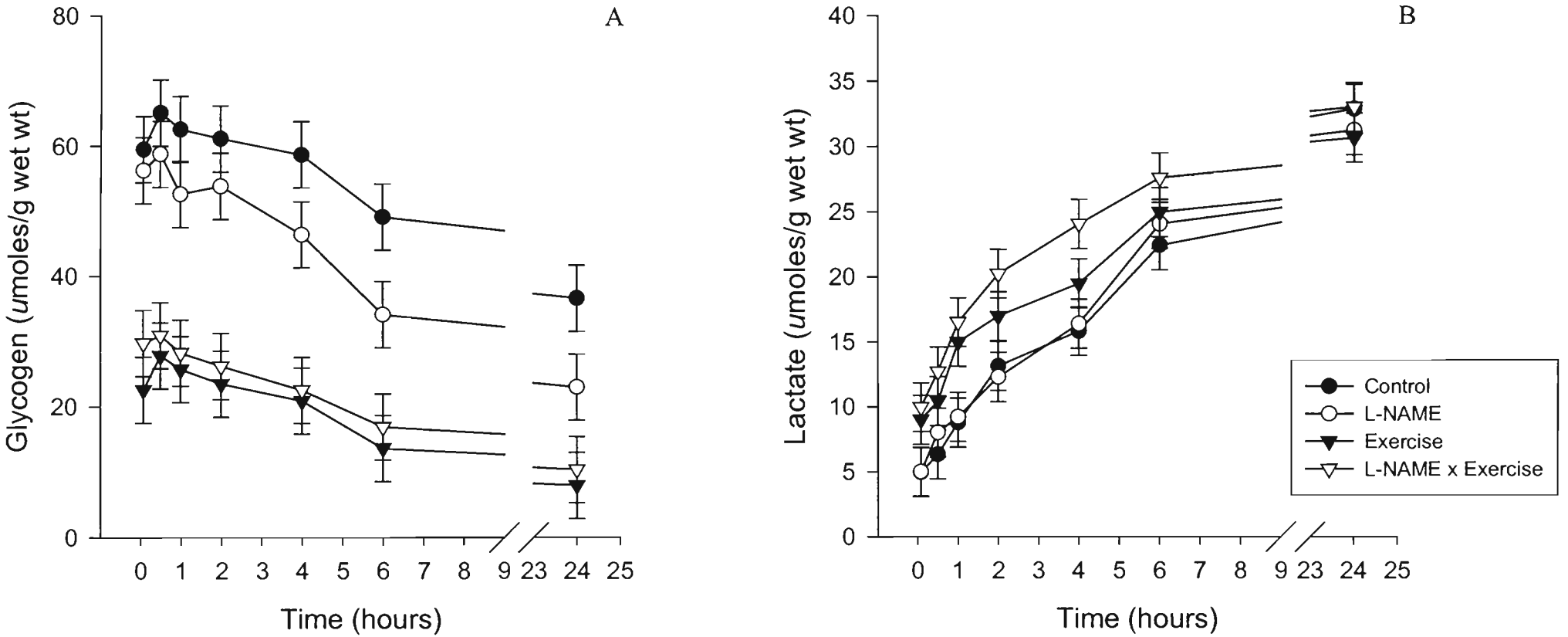


Figure 6.2: The effect L-NAME infusion (L), exercise (E) and time on SM glycogen (A) and lactate (B) concentrations post-slaughter. A) $P<.001$ for the interaction between L x E (Figures A and B). $P>.10$ for the interactions between L or E x T and the interaction between L x E x T (Figures A and B).

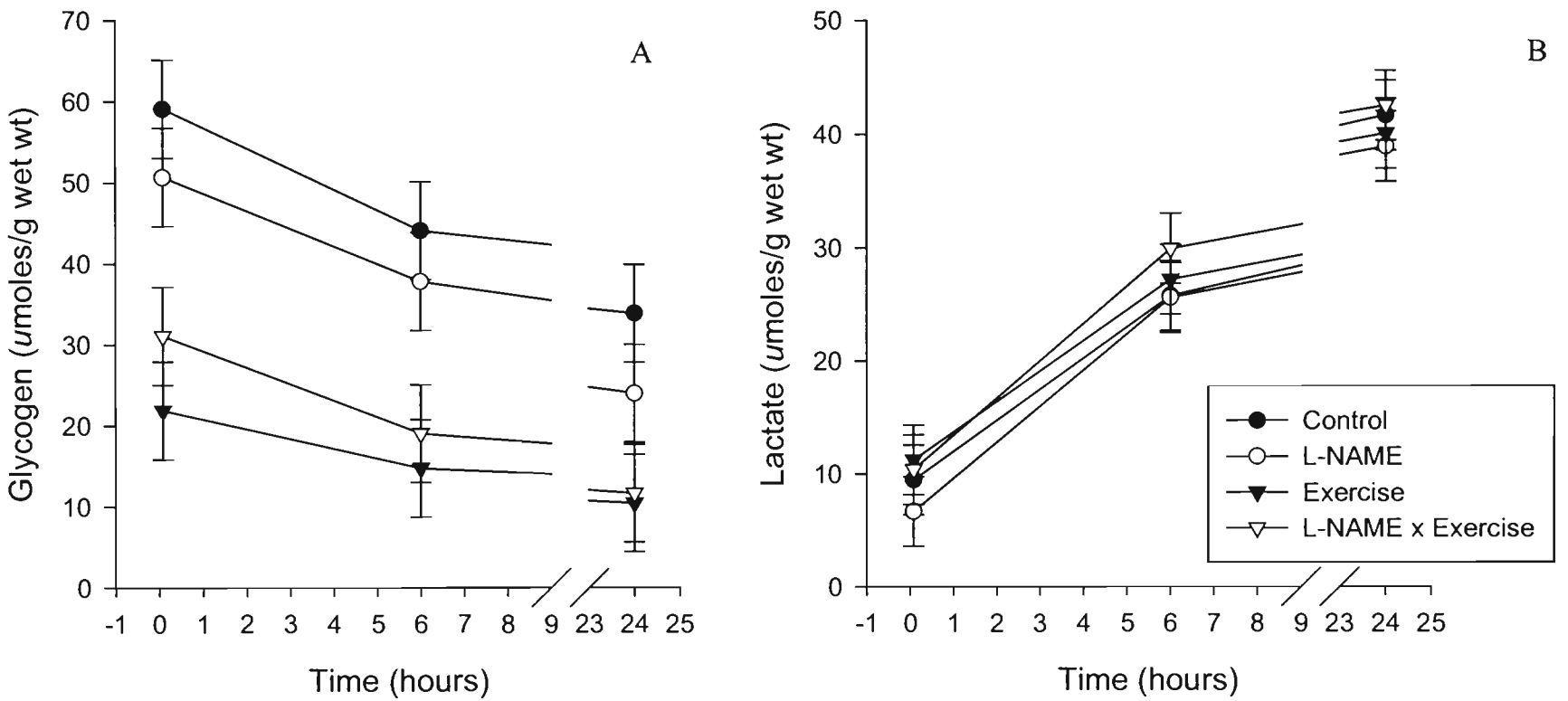


Figure 6.3: The effect of L-NAME infusion (L), exercise (E) and time on LTL glycogen (A) and lactate (B) concentrations post-slaughter. A) P=.008 for the interaction between L x E. B) P=.018 for the effects interaction between L x E. P>.10 for the interactions between L or E x T and the interaction between L x E x T (Figures A and B).

Infusion of L-NAME reduced LTL (6.17 v. 6.11 ± .025 units for 0 v. 30mg/kg L-NAME, P<.05) but not SM pH (6.10 v. 6.12 ± .020, P=.39, Figures 6.4A, 6.4B). Exercise reduced pH in LTL (6.17 v. 6.10 ± .025, for no v. yes exercise, P=.005) and SM (6.19 v. 6.04 ± .02, P<.001). No interactions between L-NAME and exercise were observed in either muscle (P=.73 and .17 for LTL and SM, Figures 6.4A and B). Carcass pH fell with time from initial levels of approximately 6.70 to an ultimate pH of approximately 5.50 at 24 hours post-mortem (P<.001 for LTL and SM). While LTL and SM pH fall with time was unaffected by L-NAME infusion (P= .73 and .99 for LTL and SM) and exercise in the LTL (P=.17), pH fall between exercised and non-exercised lambs converged approximately 6 hours post-mortem in the SM (P=.045). No interactions between L-NAME or exercise and time were observed (P=.92 and .97 for LTL and SM). While the slope (rate) of pH fall was not affected by exercise (P>.10, Table 6.2), higher rigor temperatures were observed in the SM (P<.01), while the LTL was unaffected (P>.10). Reductions in LTL and SM pH following exercise were not reflected in an increased rate of pH fall (P>.10, Table 6.2). Rigor temperature and the rate of pH fall were unaffected by L-NAME infusion in either muscle (P>.10 for both) and no interactions between L-NAME and exercise were observed on rigor temperature and pH fall were observed (P>.10).

Infusion of L-NAME did not affect the temperature of the LTL (18.0 v. 17.6 ± .29°C, P=.14) and SM (21.8 v. 21.8 ± .31, P=.87) overall. Exercise increased the temperature in the SM (21.3 v. 22.3 ± .31, P<.001), while LTL temperature was unaffected (17.9 v. 17.7 ± .29, P=.61). Infusion of L-NAME reduced LTL temperatures in non-exercised lambs (P=.016, Figure 6.5A), while the SM temperature was unaffected by L-NAME infusion (P=.20, Figure 6.5B). The

temperature decreased with time from approximately 37°C 30 minutes post-slaughter to approximately 9°C after 6 hours of refrigeration at 2°C ($P<.001$ in LTL and SM). The decline in LTL and SM temperature with time post-mortem was not affected by either L-NAME infusion or exercise ($P>.10$ for LTL and SM) and no interactions between L-NAME, exercise and time were observed ($P>.10$ for LTL and SM).

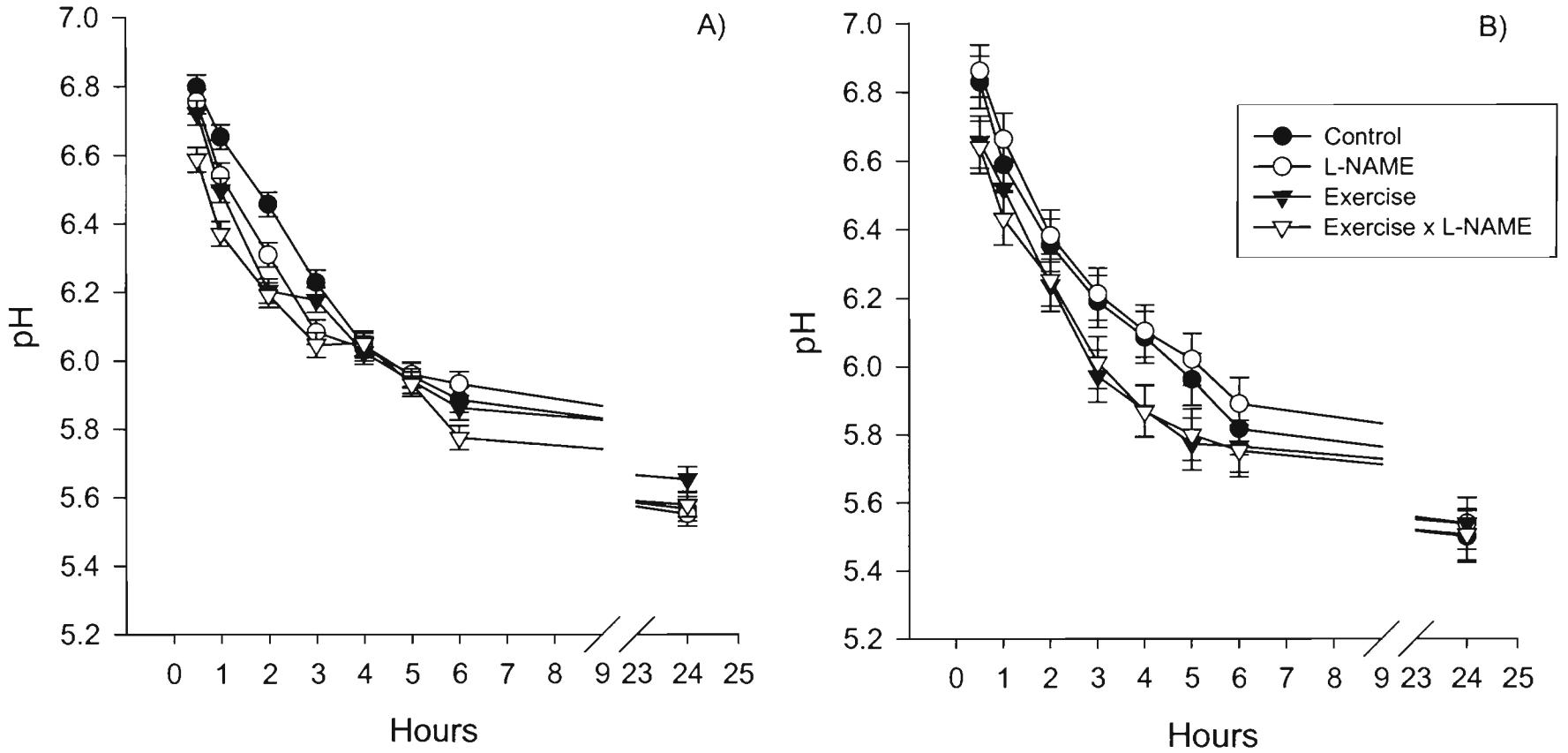


Figure 6.4: Decrease in A) LTL and B) SM pH post-mortem following L-NAME infusion (L) and exercise (E). A) P=.73 for the interaction between L x E. P>.10 for the interaction between E x T. B) P=.17 and .045 for the interactions between L x E and E x T respectively. P>.10 for the interactions between L x T and the interaction between L x E x T (A and B).

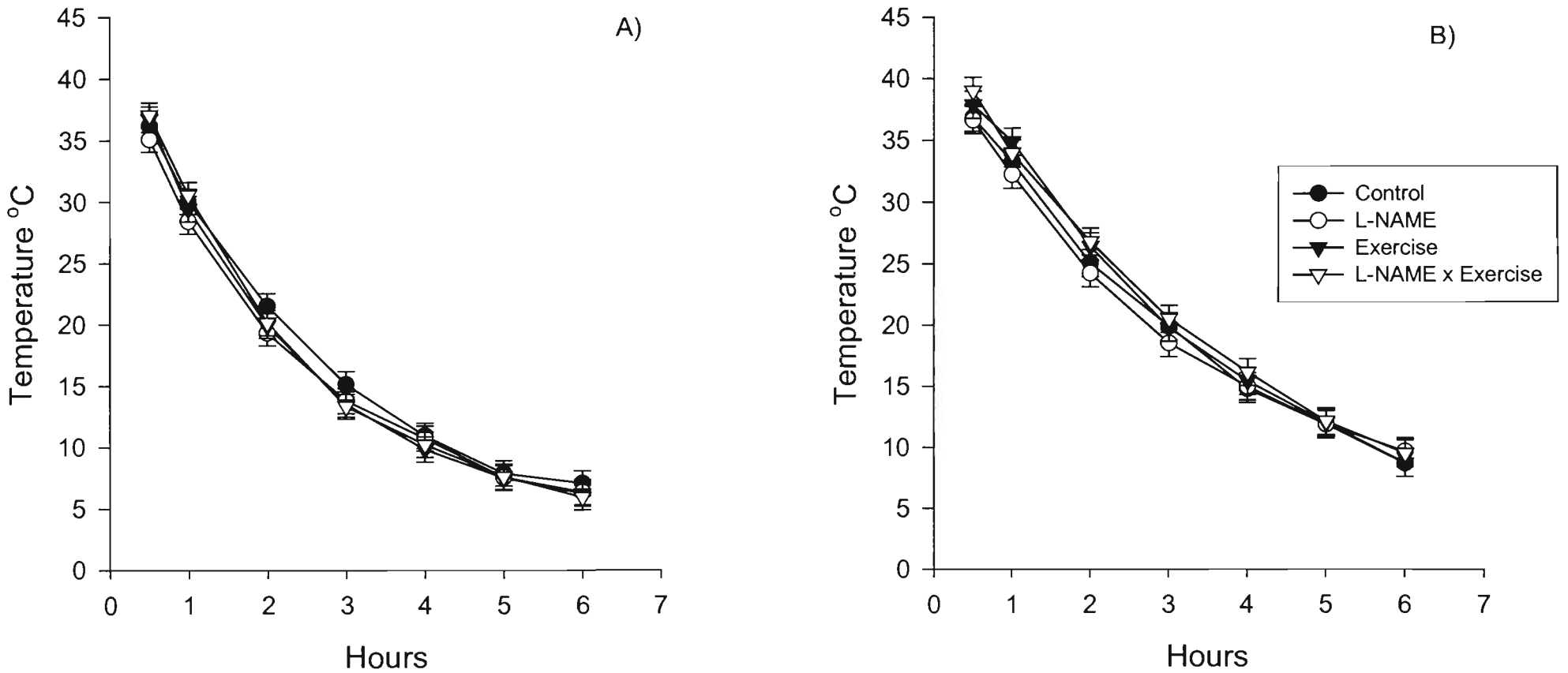


Figure 6.5: Decline of (A) LTL and (B) SM temperature post-mortem following pre-slaughter infusion of L-NAME (L) or exercise (E). A) P=.016 for the interaction between L x E. B) P=.20 for the interaction between L x E. P>.10 for the interactions between L or E x T and the interaction between L x E x T (A and B).

Table 6.2: Rigor temperature, rate of pH fall and sarcomere length, of ovine LTL and SM muscles from lambs with L-NAME (0 v. 30mg/kg) or exercise (no v. yes) treatments pre-slaughter.

Exercise	No		Yes			Significance		
L-NAME	0	30	0	30	SED	L	E	L x E
LTL								
Rigor temp (°C)	12.7	12.4	12.6	13.4	3.39	.93	.85	.82
pH rate (pH units/h)	-.168	-.147	-.131	-.133	.0234	.59	.14	.46
Sarcomere length (μm)	1.75	1.69	1.85	1.79	.061	.19	.037	.99
SM								
Rigor temp (°C)	15.2	13.9	18.8	19.4	2.18	.82	.007	.53
pH rate (pH units/h)	-.168	-.167	-.169	-.162	.0159	.73	.88	.80
Sarcomere length (μm)	1.82	1.85	1.85	1.87	.037	.35	.31	.73

The WBSF was reduced by L-NAME infusion in the LTL (9.8 v. 8.6 kg/cm^2 ± .37, P<.01, Table 6.3), but not SM (7.0 v. 7.2 ± .31, P>.10). Conversely, exercise increased WBSF in both LTL (8.7 v. 9.6 ± .37, P<.05) and SM muscles (6.6 v. 7.6 ± .31, P<.01), indicating tougher meat. While no interaction between L-NAME and exercise was observed in the LTL (9.5, 8.0, 10.1 and 9.2 ± .53 for control, L-NAME, exercise and L-NAME x exercise respectively, P>.10), L-NAME increased WBSF of non-exercised SM to levels intermediate between exercised-toughened and non-exercised muscles (6.2, 7.0, 7.8 and 7.4 ± .44, P<.05). The WBSF of both muscles declined significantly with time, indicating significant tenderisation between 1 and 3

days post-mortem (P<.001 for both). The reduction in WBSF with time was not influenced by L-NAME or exercise in LTL (P=.39 and .56 for L-NAME and exercise, respectively) or SM muscles (P=.27 and .56), nor was any interaction between L-NAME, exercise and time observed (P=.84 and .68 for LTL and SM respectively).

Sarcomere length was not influenced by L-NAME infusion (1.8 v. 1.7 μm, P>.10 and 1.83 v. 1.86 ± .026, P>.10 for LTL and SM respectively). Exercise increased sarcomere length in the LTL (1.72 v. 1.82, P<.05), but not SM (1.83 v. 1.86, P>.10). No interaction between L-NAME and exercise was observed on sarcomere length (Table 6.2).

Table 6.3: Warner Bratzler shear force (kg/cm^2) of ovine LTL and SM muscles 1 and 3 days post-slaughter from lambs infused with L-NAME (0 v. 30 mg/kg) or exercise (no v. yes) treatments pre-slaughter.

Exercise (E)	No		Yes			Significance[1]			
L-NAME (L)	0	30	0	30	SED	L	E	L x E	T
LTL									
1	10.7	9.3	11.0	10.4	0.75	.002	.017	.39	<.001
3	8.3	6.6	9.2	8.0					
SM									
1	7.0	7.7	9.0	8.1	0.63	.48	.003	.044	<.001
3	5.3	6.4	6.6	6.6					

[1]Interactions between L-NAME, exercise and time (T) were not significant (P>.05).

Water holding capacity (WHC) measurements (drip, purge or cook losses) were unaffected by L-NAME infusion (P>.10 for all, Table 6.4). Exercise did not affect drip loss in either muscle (P>0.10), but resulted in a large increase in purge and cook losses in both LTL and SM muscles (P<0.05 for all, Table 6.4). No interactions were observed between L-NAME and exercise in LTL and SM muscles for drip, cook or purge loss (P>.05 for all). Cook loss increased with time post-mortem (P<.001), but no interactions between L-NAME, exercise and time were observed (P>.05, Table 6.4).

Table 6.4: Drip, purge and cook loss (%) of LTL and SM muscles from lambs with L-NAME (0 v. 30mg/kg) or exercise (no v. yes) treatments pre-slaughter.

	Exercise (E)	No		Yes			Significance[1]			
	L-NAME (L)	0	30	0	30	SED	L	E	L x E	T
	Day									
Drip Loss (%)										
LTL	1	1.18	1.15	1.16	1.16	.136	.89	.93	.83	-
SM	1	1.01	.95	.99	1.05	.081	.91	.48	.24	-
Purge Loss (%)										
LTL	3	3.61	3.33	3.89	4.56	.597	.64	.086	.25	-
SM	3	2.24	2.17	3.20	3.28	.370	.98	<.001	.78	-
Cook Loss (%)										
LTL	1	32	30.8	35.7	35.5	1.04	.35	<.001	.93	<.001
	3	34.1	34.3	38.8	38.2					
SM	1	32.7	32.5	36.8	35.9	1.08	0.29	<.001	.84	.004
	3	33.7	33.6	39.0	37.9					

[1]Interactions between L-NAME, exercise and time (T) were not significant (P>.05).

The surface colour (L*, a* and b* values) of LTL and SM muscles was unaffected by L-NAME infusion, nor was any interaction between L-NAME and exercise, or L-NAME, exercise and time observed (P>.10, Table 6.5). Exercise increased the surface lightness (L*) in LTL (32.0 v. 32.9 ±.33 units, P<.001) and SM muscles (32.7 v. 33.2 ± .36, P<.001), indicating lighter coloured meat. Surface lightness increased with time between 1 and 3 days in both LTL and SM muscles (P<.001). The time dependent increase in L* values tended to be lower in exercised LTL muscles (30.5, 32.7 at non-exercised and exercised at 1 day and 33.6, 34.6 ± .46 after 3 days respectively, P=.069). Exercise increased a* values (redness) in the LTL (16.9 v. 17.9 ± .28, P<.001). This also occurred in the SM, but the increase was smaller in magnitude (18.2 v. 18.6 ± .23, P=.083). Surface redness increased with time post-mortem (P<.001 for both), and this was unaffected by exercise (P>.05). The increase in LTL and SM b* values with exercise indicates a reduction in yellow pigmentation in the meat (6.25 v. 6.85, P<.05 and 6.91 v. 7.59, P<.01 for LTL and SM respectively). Yellow pigmentation reduced with time post-mortem in both LTL and SM muscles (P<.001 for both), and this was unaffected by exercise (P>.05, Table 6.5).

Attempts to detect of μ- and m-calpain autolysis by Western blotting were unsuccessful, possibly due to a lack of antibody specificity.

Table 6.5: Colour (Minolta chromameter L*, a*, b*) of LTL and SM muscles 1 day post-slaughter from lambs infused with L-NAME (0 v. 30 mg/kg) exercised immediately pre-slaughter (no v. yes).

	Exercise (E)	No		Yes			Significance[1]			
Muscle	L-NAME (L)	0	30	0	30	SED	L	E	LxE	T
	Day									
LTL L*	1	30.8	30.3	32.8	32.7	.65	.65	<.001	.44	<.001
	3	32.9	34.2	34.6	34.6					
a*	1	15.1	15.5	16.9	16.9	.55	.34	<.001	.93	<.001
	3	18.4	18.6	19.0	19.2					
b*	1	4.6	4.8	5.5	8.0	.40	.28	.007	.97	<.001
	3	7.6	7.9	8.0	8.2					
SM L*	1	32.8	32.4	34.5	34.2	.50	.32	<.001	.60	.73
	3	32.8	32.9	34.2	33.5					
a*	1	17.2	17.5	17.8	17.9	.45	.71	.083	.71	<.001
	3	19.4	18.8	19.4	19.3					
b*	1	6.2	6.3	7.0	7.0	.40	.87	.002	.38	<.001
	3	7.8	7.3	8.1	8.3					

[1]Interactions between L-NAME, exercise and time (T) were not significant (P>.05).

6.6 Discussion

Plasma glucose concentrations before exercise were not affected by infusion of L-NAME. Exercise significantly increased plasma glucose concentrations, whereas NOS inhibition significantly reduced plasma glucose concentrations in exercised lambs, supporting hypothesis 5. The inhibitory effect of L-NAME on exercise-induced hyperglycaemia indicates that NO influences glucose homeostasis during exercise. This suggests that infusion of 30 mg/kg L-NAME inhibited systemic NO production as observed in Chapter 5 (Figure 5.1). The interaction between NOS inhibition and exercise indicates that increases in plasma glucose during exercise are influenced by NO, supporting hypothesis 5. While this experiment did not quantitate the origin of reduced plasma glucose concentrations, two possible mechanisms are reduced hepatic glucose release or increased muscle glucose uptake. It has been proposed that NO influences glucose homeostasis by mediating liver glycogenolysis (Alexander, 1998). This has been demonstrated in isolated perfused rat livers, where NO has been observed to inhibit hepatic glycogenolysis (Borgs et al., 1996) and inhibition of the glycogenolytic effect of platelet-activating factor (Moy et al., 1991). Nitric oxide has also been observed to inhibit glycogen synthesis in isolated rat hepatocytes by inhibiting the conversion of inactive glycogen synthase *b* isoform to active glycogen synthase *a* (Sprangers et al., 1998). Since infusion of L-NAME in the present study was via the jugular vein, inhibition of NOS was not specific to skeletal muscle and it is possible the L-NAME may have inhibited hepatic NOS. If L-NAME inhibited hepatic NOS and glycogenolysis in this experiment, it is possible that reduced hepatic glucose release contributed to the reduction in plasma glucose concentrations. Alternatively, reduced plasma glucose concentrations during NOS inhibition may be due to increased tissue glucose uptake, as indicated in Chapter 5.

Since skeletal muscle is a major site of glucose utilisation (Dunshea et al., 1995, Ferrannini et al., 1985), reductions in exercise-stimulated hyperglycaemia with NOS inhibition are likely to be a result of increased peripheral glucose uptake by exercising muscle. Roberts et al. (1997) hypothesised that insulin- and exercise-stimulated glucose transport in muscle are separate and that exercise stimulated glucose transport is NO mediated. This was supported by Bradley et al. (1999) who observed decreased glucose uptake with infusion of NOS inhibitors in exercising humans. While this runs counter to the present results, both experiments support the involvement of NO in exercise-stimulated glucose uptake. Furthermore, results from Chapter 5 indicate increased glucose uptake with NOS inhibition. Increased insulin sensitivity has also been observed with NOS inhibitors (Butler et al., 1998, Balon et al., 1999), despite insulin concentrations decreasing (Balon et al., 1999) or being unchanged (Chapter 5). However, other studies maintain that NO-mediated skeletal muscle glucose uptake is independent of insulin and contraction (Etgen et al., 1997, Higaki et al., 2001).

Inhibition of NOS pre-slaughter significantly decreased muscle glycogen concentrations post-slaughter, while concurrently increasing lactate concentrations in the SM, indicating that in the SM muscle at least, NO inhibits glycolysis. Increases in glycolysis following exercise were not influenced by NOS inhibition, rejecting hypothesis 1. The increase in muscle glycogenolysis and glycolysis observed after NOS inhibition was of a lesser magnitude than observed after exercise, which halved glycogen concentrations, supporting hypothesis 4. The restriction of the glycogenolytic effect of NOS inhibition to non-exercised lambs is most likely due to the overriding effect of the large increases in glycogenolysis associated with exercise.

Therefore, these data indicate that NO reduces glycogen depletion in LTL and SM muscles.

While NO has been demonstrated to play an inhibitory role in hepatic glycogenolysis and glycogen synthesis, little is known about the effects of NO on muscle glycogenolysis. Inhibition of basal and stimulated glycogen synthesis by SNP in rat *soleus* muscles has been observed (Young and Leighton, 1998b) and inhibited insulin-stimulated, but not basal glycogen synthesis (Young et al., 1997). Together, these results support a similar role for NO in hepatic and skeletal muscle glycogen metabolism. However inhibition of cGMP disposal in rat *soleus* muscle, increased glycogen synthesis in insulin-sensitive, but not in insulin-insensitive muscle (Young and Leighton, 1998a). Since cGMP is a second messenger of NO, these data support a role for NO in skeletal muscle glycogenesis. In the same experiment, SNP did not alter glycogen synthesis, indicating that an indirect mechanism exists. While little is known about the effect of NO on muscle glycogenolysis, the glycogenolytic effect of NOS inhibition may be via inhibition of glycogenesis while glycogenolysis remains unchecked. This does not take into account increases in glycolysis induced by NOS inhibition, indicating that NO influences processes involved in glycogenesis and glycogenolysis.

Infusion of L-NAME increased muscle glycogenolysis and lactate production, particularly in the SM, indicating that NO inhibits glycogenolysis, at least in this muscle. However, other experiments using pharmacological NO donors have observed increased lactate production in skeletal muscle (Young et al., 1997, Young and Leighton, 1998a), in contrast to the effect of L-NAME in this experiment. The

differences may in part be attributed to substantial methodological differences between the experiments. The experiments by Young and co-workers investigated the role of NO on glucose metabolism and insulin sensitivity in buffered muscle strips, under controlled pH and temperature conditions, while the current experiment investigated differences in muscle glycolysis in the post-mortem environment.

It is not known how L-NAME increases the degree of glycolysis, but other experiments have indicated that NO influences glycolysis and respiration. Since NO has been observed to inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mohr et al., 1996, Mohr et al., 1999), it is possible NOS inhibition may increase glycolysis via removal of NO-mediated GAPDH inhibition. The expression of NOS in mitochondria indicates a role for NO in influencing mitochondrial metabolism (Giulivi et al., 1998, Tatoyan and Giulivi, 1998, Brown, 2000). Indeed, NO-mediated inhibition of mitochondrial complexes I (Borutaite et al., 2000) and IV (Cleeter et al., 1994, Brown, 2000, Brunori, 2001) of the electron transport chain has been observed. While it is unlikely to have a significant impact on meat quality, inhibition of creatine kinase activity by NO has been observed (Gross et al., 1996, Arstall et al., 1998, Kaasik et al., 1999). Speculatively, a feedback loop between NO and glycolysis may exist, where contraction increases glycolysis and NOS activity and NO in turn inhibits glycolysis in what may form a component of muscular fatigue. If this were so it may explain why NOS inhibition increased glycolysis above rates observed with exercise alone.

Protein was precipitated from muscle homogenates with PCA in this experiment. While PCA is frequently used to extract glycogen from muscle (Passonneau and

Lowry, 1993, Sanz et al., 1996, Rhee and Kim, 2001), some experiments have observed that PCA extracts 15-25% of total muscle glycogen (Jansson, 1981). The low recovery of muscle glycogen is due to precipitation of proglycogen, which has a higher relative protein content compared to macroglycogen. Proglycogen is an intermediate between the synthesis and degradation of macroglycogen (Gregory, 1996) and represents a more metabolically active pool than macroglycogen (Graham et al., 2001, Schafer et al., 2001). A comparison between values presented in this experiment and some other experiments on lamb glycogen concentrations measured soon after slaughter in lambs from southern Australia are presented in Table 6.6. Glycogen concentrations measured in this experiment compare favourably with glycogen concentrations from most other experiments where HCl was used to precipitate protein. Due to the similarity in glycogen concentrations between experiments and methods, it appears that glycogen loss was minimal. Any glycogen loss is likely to be derived from the more metabolically active proglycogen pool, which is more likely to be susceptible to acute changes and the glycogenolytic effect of NOS inhibition. Therefore, any differences in glycogen concentrations due to using PCA extracted glycogen represent an understatement of the glycogenolytic effect of NOS inhibition.

Table 6.6 Comparisons between glycogen concentrations from necropsies removed from ovine muscles post-slaughter and homogenised in HCl or PCA.

	Acid used	Muscle	Approx time post-slaughter	Approx. glycogen (μmoles/g)
Chapter 6	PCA	LTL	5 min	55.5
	PCA	SM	5 min	66.6
(Warner and Pethick, 2000)	HCl	LTL	15 min	53.3
(Gardner, 2001)	HCl	SM	<30 min	55-66
(Pethick and Rowe, 1996)	HCl	SM	10 min	83-110

Since injection of arginine analogues has been observed to increase toughness in beef LL (Cook et al., 1998), hypothesis 2 postulated that pre-slaughter NOS inhibition would mimic this effect. However, hypothesis 2 was not supported by this experiment since NOS inhibition improved tenderness in the LTL. The differential effects of arginine analogues between the two experiments indicate that injection into meat and pre-slaughter infusion of arginine analogues respectively affect meat quality via different mechanisms. While NOS inhibition increased LTL pH fall independently of glycolysis, the reduction of LTL pH with NOS inhibition was short lived, small in magnitude and less than the pH fall of exercised muscles. Furthermore, NOS inhibition did not affect the rate of pH fall, or the rigor temperature. Therefore, it is unlikely that the reduction in pH after NOS inhibition

infusion significantly influenced subsequent changes in meat quality, particularly since the more rapid decrease in pH associated with exercise resulted in tougher, not more tender meat. Since NO induces vasodilation via relaxation in smooth muscle, it was hypothesised that NO may also relax skeletal muscle, and therefore, L-NAME might reduce sarcomere length. Hypothesis 3 was not supported, since L-NAME did not influence sarcomere length, indicating that NO, at least post-mortem, is not involved in muscle relaxation. Therefore, the improvement in meat tenderness with NOS inhibition was likely to be due to increased proteolytic activity, since NOS inhibition reduced WBSF independently of sarcomere length, water holding capacity and pH.

The proteolytic enzyme calpain is thought to be the principal tenderising enzyme in meat (Dransfield, 1993, Koohmaraie, 1994). Following activation, autolysis of calpain occurs, cleaving the 28kDa fragment from the molecule (Cong et al., 1989, Cong et al., 1993, Baki et al., 1996). Therefore, Western Blotting was used to determine the degree of calpain autolysis by the migration distance of calpain domain III and therefore the level of *in vivo* calpain activity. However, attempts to detect μ- and m-calpain domain III, the most highly conserved calpain domain (Cong et al., 1989), were unsuccessful, possibly because of a lack of specificity of the primary antibodies to ovine calpain. The primary antibodies used were specific for porcine, human and rat calpain and despite calpain being highly conserved between species (Nonneman and Koohmaraie, 1999), attempts to detect calpain by western blotting were unsuccessful.

Exercise increased the shear force of LTL and SM muscles, indicating tougher meat. Exercise has been observed to elevate skeletal muscle NO production (Jungersten et al., 1997, Roberts et al., 1999, Shen et al., 2000). The involvement of NO in exercise toughening of meat was not supported by the results presented in this experiment, since NOS inhibition did not reduce the WBSF of exercise-toughened meat. The involvement of NO in increasing exercise-induced toughness may not be excluded, since more rapid pH decline, increased temperature post-slaughter and reduced water holding capacity confound this result. A similar experiment examining the interaction between acute exercise and post-slaughter electrical stimulation (ES) showed that while exercise alone did not affect lamb LD pH and tenderness, toughening occurred when ES was applied in conjunction with exercise (Daly et al., 1995). From this it was postulated that altered muscle biochemistry due to exercise and ES resulted in tenderness reductions, specifically ante-mortem activation of calpains and subsequently, reduced activity post-mortem.

The improvement in LTL tenderness following L-NAME infusion was not observed in the SM. Instead, L-NAME increased the SM WBSF to intermediate values, higher than controls, but lower than exercise-toughened values. The significance of this result is not clear since WBSF of the L-NAME infused lambs was not significantly different to either group, but this result does not indicate that L-NAME served as a toughening agent in the SM.

The increased LTL sarcomere lengths in exercised lambs most likely reflect shortening of control muscles. It is likely that this is observed in the LTL and not the SM due to the fact that the LTL is a long, flat peripheral muscle, while the SM is

considerably deeper in the carcass, providing better insulation during chilling. Muscle shortening occurs when the pre-rigor temperature falls below approximately 13°C (Locker and Hagyard, 1963). Since the rigor temperatures of the LTL were below 13°C (except for the L-NAME, exercise group), these data indicate mild cold shortening in the LTL that was more prominent in non-exercised lambs. Heat toughening in the SM is not expected, as at the temperatures observed (19°C), minimum shortening is expected (Locker and Hagyard, 1963).

While the acute exercise model used in this experiment toughened LTL and SM muscles, other experiments using more exhaustive exercise-stress models appeared to result in DFD meat (Chrystall et al., 1982). Conversely, lower pH values of exercise lambs relative to non-exercised lambs in this experiment reflected an initial reduction in pH, particularly in the SM, with no change in the rate of pH decline. Exercise also increased meat surface lightness and cook and purge loss from LTL and SM muscles, indicating increased protein denaturation and reduced membrane integrity. Elevated post-mortem temperature has been associated with increased protein denaturation (Betchel and Parrish, 1983). It is well-known that a fast muscle pH fall at high temperature increases protein denaturation, often resulting in a pale meat surface, as evidenced by pale, soft, exudative (PSE) pork (Briskey and Wismer-Pedersen, 1961, Warner, 1994). In addition increased Ca^{++} influx associated with exercise may activate Ca^{++}-sensitive cellular phospholipases (Gissel and Clausen, 2001), reducing membrane integrity and contributing to the increased purge and cook loss of exercised muscles.

6.7 Conclusion

These data indicate that NO plays a role in the conversion of muscle to meat, influencing muscle glycolysis and possibly proteolysis. The reduction in plasma glucose concentrations after NOS inhibition in exercised lambs indicates that NO is involved in stimulating glucose release during exercise. This also indicates that NOS activity is increased during exercise-stress. While the glycogenolytic and glycolytic effects of NOS inhibition were observed independently of exercise, increases in muscle lactate synthesis after NOS inhibition were larger in exercised lambs, indicating that NO inhibits exercise-induced increases in glycolysis. Importantly, NOS inhibition improved meat tenderness without influencing sarcomere length, pH fall, WHC or colour parameters indicating a mechanism whereby NO inhibits muscle proteases.

Chapter 7: General discussion

Meat quality is linked to an animal's physiological status before slaughter and a range of processing factors such as electrical stimulation and chilling rate. While not without limitations, there can be far greater control over carcass conditions post-mortem than over the physiological state *in vivo*. Animal stress has been identified as a common industry event leading to conditions such as "dark cutting or pale soft exudative" meat, tougher meat and reduced consumer acceptance. The term "stress" is poorly defined and can be the result of numerous stimuli. Rather than focus on wide-ranging and highly varied responses to stress, the work reported in this thesis has focused on the effects of NO, which was postulated to play a multi-faceted role in the physiological response to stress and influence biochemical determinants of meat quality.

Experiments in this thesis investigated the influence of NO in hot boned meat quality, Ca^{++} homeostasis, muscle metabolism and the inter-relationship between exercise stress and meat quality. The chemical donors of NO, SNP and NONO were used to study the effects of increased NO concentrations on meat quality in Chapters 3 and 4. In Chapters 3, 5 and 6, the substrate inhibitor L-NAME was used to determine the influence of endogenous NO production on muscle metabolism and/ or meat quality. The effects by which NO may influence meat quality is summarised in Figure 7.1. The first section of the general discussion will examine the effects of NO on metabolism, followed by the influence of NO on meat quality.

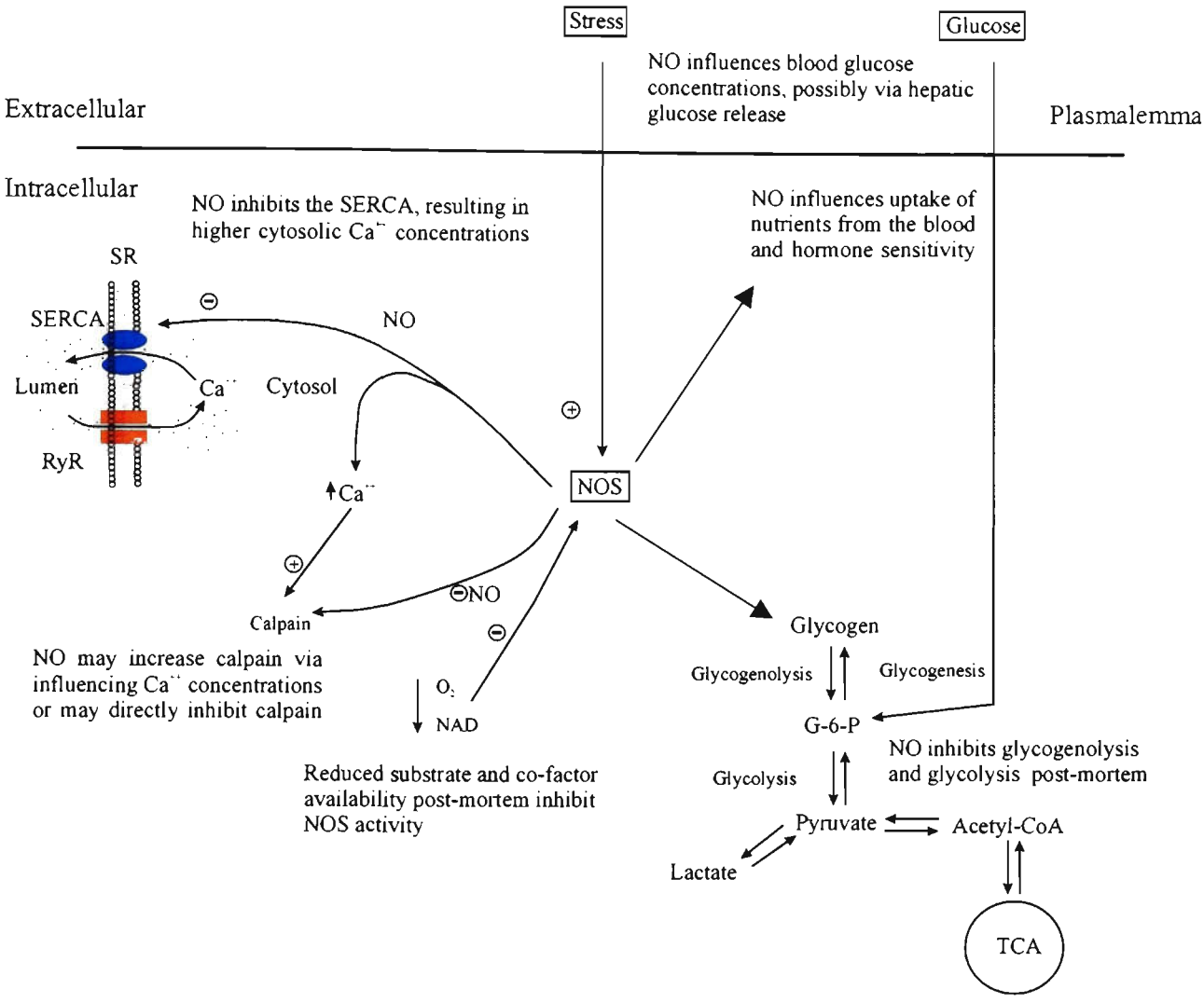


Figure 7.1: Summary of mechanisms by which NO may influence muscle metabolism and meat quality.

7.1 The effects of NO on metabolism

7.1.1 Carbohydrate metabolism

Inhibition of NOS increased glucose AVD, indicating NO may inhibit glucose uptake (Chapter 5). This response was not associated with a change in plasma insulin concentrations, so it was concluded that inhibition of NOS may increase insulin sensitivity. This has been previously reported by Balon et al. (1999), who observed increased muscle insulin sensitivity independent of systemic insulin concentrations in

a chronic model of L-NAME administration in rats. Since NOS is expressed in the sarcolemma, it has been postulated that NO forms part of a secondary signalling cascade. This has been demonstrated by increased expression of the insulin specific glucose transporters on cell surfaces after incubation with pharmacological NO donors (Etgen et al., 1997). The effects of insulin and NO may also be additive and it has been postulated that insulin and NO mobilise different pools of the skeletal muscle specific glucose transporter GLUT-4 (Hayashi et al., 1997). Collectively, these studies support the conclusions reached in Chapter 5, that NO can influence glucose uptake independently of insulin concentrations (Figure 7.1).

Increases in systemic glucose concentrations are principally regulated by rates of hepatic glucose release. Since NO has been observed to stimulate liver glycogenolysis (Borgs et al., 1996), it was proposed in Chapter 6, that systemic inhibition of NOS with L-NAME would decrease systemic glucose concentrations. This hypothesis was supported, but L-NAME reduced plasma glucose concentrations in exercised lambs only. Since the inhibitory effect of L-NAME is expected to be proportional to NOS activity, it was concluded that NO only influences stimulated rates of glucose release (Figure 7.1). This may be expected due to increased hepatic glucose output to maintain blood glucose concentrations during exercise. However, it is not known if increased hepatic glucose release during exercise corresponds to increased hepatic NOS activity.

Little is known about the effects of NO on skeletal muscle glycogen metabolism. Although initial muscle glycogen concentrations post-slaughter were not affected by NOS inhibition 135 minutes pre-slaughter, depletion of glycogen in the LTL and SM

post-slaughter was increased by NOS inhibition, indicating that NO may influence glycogenolytic rate in muscle (Figure 7.1). Possible mechanisms by which L-NAME could increase muscle glycogenolysis exist on cellular and endocrine levels. It is possible that increased skeletal muscle glycolysis following NOS inhibition is a product of increased insulin sensitivity as this will in turn increase the activity of glycogen phosphorylase and glycogen synthesis (Komuniecki et al., 1982, Halse et al., 2001). While it is not likely that NOS inhibited increases in glycogenolysis were driven by insulin, the involvement of other glycogenolytic hormones cannot be excluded. For example, the NO donor SNAP has been observed to be inhibit hepatic glucagon-stimulated glycogenolysis (Alexander, 1998).

In muscle post-slaughter glycogen is converted to lactate via glycolysis. It was concluded in Chapter 6 that NO spared muscle glycogen from degradation, as NOS inhibition increased glycolysis. While it has been reported elsewhere that muscle NOS activity is increased by exercise (Jungersten et al., 1997, Roberts et al., 1999, Tatchum-Talom et al., 2000), the glycogenolytic effect of NOS inhibition was not observed in exercised lambs, but rather, in control lambs. It is proposed that NOS inhibition did not increase glycolysis above levels induced by exercise alone. Since the glycolytic effect of L-NAME was observed for as long as 6 hours post-mortem, these data indicate that the influence of NO production pre- and/ or post-slaughter plays a significant role in inhibiting glycolysis in the lamb carcass.

7.1.2 Fat metabolism

Inhibition of NOS by infusion of L-NAME resulted in increased hind-limb lipolysis independent of circulating insulin concentrations (Chapter 5). It was concluded that

increased lipolysis most likely resulted from one of or a combination of reduced insulin sensitivity or increased activity of LPL and/ or HSL. The metabolic changes in the ovine hind-limb did not appear to be solely mediated by changes in insulin sensitivity, as increased glucose uptake and lipolysis would require increased and reduced sensitivity respectively. It is possible that increased and decreased responses to insulin are mediated by NO on a cellular level. As discussed in Chapter 5 the cellular response to insulin is partly mediated by NO (Young and Leighton, 1998a, Kahn et al., 2000, Shankar et al., 2000) and the expression of NOS isoforms varies between skeletal muscle and adipose tissue. While nNOS is the most prevalent isoform in skeletal muscle, it is not expressed in adipose tissue (Ribiere et al., 1996, Elizalde et al., 2000). If infusion of L-NAME inhibits NOS isoforms in adipose and skeletal muscle, the resulting inhibition may propagate divergent responses to endocrine hormones between tissues. As the pathways through which NO influences fat metabolism in the hind-limb were not investigated more thoroughly in this thesis, they have been excluded from Figure 7.1.

How NO-mediated changes in muscle fat metabolism might affect meat quality remains untested. Other experiments investigating the influence of NO on LPL and HSL have focused on acute mediation. In Chapter 6, acute changes in lipolysis approximately 2 hours pre-slaughter are less likely to influence meat quality than, for example chronic changes to fat deposition, particularly the deposition of intramuscular fat resulting in marbling. Elizalde et al. (2000) observed increased expression of eNOS in obese human adipose tissue. The increased eNOS expression contrasted with decreased HSL expression in the same individuals, indicating that NO is capable of acute and chronic regulation of fat metabolism. Furthermore, plasma

NO_x increased with obesity, indicating higher levels of NO synthesis in obese individuals (Choi et al., 2001). In other experiments, chronic administration of NOS inhibitors increased fat deposition via reduced fatty acid oxidation (Khedara et al., 1996, Khedara et al., 1999). Collectively these data indicate that NO is involved in acute and chronic fat metabolism.

7.1.3 Calcium homeostasis

Cytosolic Ca^{++} concentrations regulate many processes in muscle fibres. In relation to meat quality, cytosolic concentrations of Ca^{++} rise post-mortem due to the inability of the SR and mitochondria to accumulate Ca^{++} ions due to ATP depletion (Cornforth et al., 1980). This has been purported to degrade myofibrillar structure and improve meat tenderisation due to activation of the Ca^{++} dependant protease calpain (Dransfield, 1993, Koohmaraie, 1994), or by ionic interactions resulting in weakening of the sarcomere Z-disc (Takahashi et al., 1987, Takahashi, 1992). The SR represents the major site of muscle fibre Ca^{++} sequestration and SERCA and RyR effect the uptake and release of Ca^{++} from the SR lumen.

Since SERCA, RyR and hence cytosolic Ca^{++} concentrations are influenced by NO, it was hypothesised that NO mediated regulation of Ca^{++} uptake and release may provide a mechanism to influence enzymatic and ionic tenderisation of meat. Exposure of purified SR membranes to pharmacological donors of NO in Chapter 4 reduced Ca^{++} sensitivity of the SERCA, indicating reduced sequestration of Ca^{++} into the SR. It was hypothesised that increasing cytosolic Ca^{++} concentrations with NO may activate muscle proteolysis and tenderisation (Figure 7.1). The RyR was not affected by NO exposure in Chapter 4. It was concluded that this was most likely

because the RyR was not stimulated, and therefore inactive. Numerous other experiments have reported either activation or inhibition of RyR by NO (Meszaros et al., 1996, Suko et al., 1999, Hart and Dulhunty, 2000). Opening of the RyR rapidly releases Ca^{++} sequestered within the SR, substantially increasing cytosolic Ca^{++} concentrations and presumably a significant increase in Ca^{++} dependent muscle fibre proteolysis. However, since the rates of release of Ca^{++} from the SR were low, it was concluded that under the experimental conditions, NO did not increase RyR opening by itself, but NO may influence RyR opening when stimulated by another means.

7.2 The effect of NO on meat quality

7.2.1 The post-slaughter role of NO in determining meat quality

While NO has long been recognised for the importance of the role it plays during meat curing with nitrates (Cornforth, 1996), little is known of the involvement of NO in fresh meat quality. Cook et al. (1998) examined the effect of injection of pharmacological donors of NO and inhibitors of NOS on meat quality. While demonstrating that NO did affect meat tenderness, many questions remained unanswered. The concentrations of pharmacological donors and inhibitors used (100mM) were supraphysiological, compared to the basal activity of NOS in skeletal muscle, which is approximately 10 picomolar/mg/min (0.01μM) (Stamler and Meissner, 2001). However, Cook et al. (1998) determined that free NO concentrations were approximately 2.5μM near slaughter by use of a selective NO electrode. Even in the presence of an inhibitor cocktail, concentrations of free NO 24 hours post-slaughter in bovine LD were still 1μM. The contribution of endogenous NO synthesis to lipid oxidation is minimal (Brannan et al., 2001), but injection of

1mM SNP in Chapter 3 caused greater than a 10-fold increased in lipid oxidation (TBARS), demonstrating the large excess of NO generated by SNP at significantly lower concentrations than those used by Cook et al. (1998).

Chapter 3 examined the dose response relationship between NO donors and NOS inhibitors and meat quality in the hot boned beef LL. Concentrations of NO donors and NOS inhibitors at 1, 10 and 100mM were injected into hot boned beef LL muscles and meat quality assessed between 1 and 14 days post-mortem. Unlike the experiment of Cook and co-workers, no effects of NO donors or NOS inhibitors on meat tenderness as assessed by WBSF were observed. However, even at 1mM, the lowest concentration of SNP was most likely supraphysiological. The differences between results presented in this thesis and the significant differences observed by Cook et al. (1998) may be partly due to methodological differences between the two experiments. This includes different NO donors, times of injection post-mortem and injection systems, But the point remains that the levels of NO used by Cook et al. (1998) were supraphysiological and therefore do not represent endogenous NO synthesis.

It was hypothesised in Chapter 3 that the activity of NOS is attenuated post-mortem, since the activity of NOS is tightly regulated, requiring 2 substrates (arginine and NADPH) and 5 co-factors (heme, BH_4, FAD, FMN and calmodulin). This has been supported by Brannan and Decker (2002), who, while observing that NOS activity from pork, trout and chicken muscle was active under conditions of post-mortem pH and temperature fall, postulated that this was actually an artefact of the assay itself. Their assay measured NOS activity under "ideal" conditions, with all co-factors and

substrates in ample supply and therefore did not represent endogenous activity. Brannan and Decker (2002) also concluded that availability of these substrates and in turn NOS activity in post-mortem skeletal muscle was limited (Figure 7.1). It was concluded from the results of Chapter 3 and by Brannan and Decker (2002) that true quantification of the effects of NOS and NO on meat quality were best conducted after pre-slaughter interventions, rather than post-slaughter manipulation.

7.2.2 The pre-slaughter role of NO in determining meat quality

To study the role of NO in determining meat quality, L-NAME was infused 135 minutes pre-slaughter. Plasma NO_x concentrations in Chapter 5 were lowest approximately 60 minutes after L-NAME infusion, indicating that NOS was inhibited after this point. The interaction between exercise and NOS inhibition was tested, since exercise increases skeletal muscle NOS activity. It was also hypothesised that muscle NOS activity was elevated under conditions of pre-slaughter stress as a routine part of handling livestock under commercial conditions. Since NOS inhibition improved tenderness in Chapter 6, independent of pH fall, sarcomere length or water holding capacity, it was concluded that NO inhibits protease activity in skeletal muscle. Proposed mechanisms included antagonism of direct inhibition of calpain with NOS inhibition or a shift in calcium homeostasis (discussed in section 7.2, Figure 7.1). The involvement of Ca^{++} in NOS inhibition-mediated tenderisation is not clear since the effect of NOS inhibition on Ca^{++} concentrations is unknown. The hypothesis that NOS inhibition increases cytosolic Ca^{++} concentrations does not conform to results from Chapter 4 where NO appeared to increase Ca^{++} concentrations. It is expected that inhibition of NOS will antagonise this effect. The assay conducted in Chapter 4 while linking NO to Ca^{++} homeostasis did not replicate

pH, temperature and substrate concentrations found in the carcass and the relationship between NO and cytosolic Ca^{++} concentrations in the carcass can only be speculated upon.

Since calpain is theorised to be the principal protease involved in tenderising meat (Koohmaraie, 1994, Dransfield, 1994), inhibition by NO could increase the toughness of meat (Figure 7.1). Michetti et al. (1995) reported that inhibition of rat skeletal muscle calpain by NO was isoform- and pH-dependent. At neutral pH, m-calpain was inhibited by SNP and μ-calpain was unaffected, while at acidic pH's the opposite was true. This result is significant for meat quality, since tenderisation occurs at acidic pH's and μ-calpain is proposed to be the most active calpain isoform post-mortem (Uytterhaegen et al., 1994). Michetti et al. (1995) proposed that NO inhibited calpain directly, via oxidation of critical cysteine sites. Since inhibition could be reversed by addition of competitive thiols in the form of dithiothreitol, they concluded that calpain was inhibited by S-nitrosylation of cysteine residues. Furthermore, NO may influence Ca^{++} dependent proteolysis via influencing Ca^{++} homeostasis, as demonstrated in Chapter 4. If, as proposed in Chapter 4, NO increases cytosolic Ca^{++} concentrations, this may in turn indirectly improve meat tenderness via activation of the Ca^{++} dependant protease calpain or by directly weakening myofibrils (Takahashi et al., 1987, Takahashi, 1992). However, increased cytosolic Ca^{++} concentrations would require increased NO concentrations, whereas L-NAME inhibits NO production. Collectively, these data do not indicate that improved tenderness with L-NAME was due to a Ca^{++}-dependent mechanism, but the possibility cannot be excluded, since the assay conducted in Chapter 4 did not replicate carcass conditions.

Increased Ca^{++} influx associated with exercise has been proposed to increase protease calpain activity in livestock pre-slaughter (Daly et al., 1995). Increases in plasma amino acid and urea concentrations have been observed in humans after semi-acute bouts of exercise (Rennie et al., 1980, Graham et al., 1991), indicating increased proteolysis during exercise. Exercise-induced increases in proteolysis have been attributed to non-lysosomal and lysosomal pathways (Salminen and Vihko, 1981). Since increases in cathepsin activity occur too late to account for the acute loss of myofibrillar protein post-exercise (Belcastro et al., 1985), it is unlikely that the lysosomal pathway is responsible for acute increases in skeletal muscle proteolysis post-exercise (Kasperek and Snider, 1989). Alternatively, it has been proposed that calpain is responsible for acute increases in skeletal muscle proteolysis post-exercise (Belcostro, 1993). Since calpain activity in C2C12 cells has been increased by adrenaline (Ertbjerg et al., 2000), it is possible that both exercise and stress increase muscle calpain activity. Daly et al. (1995) speculated that since activation parallels autolysis of calpain, pre-slaughter exercise-stress may reduce the total calpain activity in muscle pre-slaughter, resulting in tougher meat. Speculatively, if such a system were in place, concomitant increases in NOS activity may contribute to inhibition of calpain. Furthermore, increased Ca^{++} influx to the cytosol associated with exercise may activate Ca^{++}-sensitive cellular phospholipases (Gissel and Clausen, 2001), reducing membrane integrity and contributing to increased drip loss.

Inhibition of NOS in Chapter 6 increased glycogen depletion and lactate production, indicating that NO inhibits glycogenolysis and glycolysis. The effect of NOS inhibition of on muscle glycolysis did not occur pre-slaughter, as indicated in Chapter 5, and since glycogen depletion after NOS inhibition did not occur in Chapter 6.

Inhibition of NOS only occurred post-mortem, indicating that NO may only influence glycolysis when it is stimulated by other means, possibly as part of a feed-back loop. Since NO inhibits the glycolytic enzyme GAPDH, it was proposed in Chapter 6 that this may be a mechanism by which NOS inhibition increases glycolysis (Figure 7.1). Inhibition of NOS pre-slaughter did not influence initial or final glycogen and lactate concentrations, but rather the rate of metabolism in muscle post-mortem. Rates of glycogen depletion post-slaughter often influence meat quality by increasing or decreasing the rate of pH fall, as observed with DFD and PSE meat. The increased levels of glycolysis observed in Chapter 6 following NOS inhibition did not affect pH fall, and therefore the contribution to meat quality is unclear.

7.2.3 Effects of NO on meat colour and lipid oxidation

The pink appearance of cooked cured meat is due to the formation of nitrosomyoglobin, which occurs via the non-enzymatic reaction of NO from sodium nitrite and nitrate with the meat pigment myoglobin (Hultin, 1985). There is no evidence in these results indicating similar reactions occurring between enzymatic NO production and myoglobin in meat. However, since NO is synthesised at lipid membranes and is lipid soluble, it is possible that NO is involved in lipid oxidation and meat rancidity. There was evidence for this in Chapter 3, where the injection of the NO donor SNP resulted in a large increase in lipid peroxidation. The applicability of this result to normal ageing processes during storage was dismissed since it was postulated that concentrations of NO were supraphysiological. Brannan et al. (2001) hypothesised that peroxynitrite, formed from the reaction between NO and O_2^- is an important initiator of lipid oxidation in muscle foods after they observed an increase in lipid oxidation with 2mM SIN-1 (donor of NO and O_2^-). The contribution of NO to

lipid oxidation and rancidity in meat is still not clear, particularly since the concentrations of SIN-1 used by Brannan and co-workers was also supra-physiological and peroxynitrite-induced oxidation is inhibited by low pH and increasing CO_2 concentrations (Brannan et al., 2001). In addition, it is likely that the activity of NOS is short-lived post-mortem, limiting its capacity to induce lipid oxidation.

7.3 Further research

Data in this thesis have demonstrated involvement of NO in influencing muscle carbohydrate and fat metabolism, calcium sensitivity of the SR and meat tenderness. The mechanism by which NO influences muscle metabolism is not clear, but interactions with endocrine hormones, substrate transporters and metabolic enzymes have been implicated. The role of NO in regulating insulin sensitivity needs to be further investigated, similar experiments conducted in rats have observed decreased insulin secretion, but increased sensitivity (Balon et al., 1999). The contribution of potential perturbations in blood flow on insulin sensitivity and glucose uptake also needs to be quantified.

While experiments conducted in Chapters 5 and 6 focused on the interactions between NO and carbohydrate metabolism, apparent increases in lipolysis were observed in Chapter 5. This may have significant ramifications for the potential for manipulating fat distribution, marbling and hence commercial value of livestock. The mechanism whereby NO increased hind-limb lipolysis needs to be identified, whether it is induced on a cellular level by regulation of HSL and LPL activity or by alterations to hormone sensitivity. It is likely that NOS in adipose and muscle tissues are

differentially regulated, implicating direct regulation of HSL and LPL by NO. The involvement of NO in chronic fat metabolism warrants investigation, since if NO influences lipolysis, it is likely that it also has a role in fat deposition, which can significantly influence the commercial value of a carcass. Altered sensitivity or secretion of lipolytic hormones leptin, insulin and growth hormone have all been observed to be regulated by NO. However, many existing experiments have been conducted in monogastrics, whose hormone profiles, sensitivities and responses are likely to differ from ruminants.

Since inhibition of NOS with L-NAME was observed to improve tenderness independent of changes in pH, temperature and sarcomere length in Chapter 6 in the LTL muscle, it was surmised that L-NAME removed NO-mediated inhibition of proteolytic enzymes. It was further concluded that this was most likely due to inhibition of calpain, since NO is capable of oxidising regulatory thiol residues. Whether this reaction occurs post-mortem needs to be quantified as this could provide an important mechanistic link between events increasing NOS activity (eg. stress), inhibition of calpain and increased toughness. This may be further assisted by quantification of the effect of NO on protein turnover *in vivo*.

The effect of NO on calcium homeostasis was evaluated *in vitro* by SR uptake, release and ATPase assays. Recently microprobes have been used to ascertain free Ca^{++} in the carcass (Hopkins and Thompson, 2001, Hopkins and Thompson, 2002). Use of microprobe techniques could be used to validate *in vitro* experiments and determine the effect of NO on free Ca^{++} in the carcass.

7.4 General conclusion

Results presented in this thesis have demonstrated that NO is involved in regulating physiological and biochemical factors important to meat quality (Figure 7.1). This was evidenced in the involvement of NO in muscle carbohydrate and lipid metabolism. Specifically NO appears to be inhibitory of muscle glucose uptake and lipolysis. While the exact mechanism by which the effects of NOS inhibition on cellular metabolism are not known, it was postulated that they were a combination of altered hormone sensitivity and changes in cellular regulation. Furthermore, NOS activity appears to be increased by exercise-stress, supporting a role for NO in ante-mortem stress responses.

While it is hypothesised that the activity of NOS is limited post-mortem, the effects of pre-slaughter NOS inhibition were long-lived and included changes in post-mortem muscle metabolism and meat quality. Post-mortem metabolic effects of NO were evident in increased rates of glycolysis post-mortem upon NOS inhibition, indicating NO is inhibitory of muscle glycolysis. Long-term effects of NO on meat quality were observed when L-NAME improved tenderness in the LTL muscle, indicating that NO inhibits meat ageing. This was postulated to be through altered Ca^{++} metabolism or inhibition of calpain directly via nitrosylation or indirectly via Ca^{++} concentrations.

Therefore, these results support the thesis hypothesis, that NO influences physiological determinants of meat quality.

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