A PHYSIOLOGICAL PERSPECTIVE:
ELECTRICAL STIMULATION OF POST-MORTEM MUSCLE

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2016
A physiological perspective: Electrical stimulation of post-mortem muscle
Declaration

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

.................................................................

Paula Irene Fuller
A physiological perspective: Electrical stimulation of post-mortem muscle
Electrical stimulation of carcasses is a mainstay processing technique within the sheep meat industry. A primary use is to accelerate tenderisation of meat and to reduce the variation observed in its end products. Technological advances have resulted in a new generation of medium voltage electrical stimulation (MVES) units, which offer superior outcomes to previous high- and low-voltage systems. These new units allow precisely controlled electrical inputs to individual carcasses, thus providing processors with greater control over end product outcomes. Yet even with these new systems, significant variation in meat quality still remains. This variation likely results from an unidentified interaction between the electrical input and the carcass, as previously used electrical parameters were empirically defined, based on processor experience rather than an integrated understanding of the applied parameters and the induced response in the post-mortem muscle. Thus a scientific understanding of how the electrical input interacts with post-mortem muscle to improve meat quality is required. Given the use of electrical stimulation stems from its ability to engage muscle activity, this thesis sought to examine the effect of MVES on post-mortem muscle function using contractile characteristics as a means to identify how this type of system imparts its beneficial effects.

The first experimental chapter (Chapter 2) examined the effect of various MVES parameters on dressed sheep carcasses (under commercial processing conditions), using the ΔpH of the M. longissimus thoracis et lumborum as a means by which to define carcass response and determine which electrical parameters were most effective. We observed that stimulation parameters incorporating longer pulse widths (i.e. 5ms) or those with a modulated (increasing) frequency across the electrodes produced the
largest carcass response (i.e. largest $\Delta$pH). Interestingly, the effect of this electrical
stimulation on pH was influenced by hot carcass weight, with lighter carcasses (<23Kg)
more responsive to all stimulation parameters tested, in contrast to heavier carcasses
that did not distinguish between the different electrical inputs. As part of this study, we
also determined that pre-stimulation pH is an adequate marker of carcass responsiveness
to MVES stimulation, offering a way in which to determine how carcasses will respond
to the applied current. In a concurrent study, we took advantage of the positioning of the
stimulation system in order to compare the effect of MVES on muscle pH within the
immediate stimulation period (i.e. within five minutes) compared to a time period more
reflective of that in the literature, documenting a significant difference. This, in
conjunction with showing a significant difference between pH measuring techniques,
highlights the requirement for consideration of both the timing and methodology when
comparing pH responses to MVES.

Having determined the parameters that produced the biggest effect on carcass pH, the
second experimental chapter (Chapter 3) undertook a closer examination as to how
these parameters affected the contractile properties of post-mortem muscle. This was
achieved by establishing an isolated nerve-muscle electrophysiology rig to examine
their influence on muscle in a controlled environment. The electrical parameters
identified in the previous chapter were examined, with a particular interest in the effect
of longer pulse widths and modulated frequency on muscle contraction given their
positive effect on $\Delta$pH. The contractile characteristics of two different types of sheep
muscle [$M. semitendinosus$ (ST) and $M. semimembranosus$ (SM)], were examined. This
study revealed that electrical stimulation with longer pulse widths produced a greater
contractile response in terms of both peak tension and the overall amount of contraction.
Muscle response to modulated frequency stimulation was similar to that elicited by
longer pulse widths, but likely arose from enhanced activity of different contractile elements. Comparison of the different muscle responses to simulated MVES showed that overall, the oxidative-glycolytic SM muscle produced a larger contractile response than the glycolytic ST, but did not distinguish between the electrical parameters. This study also revealed a difference in the way in which the electrical impulse was transmitted through the muscle bundle, i.e., direct stimulation of muscle vs. nerve-mediated (stimulation of residual nerve activity). It appeared that more of each parameter was transmitted via the nerves in SM, but more directly transmitted in ST.

The last observations from Chapter 3 were particularly interesting, as it suggested that electrical stimulation transmitted via the nervous system may a viable option for use within the MVES set up. This possibility would provide another avenue for whole carcass stimulation, as electrical transmission via the nervous system generates a consistent and more homogenous decline of pH in all carcass muscles. Thus the last experimental chapter (Chapter 4) examined if, and to what extent, post-mortem muscle could respond to nerve-mediated electrical stimulation. This study showed that under a defined post-mortem environment (i.e., anoxic conditions), post-mortem sheep muscle does retain contractile activity in response to nerve-mediated electrical stimulation, but in a muscle-specific manner - both in the magnitude of contraction and the ability to distinguish between stimuli frequencies. We also investigated the role of several neuromodulators in muscle contraction under these conditions, specifically those that are known to mediate muscle contraction arising from nerve stimulation (ATP, NO and CGRP). The neuromodulators ATP and NO appear to affect muscle response to nerve stimulation at the level of neurotransmission in ST, in contrast to E-C coupling processes in SM. These results suggest that post-mortem SM muscle is better able to respond to nerve-mediated parameters, and this response is likely directed by the
physiological properties that dictate normal muscle function (i.e., the type of innervation). Overall this study suggests that nerve-mediated electrical parameters may be a viable option for incorporation into commercial processing environment if optimised in a muscle-specific manner, with the most advantage likely gained for the processing of muscle groups containing oxidative-glycolytic fibre types.

In summary, this thesis has provided a number of insights into the mechanisms by which various MVES inputs influence post-mortem muscle function. Identification of the biological changes that occur within stimulated nerve and muscle in post-mortem tissue may be used to refine electrical stimulation parameters for specific carcass applications to enhance overall meat quality. Furthermore, we identified a potential role for nerve-mediated responses as part of the stimulation strategy. It is hoped that these findings contribute to a more sophisticated understanding of the use of electrical stimulation, and may provide the commercial sector further opportunities for refining this technology for the benefit of both the producer and consumer.
This candidature has endured through the ages...at last, its time has come.
To my principle supervisor, Prof Jacqueline Phillips - words do not do justice for the guidance, encouragement, support, and never-ending patience you have given me over this lifetime. You have gone above and beyond the requirements of a PhD supervisor, and both this thesis and I are all the better for it. I couldn’t have done it without you. I wouldn’t want to have done it without you. I would also like to profusely thank Prof David Pethick for his support, enthusiasm, and wonderful opportunities to meet many excellent researchers in the field. I thank Dr Kate Creed for sharing her time, equipment, and extensive knowledge of muscle physiology, and assistance in establishing the electrophysiology rig. This project would not have been possible without Dr Kelly Manton-Pearce, to whom I owe many, many thanks, for not just the considerable assistance with all facets of organizing and executing the abattoir study, but also her willingness and immeasurable patience to explain the unknown world of meat research to me – she made an incomprehensible challenge doable. And with style (think hairnet, wellies, and pearls).

I am also very grateful to the following: The management and staff at WAMMCO for their support and assistance in accommodating our experimental wishes. Mr Kim Thomas, Murdoch Farm Manager, and his staff for assisting with all sheep work, whose accommodating approach and unflappable manner was much appreciated. The Molecular Neurobiology lab members whose support I’ve greatly appreciated along the way. Particular thanks go to Steve Callahan for assistance with IT/electrical recordings, Rhonda Loxley for sharing her extensive lab knowledge, and Andrew Williams and Courtney Reddrop for technical assistance with muscle recordings. Special mention to Gael Gibbs and Anna Barron for their enduring friendships, which have provided much comfort in times of need and times of joy. I thank the Australian Sheep Industry CRC and Meat and Livestock Australia for financial support and professional development opportunities.

I would like to thank my family and friends, here and gone, for their unlimited patience, understanding and unwavering support. A special thanks to Pamela and Jody, Martin and Cathy, who have lovingly and patiently supported me in this endeavor from the beginning. To the feline fuzzy kids, whose companionship was constant (especially on the keyboard). And to Kim, my rock – whose belief in me finishing has been as steadfast as his promise to never let my teacup run dry (110% commitment on both accounts).

“It always seems impossible until it is done”
Nelson Mandela
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>β,γ-ATP</td>
<td>beta-gamma-imidio-adenonine triphosphate</td>
</tr>
<tr>
<td>ΔpH</td>
<td>change in pH level</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP(s)</td>
<td>action potential(s)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve: in reference to the total amount of contraction</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ions</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>calcium ATPase (sarco/endoplasmic reticulum Ca²⁺-ATPase, SERCA)</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>CGRP₈₋₃₇</td>
<td>CGRP inhibitory peptide (fragment containing peptides 8-37)</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>DHPR(s)</td>
<td>dihydropyridine receptor(s)</td>
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<tr>
<td>E-C</td>
<td>excitation-contraction (coupling process)</td>
</tr>
<tr>
<td>EDL</td>
<td><em>M. extensor digitorum longus</em></td>
</tr>
<tr>
<td>(g)</td>
<td>grams</td>
</tr>
<tr>
<td>(g.s)</td>
<td>grams per second</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HVES</td>
<td>high voltage electrical stimulation</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz; measurement of frequency</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ions</td>
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<td>[K⁺]ᵢ</td>
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<td>[K⁺]ₒ</td>
<td>extracellular potassium concentration</td>
</tr>
<tr>
<td>Kₐ₅</td>
<td>ATP-sensitive potassium channel</td>
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<tr>
<td>L-NAME</td>
<td>N⁵-nitro-L-arginine methyl ester hydrochloride</td>
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<tr>
<td>LL</td>
<td><em>M. longissimus thoracis et lumbarum</em></td>
</tr>
<tr>
<td>LVES</td>
<td>low voltage electrical stimulation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>MU(s)</td>
<td>motor unit(s)</td>
</tr>
<tr>
<td>MVES</td>
<td>medium voltage electrical stimulation</td>
</tr>
<tr>
<td>Na^+/K^+-ATPase</td>
<td>sodium-potassium ATPase</td>
</tr>
<tr>
<td>Na^+</td>
<td>sodium ions</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>P_i</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PCr</td>
<td>phosphocreatine</td>
</tr>
<tr>
<td>P_o</td>
<td>optimal muscle fibre length</td>
</tr>
<tr>
<td>pHu</td>
<td>ultimate pH</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RYR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SM</td>
<td>M. semimembranosus</td>
</tr>
<tr>
<td>SMEQ</td>
<td>Sheep Meat Eating Quality</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>ST</td>
<td>M. semitendinosus</td>
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<td>TnC</td>
<td>troponin C</td>
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<tr>
<td>TnT</td>
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<tr>
<td>T-tubules</td>
<td>transverse tubules</td>
</tr>
<tr>
<td>VACHT</td>
<td>vesicular acetylcholine transporter</td>
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Chapter 1.

Literature Review: The life and death of skeletal muscle

The transition of living muscle through to meat is a complex process, but by considering the physiological function of muscle in its normal state of activity, through the post-mortem transitional stages, and into the final product as meat, this literature review aims to highlight pertinent areas and issues between the beginning and end stages of this process. This literature review is divided into three sections, each corresponding to an important stage of this transitional process: Part A reviews the physiological functioning of skeletal muscle and how its function is regulated by both internal and external factors; Part B highlights the changes that occur at the ‘in-between’ stage of muscle transitioning to meat, specifically the response of muscle in a declining oxygen and energy state; and Part C reviews aspects of the post-mortem period that influence, and ultimately determine, the end product quality of the meat, with the influence of electrical stimulation the main focus.

Part A. In life: physiological regulation of muscle

1.1 Muscle structure and contractile mechanisms

Skeletal muscle is an extremely heterogeneous tissue, composed of a variety of functionally diverse fibre types. These fibre types differ according to their molecular, structural, and contractile properties, all of which contribute to the versatility that is required of skeletal muscle in order to respond to the broad range of functional demands placed upon it (Schiaffino and Reggiani, 1996, Bottinelli and Reggiani, 2000).
The fundamental basis of all body movement is the contraction of skeletal muscle, which also imparts both strength and postural support (Brooks, 2003). The process of muscle contraction, from initiation of an electrical impulse in a motor neuron to the physical mechanical contraction of the muscle, can be separated into three main events: neurotransmission (propagation of an electrical signal from the motor neuron to the muscle membrane), excitation-contraction coupling process (E-C coupling; transmission of the electrical signal throughout the muscle fibre and subsequent release of Ca\(^{2+}\)), and finally the mechanical shortening of the muscle fibre via the contractile apparatus (thick and thin filaments). Each event is interlinked, yet each has different factors that contribute to its optimal functioning and inhibition. Numerous reviews have examined this area in great detail (for example, (Close, 1972, Schiaffino and Reggiani, 1996, Schiaffino and Reggiani, 2011, Greising et al., 2012), thus the below section outlines the most pertinent facts relevant to this thesis.

### 1.1.1 Structure

Skeletal muscle is made up of fascicles, bundles of individual elongated, multinucleated muscle cells (muscle fibres), as well as connective tissue, blood vessels and nerves (Figure 1.1B and 1.1C). Within each muscle fibre are hundreds of striated, rod-like elements termed myofibrils, which contain the muscles contractile machinery (Figure 1.1D). Each myofibril is comprised of partially overlapping thick (myosin) and thin (actin) filaments, organised into a repeating functional unit called the sarcomere (Figure 1.1E and 1.1F; Bottinelli and Reggiani, 2000). Each sarcomere is bordered on either end by Z-disks, which run perpendicular to the long axis and anchor the thin filaments at one end, whilst the thick filaments are connected to the sarcomeric middle by M lines. Thin filaments consist of actin monomers polymerised into two chains forming a double
helix-style structure, with each monomer containing a myosin-binding site (Close, 1972). Also present in thin filaments are the regulatory proteins of contraction, tropomyosin and troponin (Weber and Murray, 1973). Tropomyosin is a fibrous molecule that blocks myosin binding sites in muscles at rest, whilst troponin is a three-polypeptide complex that regulates myosin-actin aligning activity [one peptide attaches to the actin strand (TnI), another binds to tropomyosin (TnT), and the third contains a Ca\(^{2+}\) binding site (TnC)].

The binding of Ca\(^{2+}\) triggers muscle contraction by causing troponin to move tropomyosin aside, thereby exposing the myosin binding sites on actin molecules. Myosin thick filaments are composed of two heavy and four light chains that intertwine to form a long tail and a pivoting globular-like head containing ATPase and actin binding sites. The myosin head is of particular importance in muscle contraction, as its activity produces mechanical force by interaction with actin and ATP (Huxley, 1985). Further analyses of the myosin heads enzymatic function demonstrated that the myosin heavy chain (MHC) component is the main modulator of ATPase activity, with seven isoforms recognized to have different ATPase characteristics (Barany, 1967, Sciote et al., 1994). The actual force generating capacity of the fibre is determined by the number of myofibrils arranged in parallel (Goldspink, 1985), with the change in the length of individual sarcomeres occurring as thick and thin filaments slide past each other, mediated by cyclical interactions between the myosin heads from the myosin thick filaments and binding sites on the actin molecules of the thin filament; these structural interactions are referred to as cross-bridges (Huxley and Niedergerke, 1954).
Figure 1.1. Structure of skeletal muscle

A muscle (A) is comprised of bundles of fascicles (B), which are groups of muscle fibres (C) encased in connective tissue. Each muscle fibre, in turn, contains hundreds of myofibrils (D), containing repeats of the functional contracting units, sarcomeres (E), which are made of overlapping myosin and acting filaments (F) (King et al., 2014).
Skeletal muscle fibres also contain two sets of intracellular tubules that participate in regulation of muscle contraction: the sarcoplasmic reticulum (SR) and transverse tubules (T-tubules). The SR is a membranous interconnecting system of longitudinal tubules that surrounds each myofibril; its main role is to regulate intracellular levels of Ca\(^{2+}\) by its release and reuptake, maintaining free Ca\(^{2+}\) at levels around 1mM (Fryer and Stephenson, 1996). Terminal cisternae are sac-like structures within the SR that store the Ca\(^{2+}\), and these lie parallel to the branching network of T-tubules, which are continuations of the sarcolemma that penetrate into the cell interior, allowing conduction of the action potential into the deepest regions of the muscle cell.

### 1.1.2 Neurotransmission

Neuromuscular transmission consists of a series of events that converts a motor nerve action potential into a muscle action potential, and is confined to the neuromuscular junction (NMJ), a highly specialised contact area between the motor nerve and the muscle fibre (Figure 1.2A). This interface area functions to translate a neural-electrical impulse into a chemical signal, and then back to an electrical stimulus that initiates muscle contractions (a feat achieved in ~200\(\mu\)s; Boonyapisit et al., 1999, Ruff, 2003).

Anatomically, the NMJ is divided into a presynaptic terminal (the nerve end), the synaptic cleft, and the postsynaptic terminal (the muscle endplate and adjacent intracellular structures).

The mechanisms of neuromuscular transmission are well established. An action potential (AP), initiated by motor neurons, is propagated down a motor nerve axon via Na\(^+\) channels (Black et al., 1990). When the nerve AP reaches the NMJ, voltage-gated Ca\(^{2+}\) channels in the presynaptic membrane open, allowing an influx of Ca\(^{2+}\) from the
extracellular fluid (Figure 1.2B). The presence of Ca\(^{2+}\) inside the axonal terminal causes the release of small synaptic vesicles that contain the neurotransmitter, acetylcholine (ACh; Augustine et al., 1991). Together with ACh, other compounds (e.g. adenosine 5’-triphosphate; ATP) are co-released from the nerve terminal, with these co-transmitters modulating neuromuscular transmission in both the short- and long-term (Silinsky and Redman, 1996, Rothe et al., 2005). Acetylcholine diffuses across the cleft and attaches to ACh nicotinic receptors on the postsynaptic terminal (a trough-like part of the muscle membrane, also known as the motor endplate; Katz and Miledi, 1973). Activation of these receptors, in turn, causes activation of Na\(^{+}\) channels in the sarcolemma, which depolarizes the muscle membrane and continues the transmission of the AP (Figure 1.2B; Ruff, 1992). Simultaneously, the released ACh undergoes rapid enzymatic hydrolysis by acetylcholinesterase, which terminates the transmission of the AP by preventing continued muscle fibre contraction in the absence of additional nerve stimulation (Boonyapisit et al., 1999).

### 1.1.3 Excitation-Contraction coupling

The excitation-contraction (E-C) coupling process is a sequence of events that occurs between initiation of the muscle AP and the beginning of mechanical activity. This process couples the electrical excitation of the sarcolemma to muscle fibre contraction, and is mediated by intracellular Ca\(^{2+}\) levels (Figure 1.2C; Ebashi, 1976, Catterall, 1991). Once initiated, the sarcolemmal AP continues throughout the muscle fibre via the T-tubules, where the associated depolarisation is sensed by dihydropyridine receptor (DHPR) voltage sensors (Schneider, 1994). The DHPR receptors of skeletal muscle are modified L-type Ca\(^{2+}\) channels in which the Ca\(^{2+}\) channel function is virtually redundant because entry of Ca\(^{2+}\) into the cell is not necessary to initiate contraction, which results
Chapter 1

from a change in the membrane voltage (Dulhunty, 1992). The DHPRs colocalise in arrangements of four and are located in the T-tubules immediately adjacent to the alternative Ca\textsuperscript{2+}-release channels in the SR terminal cisternae, the homotetrameric muscle-specific ryanodine receptors (RYR; (Block et al., 1988). The E-C coupling is controlled by a unique, bi-directional interaction between the DHPRs in the T-tubules and the RYR of the SR, as activation of the DHPR subsequently leads to the activation of the RYR through direct molecular interaction, thereby transforming the voltage change across the T-tubule membrane to stimulate Ca\textsuperscript{2+} release from the SR via RYR (Schneider and Chandler, 1973). A distinct feature of the RYR is the strong inhibition of channel activity by millimolar concentrations of Ca\textsuperscript{2+}, modulation by reactive oxygen species (ROS), and the ability of ATP to stimulate channel activity even in the absence of Ca\textsuperscript{2+} (Lamb, 2000, Sun et al., 2001, Copello et al., 2002). Once Ca\textsuperscript{2+} has been released from the SR, the increase in intracellular Ca\textsuperscript{2+} initiates cross-bridge cycling and muscle contraction (Figure 1.2D). Relaxation occurs when Ca\textsuperscript{2+} is pumped back into the SR by Ca\textsuperscript{2+}-ATPase pumps (sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase, SERCA), which are the main transporters of Ca\textsuperscript{2+} out of skeletal muscle (Fill and Copello, 2002). SERCA-mediated Ca\textsuperscript{2+} reuptake controls the rate of muscle relaxation and is responsible for maintaining a 10,000-fold calcium gradient across the SR membrane (Martonosi, 1984, Rossi and Dirksen, 2006).

1.1.4 Mechanical contraction

The increased Ca\textsuperscript{2+} levels in the muscle fibres result in the sliding of myosin and actin myofilaments, which produces a shortening of the sarcomeres, and in turn, contraction of the muscle fibre. Muscle fibres change length via a mechanism whereby actin and
myosin filaments extend their amount of overlap, with the sliding force generated by repetitive interaction of myosin cross-bridges with the actin filaments, resulting in the thick filaments being ‘pulled’ along the thin filaments in the appropriate direction (Figure 1.2E; Huxley, 1985). This process is part of a cycle driven by ATP hydrolysis, and is known as the power stroke, with each stroke of the cross-bridge hydrolysing one molecule of ATP (McLester, 1997). The rate at which ATP is hydrolysed dictates the rate of cross-bridge cycling, i.e., how fast the thick and thin filaments slide past each other, and thus how rapidly a muscle can shorten. This process, in turn, is determined by the myosin ATPase activity that is established by the MHC isoform of the fibre (Barany, 1967).

The cross-bridge cycle begins with the release of Ca$^{2+}$ from the SR via RYR that results in Ca$^{2+}$ binding to troponin, which is attached to the actin filament and tropomyosin (Figure 1.2D and 1.2E). Troponin then changes shape and pulls tropomyosin away from the myosin-binding sites on the actin filament. Once the myosin-binding site on the actin filament is exposed, a contraction cycle begins. When ATP binds to the myosin head the ATPase hydrolyses ATP, forming ADP and inorganic phosphate. Hydrolysis of ATP energises the myosin heads to bind to the unblocked myosin-binding site on actin, releasing the inorganic phosphate from the myosin head. The release of the inorganic phosphate starts the power stroke, which is the rotation of the myosin head that pulls the thick filament toward the centre of the sarcomere. During the power stroke, ADP is released from the myosin head, but the myosin head remains attached (forming a rigor complex). Finally, another ATP molecule binds to the myosin ATP-binding pocket, releasing the myosin head from actin, and the contraction cycle begins again (Holmes and Geeves, 2000). The contraction cycle continues as long as intracellular Ca$^{2+}$ levels remain high. As the
Figure 1.2. Mechanisms of muscle contraction

Summary of the four main events in contracting muscle. (A) Motor neurons in spinal cord innervate groups of muscle fibres, transmitting APs down their axons that terminate at the NMJ. (B) At the NMJ, neurotransmission occurs between nerve and muscle via release of neurotransmitter, ACh. Binding to the ACh postsynaptic receptor activates Na\(^+\) channels, which depolarises the muscle membrane. (C) Propagation of the AP along the muscle membrane and down the T-tubules involves many ion channels (e.g., Na\(^+\), K\(^+\), Na\(^+\)/K\(^-\)-ATPase). Changes in membrane voltage engage DHPRs (yellow), which open RYRs (grey) on the SR to release Ca\(^{2+}\). This E-C coupling links action potentials to Ca\(^{2+}\) release to shortening of myofilaments. (D) Ca\(^{2+}\) binding to troponin initiates the cross-bridge cycle whereby myosin binds to actin (1) and is ‘pulled’ along by hydrolysis of ATP [(during the power stroke (2)]. The myosin head is then stuck on actin [rigor (3)] until ATP binds to myosin and it releases from actin (4), ready to start the cycle again when there is appropriate Ca\(^{2+}\) levels (5). (E) The sliding filaments result in the shortening of muscle fibres, i.e. muscle contraction. Figure adapted and created from Rowland, 1982, Cairns et al., 2009, MacIntosh et al., 2012, Spudich, 2012, Gonzalez-Freire et al., 2014.
intracellular Ca\textsuperscript{2+} levels drop due to removal by the Ca\textsuperscript{2+}-ATPase, tropomyosin blocks the myosin-binding sites on actin and the muscle fibre relaxes.

1.2 Characteristics of muscle fibres that define muscle function

Muscles are composed of varying proportions of different fibre types that enable flexibility in force production, thus allowing muscles to respond to their diverse roles. The functional properties of each fibre type are determined by the innervation pattern, which in turn defines its biochemical, morphological (channels/receptors), and physiological (neuromodulator) properties (Bottinelli and Reggiani, 2000).

1.2.1 Innervation type

Skeletal muscles are innervated by motor neurons located in the brainstem or ventral horn of the spinal cord, with the motor neuron and the muscle fibres it innervates collectively called a motor unit (MU; Liddell and Sherrington, 1925, Buller et al., 1960, Burke et al., 1973, Buchthal and Schmalbruch, 1980, Nemeth et al., 1986). Whilst a single motor neuron innervates many muscles fibres, each muscle fibre is innervated by only one motor neuron; groups of MU work together to coordinate the contractions of a single muscle, with all units that subserve a single muscle considered a motor unit pool (Liddell and Sherrington, 1925, Enoka, 1995).

Motor units are characterised by the firing pattern of the motor neuron and subsequent mechanical properties of its associated muscle fibres. Early studies classified mammalian MU into two functional groups: a homogenous group of slow contracting and non-fatigable units (slow-twitch fatigue resistant; S) and a heterogenous group of fast-contracting units with various degrees of fatigability (Figure 1.3; Burke,
Based upon criteria such as the twitch time to peak tension, “sag” of an unfused tetanic contraction, and fatigue upon repetitive tetanic activation, the fast contracting MU can be further sub-grouped into fast fatiguing fast-twitch (type FF), intermediate fatigue resistant fast-twitch (FInt), and fatigue-resistant fast-twitch (type FR; Figure 1.3; Burke, 1967, Burke et al., 1973).

Neural control of muscle contractile properties is dictated by the size of the MU, the number of MU’s recruited and the discharge rate of the MU group, with each factor contributing to the amount of muscle force generated. As such, type S MUs have the smallest axon size and lowest excitation threshold, making them more easily activated than fast types, which increases in the order of S<FR<F<FF (Granit et al., 1957, Eccles et al., 1958). Muscles innervated with fewer MUs generally produce fine gradation of force (e.g. the rectus lateralis muscle of the human eye contains approximately 5 fibres per unit, whilst the medial gastrocnemius of leg muscle has up to 2000 fibres; (Feinstein et al., 1955, Enoka, 1995). Finally, the discharge pattern of the motor neuron influences the overall force produced by the muscle as this relates to the optimal mechanical tension e.g. in rat soleus muscle (slow twitch muscle – type S MU) discharge occurs in the range of 10-50 Hz, in contrast to the extensor digitorum longus (fast twitch muscle – type FF MU), which discharges between 40-160 Hz; (Burke, 1981, Hennig and Lomo, 1985, Kernell, 2003).

The classification of muscle fibres types based on histochemical staining profiles or MHC immunoreactivity generally corresponds to the contractile and fatigue properties of the motor units that they comprise (Figure 1.3; Dum and Kennedy, 1980, Greising et al., 2012). Muscle fibres classified as type I comprise type S MUs; whilst types IIA, IIX and IIB comprise type FR, FInt, and FF MUs, respectively (Burke et al., 1973, Sieck et al., 1996, Pette and Staron, 2000). The results of combined physiological
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and histochemical studies have demonstrated that muscle fibres of the type S MUs tend to be slow and highly resistant to fatigue, thus they can keep up a series of repeated contractions with little loss of force. At the other extreme, fibres of type FF MUs are fast but more fatigable, and are active at higher frequencies but for shorter periods of time (Storella and Baker, 1988). Morphological differences of the NMJ have also been noted between fibre types, with 3D confocal microscopy showing that nerve terminal and endplates increased in complexity moving from type I to type IIB fibres (Prakash et al., 1996). As such, the transmitter release characteristics also differ between fast and slow fibres, having adapted to the different patterns of nerve impulses they receive in vivo (Lev-Tov and Fishman, 1986, Ruff, 2003).

Figure 1.3. Correlation between motor unit classification and fibre type groupings

Classification of motor units (MU) based on contractile and fatigue properties of the innervated muscle fibres (Greising et al., 2012).
1.2.2 Biochemical properties

The physical properties of each fibre type are highlighted in Table 1.1. Type I and IIA fibres are generally smaller and have a higher mitochondrial density and oxidative capacity than type IIX and IIB, which in part accounts for the greater fatigue resistance displayed by these fibres. In addition, isolated mitochondria from different fibre types have been shown to vary substantially in their functioning capacity and regulation of oxygen consumption (Crow and Kushmerick, 1982, Jackman and Willis, 1996, Saks et al., 1996, Gueguen et al., 2005b, Picard et al., 2008). Type I and IIA fibres are also supplied with an extensive vascular system and are functionally involved in endurance activities and posture maintenance. Conversely, type IIX and IIB fibres are large fibres that possess an extensive sarcoplasmic reticulum for rapid release of Ca$^{2+}$ to initiate contraction, and large amounts of glycolytic enzymes used in energy production by the glycolytic process, specialised in producing rapid and powerful movement (Close, 1972, Schiaffino and Reggiani, 1996). Studies using permeabilised fibre preparations have also shown that type I and IIA fibres generate less force per cross-sectional area (specific force) and produce slower contractions than type IIX and IIB (Reiser et al., 1985), with the latter functional property related to the speed of the myosin ATPase (Bottinelli et al., 1994, Sieck et al., 1995, Schiaffino and Reggiani, 1996). The rate at which the different MHC isoforms hydrolyse ATP varies significantly, with type II fibres utilising ATP 3-fold faster than type I; however it has also been shown that ATPase activity varies 2-3 fold among fibres of the same MHC composition (Bottinelli et al., 1994, Stienen et al., 1995, Bottinelli and Reggiani, 2000). As such, the rate of ATP consumption rate during cross-bridge cycling is a major determinant of the mechanical performance of skeletal muscle fibres (Barany, 1967, Han et al., 2001).
Table 1.1. Relative magnitude of biological characteristics for individual fibre types (low, intermediate, high)

<table>
<thead>
<tr>
<th>Property</th>
<th>Type</th>
<th>Slow oxidative (I)</th>
<th>Fast oxidative glycolytic (IIA)</th>
<th>Fast glycolytic (IIX/IIB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffering capacity</td>
<td>low</td>
<td>high</td>
<td>very high</td>
<td></td>
</tr>
<tr>
<td>Capillary density</td>
<td>high</td>
<td>intermediate/high</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Contraction speed (mATPase activity)</td>
<td>slow</td>
<td>intermediate/fast</td>
<td>fast</td>
<td></td>
</tr>
<tr>
<td>Fatigue resistance</td>
<td>high</td>
<td>intermediate</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Fibre diameter</td>
<td>small</td>
<td>intermediate</td>
<td>large</td>
<td></td>
</tr>
<tr>
<td>Force-generating capacity</td>
<td>low</td>
<td>intermediate</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>low</td>
<td>intermediate</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial density</td>
<td>high</td>
<td>high</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Myoglobin content</td>
<td>high</td>
<td>high</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>high</td>
<td>high</td>
<td>low</td>
<td></td>
</tr>
</tbody>
</table>


The expression of different fibres types is species-dependent, particularly for type IIX and IIB. Type IIB fibres are found predominantly in smaller mammals (rat, mice, etc), whilst the limb and trunk muscles of sheep, cattle and other ruminants express type IIX and little, if any, type IIB (Maccatrozzo et al., 2004, Greenwood et al., 2007, Hemmings et al., 2009). There are exceptions to this, with studies revealing that pigs and llamas express the IIB isoform (Schiaffino et al., 1989, Pette and Staron, 2000, Lefaucheur et al., 2004, Reggiani and Mascarello, 2004). Aside from differing in the type of fast MHC isoform expressed, other variations displayed between species include the total number of fibres, and the proportion and spatial distribution of fibre types in a given muscle. It is documented that body size is much more related to the total number of fibres than the fibre cross-sectional area (Plaghki, 1985), and further, there is a positive correlation
between body size and the proportion of slow twitch type I fibres (Close, 1972, Lefaucheur and Gerrard, 2000, Pellegrino et al., 2003).

1.2.3 Physiological

Optimal functioning of muscle is highly dependent upon the correct movement of various ions into and out of the cell, with the movement of Na$^+$ and K$^+$ concentrations pertinent to membrane excitability, and Ca$^{2+}$ concentrations important for sustained function of contractile elements. The molecular pumps that return these ions to their appropriate compartments are essential for sustaining muscle contraction (as reviewed by (Berchtold et al., 2000, Clausen, 2003, Zhu et al., 2013). Notably, there are significant differences between fibre types in the activity of these ions and their pumps.

Within skeletal muscle, the maintenance of the membrane potential, and thus excitability, is performed by Na$^+$/K$^+$-ATPase pumps. The movement of an AP along the membrane results from the concomitant efflux of K$^+$ from the muscle cell, with the increased influx of Na$^+$ during muscle contraction resulting in depolarisation. If not removed efficiently, the buildup of extracellular potassium ([K$^+$]$_o$) leads to a loss of excitability and inhibition of muscle contraction (Clausen et al., 2004). This is an important problem for fast twitch fibres, given the release of intracellular potassium ([K$^+$]$_i$) is four fold larger in fast twitch vs. slow twitch fibres, thus making them more susceptible to contractile inhibition (Matar et al., 2000, Clausen, 2008). The electrogenic action of the Na$^+$/K$^+$-ATPase reverses the depolarisation that occurs during both normal excitation and aberrant muscle activity, such as during anoxia or in diseased muscles (Clausen, 2003). Studies that have measured the density of this pump in the sarcolemma (by measuring ouabain binding) revealed a muscle type bias, with fast-twitch fibres containing approximately 20% more pumps than slow-twitch ones.
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(Everts and Clausen, 1992, Fowles et al., 2004). This higher proportion of Na⁺/K⁺-ATPase pumps is likely associated with their greater susceptibility to aberrant function due to increased [K⁺]o from high frequency stimulation (Clausen et al., 2004, Juel, 2009). Interestingly, the activity of this pump is also influenced by several hormones, including calcitonin gene related peptide and adrenaline, which have been shown to augment force production after inhibition by high [K⁺]o (Clausen et al., 1993, Clausen, 2000, Macdonald et al., 2008). Another important regulator of [K⁺]o is the ATP-sensitive K⁺ channel (Kₐₜₚ). This channel regulates cellular K⁺ levels in accordance with the energy state of the cell, thereby linking membrane excitability with ATP availability as a means to preserve contractile function based on the metabolic state of the cell (Kristensen and Juel, 2010, Zhu et al., 2013). Similar to the Na⁺/K⁺-ATPase, Kₐₜₚ is thought to be more important in maintaining contraction in glycolytic fibres, given the increased susceptibility to K⁺-induced inhibition.

Calcium signaling is another highly important process in muscle contraction, with most steps in contraction associated with some form of Ca²⁺ activity. Differences between fibres types extend to this pump also, with a five fold difference in density observed between fast- vs. slow-twitch fibres (Lytton et al., 1992, Wu and Lytton, 1993). The need for such a greater density of Ca²⁺ pumps lies in the increased density of DHPRs, which is three to five fold greater in fast- vs. slow-twitch fibres (Delbono and Meissner, 1996). The faster decline of Ca²⁺ transients in fast fibres results from combined action of increased Ca²⁺-ATPase density and also greater cytoplasmic Ca²⁺ buffer, such as parvalbumin (present at about 1mM in fast fibres, but virtually absent in slow fibres; Dulhunty and Gage, 1983, Gundersen et al., 1988).
1.2.4 Neuromodulators

The secretion of modulatory substances in synchrony with primary neurotransmitters has long been proposed as an important feature of synaptic control (White and Macdonald, 1990, Worden, 1998). The many types of modulatory influences, from soluble factors to second messenger systems to specific proteins in both nerve and muscle, contribute both individually and synergistically to neuromuscular function. Important neuromodulators that influence the overall muscle contractile process include adenosine 5’-triphosphate (ATP), nitric oxide (NO), and calcitonin gene related peptide (CGRP). The overall influence these modulators have on muscle contraction differs depending if the site of action is pre- vs. postsynaptic.

Purinergic signalling – ATP

In skeletal muscle, ATP has multiple roles in the contractile process. In addition to the aforementioned roles in providing energy for the cross-bridge movement and the Ca$^{2+}$-ATPase pump during muscle contraction, ATP also modulates neuromuscular neurotransmission (Ralevic and Burnstock, 1998). During nerve stimulation, ATP is released along with ACh from the nerve terminal into the synaptic cleft, where it can reach a (transient) estimated concentration of 30-300µm, and once in the synaptic space it is degraded to adenosine via the ectonucleotidase cascade (Silinsky, 1975, Smith, 1991, Redman and Silinsky, 1994, Ribeiro et al., 1996). ATP is also released from the muscle fibre upon contraction, with reports that up to 60% of released ATP is derived from this source in a frequency-dependent manner (Smith, 1991, Henning, 1997, Vizi et al., 2000, Santos et al., 2003).

The diverse role of ATP in skeletal muscle contraction is mediated by the pre- and postsynaptic locations of the P2 family of cell-surface purine receptors, which allow
for its bifunctional role in being able to potentiate and depress transmitter (ACh) release. ATP acts presynaptically to potentiate transmitter release, with activation of the presynaptic purinergic P2X7 receptors increasing non-specific cation conductance across the membrane, leading to increased Ca^{2+} entry (Deuchars et al., 2001, Ryten et al., 2001, Grishin et al., 2005, Moores et al., 2005). Postsynaptically, the effects of ATP on muscle are mediated by the inhibitory G-protein coupled P2Y subtype of purinergic receptors, which act principally through phospholipase C to inhibit transmitter release via inhibition of voltage-gated ion channels (Choi et al., 2003, Santos et al., 2003, Grishin et al., 2005, Burnstock, 2007). The action of ATP at the synapse also influences the activity of channels and pumps located in the sarcolemmal membrane, such as K_{ATP} channels and ATPases (MacLennan et al., 1997, Zhu et al., 2013); see section 1.2.3).

**Nitrergic signalling - NO**

Nitric oxide is a ubiquitous cell-signaling molecule involved in numerous physiological and pathophysiological processes. Skeletal muscle is a major source of NO in the body, with this endogenous modulator involved in the regulation of muscle contraction, neuromuscular transmission, mitochondrial respiration, blood flow and metabolism (Nakane et al., 1993, Reid, 1998, Grozdanovic and Baumgarten, 1999, Mukhtarov et al., 2000, Kaminski and Andrade, 2001). Its synthesis from molecular oxygen and L-arginine is mediated by a striated muscle-specific variant of neuronal nitric oxide synthase (nNOSμ; Silvagno et al., 1996, Kaminski and Andrade, 2001), with another constitutively expressed isoform, endothelial NOS (eNOS), thought to influence skeletal muscle contraction through its action on mitochondrial and vasculature function (Kroncke et al., 1998, Reid, 1998, Grozdanovic, 2001).
As NO is a short-living free molecule, its action is limited to the vicinity of the production site, linking the localisation of nNOSμ closely to its site of action. Within striated fibres, nNOSμ is localised in high concentrations at the postsynaptic surface of the NMJ (Grozdanovic et al., 1995). Several studies have also reported the cytoplasmic expression of nNOS in the presynaptic terminal of the NMJ (Ribera et al., 1998, Rothe et al., 2005). Whilst nNOSμ has been found in both fast- and slow-twitch fibres (Kusner and Kaminski, 1996, Chao et al., 1997), the enzyme is particularly abundant in type II fibres in most mammals (Kobzik et al., 1994); a more homogenous distribution between fibre types is seen in human muscles compared with other mammals (Grozdanovic et al., 1995). In comparison, the eNOS isoform is limited to the mitochondria and skeletal vasculature (Kobzik et al., 1995). Both nNOS and eNOS are termed constitutive, such that they continually generate NO at relatively low levels to regulate inter- and intracellular signaling events, however during muscle contraction the concentration of NO in muscle can increase by 200%, suggesting further involvement in the contractile process (Balon and Nadler, 1994, Murrant and Reid, 2001).

Similar to the activity of ATP, NO targets are found on both sides of the NMJ, however the effects of NO are mediated by different mechanisms. The presynaptic effects of NO are predominantly mediated via an indirect second messenger pathway involving cyclic guanosine monophosphate (cGMP). Activation of soluble guanylate cyclase, located at the NMJ (Schmidt et al., 1993) increases cGMP formation that in turn regulates protein kinase G activity, protein phosphorylation and numerous other biological processes (Arnold et al., 1977, Schmidt et al., 1993, Kobzik et al., 1994). The result of the NO-cGMP pathway is inhibition of ACh release from presynaptic terminals (Lindgren and Laird, 1994, Ribera et al., 1998, Mukhtarov et al., 2000), suggesting that increased NO released from the muscle acts as a retrograde messenger, mediating
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activity-dependent synaptic suppression at the NMJ (Kaminski and Andrade, 2001).

Interestingly, the eventual outcome appears to be dependent upon contraction pattern
and frequency (Murrant et al., 1997, Thomas and Robitaille, 2001, Stojanovic et al.,
2005), as evidenced by studies that found low frequency stimulation leads to the
production of NO at the synapse and depression of transmitter release via a cGMP-
dependent mechanism (Etherington and Everett, 2004), whilst high frequency-induced
NO effects are mediated in a cGMP-independent manner (Thomas and Robitaille,
2001).

In contrast to the indirect pathway above, the postsynaptic actions of NO within
the muscle fibre are mediated directly through modification of various regulatory
proteins. Nitric oxide was originally shown to inhibit muscle contraction, as Kobzik et
al. (1994) and others showed that pharmacologic blockade of constitutive NOS activity
in rat muscle fibre bundles increased twitch and subtetanic maximal force production,
whereas NO donors, such as sodium nitroprusside (SNP), decreased force production
(Kobzik et al., 1994, Morrison et al., 1996). This NO-induced depression of contractile
force involves reversible oxidation of the protein thiols of myosin ATPase (decreasing
cross-bridge recruitment), nitrosylation of iron-sulphur clusters of mitochondrial
respiratory chain complexes (affecting energy production), and oxidative modification
of labile cysteine residues on ryanodine receptors (reducing the rate of Ca\textsuperscript{2+} release from
skeletal muscle SR; Kobzik et al., 1994, Stamler, 1994, Meszaros et al., 1996, Reid,
1996, Perkins et al., 1997, Klebl et al., 1998, Reid, 1998). However, the regulation of
Ca\textsuperscript{2+} signaling by NO is complex, as NO exhibits a biphasic action on Ca\textsuperscript{2+} release
which is dependent upon NO concentration, whereby low levels of NO inhibit
oxidation-induced activation, while high concentrations of NO increase the probability
Chapter 1

of opened \( \text{Ca}^{2+} \) channels thus increasing \( \text{Ca}^{2+} \) efflux from the SR (Murrant and Barclay, 1995, Aghdasi et al., 1997).

The actions of NO are also dependent upon its interaction with reactive oxygen species (ROS). These free radicals are produced in muscle along with NO, with a similar increase in concentration during contraction (Davies et al., 1982, Reid et al., 1992). The ROS produced by skeletal muscle promote E-C coupling and appear to be obligatory for optimal contractile function, as ROS depletion depresses twitch responses, whilst adding ROS to a muscle preparation has the opposite effect (Reid et al., 1993). Nitric oxide signaling within muscle is likely to be influenced by ROS, as NO readily undergoes electron exchange reactions with ROS and competes for the same redox-sensitive molecular targets (Reid, 1998, Reid et al., 1998). Further, studies have shown that NO opposes the action of ROS by inhibiting E-C coupling, as the effectiveness of this event varies among skeletal muscles according to the activity of endogenous NOS such that muscles with the highest NOS activity have the least effective E-C coupling and vice versa (Reid et al., 1993, Kobzik et al., 1994).

**Peptidergic signalling - CGRP**

The action of peptides on contractile function must also be considered when investigating modulatory substances in skeletal muscle fibres. Calcitonin gene-related peptide is a well-conserved 37 amino acid neuropeptide that is broadly distributed in the peripheral and central nervous, with wide ranging biological actions influencing vasodilation (Brain et al., 1985), the immune system (Bulloch et al., 1998) and neurotransmission in central and peripheral synapses (Bishop, 1995, Changeux et al., 1992). CGRP was among the first neuropeptides shown to coexist with ACh in spinal cord and brainstem motor neurons (Rosenfeld et al., 1983, Gibson et al., 1984). It is
synthesised in the motor neurons and travels anterogradely down the axon to the NMJ, where it appears to colocalise with vesicular ACh transferase (VChT; Wang et al., 2013). The levels of CGRP mRNA appear to vary between different spinal motor nuclei in situ, suggesting that the levels of CGRP present in individual motor neurons are related to the type of muscle unit that is innervated by the respective motor neuron (Micevych and Kruger, 1992, Popper et al., 1992, Piehl et al., 1993). Thus it has been claimed that there is a relationship between the MHC subtype composition of different muscles and the CGRP mRNA expression in innervating motor neurons (Blanco and Sieck, 1992).

Similar to ATP and NO, the release of CGRP upon stimulation influences events at both the NMJ and muscle fibre (Csillik et al., 1992). Its indirect modulatory actions are mediated through the increased expression and desensitization of ACh receptors (Fontaine et al., 1986, New and Mudge, 1986), and decreased acetylcholinesterase levels (Boudreau-Lariviere and Jasmin, 1999). In contrast, CGRP has been shown to directly potentiate contractile activity through its postsynaptic receptor (Takami et al., 1985, Ohhashi and Jacobowitz, 1988, Popper and Micevych, 1989). The binding of CGRP to these G protein-coupled receptors stimulates adenylate cyclase activity, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP), which is a potent intracellular second messenger and stimulator of Na⁺-K⁺ ATPase (Takami et al., 1986, Uchida et al., 1990, Clausen et al., 1993, Wimalawansa, 1996). These pumps play an important role in the recovery of muscle excitability from muscle fatigue caused by elevated K⁺ levels (Andersen and Clausen, 1993, Macdonald et al., 2008). CGRP is also involved in maintaining E-C coupling through stimulating Ca²⁺ reuptake via Ca²⁺-ATPase (Vega et al., 2011).
1.2.5 Metabolism

In skeletal muscle ATP is a major form of energy, which is used in a variety of processes and reactions including cross-bridge movement and detachment, and operation of ATP-mediated pumps (e.g., Ca\(^{2+}\)-ATPase, Na\(^+\)/K\(^+\)-ATPase). Hydrolysis of ATP produces ADP and inorganic phosphate (Pi), which are involved in further intracellular signaling or neuromodulator roles. After ATP is hydrolysed, it is rapidly regenerated via either interaction of ADP and phosphocreatine (PCr), or from the two main metabolic processes within muscle, glycolysis and the tricarboxylic acid (TCA) cycle. Muscle cells store more PCr than ATP, and the efficiency of the PCr-ATP reaction, catalysed by creatine kinase, is such that the overall amount of ATP in muscle cells changes very little during the initial period of contraction. The ATP demand in working muscle is tightly coupled to mechanical output and depends on numerous variables, such as the type of contractile activity, the intensity and duration of contractions, and the fibre type composition of the muscle (Crow and Kushmerick, 1982, Bergstrom and Hultman, 1988, Newham et al., 1995, Hochachka and McClelland, 1997, Hogan et al., 1998, Stary and Hogan, 2000).

During glycolysis, glucose is broken down to pyruvate and releases ATP (Alberti, 1977). Under aerobic conditions, pyruvate undergoes oxidation by the TCA cycle to form large amounts of ATP; however under anaerobic conditions, pyruvate is converted to lactic acid, which in turn dissociates into H\(^+\) and lactate at physiological pH levels (Stackhouse et al., 2001). The increase of H\(^+\) within the muscle lowers the pH, which is known to hamper muscle function by decreasing Ca\(^{2+}\) release from the SR, decreasing the sensitivity of troponin C to Ca\(^{2+}\), and interfering with cross-bridge cycling (Rousseau and Pinkos, 1990, Allen et al., 1995, Chin and Allen, 1998). Muscles operate within a limited pH range, and this is a highly influential factor in many
processes. Changes in muscle fibre pH occur through a variety of mechanisms, such as the breakdown of PCr (absorbs protons; Amorena et al., 1990), hydrolysis of ATP (releases protons; Smith et al., 1993) and dissociation of lactic acid to H\(^+\) and lactate, with this latter mechanism cited as the main reason for intracellular acidosis (Robergs et al., 2005). The magnitude of intracellular acidosis reflects the interplay between the production of acid metabolites (e.g. CO\(_2\), lactate, H\(^+\)), pH buffering by the cell (e.g., via histidine proteins) and the activity of intracellular pH regulating mechanisms (e.g. proton pumps). A decline in muscle pH has multiple actions on cell function, and observations from whole muscle preparations represent a complex summation of these effects. In whole muscles, the activation pattern and the muscle fibre types are usually heterogenous, and studies suggest that the change in intracellular pH varies from cell to cell (Allen et al., 1995), with up to 0.8 pH units difference (Fitts, 1994). One important source of variation between fibre types is the relative contribution of glycolytic vs. oxidative phosphorylation enzymes. For example, studies involving *Xenopus* muscle have revealed that type II fibres have a smaller change in intracellular pH than type I in response to fatiguing stimulation (Westerblad and Lannergren, 1988). The buffering capacity of different fibres also varies, providing another source of pH variation (Roos and Boron, 1981). In living cells, many membrane processes, metabolic reactions and contractile processes are influenced by pH, thus it is important for cell function that pH is kept at an optimal level (Juel, 1996). Muscle fibres have many mechanisms that allow the internal levels of H\(^+\) and thus intracellular pH, to be kept in check, such as the Na\(^+\)/H\(^+\) exchange system, bicarbonate dependent system, lactate/proton co-transport system, protein buffering systems (Juel, 1995, Abe, 2000). Interestingly it appears that muscle fibre types differ in their main buffering mechanisms. For instance, Juel (1991) showed that slow-twitch muscles have approximately 50% higher lactate/proton transport.
capacity than fast-twitch, indicating a more efficient pH regulation in oxidative fibres.

In contrast, fast twitch muscles were shown to have a higher proton buffering capability
due to histidine-related compounds (e.g. carnosine; Davey, 1960, Juel, 1996).

Numerous studies have investigated the effect of pH on the contractile activity
of skeletal muscle, as the decrease in muscle pH is a generally accepted cause for
muscle fatigue (Westerblad et al., 2002, Juel et al., 2004), and intracellular acidosis has
been shown to affect different aspects of the contractile activity, such as reducing the
maximal Ca\(^{2+}\)-activated force and Ca\(^{2+}\) sensitivity of myofibrils, slowing the shortening
velocity and prolonging relaxation (Metzger and Moss, 1987, Cooke et al., 1988,
Westerblad and Allen, 1993). Muscle pH generally falls during repetitive
activity/stimulation with a simultaneous decline in muscle performance (Allen et al.,
1995). Intracellular pH tends to fluctuate around pH 7.0-7.2, however during moderate
to high intensity exercise this can fall to around pH 6.3-6.5 (Fitts, 1994, Juel, 1996).
Functional tests using skinned muscle preparations (at 12\(^\circ\)C) revealed that a drop in pH
from 7.0 to 6.5 reduces isometric force by approximately 35% (Chase and Kushmerick,
1988, Cooke et al., 1988). In contrast, studies performed between 25-37\(^\circ\)C did not find
such a dramatic effect of reduced pH on the maximum isometric tension generated,
suggesting that with decreasing temperature, the effect of pH on maximum tension

1.3 Electrical stimulation of skeletal muscle

At its most basic level, the contraction of a muscle fibre caused by a single AP is known
as a twitch, the smallest and shortest activation of a muscle fibre. The muscle twitch
lasts much longer than the nerve AP, but is too brief for whole muscle action, thus trains
of repetitive APs are required to evoke whole muscle movements (Figure 1.4a;
If the APs occur at a rate similar to the twitch duration, a repeated series of twitches is seen. If the AP rate is increased (e.g., 30 Hz), the twitches ‘summate’, i.e. they grow into each other and the peak force is increased (Figure 1.4b). This occurs when the rate of Ca\(^{2+}\) released from the SR into the cytosol exceeds the rate of Ca\(^{2+}\) removal, resulting in an increase in peak tension. The more Ca\(^{2+}\) there is in the cytosol, the more is bound to troponin, which moves tropomyosin thereby exposing more myosin-binding sites on actin. The more sites exposed, a greater number of cross-bridges can participate in the sliding process, thus developing greater tension. Henceforth, over a given range of frequencies, an increased AP rate produces increased force. Above a given rate however (>60 Hz), the twitches fuse and produce a tetanic contraction with no further increase in force, arising from complete Ca\(^{2+}\) saturation of troponin such that all myosin-binding sites are continually exposed (Figure 1.4c; Enoka and Stuart, 1992, Gregory et al., 2007).

Electrical stimulation of the nervous system and surrounding musculature is a useful tool to aid in understanding the integrative response of the nervous system and surrounding skeletal muscle. The components of electrical stimulation known to dictate contractile activity include voltage/amplitude, frequency and pulse duration, with the former aspects being the most studied in both animals and humans (Bridges et al., 1991, Lieber and Kelly, 1993). Much of its known effects stem from neurorehabilitation studies, where it is used to restore neurological function and facilitate contraction of damaged and denervated skeletal muscle (Alon et al., 1983, Petrofsky, 1991, Gorgey and Dudley, 2008).
Figure 1.4. Myogram schematic of muscle contraction response to increasing stimuli

A single action potential (lasting 1-2 ms) produces a single muscle twitch contraction (lasting 20-200 ms). (a) A single twitch occurs when Ca\(^{2+}\) is released from the SR, causing myofilaments to slide past each other, and the Ca\(^{2+}\) is then actively pumped back into the SR. (b) If a stimulus is applied before the complete removal of Ca\(^{2+}\), the muscle contracts again but generating more force; (c) this continues until complete Ca\(^{2+}\) saturation of all troponin, which produces tetanus and no further increase in force production. The force produced from a muscle contraction is also correlated with the number of nerve fibres excited. Figure adapted and created from Henneman et al., 1974, Booth et al., 1997.

The various components of an electrical input subtly influence contractile activity by targeting different aspects of the contraction process. An increase in the stimulation voltage and/or amplitude results in recruitment of additional motor units and stronger contraction, ultimately resulting in greater force generation (Wong, 1986, Adams et al., 1993). Increased frequency results in an increase in force development via summation of twitches, and operates by influencing Ca\(^{2+}\) kinetics such that at lower frequencies less
Ca$^{2+}$ is released from the SR, resulting in fewer cross-bridge attachments and thus less force (Fitts, 1994). This is effective up to approximately 60 Hz, at which point tetanic contraction occurs, with no further increase in force (Lieber and Kelly, 1993, Scott et al., 2006). This plateau is due to Ca$^{2+}$ uptake being the rate-limiting step while muscle cross-bridge cycling is occurring at a maximal rate, resulting in the maximum force per muscle fibre in the motor unit (Endoh, 2004). Accordingly, energy expenditure is higher during high frequency stimulation, generally resulting in greater muscle fatigue than low frequency stimulation (Gregory and Bickel, 2005). However, sub-tetanic increases in stimulation frequency increase force output by maximising the amount of force each individual motor unit can produce without affecting recruitment (Binder-Macleod et al., 1995).

Force output by muscle is also affected by altering pulse duration. Although the relationship between muscle output and pulse duration is an accepted phenomenon, the mechanisms that dictate this response are understudied (Gregory and Bickel, 2005). It has been suggested that longer pulse durations increase recruitment by more easily over-coming resistance to current flow, thereby facilitating motor unit recruitment and resulting in greater force output. Similar to the effect of frequency, increasing pulse duration has limitations with the most effective responses produced at ≤ 5 ms (Hultman et al., 1983). Several studies have shown that the threshold difference between different nerve fibres is dependent upon pulse duration (Gorman and Mortimer, 1983), such that shorter pulse duration provides greater selectivity between fascicles, allows activation of more fibres in a localised area, and reduces the spread of stimulation to adjacent fascicles (Grill and Mortimer, 1996). Accordingly, studies have shown that decreasing pulse duration decreases not only the force, but also the amount of muscle (i.e., number
of fibres) activated, whereas decreased frequency reduces force output but not the area activated (Gorgey et al., 2006).

**Part B. In transition: not quite muscle, not yet meat**

From the previous literature, it is evident that for optimal muscle functioning, the control and regulation of all intrinsic and extrinsic processes is highly organised, and these processes are best operated within a specific range of physiological conditions (relating to pH, temperature, oxygen concentration and energy supply; Forest et al., 1975). Yet skeletal muscle is one of the body’s most adaptable tissues, in terms of its flexibility to operate in a wide range of environments that may be actually quite detrimental to its functioning, such as those produced by exercise, ageing, trauma, hibernation, and even death (Alberti, 1977, Boutilier, 2001). When the homeostatic balance of skeletal muscle is disrupted by a reduction in blood flow (ischaemia) or oxygen supply (hypoxia/anoxia), one of the first responses is alteration of the metabolic energy supply to ensure continual ATP production (Connett et al., 1990). This metabolic shift from aerobic to anaerobic glycolysis involves pyruvate being converted to lactic acid, which produces only 2 units of ATP, compared to aerobic glycolysis, in which pyruvate is used in the TCA cycle and approximately 30 ATP units are produced (Alberti, 1977). The accumulation of lactic acid results in a lowering of muscle pH, due to dissociation of lactic acid into H⁺ ions and lactate, and consequently the net result is a reduction in ATP generation and an increased acidic environment, both of which interfere with the normal functioning of muscle contraction in several ways (Stackhouse et al., 2001).
1.4 Oxygen going....

Hypoxia interferes with muscle function through altered muscle energetics, impaired neuromuscular transmission and/or changes in the E-C coupling process, all of which result in reduced muscle strength (Dousset et al., 2001). However existing data on the specific hypoxic effects on muscle force generation are often contradictory, with some authors reporting no change in contractile force generation due to hypoxia (Caquelard et al., 2000, Degens et al., 2006, Howlett and Hogan, 2007), whereas others report a significant decrease (Badier et al., 1994, Dousset et al., 2001). In addition, it remains unclear as to which aspect of the contractile process is most affected, the muscle or NMJ, as some studies report the peripheral nerve is more susceptible to ischaemia than skeletal muscle, whilst others have shown that muscle is more vulnerable to ischaemic injury (Korthals et al., 1985, Chervu et al., 1989). Regardless of which is most affected, a reduction in oxygen slows down the propagation of APs as a result of dysfunctional ion channels and pumps (Enoka and Stuart, 1992). Maintenance of the intracellular environment requires the redistribution of Na\(^+\) and K\(^+\) by ATP-dependent pumping systems, such as Na\(^+\)/K\(^+\)- and Ca\(^{2+}\)-ATPases, and a decline in ATP levels leads to a failure of these ATPases. Thus the passive Na\(^+\) and K\(^+\) fluxes overwhelm the capacity of the Na\(^+\)/K\(^+\) pumps and channels, resulting in a net intracellular Na\(^+\) gain and net K\(^+\) loss, and an ensuing loss of membrane excitability (Green et al., 1999, Clausen, 2003). This membrane depolarisation coincides with an uncontrolled release of Ca\(^{2+}\) (Fredsted et al., 2005, Fredsted et al., 2007). The rise in free cytosolic intracellular Ca\(^{2+}\) concentration results in the activation of Ca\(^{2+}\)- dependent phospholipases and proteases that further hasten the rate of membrane depolarization. Left unchecked, this condition leads to uncontrolled cellular swelling and cell necrosis (Hochachka, 1986, Boutilier, 2001). Downstream of the NMJ and sarcolemma, changes that occur within the muscle milieu
during acute hypoxia (e.g., pH, ADP, P\textsubscript{i}, lactate) alter muscle function by having a
direct effect on the contractile apparatus, including a decline in Ca\textsuperscript{2+} sensitivity of the
contractile proteins (Brotto et al., 2000). Observations from exercise studies have shown
prolonged muscle activity (which induces an hypoxic environment) results in a loss of
force, which is most prevalent at lower stimulation frequencies (10-20 Hz vs. 30-100
Hz; (Shee and Cameron, 1990, Sandiford et al., 2004). The lack of hypoxic effect on
muscle force at higher frequencies suggests membrane excitability may not have been
greatly perturbed (Jones, 1996). The reductions in force at low frequencies of
stimulation after exercise are consistent with impaired Ca\textsuperscript{2+} cycling by the SR, with both
release and reuptake disturbed due to acidification (Westerblad et al., 1991).

The overall metabolic and contractile response of muscle to oxygen-limiting
conditions and decreased pH levels is dependent upon the muscle fibre type
composition, with several studies reporting that hypoxic slow-twitch muscle has a lower
intracellular pH level and ATP concentration than fast-twitch glycolytic fibres
(Carvalho et al., 1997, Ginneken et al., 1999).

The alterations associated with neuromuscular transmission at the NMJ and the
E-C coupling processes under conditions of oxygen deficit are further complicated by
the actions of various neuromodulators, such as NO (Barclay et al., 1990, Gautier and
Murariu, 1999, Andrade et al., 2001). For example, the overall effects of NO on E-C
coupling and force production are dependent upon the level of tissue oxygenation, as
shown by Eu et al., (2003), who used an isolated muscle preparation of mouse extensor
digitorum longus (EDL) muscle to reveal that NO influence is facilitatory under low
oxygen tension, yet inhibitory when oxygen levels are high. Ultimately however, it is
the ATP status of the cell that determines the outcome, for if blood and oxygen flow is
restored to the tissue before ATP runs out, the muscle can resume most-to-all normal
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During this period the muscle is still responsive to external influences, thus remaining malleable. However, once ATP levels are exhausted, as occurs after death, irreversible changes take place in the muscle cells that result in muscle becoming meat.

1.5 Oxygen gone: Rigor mortis

After the cessation of blood and oxygen flow following the death of an animal, muscles continue to function for a period of time, using up the remaining glycogen stores to maintain cell viability and contractile activity (Forest et al., 1975). During this early post-mortem period, skeletal muscle remains metabolically active and although it varies between species, this activity may be in excess of 15 hours (Bendall, 1978). Importantly, the oxygen levels aren’t completely depleted immediately after exsanguination; rather, mitochondria are still functional up to 96 hrs after death, thereby providing both aerobic and anaerobic means of ATP production (England et al., 2013).

With depletion of glycogen and a concomitant increase in lactic acid levels, the pH of muscle decreases to a level that inhibits the enzymes involved in glycolysis, and ATP production ceases. When ATP falls below a critical level of 1µM/g, there is insufficient ATP to dissociate the actin and myosin filaments from the actomyosin cross-bridge formation, thus preventing the muscle from relaxing (Asghar and Henrickson, 1982). Rigor is the term applied to individual muscle fibres becoming depleted of ATP, which occurs progressively as a succession of individual fibres run out of ATP and each produces a rigor contracture (Jeacocke, 1984). Rigor mortis refers to the muscle stiffness that occurs after all the muscle fibres enter rigor (Hwang et al., 2003). The amount of glycogen stored in the muscle before death dictates the amount of muscle contraction post-mortem and also the rate at which muscle pH drops; consequently, rigor does not occur simultaneously across all fibres in a muscle, even
amongst those of identical fibre type, resulting in a heterogeneous pH decline with the muscle (Hintz et al., 1982, Jeacocke, 1984).

The post-rigor contraction of muscle is degraded by Ca$^{2+}$-dependent proteases and phospholipases, which are activated by the increasing concentration of cytosolic Ca$^{2+}$ stemming from uncontrolled Ca$^{2+}$ release by the SR. Activated proteases, such as calpains, degrade myofibrillar and cytoskeletal proteins, whilst phospholipases induce damage to the sarcolemma (Duncan and Jackson, 1987, Goll et al., 1992, Koohmaraie, 1992, Belcastro, 1993). Increased cytosolic Ca$^{2+}$ may also augment the production of ROS, leading to peroxidation of the membrane lipids and thereby contributing to damage of the cell membrane (Reid and Li, 2001). The ensuing increase in membrane permeability allows passive influx of extracellular Ca$^{2+}$ down its electrochemical gradient and intracellular enzymes (e.g. lactic acid dehydrogenase, creatine kinase) to leak out, resulting in further cell damage and ultimately cell death (Fredsted et al., 2007).

**Part C. In death: influencing post-mortem muscle function**

Within the meat industry there is a need to produce consistent, high quality meat products, as variability in eating quality is the main source of consumer complaint. In particular, reducing the variability in the tenderness of the end product has been the subject of much research (Tarrant, 1998, Pethick et al., 2007, Warner et al., 2010, Pethick et al., 2011). Previously, there was little industrial regulation by producers and processors in terms of the eating quality of sheep meat products, leading to unacceptable variation. As a result, an overhauling review of industry processes for the Australian sheep meat market saw the implementation of the Sheep Meat Eating Quality
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(SMEQ) research program by Meat and Livestock Australia, an overall approach that sought to define the best practice procedures for every part of the industry through identification of critical control points for eating quality (Thompson, 2002, Young et al., 2005). These critical points were identified throughout the supply chain, with the aim of guaranteeing the eating quality of the end product by decreasing the variability. This aim of delivering a consistent quality of sheep product into the market place has resulted in substantial changes to the sheep production and processing chain, with producers required to optimise the way in which they breed, handle and market their animals to slaughter, and processors needing to adopt new technologies and strategies to ensure that every carcass achieves maximum eating quality (Young et al., 2005, Anon, 2006). This latter point concerning the processor is particularly important, as the commercial environment in which the meat industry operates has undergone significant changes in the past 20 years. Not only is reduced variability in eating quality and increased tenderness required, but the meat industry must also provide a wider range of product choice and branding, and satisfy higher expectations of product quality for both local and international markets.

Since muscle is intrinsically a highly organised and complex structure, it is likely that the determinants of optimum meat quality are also going to be multifactorial and complex, encompassing the molecular to the mechanical. Of all critical factors, the management of the animal immediately before slaughter and during the first 24 hours post-slaughter is by far the most influential in the context of meat quality/tenderness (Ferguson et al., 2001, Simmons et al., 2006). Studies have shown that whilst factors such as nutrition and selective breeding may be used as in vivo strategies to optimise
meat quality, all efforts may be in vain if, during the conversion of muscle to meat post-mortem, processing factors are suboptimal (Butchers et al., 1998, Maltin et al., 2003).

1.6 Important pre-slaughter factors

The metabolic and physiological status of the animal just before exsanguination heavily influences downstream post-mortem activities. As such, pre-slaughter management is largely aimed at reducing the amount of glycogen lost in muscles prior to slaughter, as this strongly influences post-slaughter events. The pre-slaughter glycogen concentration determines the amount of lactic acid produced and thus the amount the pH can decrease by. The amount of post-mortem contractile activity is also influenced by this, as pre-slaughter glycogen concentration defines the amount of anaerobic metabolism and subsequent muscle work that can occur. The genetic breed and nutritional state of the animal influences this glycogen status, as certain breeds are more susceptible to stress experienced between the farm gate and abattoir including transport and lairage issues (Gardner et al., 1999, Jacob et al., 2005). Minimisation of glycogen loss is also aimed at reducing the incidence of dark cutting, whereby meat is characterised by darker colour, higher water-holding capacity and potentially increased toughness (Ferguson et al., 2001).

1.7 Important post-mortem factors that influence effect of electrical stimulation

Many factors must be taken into account when trying to optimise the post-slaughter processes, with much research focused on how post-mortem processing is influenced by transport/lairage period, animal stress, carcass suspension, electrical input and chilling method (Ferguson et al., 2001, Jacob et al., 2005, Thompson et al., 2005, Warner et al.,
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However, refinement of these processes relies on a good understanding of muscle physiology and the alterations that take place when muscle is converted to meat, and indeed the importance of this transition period has been noted by several recent reviews (Maltin et al., 2003, Ouali et al., 2006, Park et al., 2010, England et al., 2013, Adeyemi and Sazili, 2014, Ferguson and Gerrard, 2014).

1.7.1 pH/temperature interaction

A number of metabolic and structural changes occur when muscle is converted to meat. Anaerobic glycolysis generates lactic acid, which produces a gradual decline from approximately pH 7 in living muscle to a pH 5.6-5.7 within 6-8 hours post mortem, and then to an ultimate pH (pHu) of about 5.3-5.7, which is reached approximately 24 hours post-mortem (Figure 1.5; Forest et al., 1975, Maltin et al., 2003). Under normal conditions, this decline occurs over a period of hours. If, however, carcass temperatures fall below 10-15°C in the early post mortem period, when lactate accumulation has not yet lead to major reduction in pH levels (i.e., pH>6) and there is sufficient residual ATP, the muscle will contract severely leading to tougher meat (commercially referred to as cold shortening; Locker and Hagyard, 1963, Marsh and Leet, 1966, Tornberg, 1996). In contrast, the opposite occurs if the pH drops too rapidly (heat shortening), whereby acidic conditions are created before the natural body heat, and heat of the continuing metabolism has dissipated (muscle pH <6 whilst the carcass temperature >35°C), creating detrimental conditions for the proteolytic enzymes involved in the ageing of meat (Koohmaraie et al., 1986, Devine et al., 1999). These conditions are dependent upon the degree of pH decline and the rate at which it occurs, as the rate at which lactic acid accumulates can have serious implications for numerous meat quality characteristics including tenderness, colour, drip-loss and shelf-life. The SMEQ
program has identified a pH/temperature window as a critical control point for achieving maximum eating quality, whereby muscle reaches pH 6 when carcass temperatures are 18-25°C (Figure 1.5; Thompson, 2002, Thompson et al., 2005).

**Figure 1.5. pH/temperature window showing heat/cold shortening scenarios**

The pH/temperature window, as recommended by Meat Standards Australia to optimise decline in pH relative to the temperature of the muscle. The solid line represents an optimal rate of decline, whilst the dashed and dotted lines represent cold- and heat-shortening scenarios, respectively. Figure adapted from Thompson, 2002.
1.7.2 Fibre type

As highlighted in the previous sections, skeletal muscles are characterised by a complex and heterogeneous composition, which makes it difficult to unequivocally establish specific pathways involved in post-mortem biochemical changes, and ultimately affecting meat quality. It is, however, generally accepted that fibre type composition is related to the final meat quality and its associated variability, in conjunction with other muscle components such as intramuscular fat or connective tissue content. Fibre type affects tenderness and ageing rate, juiciness and flavour intensity, colour stability and post-mortem proteolysis (Dransfield and Jones, 1981, Ouali and Valin, 1981, Valin et al., 1982, Renerre and Labas, 1987, O'Halloran et al., 1997).

The extent of the pH fall within muscles is dependent upon the capacity of all the muscle fibres to produce lactic acid, which in turn is dependent upon its fibre type composition (Monin and Sellier, 1985, Valin, 1988, Maltin et al., 2003). It has thus been suggested that muscle with a high proportion of anaerobic fibres, and hence greater ability to produce lactate, has a higher probability of being poor eating quality due to the more rapid accumulation of lactate, decline in pH and a lower pHu (Talmant and Monin, 1986). In support of this, a slower rate of decline may be associated with a more oxidative fibre type composition within the muscles (Klont et al., 1998). In response to growth and an increase in body weight, muscle metabolism in heavier animals may become more oxidative, where greater fatigue-resistant fibre types may be required and thus predominate in muscles (Zerouala and Stickland, 1991). Furthermore, in contrast to glycolytic fibre types, oxidative fibres are more fatigue resistant and have a lower glycolytic capacity that may diminish their ability to metabolise anaerobically.
in the post-mortem environments, resulting in slower rates of pH decline (Farouk and Lovatt, 2000).

1.7.3 Electrical stimulation

The processing sector of the meat industry controls the vital step of converting muscle tissue into meat. The period between slaughter and the onset of rigor, a time when nerve and muscle are still conducive to external influences, offers a unique opportunity to influence the commercial value of meat, both beneficially and adversely. These opportunities to manipulate post-mortem events include the use of alternative carcass suspension, controlled chilling, and hot boneing (Wang et al., 1994, Pisula and Tyburcy, 1996, Ferguson et al., 2001). In particular, the use of controlled chilling has been widely studied as a way to manipulate post-mortem pH and avoid cold-shortening (Devine et al., 2002). However it has been the application of electrical stimulation that has had the most profound impact in terms of defining commercially important attributes of meat, and hence increasing financial return to the meat industry (Ferguson et al., 2001, Devine et al., 2004, Hopkins et al., 2008).

Electrical stimulation has long been a standard commercial processing technology, with applications including pre-slaughter stunning, immobilisation and manipulation of pH decline (i.e., decrease in carcass muscle pH). Its use in commercial processing is based on the application of an electrical current to the carcass, which causes muscles to contract, thus accelerating post-mortem glycolysis by producing a rapid drop in muscle pH (ΔpH) followed by an increase in the subsequent rate of pH decline (Figure 1.6; Chrystall and Devine, 1978, Asghar and Henrickson, 1982, Ferguson and Gerrard, 2014). This combined effect ensures muscles enter rigor mortis before muscle
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temperature falls to values conducive to cold shortening and toughening (Swatland, 1981). Upon completion of rigor, the ageing process commences at an elevated temperature, resulting in faster ageing and tenderisation (Dransfield et al., 1992).

Electrical stimulation has additional means of increasing tenderness through physical disruption of the myofibrillar matrix and accelerating the proteolysis of myofibril and cytoskeletal proteins (Savell et al., 1978, Uytterhaegen et al., 1992, Ho et al., 1996, Hwang et al., 2003). This later process results from enhanced activity of the calpain system, as electrical stimulation has been shown to hasten the increase in intracellular Ca\textsuperscript{2+} levels, thus initiating the activity of \( \mu \)-calpain protease sooner (Ferguson et al., 2000, Geesink et al., 2001b, Hwang and Thompson, 2001, Goll et al., 2003).

![Figure 1.6. The mean effect of electrical stimulation on post-mortem pH over time](image)

**Figure 1.6. The mean effect of electrical stimulation on post-mortem pH over time**

*Representation of post-mortem pH/time profiles resulting from electrically stimulated (solid line) and non-stimulated (dashed line) carcasses. The increase in pH decline during the stimulation period is followed by acceleration in the rate of pH decline after it Ferguson and Gerrard, 2014.*
Two main types of electrical systems have been previously employed to enhance meat quality, each operating via a different biological pathway. High voltage electrical stimulation (HVES) systems are based on the application of high voltage inputs to carcasses (> 1000V) and impart an effect by directly depolarising the muscle cell membrane, resulting in significant myofibrillar breakage (Morton and Newbold, 1982, Sorinmade et al., 1982). This type of system was first used commercially by New Zealand sheep and cattle producers as a way of ensuring tenderness for their overseas frozen market (Carse, 1973, Chrystall and Hagyard, 1976). The use of HVES application has been widely incorporated, with many studies reporting efficient tenderness outcomes for both sheep and beef meat (Elgasim et al., 1981, Devine et al., 2001, King et al., 2004). However, HVES poses significant risks with regards to operator safety, and high costs associated with safe installation and overall running costs. An alternative system is low voltage electrical stimulation (LVES; <100V), which has also been shown to produce similar beneficial effects on meat quality; however its use is limited to the early post-mortem period as it requires a functional nervous system in order to produce muscle contractions (Morton and Newbold, 1982, Taylor and Martoccia, 1995, Polidori et al., 1999). Regardless of the system, however, both are generally sub-optimal in meeting the expanding and changing needs of meat processors. This particular issue has been the focus of much research, resulting in the development of a more flexible and efficient medium voltage electrical stimulation (MVES) system, which is discussed in the next section.

In conjunction with the type of stimulation system used, the other factor most affecting the end product quality is the specific electrical parameters chosen to stimulate carcasses. Indeed, just as different components of an electrical input have specific effects on living muscle, as outlined in Part A of this review, they also individually
influence post-mortem muscle to the extent that the magnitude of ΔpH and the post-stimulation rate of pH decline is contingent upon the electrical parameters used. Over the years, many studies have examined different components of the electrical input, including voltage/current, frequency, pulse duration, waveform and total duration of stimulation (Carse, 1973, Chrystall and Devine, 1978, Bendall, 1980, Bouton et al., 1980, Rashid et al., 1983b, Butchers et al., 1998, Hwang et al., 1999); however, variation in the experimental paradigm has made it difficult to draw conclusive comparisons. This has contributed to an empirical definition of the protocols implemented commercially, with use of electrical stimulation based on experience rather than an integrated understanding of the applied electrical parameters and the induced response in post-mortem muscle. This lack of integrated knowledge is evident by the inconsistent results observed in electrically stimulated carcasses within the commercial setting, for whilst the benefits of electrical stimulation are well known, its use imposes risks of both under- or over-stimulation of carcasses, resulting in deterioration of meat quality (including issues such as colour defects, tough meat and increased drip-loss; Hwang et al., 2003). Therefore, although electrical inputs are used to reduce variation in meat quality, the substantial inter- and intra-carcass variation that exists is not always circumvented using current technology (Bendall et al., 1976, Chrystall et al., 1980, Devine et al., 1984a, Solomon, 1988, Hertzman et al., 1993, O'Halloran et al., 1997). This is exemplified by reports that muscles comprised of more oxidative fibre types are less responsive to electrical stimulation (Devine et al., 1984a) and it is therefore possible that heavier carcasses, which are more oxidative in their metabolism, may not experience as intense a response to electrical stimulation as smaller carcasses, which are more glycolytic in their metabolism.
Medium voltage electrical stimulation (MVES)

Ongoing research into the use of electrical stimulation within the sheep meat processing industry has continued to redefine its role in manipulating carcass qualities, with the aim of addressing the aforementioned problems (i.e. reducing variability and enhancing meat quality; (Pearce et al., 2006, Simmons et al., 2006, Hopkins et al., 2008, Simmons et al., 2008, Toohey et al., 2008, Pearce et al., 2009, Pearce et al., 2010). One of the major advances in the field has been the development and uptake of MVES units, which are designed to impart the same type of response observed with traditional high voltage stimulation, but without the associated danger and cost (Shaw et al., 2005; Devine et al., 2004). This is achieved by incorporating shorter pulse widths and medium-range voltages into the stimulation paradigm, which is delivered through segmented electrodes. These segments allow for the applied current to be controlled by a computer-regulated feedback system based on individual carcass resistance, thereby delivering precise amounts of stimulation to each individual carcass (Hopkins et al., 2008). As such, this constant current system produces a range of voltages that is dependent upon the carcass resistance (peak voltage of 300V and peak current of 5 amps.) This set up allows greater control over the targeted electrical application, and as such provides an opportunity to thoroughly examine each component of the overall electrical input, without the associated dangers or restrictive requirements of previous electrical systems. Indeed, optimisation studies from commercial plants that have installed these new units have shown that electrical parameters that were not technically feasible with previous electrical systems can induce a superior carcass response (i.e. large ΔpH and better tenderness outcomes, in combination with controlled chilling of approximately 2°C; Pearce et al., 2006, Pearce et al., 2009). In particular, increased pulse widths and modulated frequencies (across the electrode segments) were observed to generate the
A physiological understanding of post-mortem muscle stimulation

best responses (Pearce et al., 2009). Further to this, there is potential to tailor the MVES parameters in individual commercial plants to both the specific techniques used by the plant (e.g. hot boneing) and to market specifications (e.g. slow pH decline for domestic vs. fast pH decline required for frozen overseas market; Pearce et al., 2006, Pearce et al., 2009, Pearce et al., 2010). Ultimately, however, the way in which this electrical application interacts with post-mortem muscle function and brings about an enhanced response in carcasses remains speculative.

1.8 Project rationale

Two major issues need to be addressed in the course of refining these electrical stimulation technologies. Firstly, development of a physiological understanding of the interaction between the electrical input and the induced response in post-mortem muscle; and secondly, integrating this biological information into automated computer-controlled functions to give the processor greater control over the end product quality (Hopkins et al., 2008). This also highlights questions as to what type of carcass indicator could be used to ascertain how responsive an individual carcass is likely to be to a specific electrical input, which in turn could then be used to modulate the applied current in order to achieve a pre-defined carcass response. Early studies by Chrystall and Devine highlighted the relationship between the biochemical response of stimulated post-mortem muscle and the mechanical tension generated (Chrystall and Devine, 1978, Chrystall et al., 1980, Devine and Chrystall, 1984). This idea was added to in more recent studies of Simmons et al. (2008), who revealed that indirect measurements of whole carcass contractile responses may potentially be used as an indicator of carcass responsive to electrical stimulation, and thus be used as an indirect measure of pH status. Indeed, they suggest that the response characteristics of post-mortem muscle may
be used to select appropriate parameters (e.g. specific frequency, waveform, voltage, pulse width) to selectively stimulate muscle and produce a specific response (Simmons et al., 2006). This is a particularly compelling idea that warrants further investigation, particularly for the new MVES systems. However, as the research reviewed here reveals, few studies have examined electrical stimulation beyond the whole carcass perspective. Yet this is an area where a better integration of muscle physiology concepts, as highlighted in Part A and B of this review, would undoubtedly benefit traditional meat research, particularly in the context of applying electrical currents and manipulating carcass response (Adeyemi and Sazili, 2014). Such knowledge would aid in creating opportunities to produce electrical parameters that are tailored for specific applications, subsequently generating more consistent carcass responses. In particular, there has been no direct examination of the contractile characteristics of muscle in response to new generation MVES, as a means of identifying and understanding the variation observed with its use in meat processing. It is hoped that by examining this area, this thesis will provide insight into the underlying physiological response of post-mortem muscle to varying electrical inputs.

### 1.8.1 Thesis Aim and Hypothesis

The aim of this thesis was to examine the effect of MVES on post-mortem muscle function. Using the induced contractile characteristics as a means of understanding how particular MVES parameters interact with post-mortem muscle, this thesis sought to examine the hypothesis that the commercially observed superior effect of MVES on meat properties results from an enhanced muscle response, one that optimally engages nerve- and muscle-mediated pathways in a physiologically relevant way. In order to investigate this, the first study examined the effect of different MVES parameters on
A physiological understanding of post-mortem muscle stimulation carcass pH in a commercial processing environment. To delineate the influence of individual electrical parameters on muscle contractile properties, commercially important muscles were then examined *in vitro* in the second study, using an experimental system (isolated nerve-muscle preparation) that generated comparable electrical stimulation settings to those used in the abattoir. In the final study, further physiological evaluation of electrical paradigms were performed, specifically examining the role of nerve-mediated electrical stimulation in the post-mortem environment, and the subsequent contribution of common neuromodulators to the overall contractile activity.
2.1 Introduction

The value of electrical stimulation in enhancing meat tenderness is well recognised, as the benefits of stimulation in reducing the variability in sheep meat eating quality brings significant economic advantage (Devine et al., 2004, Hopkins and Toohey, 2006). Electrical stimulation hastens the process of rigor mortis by producing an accelerated pH fall (ΔpH; difference between pre- and post-stimulation pH) in muscle immediately after stimulation, followed by an increase in the rate of pH decline. Within Australia, a new approach to stimulation has been developed, which is being increasingly implemented in sheep processing plants (Shaw et al., 2005, Pearce et al., 2006, Toohey et al., 2008, Pearce et al., 2010). Medium-voltage electrical stimulation (MVES) units have been designed to impart the same type of response observed with traditional high voltage stimulation, but without the associated danger and cost (refer section 1.7.3; Devine et al., 2004). These factors enable a MVES unit to be positioned anywhere along the slaughter chain and has increased the adoption rate of electrical stimulation in commercial processing (Toohey et al., 2008). This impetus is further driven by the need for sheep meat products to reach critical pH/temperature windows related to the market for which they are destined, with these windows dependent upon the Sheep Meat Eating Quality (SMEQ) guidelines for optimal eating quality (refer Figure 1.5; Thompson et al., 2005, Pearce et al., 2006, Pethick et al., 2006, Toohey et al., 2006). Optimisation of the stimulation parameters used to obtain these specific carcass outcomes requires in-house testing, as factors such as the location of the stimulation unit, speed of the processing chain, chilling regime etc, influence the stimulation outcomes (Pearce et al.,
A preliminary validation study into the effectiveness of the MVES post-dressing unit at a West Australian abattoir demonstrated that only 42% of the carcasses met the SMEQ pH-temperature guidelines (Pearce et al., 2006). To improve the percentage of carcasses reaching the required pH/temperature window, alternative approaches to stimulation need to be considered, including varying frequency and modulation of stimulation on/off times (Pearce et al., 2006). However, the mechanism by which these parameters impart such affects remains unknown. As a first step to address this question, a specific goal of this study was to examine the impact of these MVES electrical components on sheep muscle pH.

It has been recognised, however, that regardless of the existing type of electrical stimulation system used, inherent variation remains between carcasses and their response to stimulation; and this will continue to be problematic for processors if not addressed by optimisation of stimulation parameters (Hollung et al., 2007, Hopkins and Toohey, 2008). Yet without a means of distinguishing ‘highly responsive’ carcasses from ‘low responsive’ ones, processors are forced to apply the same stimulation to all carcasses, even though some could benefit from more electrical stimulation whilst others are over-stimulated. For instance, stimulation of carcasses with faster metabolic rates will result in a quicker drop in pH that may result in the carcass overshooting the optimal pH/temperature window (Thompson et al., 2006). Furthermore, given the need for processors to supply different markets, the existence of a biomarker that could be used to predict how individual or consignments of carcasses are likely to respond to specific stimulation parameters would be extremely beneficial, allowing processors to tailor the stimulation regime to suit both carcass and market optimals (Pearce et al., 2006). In an attempt to identify such a marker, previous studies have examined the pre-
stimulation pH of carcasses (Chrystall and Devine, 1978, Ferguson et al., 2008), whilst others have suggested the potential of muscle colour, electrical conductivity and even glycogen concentration as indicators of carcass responsiveness (Byrne et al., 2000, McGeehin et al., 2001, Lepetit et al., 2002, Ferguson et al., 2008). In addition to pre-stimulation pH, a number of studies suggest that the pH immediately post-stimulation is a good candidate for predicting subsequent carcass quality and meat tenderness attributes, as the muscle pH has both direct and indirect influences on several characteristics of meat, such as colour, water-holding capacity, tenderness, juiciness and flavour (Kauffman et al., 1993, Simmons et al., 2008). A component of this, the \( \Delta \)pH, is used to determine the effectiveness of electrical stimulation, as the extent of the pre- vs. post-stimulation pH difference is highly dependent upon the applied electrical parameters (Bendall et al., 1976, Chrystall and Devine, 1978). Given the importance of pH as an indicator of meat quality, and of the significant influence of stimulation on pH, precision is required both in the timing of when this characteristic is measured after stimulation, and in the methodology used to record it. For instance, the initial post-stimulation pH measurement is reported in the literature anywhere from <10min to >40min after the application of an electrical current, which creates great difficulty in trying to delineate what and how the electrical component in question affects muscle, as the later the pH is recorded, the more degradative proteolytic processes interfere with the end pH measurement (via changes in ion concentration from the degradative processes; Pearce et al., 2006, Ferguson et al., 2008, Jacob et al., 2008, Toohey et al., 2008). Another aim of this study, therefore, was to determine if pre-stimulation pH is a reliable indicator of carcass responsiveness to MVES and further, determine the impact of time post-stimulation on pH values.
In addition to the timing of pH measurement, variation exists between methods have been developed to record muscle pH of carcasses. Two of the most commonly used techniques are the iodoacetate homogenate method and the use of pH probes. The iodoacetate method involves the removal of a small muscle biopsy from the carcass and immediate homogenisation in iodoacetate (Bendall, 1973). This process stops glycolysis in the muscle (by inhibiting GAPDH), ensuring that the pH recorded accurately reflects the muscle pH at the time of biopsy. This method was routinely utilised until the development of pH probes, which can take a direct pH recording from the muscle (Carter et al., 1967). Both methods have been used extensively in meat science research, although their comparability remains questionable (Bager and Petersen, 1983, Dutson, 1983). We therefore sought to compare the pH measurements using the two different methods.

2.1.1 Objectives

The hypothesis of this study was that electrical stimulation using a MVES unit would produce a significant decrease in carcass muscle pH. As such, this study sought to determine the response of sheep carcasses to different electrical parameters of a MVES system, using the ΔpH of the *M. longissimus thoracis et lumborum* (LL) muscle as a means to define carcass response and determine which electrical input was most effective. As part of this study, we also determined if pre-stimulation pH remains an adequate marker of muscle responsiveness to MVES. Another objective was to examine pH differences when recorded at different time points after stimulation. Finally, we investigated the magnitude of difference in muscle pH measurements when using the pH probe as compared to the iodoacetate methodology.
2.2 Materials and Methods

2.2.1 Experimental Outline

Experiment 1
Experiment 1 examined the effect of different MVES treatments (varying frequency, pulse width and current) on the ΔpH and temperature of the LL muscle as measured using a glass pH probe combined with temperature logger. The biggest, positive ΔpH value was used to note the treatment with the greatest stimulation response, as defined by ΔpH = pH₁-pH₂, where pH₁ is the pre-stimulation pH and pH₂ is the post-stimulation pH. Positive values indicate a decrease in pH whilst negative values indicate an increase in tissue pH.

Experiment 2
Experiment 2 examined how much carcass pH differed when measured at different time points after stimulation. The ΔpH was determined by measuring pH before electrical stimulation [(1) pre-stimulation: <5 min before entering stimulation tunnel] and compared to pH measurements at two time points after stimulation [(2) post-stimulation: within approximately 10 min of stimulation, approximately 30 min post-slaughter; and (3) in the chiller: within 40-60 min of stimulation, approximately 60-80 min post-slaughter]. These measurements were recorded from the first sampling date only, using a pH probe coupled to a temperature logger, and tested a subset of electrical parameters.
Experiment 3

Experiment 3 compared carcass pH measurements recorded by two commonly used techniques, the glass spear-tipped portable pH probe, and the iodoacetate method. Both probe and biopsy recordings were taken from the same carcass pre-stimulation.
2.2.2 Animals

All animals used in this study were transported to and housed at the Western Australian Meat Marketing Co-operative Limited (WAMMCO Pty Ltd) abattoir, Katanning, Western Australia. The collection of this data was approved by Murdoch University Ethics Committee and management of WAMMCO abattoir. The sheep included in this study were of varying backgrounds (the majority were Merino, Poll Dorset, Merino x Poll Dorset), sourced from the abattoirs daily kill sheets.

2.2.3 Electrical stimulation

The WAMMCO Katanning abattoir uses a post-dressing system on the production chain (Realcold Milmech Pty Ltd., Queensland, Australia). The stimulation bar is comprised of 6 electrodes interspaced by electrical isolators, providing carcasses with a total contact time of 34 sec. The rubbing bar is positioned to contact the underside of the front limbs of carcasses hung by the calcaneal tendon (Achilles). All carcasses enter the stimulation tunnel approximately 15-25 min after slaughter as part of the processing chain, including those that were unstimulated (no electrical current was applied to these control carcasses).

Large consignments (greater than 200 sheep) were chosen and sheep were randomly assigned to an electrical stimulation treatment group. Due the logistics of alternating the stimulation treatment between groups on a moving processing chain, consignments of this size were required to ensure multiple batches of sheep from the same source could be examined (with each batch testing all of the allocated treatments). Seven sampling periods were undertaken over a ten-month period (October 2005 - July 2006). Table 2.1 outlines sample date, number of consignments, number of sheep used and treatments.
tested. Each sampling date tested between four to six consignments (with the exception of the first sampling date; see Table 2.1). Within each consignment seven treatments were tested (with the exception of consignments tested on sampling dates 1 and 7), using eight consecutive carcasses per treatment. Table 2.2 outlines the electrical parameters of each stimulation treatment used. It should be noted that as the MVES unit was only newly installed in the abattoir, a broad range of electrical parameters were initially tested in order to provide a baseline of muscle pH responses to MVES (only in sampling date 1, 17/10/2005). These parameters were based on the optimisation studies of Pearce and colleagues (2006). The preliminary data (not shown) indicated that treatments 1-6 (Table 2.2) had superior effects on pH decline and were thus chosen for all subsequent experiments.

Table 2.1. The number of consignments and sheep used

<table>
<thead>
<tr>
<th>Sampling date</th>
<th># Consignments</th>
<th># Sheep</th>
<th>Treatment no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 17/10/2005</td>
<td>7</td>
<td>299</td>
<td>0-6 (plus other optimizing parameters)</td>
</tr>
<tr>
<td>2. 17/01/2006</td>
<td>4</td>
<td>216</td>
<td>0-6</td>
</tr>
<tr>
<td>3. 25/01/2006</td>
<td>5</td>
<td>280</td>
<td>0-6</td>
</tr>
<tr>
<td>4. 13/03/2006</td>
<td>6</td>
<td>328</td>
<td>0-6</td>
</tr>
<tr>
<td>5. 22/03/2006</td>
<td>5</td>
<td>279</td>
<td>0-6</td>
</tr>
<tr>
<td>6. 30/03/2006</td>
<td>5</td>
<td>279</td>
<td>0-6</td>
</tr>
<tr>
<td>7. 20/07/2006</td>
<td>6</td>
<td>240</td>
<td>0, 2, 3, 6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>38</strong></td>
<td><strong>1921</strong></td>
<td></td>
</tr>
</tbody>
</table>

# consignments: number of batches of sheep tested, with each batch testing all of the outlined electrical treatments; # sheep measured: total number of sheep carcasses tested on that date; Treatment no: treatment protocols tested on that sampling date (see Table 2.2).
Table 2.2. Electrical parameters of the MVES treatments examined

<table>
<thead>
<tr>
<th>Tmt no</th>
<th>Stimulation Treatment</th>
<th>Frequency (Hz)</th>
<th>Pulse Width (ms)</th>
<th>Current (mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No stimulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>15</td>
<td>1</td>
<td>800</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>15</td>
<td>2.5</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>15</td>
<td>5</td>
<td>800</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>15</td>
<td>1</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>15</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>15/15/20/20/25/25</td>
<td>1</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>Tmt used during</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>preliminary testing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>1</td>
<td>750</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td>1</td>
<td>750</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>1</td>
<td>750</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>3</td>
<td>750</td>
</tr>
<tr>
<td>15/15/20/20/25/25</td>
<td></td>
<td>1</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>25/25/20/20/15/15</td>
<td></td>
<td>1</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>25/25/20/20/15/15</td>
<td></td>
<td>3</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>25/25/20/20/15/15</td>
<td></td>
<td>5</td>
<td>750</td>
<td></td>
</tr>
</tbody>
</table>

_Treatment 0 was the unstimulated control. Treatments 1-5 had a constant current, pulse width and frequency across the six electrode segments of the MVES unit, whereas Treatment 6 had varying frequencies (the frequency of each electrode is shown in order consecutively). Tmt no: treatment number. Treatments in the bottom half of the table were tested only in a preliminary trial performed during the first sampling date. These parameters, in combination with Tmt 1-6, were tested as the MVES system was only newly installed and required optimization (based on previous optimization study of Pearce et al., 2006)._
2.2.4 Measuring carcass pH and temperature

The pH and temperature measurements were conducted on the right portion of the LL muscle, at the caudal end over the lumbar-sacral junction. Initial measurements were recorded within 5 min prior to stimulation. Carcasses were tagged with identifying numbers whilst the pH and temperature measurements were recorded, and then passed through a ViaScan machine, which assessed the weight, fat score and total lean yield of the carcass, then washed before passing through the stimulation tunnel. The 10 min post-stimulation pH and temperature recordings were performed in the unchilled sorting area before carcasses were assigned to chillers. The additional pH measuring time point (40-60 min post-stimulation) for Experiment 2 was made when the carcasses were stored in a refrigerated room.

Probe method - pH and temperature recording

A pH-temperature data logger (WP-80, TPS Brisbane Australia) with a glass body, spear-tipped probe (Mettler Toledo, USA), coupled with a temperature probe was used to determine muscle pH and temperature. The pH meter was calibrated before use and at regular intervals using buffers of pH 4 and pH 7 at the ambient temperature of the slaughter floor. The depth of penetration was approximately 1/3rd into the muscle (adjusting for differences in muscle size between carcasses). We were able to record a stable pH reading from the moving carcasses (which were moving at line speed) within 15-20sec, and only kept measurements on those carcasses that produced a stable recording within this time frame.
Iodoacetate method - pH and temperature recording

An iodoacetate method adapted from Dransfield et al., (1983) was used only for Experiment 3. Muscle samples were taken from the LL at both pre- and 10 min post-stimulation time points. A small bore hole (approximately 0.3cm) was drilled into the tissue just above the incision made for the pH probe and approximately 0.2g of muscle was removed using a 12V purpose-built, portable muscle biopsy gun (designed by Dr Graham Gardner, Murdoch University, Western Australia). Muscle temperatures were recorded for each carcass using a standard temperature probe as described above. Muscle samples were snap frozen in liquid nitrogen immediately after collection and stored at -80°C until further testing in the laboratory. This process involved homogenizing approximately 0.1-0.2g of frozen muscle for 20-30 seconds in 1ml of iodoacetate buffer (5mM sodium iodoacetate and 150mM potassium chloride adjusted to pH 7 with potassium hydroxide at 4°C). Samples were allowed to equilibrate to approximately 25°C and then pH was measured using the aforementioned pH-temperature data logger (to ensure consistency between equipment used).

pH data adjustment

Initial analysis involved removal of pH outliers, defined by being three or more standard deviations above the mean. The rate of pH decline following stimulation varies on a cooling carcass, as the biochemical reactions that drive the fall in pH decline with declining temperatures. In order to compare post-stimulation measurements between treatments it was therefore necessary to correct for cooling effects and adjust the rates of pH decline to a common temperature (Bruce et al., 2001). Thus before the pH data was analysed, it was adjusted to an average carcass temperature of 25°C, using the formula adapted from (Bendall and Wismer-Pedersen, 1962) whereby:
\[ \text{pHadj} = \text{pHunadj} + (\text{Temp}_{\text{measured}} - \text{Avg temp}) \times 0.01 \]

\( \text{Temp}_{\text{measured}} \) is the temperature of the carcass when pH was recorded or muscle biopsy taken.

### 2.2.5 Statistical analysis

All data was analysed using the Statistical package for Social Sciences (SPSS, version 11; SPSS Inc., Chicago, Illinois, USA) and graphs constructed using GraphPad Prism (version 5.03, GraphPad Software, Inc.).

Experiment 1: Differences in the general characteristics of carcasses (pre-stimulation pH and temperature, and carcass weight) across sampling dates were determined by a one-way analysis of variance (ANOVA), with sampling date as the fixed variable. A Tukey HSD post hoc test was used to differentiate sampling date effects, with significance set at \( p<0.05 \). Post-stimulation information was also analysed using a one-way ANOVA, with treatment as the fixed variable, and sampling date and carcass weight as a covariate. Tukey HSD post hoc tests determined treatment effects on the post-stimulation pH, post-stimulation temperature, \( \Delta \text{pH} \) and the \( \Delta \text{temperature} \). Multiple regression analysis was used to examine the relationship between pre-stimulation pH and \( \Delta \text{pH} \) (after adjusting for treatment and date parameters).

Experiment 2: A mixed between-within subject ANOVA (combination of a three-way and repeated measures ANOVA) was performed to identify differences in pH and temp when measured at the 3 different time points along the process line described above [time points (1), (2), (3)].
Experiment 3: Differences between the probe vs. iodoacetate pH sampling methods were analysed by paired T-test (data from the same carcass). Covariates included sampling date, consignment number and hot carcass weight.
2.3 Results

2.3.1 Experiment 1: The effect MVES on carcass pH

This experiment examined the response of sheep carcasses to different MVES settings, using the ∆pH of the LL muscle as a means to define carcass response and determine which setting was most effective. Of particular focus was the influence of pulse width and stimulation frequency.

Carcass muscle characteristics

The characteristics of sheep carcasses sampled are shown in Table 2.3. There were significant differences in LL pH, temperature and weight between sampling dates (p<0.05). The lowest pH (pre-stimulation) values occurred in the March sampling dates (avg. pH 6.83 ± 0.01) and the highest occurred during January sampling dates (avg. pH 7.13 ± 0.01; p<0.05). Carcasses examined during the March sampling dates also averaged the highest carcass temperatures (avg. 38°C) and lowest carcass weights (avg. 21.6 ± 0.1 Kg). Daily forecast temperatures were taken from the Bureau of Meteorology recordings at the Katanning weather station.
### Table 2.3. Carcass and LL muscle characteristics and sampling day temperatures

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Pre-stimulation pH</th>
<th>Pre-stimulation temperature (°C)</th>
<th>Weight (kg)</th>
<th>Fat score</th>
<th>Min pH</th>
<th>Max pH</th>
<th>Ambient air temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 17/10/05</td>
<td>6.91 (0.01)A</td>
<td>29.9 (0.2)A</td>
<td>23.1 (0.2)A</td>
<td>3</td>
<td>6.28</td>
<td>7.52</td>
<td>10.7 16.7</td>
</tr>
<tr>
<td>2. 17/01/06</td>
<td>7.13 (0.01)B</td>
<td>34.9 (0.2)B</td>
<td>23.4 (0.2)A</td>
<td>3</td>
<td>6.47</td>
<td>7.56</td>
<td>16.0 30.3</td>
</tr>
<tr>
<td>3. 25/01/06</td>
<td>7.13 (0.01)B</td>
<td>34.8 (0.2)B</td>
<td>22.9 (0.2)A</td>
<td>3</td>
<td>6.22</td>
<td>7.52</td>
<td>19.0 25.7</td>
</tr>
<tr>
<td>4. 13/03/06</td>
<td>6.83 (0.02)C</td>
<td>37.4 (0.1)C</td>
<td>20.2 (0.1)B</td>
<td>2</td>
<td>6.27</td>
<td>7.53</td>
<td>8.4 29.0</td>
</tr>
<tr>
<td>5. 22/03/06</td>
<td>6.85 (0.02)C</td>
<td>38.1 (0.1)D</td>
<td>21.9 (0.1)C</td>
<td>3</td>
<td>6.27</td>
<td>7.50</td>
<td>16.6 24.3</td>
</tr>
<tr>
<td>6. 30/03/06</td>
<td>6.79 (0.02)C</td>
<td>37.8 (0.1)C</td>
<td>23.2 (0.2)A</td>
<td>3</td>
<td>6.22</td>
<td>7.45</td>
<td>7.3 25.3</td>
</tr>
<tr>
<td>7. 20/07/06</td>
<td>7.02 (0.01)D</td>
<td>34.7 (0.2)B</td>
<td>23.1 (0.2)A</td>
<td>3</td>
<td>6.36</td>
<td>7.52</td>
<td>0.5 18.7</td>
</tr>
<tr>
<td>Average</td>
<td>6.94</td>
<td>35.4</td>
<td>22.4</td>
<td>-</td>
<td>6.30</td>
<td>7.51</td>
<td>-</td>
</tr>
<tr>
<td>SE</td>
<td>0.001</td>
<td>0.1</td>
<td>0.06</td>
<td>-</td>
<td>0.09</td>
<td>0.03</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented as mean ± (SEM). Values within columns not followed by the same letter are significantly different (p<0.05).
**Effects of electrical stimulation on carcass muscle pH**

The effect of electrical stimulation treatments on sheep LL muscle pH and temperature characteristics is outlined in Table 2.4. A high ∆pH value defined the treatment with the greatest stimulation response. It should be noted that although there were significant differences in the carcass characteristics between sampling dates, all electrical treatments were tested in multiple consignments from each sampling date, thereby eliminating any sampling day bias, such that when analysed by treatment groups, the pre-stimulation pH, pre-stimulation temperature, and hot carcass weight values were not statistically different.

Values for post-stimulation pH, post-stimulation temperature, ∆pH and ∆temp under treatment 0 (unstimulated) differed significantly (p<0.05) to the corresponding values of all other treatments. Treatment 2 (15 Hz 2.5ms 800mA) produced the largest ∆pH (0.44 pH units), followed by treatment 3 (15 Hz 5ms 800mA; 0.41 pH units) and 6 (15/20/25 Hz 1ms 800mA; 0.40 pH units). The treatments that produced the greatest ∆pH also resulted in the lowest post-stimulation pH values (pH 6.53 for treatment 3 and 6.54 for both treatments 2 and 6). There was little difference between post-stimulation carcass temperatures of all treatments, only differing statistically between treatment 0 (unstimulated), which produced the lowest temperature (unstimulated; 36.7°C), and treatments 5 and 6, which produced the highest carcass temperatures (37.4°C). Interestingly, the treatment that produced the lowest pH (treatment 3) also produced the lowest temperature (37.0°C) of the stimulation groups.
### Table 2.4. The effect of electrical stimulation on LL muscle pH and temperature

<table>
<thead>
<tr>
<th>Tmt no</th>
<th>Frequency (Hz)</th>
<th>Pulse width (ms)</th>
<th>Current (mA)</th>
<th>n</th>
<th>Post-stimulation pH</th>
<th>Post-stimulation temperature (°C)</th>
<th>ΔpH</th>
<th>Δtemp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td>250</td>
<td>6.83 (0.02)</td>
<td>36.7 (0.14)</td>
<td>0.13 (0.02)</td>
<td>-0.57 (0.20)</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>800</td>
<td>200</td>
<td>6.58 (0.02)</td>
<td>37.2 (0.16)</td>
<td>0.38 (0.03)</td>
<td>-0.43 (0.22)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2.5</td>
<td>800</td>
<td>252</td>
<td>6.54 (0.01)</td>
<td>37.2 (0.16)</td>
<td>0.44 (0.03)</td>
<td>-0.97 (0.20)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>5</td>
<td>800</td>
<td>260</td>
<td>6.53 (0.01)</td>
<td>37.0 (0.14)</td>
<td>0.41 (0.02)</td>
<td>-0.59 (0.20)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>400</td>
<td>200</td>
<td>6.61 (0.02)</td>
<td>37.3 (0.16)</td>
<td>0.34 (0.03)</td>
<td>-0.63 (0.22)</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1000</td>
<td>200</td>
<td>6.58 (0.02)</td>
<td>37.4 (0.16)</td>
<td>0.35 (0.03)</td>
<td>-0.71 (0.22)</td>
</tr>
<tr>
<td>6</td>
<td>15/20/25</td>
<td>1</td>
<td>800</td>
<td>260</td>
<td>6.54 (0.01)</td>
<td>37.4 (0.14)</td>
<td>0.40 (0.02)</td>
<td>-1.15 (0.20)</td>
</tr>
<tr>
<td>Avg (stimulated carcasses only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.56</td>
<td>37.3</td>
<td>0.39</td>
<td>-0.77</td>
</tr>
</tbody>
</table>

**SEM**

| SEM | 0.01 | 0.1 | 0.1 | 0.08 |

^A Average of stimulated carcasses only. Values presented are the mean ± (SEM). Values within columns not followed by the same letter are significantly different (p<0.05). ΔpH: difference between pre-and post-stimulation pH; Δtemp: difference between pre- and post-stimulation temperature.
Low-weight vs. high-weight carcasses

After examining the distribution of data by carcass weight, an interesting trend was observed (based on the approximate mean of the carcass weights across all sampling periods). Data was therefore grouped by weight and reanalysed, with hot carcass weights less than 23Kg defined as ‘low-weight’ and those equal to or higher then 23Kg defined as ‘high-weight’. Figure 2.1 reveals that the high-weight carcasses differed only between those stimulated and unstimulated, whereas low-weight carcasses statistically differentiated between the stimulation treatments. Weight stratification analysis did not influence post-stimulation carcass temperatures, with both weight groups displaying differences between treatments.
There was a significant difference between unstimulated and stimulated carcasses for both weight groups (\( ^* \) p<0.05). The response of high weight carcasses however did not distinguish between ES parameters. Low weight carcasses did produce a varied response to ES, with the largest pulse width (5ms) and modulated frequency parameters producing the biggest drop in pH (p<0.05). Significant differences between parameters of low-weight carcasses are represented as columns containing different letters (one-way ANOVA with Tukey’s HSD post hoc test, p<0.05).

Figure 2.1. The effect of MVES on LL muscle pH, grouped by carcass weight
Relationship between pre-stimulation pH and ∆pH

To determine if pre-stimulation pH was a reliable indicator of carcass responsiveness to MVES, regression analysis was used to determine the relationship between various carcass characteristics and their ability to predict ∆pH. Variables examined in the model included pre-stimulation pH, pre-stimulation temperature, weight, stimulation treatment, fat score and daily temperature.

![Graph showing the relationship between pre-stimulation pH and ∆pH](image)

The strong positive correlation (combined $r^2 = 0.63$) reveals that pre-stimulation pH is correlated with ∆pH, after adjusting for variables (temperature, weight, stimulation treatment, fat score and daily temperature).
The model revealed a strong positive relationship between muscle pre-stimulation pH and $\Delta$H, with a combined $r^2$ of 0.63 (there was no significant difference between the slopes of each treatment, $p=0.425$; Figure 2.2). Muscles with a higher pH before stimulation were likely to produce the largest drop in pH regardless of treatment. These results suggest that after adjusting for outside influential factors, pre-stimulation pH suitably predicts the $\Delta$pH.

*Unexpected observation –increased pH after stimulation (i.e., negative $\Delta$pH values)*

An unexpected observation was made during the correlation of pre-stimulation pH with the $\Delta$pH of after stimulation. The results revealed that a proportion of carcasses actually increased in muscle pH after stimulation, as indicated by the negative $\Delta$pH values in Figure 2.2. In total, approximately 18% of all carcasses that were studied had a negative $\Delta$pH, i.e., the pH had increased in the immediate post-stimulation period. Figure 2.3 shows the breakdown of those carcasses that increased in pH, shown by sampling date and stimulation treatment group. Interestingly, carcasses with increased muscle pH were not limited to any specific treatment group, and included the non-stimulated carcass group. The majority of carcasses that increased in pH post-stimulation were sampled during the autumn sampling dates.
Figure 2.3. Number of carcasses with an increased post-stimulation pH (i.e., negative ΔpH in LL muscle)

Figure represents the percentage of carcasses from each sampling date with increased pH after electrical stimulation, inclusive of unstimulated control carcasses. Each sampling date is broken down into the percentage of carcasses from each treatment.

Table 2.5 shows the pre-stimulation characteristics of carcasses (from Figure 2.3) in which the pH increased during post-stimulation period (negative ΔpH) and those that decreased pH (positive ΔpH). Due to the fact that carcasses in which the post-stimulation pH increased occurred in all treatment groups and sampling periods, the pre-stimulation characteristics of these specific carcasses were examined. Apart from the
obvious statistical differences in the ∆pH, there were also significant differences between the pre-stimulation pH and carcass temperature.

Table 2.5. Comparison of pre-stimulation characteristics between carcasses with increased (negative ∆pH) and decreased (positive ∆pH) LL muscle pH

Carcasses that increased in pH had a lower pre-stimulation pH, higher pre-stimulation temperature and were of lower carcass weight.

<table>
<thead>
<tr>
<th>Average Characteristics</th>
<th>Negative ∆pH</th>
<th>Positive ∆pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Carcasses</td>
<td>298</td>
<td>1246</td>
</tr>
<tr>
<td>∆pH</td>
<td>-0.23 (0.01)(^A)</td>
<td>0.49 (0.01)(^B)</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>21.8 (0.14)(^A)</td>
<td>22.4 (0.07)(^B)</td>
</tr>
<tr>
<td>Pre pH</td>
<td>6.57 (0.01)(^A)</td>
<td>7.04 (0.01)(^B)</td>
</tr>
<tr>
<td>Pre temperature (°C)</td>
<td>37.6 (0.14)(^A)</td>
<td>36.1 (0.08)(^B)</td>
</tr>
</tbody>
</table>

No. Carcasses: number of carcasses; pre pH: pre-stimulation pH; pre temperature: pre-stimulation carcass temperature. Values presented are the mean ± (SEM). Values across columns not followed by the same letter are significantly different (p<0.05).

In order to determine if the observation of increased ∆pH was related to carcass weight, the characteristics examined in Table 2.5 were then grouped by weight (Table 2.6) and this revealed that the carcasses with a negative ∆pH were predominantly less than 23Kg (low weight), and interestingly, these carcasses increased in temperature by 0.81°C. This conflicts with the normal biochemistry of cooling muscle, which displays an inverse relationship between pH and temperature (Bendall and Wismer-Pedersen, 1962). Thus the results of the low weight carcasses contrasts to the heavier carcasses that had an expected corresponding decrease in temperature (0.33°C) to their increase in pH (Table 2.6). Carcasses that showed the expected positive ∆pH were more evenly split between the weight groups, and also displayed the more commonly seen trend of a decrease in pH whilst increasing in temperature.
Table 2.6. Characteristics of carcasses in which $\Delta p$H increased (negative $\Delta p$H) or decreased (positive $\Delta p$H) after stimulation, as stratified by weight

Carcasses with a negative $\Delta p$H were predominately less than 23Kg. They also displayed an unusual correlation of increased carcass temperature with the increased pH.

<table>
<thead>
<tr>
<th>Weight</th>
<th>n</th>
<th>$\Delta p$H</th>
<th>$\Delta temp$</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;23Kg</td>
<td>200</td>
<td>-0.24 (0.01)$^A$</td>
<td>0.81 (0.20)$^A$</td>
</tr>
<tr>
<td>&gt;23Kg</td>
<td>91</td>
<td>-0.22 (0.02)$^A$</td>
<td>-0.33 (0.31)$^B$</td>
</tr>
<tr>
<td>&lt;23Kg</td>
<td>719</td>
<td>0.47 (0.02)$^B$</td>
<td>-0.68 (0.12)$^C$</td>
</tr>
<tr>
<td>&gt;23Kg</td>
<td>511</td>
<td>0.52 (0.01)$^C$</td>
<td>-1.83 (0.13)$^D$</td>
</tr>
</tbody>
</table>

Negative $\Delta p$H: carcasses with increase post-stimulation pH; positive $\Delta p$H: carcasses with a decreased post-stimulation pH; n: number of carcasses; $\Delta p$H: the change in pH between pre- and post-stimulation periods; $\Delta temp$: the change in carcass temperature between pre- and post-stimulation periods; numbers followed by different letters within columns are significantly different ($p<0.05$).

2.3.2 Experiment 2: pH measurement at differing time points post-mortem

In order to determine the difference in measurements recorded immediately post-stimulation and those recorded after carcasses were sorted into the chillers (which is where the majority of pH/temperature measurements are recorded in other literature studies), this study compared the pH and temperature measurements of the same carcass prior to stimulation [(1) pre-stimulation] and after stimulation at 2 time points [(2) 10 min post-stimulation; and (3) in the chiller approximately 40-60 min after stimulation). Results are presented in Figure 2.4. As was expected, LL pH declined consecutively over the 3 time points, with both measurements recorded after stimulation significantly lower than the pre-stimulation pH ($p=0.001$). Furthermore, carcass pH at the chiller time point was significantly less than that measured immediately post-stimulation ($p<0.001$). The only difference between the MVES parameters was between unstimulated vs. stimulated carcasses for the last 2 time points. Interestingly, there was an increase in carcass temperature in the immediate post-mortem period (for all
treatments including unstimulated carcasses), which subsequently decreased by the chiller time point (p=0.008).

Figure 2.4. LL muscle pH and temperature recorded at different time points (pre-stimulation, post-stimulation, chiller)

LL muscle pH and temperature as measured before stimulation, and at 2 different time points post-stimulation (post-stimulation = ~10 min after stimulation; chiller = ~ 40 min after stimulation). The pH decreased consecutively across time points, with all treatment groups of both later pH measurements less than their pre-stimulation pH counterpart (* p<0.001), and the chiller pH of all groups significantly lower than post-stimulation pH counterparts (# p<0.001). There were no differences between treatments at each point, with the exception of unstimulated vs. stimulated at the later 2 time points (∞ p<0.00). Carcass temperature increased at the post-stimulation period, including in unstimulated carcasses, relative to pre-stimulation temperature (* p<0.001), followed by a significant decrease by the chiller period, relative to post-stimulation temperature (# p<0.001).
2.3.3 **Experiment 3: Different pH measuring techniques**

A comparison of pH probe vs. iodoacetate biopsy technique to determine muscle pH indicated a significant difference between the two techniques (Figure 2.5).

Measurements from the same carcass showed that after adjusting for sampling date, consignment group and carcass weight, the probe pH reading was lower than that determined by the use of biopsy (6.83 ± 0.19 vs. 6.91 ± 0.08, respectively; paired T-test, p=0.0001). Use of the biopsy also reduced variability between animals. Sampling date was a significant covariate for measurements (p=0.001).

![Figure 2.5. Comparison of pH measure by probe vs. iodoacetate-biopsy method](image)

*Figure represents the pre-stimulation pH as measured from the same carcass by glass spear-tipped pH probe vs. a muscle biopsy treated with the iodoacetate procedure. Lines represent the mean ± 95% C.I. Biopsy measurements recorded significantly higher pH. After adjusting for sampling date, consignment group and carcass weight, there was greater variation in pH recorded by the pH probe compared with the biopsy.*
2.4 Discussion

This study found a significant increase in LL response to MVES parameters that contained longer pulse widths or modulated frequency component, as determined by the biggest changes in ∆pH. Unexpectedly, observations revealed that a proportion of carcasses had an increase in pH after stimulation. We also determined that the pre-stimulation pH of carcasses remains a viable marker of carcass responsiveness to newer MVES parameters. Finally, this study showed that when and how carcass pH is measured can have a significant impact on the data.

2.4.1 Effect of MVES on muscle pH

The future of electrical stimulation in commercial processing is embedded in its potential ability to be tailored for specific carcass types and end markets, although the main driver for electrical inputs remains its ability to reduce the variability in sheep meat eating quality (Hopkins and Toohey, 2006). Whilst the beneficial effects are well known, too much or too little stimulation can have similar detrimental effects (Hildrum et al., 1999, Geesink et al., 2001a, Hwang et al., 2003). One way to monitor the effects of electrical stimulation is through measurement of the ∆pH, the change in muscle pH from before and after stimulation. In this regard the set-up of this study was advantageous, in that by contrasting the pre- and post-stimulation pH and temperature measurements within the same carcass, intrinsic animal-related factors or biological variation arising from inter-animal comparison were accounted for, such that any ∆pH differences shown were solely the result of stimulation (Devine et al., 2002).
The main hypothesis examined in this study was that electrical stimulation using MVES would produce a significant decrease in muscle pH. We have demonstrated that longer pulse widths, i.e., 2.5 to 5ms, produces the most significant ∆pH response, followed by an increasing modulated frequency protocol. This correlates with the results of previous studies (Pearce et al., 2006, Pearce et al., 2009), which also found that using MVES with 2.5ms pulse width resulted in a significantly greater proportion of carcasses reaching the appropriate pH/temperature window (pH 6 between 18-25°C). Importantly, this parameter also produced the most tender meat (Pearce et al., 2009).

Previous studies have shown that in order to produce ∆pH values within the range we have documented (i.e., 0.40-0.44 pH units), high voltage stimulation was required (Geesink et al., 2001a, King et al., 2004). Several studies have tried to determine what key electrical components are most influential on carcass response. Bendall (1976) originally reported that only the total number of pulses delivered was an important factor hastening rigor, however, Chrystall and Devine (1978) diverged from these findings by noting that ∆pH increased markedly with increasing frequency, reaching a maximum (0.7 pH units) at approximately 16 Hz, after which it decreased with increasing frequency above 20 Hz. Numerous studies have reviewed the effects of both high and low voltage electrical stimulation systems, however few have investigated medium voltage set ups (Taylor and Martoccia, 1995, Polidori et al., 1999, Devine et al., 2001, King et al., 2004). Rashid and colleagues stimulated lamb carcasses with medium voltage (350V) and found that in combination with 10 Hz, this voltage increased pH decline, compared to low voltage (Rashid et al., 1983a, Rashid et al., 1983b). In a more recent study, Ferguson and colleagues (Ferguson et al., 2008) used MVES (300V, 14 Hz), and reported smaller ∆pH values than that reported here. This difference may lie in the different pulse widths used. Pulse width has been little studied.
in the literature and was previously thought to be of minimal influence (Bendall, 1980), although it was later suggested that longer pulse widths may actually increase glycogen use in muscle (Devine et al., 2004).

The increased responsiveness of LL muscle to increased pulse width may stem from the ability of this component to induce contraction in a greater number of muscle fibres; because this parameter determines the period during which current is applied to the tissue, it therefore determines the number and type of muscle fibres recruited (Ribchester, 2001). This is a well described phenomenon in living tissue, whereby the increased number of stimulated muscle fibres results from a greater number of activated motor units, since the longer pulse duration allows the excitation threshold in peripheral nerves to be reached, possibly by more readily overcoming resistance to current flow (Grill and Mortimer, 1996, Gorgey et al., 2006). For instance, increasing pulse duration (to approximately 4.5 msec) in the quadriceps femoris muscle of healthy human subjects has been shown to increase the number of muscle fibres activated by 40%, possibly through recruiting more fast twitch motor units which are stimulated by longer pulse durations (Alon et al., 1983, Hultman et al., 1983, Gorgey et al., 2006). Although this scenario takes place in living tissue, where different motor units have different membrane excitation thresholds, it may be the case that regardless of the functional state of the nervous system, the differential sensitivities of membrane thresholds remain between the different fibre types in the post-mortem state; therefore longer pulse widths are able to activate most, if not all the fibre types within a muscle. This would likely generate a more homogenous pH decline in each muscle as a consequence of more even muscle fibre stimulation. This hypothesis is supported by a recent study (Simmons et al., 2008), which reported that carcasses at a late post-mortem stage are more likely to
be influenced by longer pulses due to the increase in threshold response of differing muscle fibre types.

2.4.2 Inherent carcass variation

One of the biggest challenges processors face is the inherent variation in carcass responsiveness to electrical stimulation that exists between animals (Klont et al., 1998). Whilst electrical stimulation provides a means to limit the variability in meat quality, the ‘one size fits all’ approach in its application can often result in the incorrect amount of stimulation applied to a significant number of carcasses, which has detrimental effects on the end product (Thompson et al., 2005). Variation in carcass response even to MVES was illustrated by our findings in that low weight carcasses (i.e., hot carcass weight less <23kg) were more sensitive to MVES, by showing distinct responses to the different stimulation parameters trialed. In contrast, higher weight carcasses (hot carcass weight ≥23Kg) responded uniformly to these different electrical inputs. Hot carcass weight is dictated by many pre-mortem production processes, with sheep genetics largely responsible for bone, fat and muscle composition (Mortimer et al., 2010). Variation in carcass response to electrical inputs is likely associated with selection for specific phenotypic traits in live animals, as these choices then alter carcass composition that then impacts on the efficiency of the electrical stimulation (Maltin et al., 2003, O’Neill et al., 2004, Hollung et al., 2007). For instance, selection for muscling traits increases the proportion of type IIx fibres, which are associated with higher sensitivity to stimulation and thus would require less treatment to achieve optimal pH (Greenwood et al., 2006, Thompson et al., 2006). Thus our data suggests that factors influencing hot carcass weight should be taken into consideration when optimizing electrical inputs.
2.4.3 Unexpected observation – increase in post-mortem muscle pH

Unexpectedly, a proportion of carcasses showed an increased post-stimulation pH, with no effect of any specific treatment group or sampling date (including the non-stimulated control carcasses). Carcasses in which the post-stimulation pH increased had a significantly lower pre-stimulation pH value. To the best of our knowledge, this phenomenon has not been previously described in lamb carcasses. A similar finding was observed by Hollung et al., (2007) in beef carcasses, however it was not commented upon by the authors. It may be that these carcasses with a low pre-stimulation pH may have an initial build-up of lactate, such that after electrical stimulation the inherent buffering activity removes sufficient protons from within the muscle, whilst also exerting detrimental effects on contractile function, thus limiting further pH decline regardless of stimulation (Bendall, 1973, England et al., 2013, Ferguson and Gerrard, 2014). An interesting simulation study also suggested that metabolic deterioration after death has a greater alkalinizing affect at low pH levels, resulting in slight post-mortem pH increases (Vetharaniam et al., 2010). It is possible this observation has not been noted previously due the timing of the pH measurements (which were collected directly after stimulation), however it may have potential implications for the final resting pH. Carcasses with a high ultimate pH (pH>5.8; Jacob et al., 2005) result in dark-cutting meat that leads to greening during ageing, and subsequently economic loss (Jacob et al., 2005, McPhail et al., 2014). This is a concern as the increase in post-stimulation pH was recorded in approximately one quarter of the total number of carcasses sampled. Further study examining the rate of pH decline and pHu of animals is required to clarify if this immediate rise in pH post-stimulation does indeed have an impact on final pH status of meat.
2.4.4 Indicator of muscle responsiveness to ES

Given the relatively narrow margin within which electrical stimulation exerts beneficial effects, it would be advantageous for processors to have biological indicators that distinguish between highly responsive carcasses, i.e., those that require little stimulation in order to fully maximize their potential ΔpH, compared to those carcasses that require an increased dose. Thus the optimisation of potential on-production line methods for determining sheep meat quality during the early post-mortem period is regarded as a crucial area of research (Simmons et al., 2006, Simmons et al., 2008). Being able to easily determine what inputs are required to create a consistent and predictable product from a single on-line production measurement has great commercial potential. An easily accessible sampling site is imperative with rapid measurements a requirement in order to keep pace with line speeds, whilst contamination and carcass damage must be avoided. Results from this study show that regardless of the electrical parameters used, there is a strong correlation between the muscle pH prior to stimulation and ΔpH, correlating with previous studies (Newbold and Small, 1985, Ferguson et al., 2008). Pre-stimulation pH may therefore be a readily determined indicator of ΔpH in the commercial situation using MVES. Predicting the magnitude of pH fall at an early stage during glycolysis would potentially facilitate the use of specific electrical parameters to fully optimise the pH fall based on individual or batched carcass requirements. Thus this finding, in addition to the influential effect of carcass weight, strongly suggests that processors can tailor specific electrical stimulation treatments for specific groups of carcasses, depending on their pre-stimulation pH and weight, both measurements of which can be collected on the process chain prior to any electrical stimulation point on the carcass processing. This type of measurement could be incorporated into the automated onboard-computer feedback system, which is currently employed by MVES.
systems. Indeed, recent advances in processing technologies are focusing on automating these types of measurements (Simmons et al., 2008).

### 2.4.5 Measuring muscle pH - influence of when and how

Carcass pH is widely used to monitor the progress of meat tenderisation, and its correlation with many tenderness attributes makes it a good indicator of meat quality (Korkeala et al., 1986). It is therefore essential that this measurement is accurate, and factors that influence its accuracy must be given due consideration, including the time point post-mortem when the pH is measured, the technique and equipment used in the measurement, and also the position on the carcass from which pH is measured.

In many studies investigating the effects of electrical stimulation, the time point at which the first pH value is recorded varies between 40-60 min after stimulation (Hopkins et al., 2006). As the aim of this study was to identify the effect of different electrical components on muscle pH, our measurements were recorded as close as possible to immediately before and after the stimulation treatment. The timing of this recording may be associated with our previously discussed observation of increased ΔpH (section 2.4.3), and the observation that post-stimulation pH values in this study were significantly different to those determined in a concomitant study examining ΔpH using similar electrical stimulation parameters (pH measured ≥30 min post-stimulation; Pearce et al., 2006). Our results showed a significant difference between pH measurements recorded immediately post-stimulation (≤10 min) and at a later time points (40-60 min after stimulation) when carcasses have been stored in the chillers. This delay in measuring pH makes it harder to assess the specific impact of different electrical components on the immediate post-stimulation muscle pH, as the effects of degradative processes (e.g. ionic changes due to proteolysis) interferes with inferences...
that are made. However, in the majority of commercial settings (and thus published studies) it is impractical, if not impossible to measure carcass pH immediately post-stimulation. Whilst this finding may not overly impact investigations into the subsequent overall rate of pH decline, it does impact the value of the immediate post-stimulation response, and as such, must be kept in mind when examining related aspects of the immediate functional and biochemical physiology of stimulated post-mortem muscle.

The methodology used to measure carcass pH has evolved rapidly over the last decade. The widespread use of pH probes has largely superceded the technique of homogenising muscle samples with iodoacetate as a means to measure pH. Results from this study highlight a small but significant difference in pH values recorded from the same carcass using these two methods. Further, this study found that the biopsy readings were quite consistent across the sampling period, as opposed to the fluctuation in probe recordings, suggesting that whilst quicker (thus allowing work on faster processing chains), probe determination of pH is more likely to be influenced by extraneous factors, which may include operator ability, chain speed, and pH meter technology. In contrast, the biopsy method while more time- and labour-intensive, and not able to provide immediate pH values, does appear to generate a more constant, and perhaps reliable, reading of muscle pH (Dransfield et al., 1983). It is important therefore to exercise caution if comparing data recorded via different methods.

2.5 Conclusions

Overall, we have showed that the use of longer pulse widths or modulated frequency in MVES parameters increased LL response, i.e., larger $\Delta pH$. Furthermore, the effect of electrical stimulation on pH was associated with hot carcass weight, with lighter
carcasses (<23Kg) more responsive to all stimulation parameters tested. Pre-stimulation pH was demonstrated as a good indicator of LL responsiveness to MVES, with a high pre-stimulation pH predictive of a larger drop in pH in response to stimulation. These carcass variables are accessible on the production floor and may be readily incorporated into automated systems, allowing selection of MVES parameters best suited to specific animal consignments and product end points. We also documented a significant difference between pH measurements recorded immediately after stimulation with those recorded at a later time point, with the latter more representative of that typically reported in the literature. This, in conjunction with our showing a significant difference between pH measuring techniques, highlights the requirement for consideration of both the timing and methodology when comparing pH responses to electrical stimulation.
Chapter 3.

The effect of MVES on post-mortem muscle contraction

3.1 Introduction

Electrical stimulation protocols chosen for commercial settings have to date been set by the processor, with little knowledge of the mechanisms by which particular electrical paradigms affect post-mortem muscle changes. This lack of physiologically based knowledge makes it hard to fully explain how and why different electrical parameters produce varying effects on the ΔpH, as seen in Chapter 2, and also how to know how much and of what type of electrical stimulation is required to produce specific meat outcomes (Adeyemi and Sazili, 2014). This variation may in part stem from the response of post-mortem muscle to particular components of the electrical input (i.e. frequency, pulse width, current, etc), which may selectively induce and/or influence different biological pathways within the tissue. Thus knowing specifically how each of these components act upon muscle to induce contraction (and thus subsequent pH drop) would aid in avoiding the deleterious effect of under- or over-stimulation that contributes to variability in meat quality outcome (Simmons et al., 2008, Adeyemi and Sazili, 2014).

Having established the impact that MVES parameters had on sheep muscle pH in Chapter 2, this study sought to examine how these parameters influence different properties of muscle contraction at a more fundamental level. There was particular interest in how longer pulse widths and modulated frequency influence muscle contraction, given they both produced the best pH response in muscle in the previous study. Interestingly, a concomitant study examining organoleptic properties of MVES
meat (Pearce et al., 2009) showed that whilst both of these parameters induced similar ΔpH and rates of pH decline, only the longer pulse width produced significantly tender meat, suggesting these different parameters may induce a similar outcome but via different muscle mechanisms, potentially via differing contractile activity.

This study made use of isolated nerve-muscle preparations in order to examine the contractile response, which is a commonly used technique employed in basic muscle physiology studies. This approach has been used by physiologists and biochemists to study muscle contraction in the context of both normal function and disease, as it allows close examination of many muscle properties in a controlled environment, however few studies have examined this function in the context of meat (Chrystall and Devine, 1978, Klont et al., 1994). Isolated nerve-muscle preparations confer a number of advantages for this research field, as the confounding influences of carcass fat, bodily fluids, skin/fleece, etc, can be removed, in addition to removing the effects of undefined current pathways inherent with whole carcass studies (Chrystall and Devine, 1978). Data collected from these preparations can include measurements such as the peak tension produced during the stimulation period, which is indicative of the number of active cross-bridges generated during contraction period (Harkema et al., 1997, Hopkins, 2006); or the area under the force-time curve (AUC), which is a measure of the amount of contractile activity generated by muscle (associated with ATPase functioning, membrane responsiveness, Ca\textsuperscript{2+} signaling, etc; Hodgkin and Horowicz, 1960, Crow and Kushmerick, 1982, Hogan et al., 1998). Other measurements, such as the time it takes for a muscle contraction to reach its peak force and subsequently relax (rise and fall times, respectively) can be also recorded, which offers different information on the behavior of post-mortem muscle contraction (e.g. intracellular Ca\textsuperscript{2+}
signaling and ion channel activity). This type of information can provide an important insight as to how MVES influences muscle activity as a means of enhancing meat quality in the post-mortem period.

The variation in muscle response to electrical stimulation is tied to the specific biochemical and contractile properties of individual muscle groups, with different muscles known to have differing responses to electrical stimulation (Simmons et al., 2008). Thus, we examined the effect of simulated MVES settings on two different muscles, *M. semitendinosus* (ST) and *M. semimembranous* (SM), which are commercially valuable meat cuts that are also representative muscles of different fibre types (glycolytic and oxidative-glycolytic, respectively; Gardner et al., 2007, Greenwood et al., 2007). Previous studies have shown that there is a fibre type-dependent response to electrical stimulation, with fast-twitch glycolytic ST muscle of sheep reported to respond more efficiently to stimulation than the fast-twitch oxidative-glycolytic SM muscle, possibly resulting from the proportion of type IIX fibres (Devine et al., 1984b, Monin and Ouali, 1991, Thompson et al., 2006, Adeyemi and Sazili, 2014). The variation in muscle response to electrical inputs is thought to result from the various MHC composition and metabolic characteristics displayed by different fibre types, in addition to the pre-mortem glycogen status (Klont et al., 1998, Thompson et al., 2005, Hollung et al., 2007). Given that these characteristics determine the contractile properties of muscles, i.e., the amount of tension produced, the overall amount of contraction generated, the speed of contraction and relaxation, response to electrical impulses, etc, it would seem that a better understanding of how varying electrical inputs alters these contractile properties of different fibre types would
facilitate, firstly, a basis for the empirical success of MVES protocols, and secondly, aid in the refinement and optimisation of stimulation protocols for different muscle types.

This study also aimed to examine one of the fundamental concepts that differs between low and high voltage electrical stimulation, i.e. the pathway by which the electric current travels within the muscle. The stimulation of sheep carcasses commonly occurs within 20-30 minutes post-slaughter when post-dressing systems are employed (Pearce et al., 2010), and at this point on the processing chain it is generally thought that nerve activity within the carcass is no longer present (Chrystall et al., 1980, Hwang et al., 1998). Previous studies have shown that low voltage stimulation requires a functional nervous system to elicit contraction, in contrast to high voltage application which exerts its effect by depolarising the muscle cell membrane directly (Chrystall et al., 1980, Morton and Newbold, 1982). Observations from previous MVES stimulation studies suggest that the nervous system in sheep carcasses may still be functional enough to respond to certain MVES electrical parameters, at the point in time when they enter the post-dressing stimulation tunnel (Pearce et al., 2006). The potential for modulation of the applied electrical stimulation to include nerve-mediated muscle activity has important implications for meat processing, as transmitting an electrical current via the intrinsic nervous system would ultimately induce a more homogenous pH decline throughout carcass muscles, which in turn would reduce variability in end meat products. Thus this study also sought to determine how each electrical parameter induces a contractile response, i.e. direct stimulation vs. indirect (nerve-mediated).
3.1.1 Objectives

The objective of this study was to determine the effect different components of electrical stimulation parameters have on various muscle contraction properties of post-mortem sheep muscle, with longer pulse widths and modulated frequencies hypothesized to elicit a greater contractile response. Two representative muscle groups [(ST) and (SM)] were examined to determine if there was a muscle-dependent response to the varying electrical stimulation. In order to examine this, an isolated nerve-muscle electrophysiology rig was established to measure muscle characteristics including peak tension, the amount of contractile activity, the speed of muscle contraction and relaxation. The electrical parameters studied were based on those previously determined as most effective in a commercial setting (Chapter 2). The contribution of direct stimulation to the different induced contractile properties was also examined by using a pharmacological agent to inhibit the nerve-mediated component of the electrical transmission.
3.2 Materials and Methods

3.2.1 Experimental Outline

Experiment 1

Initial experiments were conducted to identify the stimulation setting that would induce the largest and most consistent muscle response (i.e., contraction) over the experimental time frame. This then acted as an intra-muscle strip control and was used to normalise all subsequent muscle responses resulting from different stimulation treatments, thus ensuring that comparing results between muscle strips after treatment only examined the treatment effect, and not intra-muscle strip variability.

Experiment 2

Experiment 2 examined how individual components of the overall stimulation parameter impacted on different characteristics of the resulting contraction in post-mortem sheep ST and SM. The electrical stimulations examined here altered one component of a parameter deemed as a ‘default’ setting (15 Hz, 1ms, 60V), and included increasing pulse width (2.5 ms and 5 ms), lower and higher voltages (30V and 100V), or a staggered, increasing frequency (15/20/25 Hz), outlined in Table 3.1. Each muscle strip was tested with three of the electrical settings, in a randomised order so as to avoid fatiguing the muscle bundle.

Experiment 3

This experiment was carried out simultaneously with Experiment 2, with the nerve-blocking agent, d-Tubocurarine (curare), added to each muscle strip after the contraction was recorded. After a 20 min incubation period, the electrical stimulation
protocol was repeated, and the difference in response revealed how much of the total contractile response was due direct electrical stimulation of the muscle (i.e. pre- vs. post-curare muscle response).

### 3.2.2 Animals

All experiments were approved by Murdoch University Animal Ethics Committee and carried out in accordance with the National Health and Medical Research Council guidelines. Sheep (Merino x poll dorset, 2005 drop (~1 yr old), mixed sex; \(n=10\)) were purchased at local saleyards. Animals were slaughtered in accordance with procedures of commercial meat processing plants. Immediately after death, two 2cm\(^3\) blocks of tissue were cut from the dorsal, proximal regions of both the ST and SM muscles and placed in pre-chilled Krebs solution (see composition below).

### 3.2.3 Contractile Measurements

*Isolated nerve-muscle electrophysiology rig set up*

Longitudinal strips of ST and SM muscle (average length x width, 22 x 3mm; average weight, 0.094g ± 0.013) were dissected and mounted approximately 1 hr after slaughter in vertical organ baths (100ml capacity) containing oxygenated Krebs solution (mmol/L: NaCl 120.0, KCl 5.0, CaCl\(_2\) 2.5, Na\(_2\)HCO\(_3\) 25.0, MgSO\(_4\) 1.0, NaH\(_2\)PO\(_4\) 1.0, and glucose 22.0), aerated with 5% CO\(_2\) in 95% O\(_2\) (pH 7.4) at 34°C. As this was the first time this technique had been used in such a research context, the current study employed an oxygenated rig set up (i.e., post-mortem muscle stimulated in solution aerated with a predominantly oxygen-based gas mixture) to set up a physiological stable environment in which to examine the effect of this novel type of experimental
protocol, thereby establishing the conditions for the subsequent experimental protocols. This set up slows, but does not completely prevent, the degradative effects of post-mortem processes (e.g., proteolysis), thus allowing examination of contractile activity.

Muscle preload force was adjusted to the optimal fibre length ($P_o$) to produce the maximal twitch force by applying 1g tension to each strip and allowing it to equilibrate for 30min. Mechanical activity was recorded isometrically with a force transducer (FT.03, Grass Instruments, Quincy, MA, USA). Data was recorded on a Powerlab Chart Recorder and analysed using LabChart v6 Pro software (ADInstruments, Sydney, NSW, Australia). Muscle strips were stimulated transmurally with custom-made Ag-AgCl ring electrodes 5 mm apart connected to Grass stimulators (Grass Instruments, USA). Electrode polarity was optimised with 0.5 sec of 2 Hz, 20 V and 0.5 ms pulses. Following equilibration, and before the use of the test parameters, the muscle was stimulated with an electrical setting that was determined in Experiment 1 (80 Hz, 0.1 ms pulse width at 60 V for 0.5 ms; section 3.3.1) to provide a control response for each muscle strip, with subsequent measurements calculated relative to this optimised response (as a percentage). This allowed comparisons between different electrical stimulation protocols and between different muscles (MacIntosh and Willis, 2000). After stimulation, the length and weight of each muscle strip was recorded. The cross-sectional area was calculated by dividing the strip weight (grams) by the strip length (cm) times the specific muscle density (1.056) to determine tension as per cross-sectional area (Mendez and Keys, 1960)

**In vitro muscle measurements**

For each stimulation treatment tested, the peak tension (grams/cm$^2$), AUC (grams/ms), time from onset of contraction to peak tension (rise time; ms) and time from peak
tension to relax to baseline (fall time; ms) of the contraction were simultaneously recorded using the Peak Parameters extension package for Chart (LabChart v6 Pro; ADInstruments, Australia). Figure 3.1 outlines the properties of a muscle contraction curve that the software measured, which ensured consistency between results. These four properties highlight the fundamental mechanisms of muscle contraction function and are appropriate for exploratory muscle studies. The peak tension produced by each muscle strip is indicative of the number of actively shortening myofibrils, which is in turn is associated with the amount of motor nerve innervation to each muscle strip, and also the efficiency and strength of synaptic neurotransmission (i.e., pre- and postsynaptic functioning, vesicle dynamics, receptor activity; (Fitts et al., 1991). The amount of contraction is defined as the area under the curve (AUC; also known as force-velocity relationship) calculated from a predefined lower threshold to peak tension. The AUC produced by each muscle bundle is associated with downstream postsynaptic activities, particularly with reference to the efficiency of E-C coupling (including membrane excitability and channel activity in the sarcolemmal membrane and T-tubules), Ca$^{2+}$ cycling dynamics (release from and re-uptake to the SR), and also qualitatively suggests the amount and speed at which ATP is used (which is dependent upon the types of myosin ATPase present). It is often used as an approximation of the work done by muscle during contraction, as it has been shown that such areas are a better measure of contractility than peak tension production (Hodgkin and Horowicz, 1960, Crow and Kushmerick, 1982, Chasiotis et al., 1987). The time taken to reach a predefined upper threshold (rise time), and the time taken to relax to a predefined lower threshold (fall time) can be used in conjunction with the AUC to determine which aspect of the E-C coupling/cross-bridge process is affected, with the rise time indicating activity of Ca$^{2+}$ release processes involving DHPR and RYR channels (also associated
with the number of myofibrils activated and speed of myosin ATPase), whilst the fall time is associated with the cells ability to re-sequester released Ca\textsuperscript{2+} back into SR, involving the Ca\textsuperscript{2+}-ATPase pump and potentially membrane repolarisation and Ca\textsuperscript{2+} buffering capacity of muscle fibre type (Close, 1972, Fitts et al., 1991, Bottinelli and Reggiani, 2000).

**Figure 3.1. Muscle properties measured by LabChart software**

Representative muscle contraction curve showing the different muscle properties that were measured. Peak tension (g) - the largest amount of tension produced from baseline; AUC (g.s) hatched area – area under the curve relates to the amount of contraction generated (measured between peak tension and lower thresholds); Rise time (ms) – the contraction time from the lower to upper threshold; Fall time (ms) – the time to relax from upper to lower threshold.
3.2.4 Electrical Stimulation parameters and outline

The electrical stimulation parameters used in this study were developed from those examined in Chapter 2 in order to produce comparable stimulation to the abattoir MVES paradigm. The electrical input used commercially is based on a constant current system which is comparable to the constant voltage system used by the in vitro rig, as there was negligible resistance produced by the muscle strip, thus making both amounts of energy relative to each other [as observed by Ohm’s law: Current (I) = Voltage (V)/Resistance (r)]. The voltages used on the isolated nerve-muscle preparations, 30V, 60V, and 100V, reflect the currents used in the abattoirs of 0.4 A, 0.8 A and 1/1.5 A, respectively. Table 3.1 outlines the electrical parameters tested, with each applied for 0.5sec. Treatment #1 (15 Hz 1 ms 60 V) was used as the ‘default’ treatment, as it was the default setting on the MVES systems in the commercial abattoir. Altering one component of the default parameter to produce the other five treatments was undertaken in order to reveal the contribution of that particular component to the overall muscle activity. The bolded element of each parameter in Table 3.1 represents the component that was altered from the baseline parameter.

Table 3.1. Summary of different electrical stimulation parameters used for in vitro experiments

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>Frequency (Hz)</th>
<th>Pulse Width (ms)</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>15</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>2.</td>
<td>15</td>
<td>2.5</td>
<td>60</td>
</tr>
<tr>
<td>3.</td>
<td>15</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>4.</td>
<td>15</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>5.</td>
<td>15</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>6.</td>
<td>15/20/25</td>
<td>1</td>
<td>60</td>
</tr>
</tbody>
</table>

Each muscle strip was stimulated with three of the above parameters, with the order and combination of electrical protocols randomized to ensure bias and muscle fatigue was minimized. Bolded represents the parameter that was altered from Tmt 1.
3.2.5 Pharmacological inhibition of nerve-mediated muscle contraction

For Experiment 3, the direct muscle stimulation component of the overall contraction was determined using curare [(+)-Tubocurarine chlorine hydrate, Sigma Aldrich, St Louis, MO, USA], a neuromuscular blocking agent that targets the ACh receptors on the postsynaptic membrane of the NMJ within muscle fibres (Willcockson et al., 2002). A final concentration of $10^{-5}$M of curare was added directly to the organ bath after the muscle was stimulated with three of the aforementioned electrical parameters. After incubation for 20min, the muscle was stimulated using the same parameters, and the direct stimulation component of each stimulation treatment was calculated as the post-curare response relative to the pre-curare contractile (pre-curare contractile response to stimulation is generated from both stimulation pathways, whilst the post-curare response is solely derived from direct stimulation; see Figure 3.2).

3.2.6 Statistical Analysis

Preliminary testing of a stimulus to ensure muscle viability over the experimental period used a one-way repeated measures ANOVA to identify if there was a significant decline in muscle response ($p<0.05$). Differences within muscle groups in response to the different electrical stimulation parameters were examined by one-way ANOVA with Tukey’s HSD post hoc test (significance set at $p<0.05$). Differences between muscle types in response to each parameter were identified with two-tailed independent $t$-tests (significance set at $p<0.05$). To examine the contribution of direct stimulation to the overall muscle contraction, a one-way ANOVA with Tukey’s post hoc test was used to identify differences between the stimuli (within a muscle; significance $p<0.05$);
differences between muscle types was determined by an independent t-test. All data was analysed and visualized using GraphPad Prism (version 5.03, GraphPad Software, Inc.).
3.3 Results

3.3.1 Experiment 1: Validation of isolated muscle-nerve preparation technique

In order to create an internal control that would allow the measurements of each muscle strip to be comparable, preliminary testing of different electrical stimulation settings revealed that muscle stimulated with 80 Hz, 0.1 ms pulse width at 60 V for 0.5 ms generated the largest, muscle response in both muscle groups. This optimal muscle response was shown to result entirely from nerve-mediated stimulation, as addition of the neuromuscular blocking agent, was observed to completely inhibit muscle response (as shown in Figure 3.2).

**Figure 3.2. Inhibition of nerve-mediated stimulation by curare**

Representative muscle tracing of SM muscle stimulated before and after the addition of neuromuscular blocking agent (curare). Horizontal bars indicate period of stimulation. A, stimulus that produced biggest consistent muscle response (80 Hz, 60 V, 0.1 ms pulse width) for a duration of 0.5 sec; B, repeat stimulation after incubation with curare ($10^{-5}$M) for 20 min. Lack of response indicates this parameter mediates a contractile response solely through nerve-mediated stimulation; C, stimulation of the same muscle preparation with a larger stimulus known to induce direct stimulation (100 Hz, 60 V, 5 ms pulse width for a duration of 0.5 sec) in the continued presence of $10^{-5}$M curare, shows a smaller response that is solely induced by direct stimulation of the muscle.
A series of time control experiments were then undertaken to determine if post-mortem muscle could produce a consistent response to this stimulation parameter over time. Muscle strips were stimulated every 7 min over the course of 1 hr, to ensure muscle viability over the experimental period (Figure 3.3 is a representative trace of muscle response). This parameter was shown to produce a consistent muscle response over a prolonged period (the decline in tension was not significant, \( p > 0.05 \)). Thus this parameter was used as a baseline measure for each muscle strip in each experiment, against which all subsequent test responses were normalized.

Figure 3.3. Viability of muscle over testing period (1 hr)

*Trace represents SM muscle stimulated at 0.5 sec at 80 Hz, 0.1 ms, 60 V every 7 min for the period of an hour. Tension (g) generated during this period was consistent (\( p > 0.05 \)), thus confirming the viability of the preparation over the experimental timeframe.*
3.3.2 Experiment 2: Effect of varying electrical components on post-mortem muscle contractile characteristics

Figures 3.4 to 3.7 represent ST and SM muscle response to the different electrical stimulation parameters. Tables 3.2 to 3.5 compare the effect of the parameters between muscle groups. Muscle characteristics examined included the peak force produced (peak tension; Figure 3.4; Table 3.2), the overall amount of contraction (AUC; Figure 3.5; Table 3.3), the time taken for muscle to generate peak tension (rise time; Figure 3.6; Table 3.4) and the time taken to relax (fall time; Figure 3.7; Table 3.5).

SM muscle contraction shows no difference to varying stimulation

The most notable observation was that there was no difference in SM muscle response between any of the stimulation settings trialed, and that this was observed across all the muscle properties measured ($p>0.05$; Figure 3.4 to 3.7). There were trends towards differences in the peak tension (between longest pulse width and both the default and lowest voltage settings; Figure 3.4), the AUC (between the longest pulse width and lowest voltage; Figure 3.5) and the rise time (between the lowest and highest voltages: Figure 3.6), but none reached significance (p values were $>0.05$ and $<0.08$). It should be noted that SM muscle produced higher variance in its responses to the stimulation than that observed for ST muscle, possibly associated with the complexity of preparing the SM muscle strips (compared to ST preparation), as the orientation of the SM muscle fibres were intricately overlapping particularly at perpendicular angles.
ST muscle contraction differs between stimuli but is dependent on the muscle property measured

In contrast, ST muscle showed a distinguishing response between the different stimulation settings, with the difference between electrical parameters dependent upon the property being measured. Peak tension (Figure 3.4) increased when the default stimulation setting (15 Hz, 1 ms, 60 V) was altered by increasing pulse width (5 ms) or modulating the frequency (15/20/25 Hz; \( p<0.05 \)). Interestingly, both these settings also produced significantly more tension than the smaller pulse width increase (2.5 ms; \( p<0.05 \)).

The larger pulse width (5 ms) also produced significantly more contraction (AUC) than the default setting (\( p<0.05 \)), however modulated frequency did not (Figure 3.5). The time taken to contract was similar for all stimuli, with the exception of the higher voltage setting, as increasing voltage (100V) above default setting produced a slower speed of contraction (\( p<0.01 \); Figure 3.6). There were no differences between stimuli for the time taken to relax (\( p>0.05 \); Figure 3.7).

Little difference between muscle groups in response to stimuli

Both muscles displayed a similar trend in response to the varying stimulation (for tension and AUC), but as mentioned above, only ST function showed statistical differences in response to the differing stimuli. Comparison of contractile function between muscles showed SM muscle produced generally a larger response for peak tension and AUC (Table 3.2 and 3.3), with no difference between muscles in rise or fall time (Table 3.4 and 3.5). Stimulation with the lowest voltage (30 V) showed the biggest distinction between muscle types, with SM producing greater tension (\( p<0.05 \)) and AUC (\( p<0.01 \)).
Interestingly the default stimulation produced different amounts of contraction between muscle groups. The use of an internal muscle strip control allowed us to normalize for the inter-muscle variability.
Figure 3.4. Effect of MVES-like electrical stimulation on peak tension

Peak tension (g) produced by muscle stimulated with different electrical parameters. Values presented are mean ± SEM, calculated as a percentage of the control response (muscle stimulated with 80 Hz 0.1ms 60V). Dark columns represent sheep ST (n = 8); Light columns represent sheep SM (n=7). Differences between stimulation parameters within a muscle group are shown by asterisks above graph columns (*p<0.05; **p<0.01).
Figure 3.5. Effect of MVES-like electrical stimulation on AUC

The amount of contraction (measured as area under the curve; g.s.) produced by muscle stimulated with different electrical parameters. Values presented are mean ± SEM, calculated as a percentage of the control response (muscle stimulated with 80 Hz 0.1ms 60V). Dark columns represent sheep ST (n = 8); Light columns represent sheep SM (n=7). Differences between stimulation parameters within a muscle group are shown by asterisks above graph columns (*p<0.05; **p<0.01).
Figure 3.6. Effect of MVES-like electrical stimulation on rise time

The time to contract (ms) by muscle stimulated with different electrical parameters. Values presented are mean ± SEM, calculated as a percentage of the control response (muscle stimulated with 80 Hz 0.1ms 60V). Dark columns represent sheep ST (n = 8); Light columns represent sheep SM (n=7). Differences between stimulation parameters within a muscle group are shown by asterisks above graph columns (**p<0.01).
Figure 3.7. Effect of MVES-like electrical stimulation on fall time

The time to relax (ms) by muscle stimulated with different electrical parameters. Values presented are mean ± SEM, calculated as a percentage of the control response (muscle stimulated with 80 Hz 0.1ms 60V). Dark columns represent sheep ST (n = 8); Light columns represent sheep SM (n=7). There were no statistically significant differences between stimulation parameters within a muscle group.
A Physiological Understanding of Post-mortem Muscle Stimulation

Table 3.2. Comparison of peak tension response (% control) between ST and SM

<table>
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<tr>
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<th>15 Hz 1ms 60V</th>
<th>15 Hz 2.5ms 60V</th>
<th>15 Hz 5ms 60V</th>
<th>15 Hz 1ms 30V</th>
<th>15 Hz 1ms 100V</th>
<th>15/20/25 Hz 1ms 60V</th>
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<td>ST</td>
<td>21.92 ± 5.4</td>
<td>24.00 ± 3.3</td>
<td>46.67 ± 7.3</td>
<td>16.31 ± 2.8</td>
<td>31.93 ± 5.1</td>
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<tr>
<td>SM</td>
<td>29.16 ± 2.3</td>
<td>40.19 ± 13.1</td>
<td>60.99 ± 7.4</td>
<td>33.92 ± 7.6</td>
<td>41.32 ± 11.3</td>
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<td>ns</td>
<td>ns</td>
<td>*</td>
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Original measurement in g

Table 3.3. Comparison of AUC response (% control) between ST and SM

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<th>15 Hz 2.5ms 60V</th>
<th>15 Hz 5ms 60V</th>
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<td>ST</td>
<td>14.00 ± 1.1</td>
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<td>SM</td>
<td>40.34 ± 8.9</td>
<td>43.77 ± 13.3</td>
<td>71.21 ± 13.4</td>
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Original measurement in g.ms

Table 3.4. Comparison of rise time response (% control) between ST and SM

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<th></th>
<th>15 Hz 1ms 60V</th>
<th>15 Hz 2.5ms 60V</th>
<th>15 Hz 5ms 60V</th>
<th>15 Hz 1ms 30V</th>
<th>15 Hz 1ms 100V</th>
<th>15/20/25 Hz 1ms 60V</th>
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<tbody>
<tr>
<td>ST</td>
<td>118.3 ± 21.8</td>
<td>745.1 ± 263.6</td>
<td>947.3 ± 342.2</td>
<td>122.8 ± 25.1</td>
<td>1568 ± 503.9</td>
<td>114.5 ± 14.4</td>
</tr>
<tr>
<td>SM</td>
<td>1127 ± 663.8</td>
<td>992 ± 499.4</td>
<td>1794 ± 542.0</td>
<td>154.8 ± 4.1</td>
<td>2174 ± 941.6</td>
<td>714.7 ± 495.9</td>
</tr>
<tr>
<td>Sig</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Original measurement in ms

Table 3.5. Comparison of fall time response (% control response) between ST and SM

<table>
<thead>
<tr>
<th></th>
<th>15 Hz 1ms 60V</th>
<th>15 Hz 2.5ms 60V</th>
<th>15 Hz 5ms 60V</th>
<th>15 Hz 1ms 30V</th>
<th>15 Hz 1ms 100V</th>
<th>15/20/25 Hz 1ms 60V</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST</td>
<td>1434 ± 842.7</td>
<td>833.5 ± 474.5</td>
<td>641.4 ± 531.7</td>
<td>1052 ± 947.3</td>
<td>275.3 ± 144.9</td>
<td>1312 ± 978.6</td>
</tr>
<tr>
<td>SM</td>
<td>569.8 ± 229.1</td>
<td>244.4 ± 84.6</td>
<td>603.8 ± 223.1</td>
<td>802.2 ± 419.7</td>
<td>308.2 ± 190.5</td>
<td>424.2 ± 159.0</td>
</tr>
<tr>
<td>Sig</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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</tr>
</tbody>
</table>

Original measurement in ms

Values presented are mean ± SEM, calculated as a percentage of the control response (muscle stimulated with 80 Hz 0.1ms 60V). Differences between muscle groups are shown as ns, not significant; *, p<0.05; **, p< 0.01. There were no differences between muscles in their response to each electrical parameter, with the exception of 30V and 60V SM producing significantly greater tension and AUC (Table 3.3, p<0.05 and Table 3.4, p<0.01, respectively). SM also produced greater AUC in response to the 'default' stimuli (15 Hz 1ms 60V; p<0.01).
3.3.3 Experiment 3: Contribution of direct stimulation to overall muscle contraction

Having determined the contractile response to each stimulation parameter in both muscle types, we then examined how much of the muscle contraction (AUC) resulted from direct stimulation of the muscle. The remainder of the response is associated with indirect stimulation pathways, i.e. stimulation of remaining nerve components within the muscle strip (motor axons and the neuromuscular junctions). Figure 3.8 reveals the contribution of direct stimulation to AUC produced by both post-mortem ST and SM muscle contraction. Table 3.6 outlines the differences between the stimulation settings within each muscle group.

Direct stimulation in ST muscle contraction

Within ST muscle, it appeared that all stimulation settings incorporated a similar proportion of direct stimulation, with approximately 60-70% of AUC response associated with direct stimulation of the muscle bundle. The exception was the lowest voltage setting (30 V), which previously produced the smallest AUC (Figure 3.5). This setting appeared to utilise significantly more direct stimulation in generating contraction when compared to the default stimulation (p<0.001) and also amongst the other settings examined here (p-values varied between <0.01 and <0.001). In contrast, the largest pulse width (5 ms), which also produced the most AUC (Figure 3.5), appeared to generate this response using the least amount of direct stimulation (~50%). This was also significantly lower than the amount of direct stimulation induced by the modulated frequency setting in ST muscle (p<0.001).
Direct stimulation in SM muscle contraction

Stimulation of SM muscle appeared to induce contraction using more of the nerve-mediated pathway. The proportion of direct stimulation utilised by SM across all settings was approximately 30-50%, suggesting the remaining response is attributable to ≥50% via nerve-mediated stimulation. There was no significant difference in the direct component between stimulation settings, with the exception of lowest voltage (30 V), which incorporated significantly less direct stimulation than the default setting (p<0.001) and the other settings (p-values varied between <0.01 and <0.001).

Differences between ST and SM muscle contraction

Overall, muscle contraction in post-mortem ST was generated via a significantly higher proportion of direct muscle stimulation (ranging between 50-90% across parameters). In contrast, SM muscle appeared to contract via a larger nerve-mediated component, as direct stimulation accounted for only 30-50% of contraction. Interestingly, stimulation with the longest pulse width parameter (5 ms) produced contraction in both muscle groups using similar proportions of both nerve and direct transmission (approximately 50% nerve/ 50% direct). The biggest distinction between muscle groups was noted for contraction produced by the lowest voltage (30 V), which utilised the biggest proportion of direct stimulation in ST muscle, but the smallest proportion of direct pathways in SM muscle.
### Table 3.6. Contribution of direct stimulation to muscle contraction: differences between parameters

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>SM</th>
<th>15 Hz 1ms 60V</th>
<th>2.5ms 60V</th>
<th>5ms 60V</th>
<th>30V</th>
<th>100V</th>
<th>15/20/25 Hz</th>
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<tr>
<td>15Hz</td>
<td>1ms</td>
<td>60V</td>
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<td>** ns</td>
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<td>15Hz</td>
<td>1ms</td>
<td>60V</td>
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<tr>
<td>15Hz</td>
<td>1ms</td>
<td>60V</td>
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<td>ns</td>
<td>** ns</td>
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<td>15Hz</td>
<td>1ms</td>
<td>60V</td>
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<td>15Hz</td>
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</table>

The amount of contraction attributable to direct stimulation of the muscle bundle, by each electrical parameter. Values presented are mean ± SEM of AUC measured in the presence of curare (10⁻⁵M post-curare), calculated as a percentage of the total pre-curare AUC response. Differences between parameters within a muscle group are shown in the Table 3.6 (darker columns, ST; lighter columns, SM) and were determined by one-way ANOVA with Tukey’s post hoc testing: ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.001. Differences between muscle groups in response to each parameter were determined by independent t-test and are represented by asterisks above columns in Figure 3.8 (**p<0.01, ***p<0.001).

### Figure 3.8. Contribution of direct stimulation to muscle contraction: differences between muscles
3.4 Discussion

This chapter examined the contractile activity of post-mortem muscle in response to electrical stimulation parameters designed to simulate those previously tested in a commercial abattoir (Chapter 2). A central finding of this study was that parameters utilising the longest pulse width or a modulated frequency produced the most significant differences in the muscle properties we examined. Both produced the biggest peak tension yet only the longer pulse width generated the greatest amount of contraction, suggesting these parameters may selectively target and/or engage different aspects of the contractile process. Interestingly these findings were only significant in post-mortem ST muscle, with post-mortem SM, while showing a similar trend, had no overall significant difference in response to the varying stimulation parameters. Muscle contraction in both muscle groups appeared to be produced via a similar proportion of direct stimulation when treated with the longest pulse width. In contrast, the remaining parameters utilised significantly more of the direct pathway when stimulating ST muscle, whilst utilising more nerve-mediated stimulation when applied to SM muscle.

3.4.1 Longer pulse widths generate maximal contractile activity

The first major finding of this study was that electrical stimulation that used long pulse widths (i.e. 5 ms) produced the biggest muscle response. The 5 ms parameter generated the most contractile activity, as evidenced by producing the largest peak tension and AUC responses. Interestingly, this parameter was also identified in Chapter 2 as producing the biggest drop in carcass pH. It is possible that the way in which new generation processing technology delivers regulated electrical input to individual carcasses, through combining separate stimulation segments with inbuilt computerised
feedback systems, creates an opportunity to exploit different elements of the electrical input, such as pulse width. This element in particular was previously under-utilised in low and high voltage systems, and this may potentially explain the now established ability of MVES to significantly enhance meat tenderness (Shaw et al., 2005, Pearce et al., 2006, Toohey et al., 2008, Pearce et al., 2009).

Both the findings of this study and those of Chapter 2 therefore support the idea that pulse width may be a critical variable in MVES (at 15Hz, 60V), consistent with insights derived from other physiology-based studies that also highlight the effect of this electrical component on muscle contraction (Gorgey and Dudley, 2008). Pulse width is the length of time a current is applied to tissue, and in conjunction with amplitude it determines the strength of a single pulse (Alon et al., 1983, Gorgey and Dudley, 2008, Bajd and Munih, 2010). As a result of the way pulse width interacts with the electrical current, the hyperbolic nature of this current strength-pulse duration relationship dictates that the current required to excite a membrane increases as pulse width decreases, (Katz, 1966, Mortimer, 1981, Grill and Mortimer, 1996). Thus short pulse widths have been shown to increase the membrane threshold difference (i.e. the amount of stimulus required to generate an AP) not only between fibres of different diameters lying at the same distance from the electrode, but also of fibres of similar size lying at different distances to the electrode (Gorman and Mortimer, 1983, Grill and Mortimer, 1996). This highlights two major challenges that arise when stimulating post-mortem muscle: (1) the problematic nature of stimulating heterogenic muscles containing fibres of varying size; and (2) ensuring an even distribution of the current to muscles outside of the electrical pathway. In contrast to short pulse durations, longer pulse widths have been shown to produce greater peak force as well as a greater amount of overall contraction (Baldwin et al., 2006, Gorgey et al., 2006, Gregory et al., 2007,
A Physiological Understanding of Post-mortem Muscle Stimulation

Gorgey and Dudley, 2008), and the results presented in this study are consistent with the findings from these published studies. A possible explanation for the effect of longer pulse width on post-mortem muscle comes from a study of denervated human muscle, which revealed that muscle in this condition exhibits both prolonged relative and absolute refractory periods due to non-functioning electrochemical gradients (similar to post-mortem muscle), and that electrical stimulation with longer pulse widths allowed the contractile apparatus time to respond to the stimulation between APs (Petrofsky, 1991). Further human studies on skeletal muscle contraction have also shown that longer pulse widths are more likely to activate sensory nerves in the skin and muscle, thereby enhancing the number of activated fibres and producing an increased muscle response in peak force (Klakowicz et al., 2006, Dean et al., 2007, Lagerquist et al., 2009), which is again consistent with the findings for sheep muscle preparations reported in this study.

3.4.2 **Modulated frequency - same but different to longer pulse width?**

The second major finding of this study related to the effect of modulated frequency on contractile activity. In Chapter 2, the effect of this parameter mirrored the effect of the 5 ms pulse width parameter, by producing a similar size $\Delta$pH, suggesting that it uses a similar amount of glycogen and/or accumulates an equal amount of lactic acid. The data presented in this chapter shows that both parameters also produce an equally large peak force, indicating a similar proportion of muscle fibres were being activated (Gorgey et al., 2006, Hopkins, 2006). However, these two parameters (pulse width vs. modulated frequency) diverge in the amount of overall contractile activity they generated. It is possible that these parameters induce a similar overall muscle response via different mechanisms, as this would allow each to produce a similar end point (similar magnitude
of pH drop as observed in Chapter 2) but by stimulating different contractile elements (as observed in AUC differences presented here). It is possible the answer lies in which ATPase each electrical parameter preferentially stimulates, and which one subsequently utilizes the majority of the available energy supply.

Within contracting skeletal muscle, the major ATP-consuming enzymes are myosin ATPase, Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, which are involved in force production, Ca\textsuperscript{2+} handling and membrane ion pumping, respectively (Homsher, 1987). In fast-twitch muscles, it was previously estimated that the cost of cross-bridge activity involving the myosin ATPase, i.e., producing muscle contractions, was approximately 70\% of the overall ATP use, with the balance being consumed by Ca\textsuperscript{2+} handling (>20\%) and membrane ion pumps (<5\%; Crow and Kushmerick, 1982, Homsher, 1987, Rall and Woledge, 1990). Such estimates however, were derived from experiments performed at low temperatures (0-20°C) and under conditions that elicit maximal force, thus introducing artifacts (Zhang et al., 2006). The use of newer chemical agents such as \textit{N}-benzyl-\textit{p}-toluene sulfonamide (BTS), a highly specific inhibitor of myosin ATPase that doesn’t interfere with Ca\textsuperscript{2+} handling, has aided in the delineation of ATPase energy consumption (Cheung et al., 2002, Shaw et al., 2003). It has been particularly useful in muscle studies since previous agents, such as 2,3-butane-dione monoxime (BDM), inhibited not only cross-bridge formation, but also Ca\textsuperscript{2+} release from RYRs in the SR (Fryer et al., 1988, Stienen et al., 1995, Tripathy et al., 1999). As such, more recent studies have found that the contractile (myosin ATPase) and non-contractile (Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/K\textsuperscript{+} ATPase) processes have near equal rates of ATP consumption in contracting muscle (Baker et al., 1994, Dentel et al., 2005, Walsh et al., 2006, Zhang et al., 2006, Barclay et al., 2007).
Leading on from the above, the possibility that modulated frequency produces a similar ΔpH to longer pulse widths (as seen in Chapter 2) via increased activity of non-contractile ATPases (compared to an increase in myosin ATPase) is supported by functional in vitro muscle studies. In contrast to maintaining a constant frequency over a period of stimulation, modulating the frequency (and in this case, increasing it) causes muscle contractions to summate and become tetanic (Russ et al., 2002, Gorgey and Dudley, 2008). This type of contraction does not use as much ATP during the contractile process via myosin ATPase as compared to when muscle undergoes twitching (whereby the myofibrils completely overlap each other to produce single muscle contractions in an ATP-expensive process), and so results in a smaller AUC (Marsh, 1985, Hogan et al., 1995), correlating with the findings presented here. Nevertheless, the non-contractile processes may ultimately consume the same amount of ATP, as the step-wise increase in frequency greatly increases the activity of both the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase pumps (Chasiotis et al., 1987, Newham et al., 1995, Dentel et al., 2005). This is supported by several studies reporting that increases in stimulation frequency are more energy consuming than maintaining a consistent frequency (Bergstrom and Hultman, 1988, Spriet et al., 1988, Foley and Meyer, 1993, Hogan et al., 1998, Russ et al., 2002).

3.4.3 Muscle-specific response to MVES-like stimulation: post-mortem ST, but not SM muscle differentiates between different electrical parameters

It is interesting to note that the differences in the muscle contraction induced by the different electrical parameters outlined above were only significant in ST muscle, with SM muscle showing no difference in the measured contractile properties between settings. This difference may arise from muscle-specific properties, particularly the type
of innervation of each muscle group (discussed below) and its responsiveness to
different electrical inputs. The parameters examined here may have over-stimulated the
post-mortem SM muscle, as we were unable to see distinguishing responses between
the parameters. This rationale is supported by the observation that the lowest voltage
(30V) produced the biggest distinction between muscle groups, with SM producing
more tension and contraction than ST. Interestingly, this electrical input was transmitted
mostly by nerve-mediated conduction in SM, in contrast to being mostly direct in ST,
suggesting threshold excitability, and thus innervation type, may be influential in post-
mortem muscle contraction. This idea correlates with the findings of previous meat
studies whereby low voltage settings revealed an inconsistent response in carcasses, and
this may be associated with differences in fibre type composition of various muscle
groups (e.g.,Chrystall et al., 1980, Morton and Newbold, 1982).

3.4.4 Direct stimulation vs. nerve-mediated responses

Electrical stimulation of skeletal muscle generally induces a contractile response via a
combination of two pathways, (1) indirectly via nerve-mediated pathways, as occurs in
physiologically normal muscle, i.e. AP travels from motor axon across the NMJ and
down the sarcolemma, initiating the E-C coupling process; and (2) directly, whereby the
electrical current directly depolarizes the voltage sensor proteins (DHPRs) in the T-
tubules, leading to Ca^{2+} release from the SR and onset of mechanical contraction (Rudel
and Taylor, 1969, Stephenson, 2006, Cairns et al., 2007). The contribution of each
pathway to the overall muscle contraction is dictated by many factors, with time post-
mortem one of the most critical factors for commercial meat stimulation.

This study observed a trend for electrical stimulation of post-mortem SM muscle
to generate contraction via a greater nerve stimulation component, in contrast to post-
mortem ST, which had a larger contribution arising from direct stimulation. This finding suggests that the type of innervation of each muscle group (i.e. properties of the motor unit) may be an extenuating influence in post-mortem muscle response to different electrical inputs. Stimulation of muscle is more efficient via nerves, as the threshold for eliciting a nerve fibre AP is 100 to 1,000 times less than the threshold for muscle fibre stimulation, and if application of external stimulation occurs over the motor point of the nerve, proximal to the neuromuscular junction, it acts to stimulate all the aborised nerve terminals of that axon thus stimulating all associated fibres (Mortimer, 1981, Sheffler and Chae, 2007). Given that the motor axons that innervate SM muscle (from fast-twitch fatigue resistant MUs) have a lower stimulation threshold than those axons that innervate ST (from fast-twitch intermediate fatigue resistant MUs), this suggests that SM muscle may be more excitable (Mendell, 2005). This correlates with the larger nerve-mediated component observed in the SM response to all stimulation parameters, and may also explain why SM trended towards a bigger contractile response than ST, due to its lower excitation threshold resulting in more axons and thus muscle spindles activated. Furthermore, it may also be associated with SM showing no difference between stimulation settings, as the SM may have been over-stimulated with the settings trialed here and is thus more receptive (and distinguishing) to electrical stimulation that utilises more of a nerve-based component.

Another interesting finding was that one of our parameters of interest, the longest pulse width (5 ms), induced contraction in both muscle types using similar proportions of both direct and nerve- mediated stimulation pathways. This setting produced the largest contractile response in both muscle groups, suggesting that longer pulse widths may be appropriate for a range of muscles regardless of their innervation unit. The nerve terminal is known to have the lowest excitation threshold, with this
activation threshold gradually increasing with distance along the sarcolemma, and down into the T-tubules, which have the highest threshold (Cairns et al., 2007, Cairns et al., 2009). Muscle studies on the excitability of different mouse muscles revealed longer pulses lower the activation in this latter region of the muscle fibre, thus engaging more actively contracting fibres (Cairns et al., 2009).

3.5 Conclusions

Overall, this study has shown that electrical stimulation with longer pulse widths produces a greater contractile response in terms of both peak tension and overall amount of contraction. Contractile response to modulated frequency stimulation was similar to that elicited by longer pulse widths, though this similarity is likely to occur from stimulation of different contractile elements (such as increased activity of non-contractile ATPases). Post-mortem sheep SM muscle demonstrated a greater capacity for nerve-mediated contractions than ST, an observation likely tied to intrinsic muscle-specific properties. These findings suggest there are opportunities to alter MVES protocols to incorporate more nerve-responsive elements, and therefore drive more homogenous responses to electrical stimulation.
Chapter 4.

The effect of nerve-mediated electrical stimulation on post-mortem muscle – contractile function and endogenous neuromodulator activity

4.1 Introduction

In order to delineate the effects of MVES on the contractile characteristics of post-mortem sheep muscle, the previous two chapters have examined both the biochemical effect (i.e., \( \Delta \text{pH} \)) of different stimulation parameters under commercial conditions (Chapter 2), and the corresponding mechanical effect (i.e., contractile properties) of these parameters in a controlled *in vitro* environment (Chapter 3). Consistent between these two studies was the use of direct electrical stimulation. However, findings from the previous chapter revealed that MVES might be conducted, in part, via the nervous system. Moreover, there appeared to be a trend for muscle-specific differences in the contribution of direct vs. nerve-mediated electrical conductance, as we observed a greater role for nerve-mediated contraction in SM muscle compared to ST muscle. This observation suggests that electrical parameters inducing nerve-mediated muscle contraction (i.e. electrical stimulation transmitted via the nervous system) may be further manipulated within the MVES set up, thus enabling processors to utilise the natural biological pathways by which normal physiological electrical transmission occurs. This would result in reduced electrical resistance within the carcass whilst ensuring peak muscle response, ultimately circumventing heterogeneous pH decline in muscles that is a known contributor to variation in end product quality. Anecdotal evidence from commercial processing supports this possibility, with observations that the nervous system retains some functionality within carcasses at the point on the processing chain when they are stimulated with MVES (*pers comm* I. Richards, Pearce...
et al., 2006). We sought to investigate this finding further, examining the responsiveness of post-mortem sheep muscle to nerve-mediated electrical stimulation. Given the lack of published literature available on the functioning of post-mortem skeletal muscle, and none with a particular purview to meat science, this study utilised the isolated nerve-muscle preparation (used in Chapter 3) to provide a controlled environment in which to determine if, and by how much, post-mortem muscle responds to nerve-mediated electrical stimuli. In contrast to the oxygenated set up used in Chapter 3, the current study was performed under anoxic conditions to simulate extended post-mortem conditions (Fredsted et al., 2005) in order to progress our understanding of nerve function under these conditions. This study sought to inform as to different characteristics of muscle contraction induced via nerves are impacted during post-mortem conditions (i.e. anoxia), using physiological-based electrical parameters that included a twitch response (2 Hz), a sub-tetanic contraction that produces partial summation of contraction (30 Hz), and a tetanic contraction, which produces complete muscle tetany (80 Hz; Patwell et al., 2004).

In examining muscle contraction that arises from nerve stimulation, the influence of endogenous neuromodulatory substances must also be taken into account. This diverse range of chemicals mediates the contractile response by regulating events between the nerve-muscle interface, and their actions are also impacted by extracellular conditions, particularly the availability of oxygen. In directly stimulated muscle fibres, these substances have less of a regulatory role as the current directly stimulates the muscle membrane rather than the NMJ. In contrast, nerve-mediated stimulation of skeletal muscle contraction is initiated solely by ACh, which propagates signal transmission across the NMJ, and neuromodulators play an influential role in its efficiency by acting
via pre- and postsynaptic mechanisms. Three neuromodulators that have a significant role in muscle contraction include adenosine triphosphate (ATP), nitric oxide (NO) and calcitonin gene related peptide (CGRP). These modulating agents differ in their point of origin and active sites, but all influence events at the NMJ and/or muscle fibre itself, thus affecting downstream contractile activity. At the NMJ, ATP acts upon the presynaptic P2X7 purinergic receptor, whose activation promotes vesicle release (Moores et al., 2005). Similarly, NO produced by the postsynaptic nNOSµ also has presynaptic effects on vesicle release, with the outcome dependent upon the contraction frequency (Murrant et al., 1997, Thomas and Robitaille, 2001, Stojanovic et al., 2005). In contrast, CGRP is colocalized presynaptically with the vesicular acetylcholine transporter (VACChT; Wang et al., 2013), but its actions are directed to its postsynaptic G protein-coupled receptor, where its binding ultimately results in stimulation of Na⁺-K⁺ ATPase (Uchida et al., 1990, Clausen et al., 1993, Wimalawansa, 1996). Their role in skeletal muscle contraction under physiological conditions is well established (e.g., Brenman and Bredt, 1997, Gordon et al., 2000, Macdonald et al., 2008), however their functioning under limited oxygen/anoxic conditions, and the subsequent impact this has on neurotransmission efficiency and contraction, remains unclear. In order to define the role of each neuromodulator in post-mortem muscle contraction, different pharmacological agents were used to inhibit the individual function of each. Firstly, a competitive analogue of ATP, adenosine 5’- (β,γ-imido) triphosphate (β,γ-ATP), was used to outline the role of ATP by competitively inhibiting ATP-utilising processes. This drug specifically targets the function of myosin ATPase and mitochondrial F₁-ATPase (Penefsky, 1974). Blocking ATP hydrolysis via this method closely resembles the situation of when ATP is limited, thereby enabling investigation into the role of ATP in contractile function without the complete deregulation of other cellular
processes, such as ion homeostasis (Yount et al., 1971). Secondly, to investigate the function of NO we used the arginine analogue, N\(^{G}\)-nitro-L-arginine methyl ester hydrochloride (L-NAME), a non-specific NOS inhibitor that affects both the nNOS\(\mu\) isoform in the sarcolemma and the eNOS isoform found within the mitochondrial membrane, thus inhibiting the two main sources of NO within skeletal muscle (Griffith and Gross, 1996). Finally, we used an inhibitory peptide used to investigate the role of CGRP in post-mortem muscle contraction. CGRP\(_{8-37}\) is an antagonistic fragment from the CGRP peptide sequence and has been shown to be a potent and selective inhibitor for CGRP receptors located at the NMJ (Poyner, 1992).

### 4.1.1 Objectives

The overall aim of this study was to evaluate how post-mortem muscle contraction (induced via nerve-mediated stimulation) is impacted under anoxic conditions, with the hypothesis that this type of muscle does retain a degree of responsiveness under such an environment. We specifically assessed the change in peak tension, amount of contraction (AUC), and the rate of contraction and relaxation induced by physiologically-based frequencies that induced a twitch response (2 Hz), a sub-tetanic contraction (30 Hz), and a complete muscle tetany (80 Hz), comparing anoxic responses to oxygenated controls. In addition, the contribution of several endogenous neuromodulators (ATP, NO, CGRP) to the contractile response was also examined, assessing the change in muscle contraction via pharmacological inhibition. Immunohistochemistry was also used to assess the expression and localization of these neuromodulators or their receptors within the muscle.
4.2 Materials and Methods

4.2.1 Experimental Outline

Experiment 1

Experiment 1 examined nerve-mediated muscle contractions under anoxic (i.e., post-mortem) conditions. Anoxic muscle responses in sheep SM and ST were compared to those derived under oxygenated conditions in the same muscle strip, using stimuli of 2Hz, 30Hz and 80Hz (all at 0.07 msec and 30V).

Experiment 2

Experiment 2 examined the role of each neuromodulator in the anoxic muscle contraction by blocking its activity with the appropriate pharmacological inhibitor. Results were gained from comparing the response of anoxic muscle contraction in the presence of inhibitors to that of contraction under just anoxia (on the same muscle strip).

4.2.2 Animals

All experiments were carried out on mixed sex crossbred Merino sheep (majority Poll Dorset cross; ~12-18 months) with the approval of the Animal Ethics Committee of Murdoch University. Animals were euthanased in accordance with procedures of commercial meat processing plants. Immediately after death, multiple 2cm³ blocks of tissue were cut from the dorsal, proximal regions of both the ST and SM muscles and placed in pre-chilled Krebs solution (of the following composition, mmol/L: NaCl 120.0, KCl 5.0, CaCl₂ 2.5, Na₂HCO₃ 25.0, MgSO₄ 1.0, NaH₂PO₄ 1.0, and glucose 22.0, equilibrated with 5% CO₂ in O₂ to give pH 7.4). Separate blocks of tissue were
collected either for fixation in 4% formaldehyde (for immunohistochemistry) or frozen in isopentane cooled in liquid nitrogen (for fibre typing).

4.2.3 **In vitro muscle contraction protocol**

The same isolated nerve-muscle electrophysiology rig and muscle set up described in Chapter 3 (Chapter 3.2.3) was used in this study. Figure 4.1 outlines the subsequent experimental protocol. After the equilibration period, a test stimulation (80 Hz, 0.07 ms, 30V) was applied to each muscle strip to ensure muscle viability and responsiveness to the nerve-mediated stimulation (at T_20). After this stimulation, the muscle strip was stimulated with a randomized order of nerve parameters (2 Hz, 30 Hz, and 80 Hz at 30V, 0.07 ms) to record a baseline response to each frequency (defined as the oxygenated control response). The Krebs solution in the organ bath was then changed to an anoxic version (i.e., aerated with 95% N₂ - 5% CO₂, as highlighted by Fredsted et al., 2005), and muscle strips were incubated in this for 20 min to induce anoxic conditions. At T₀, muscle strips were again stimulated with the previous order of nerve parameters to record the anoxic control response. The effect of anoxia on muscle contraction was determined as the percentage of T₀ response to that of the T_20 response. For pharmacological experiments, the appropriate agent was then added directly to the anoxic Krebs buffer in the organ bath after stimulation, with the tissue incubated for 20 min before again being stimulated with the same order of frequencies to produce the anoxic drug-treated response (T_20). The effect of the drug on anoxic muscle contraction was determined as the percentage of T_20 response to that of the T₀ response. At the end of each protocol, the length and weight of each muscle strip was recorded. The cross-sectional area was calculated by dividing the strip weight (grams) by the strip length
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(cm) times the specific muscle density (1.056) to determine tension as per cross-sectional area (Mendez and Keys, 1960). Each experimental group (i.e. oxygenated muscle, anoxic muscle, and anoxic muscle with each pharmacological agent) had an \( n = 3-4 \) animals, with the response of at least 4 muscle strips averaged per animal (4 organ baths were used simultaneously). If a muscle strip failed to respond to test stimulation at \( T_{20} \), it was replaced with another that had been kept in oxygenated Krebs solution on ice.

![Figure 4.1. Timeline for experimental procedure](image)

The properties of muscle contraction that were measured in this study were the same as those examined in Chapter 3 (section 3.2.3), i.e. peak tension, AUC (amount of contraction), time for contraction to peak tension (rise time) and time for muscle to relax to baseline (fall time). These four properties were used to highlight different aspects of the contractile process that are affected by the applied treatment or conditions. Table 4.1 summarises the processes/functions that each of the muscle measurements represent (summary of information detailed in Chapter 3, section 3.2.3).
Table 4.1. Muscle measurements

<table>
<thead>
<tr>
<th>Muscle measurement</th>
<th>Representative process/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak tension</td>
<td>- number of actively contracting fibres /axon innervation points</td>
</tr>
<tr>
<td></td>
<td>- neurotransmission (pre-/postsynaptic function: receptors/channels-vesicle activity)</td>
</tr>
<tr>
<td>AUC</td>
<td>- postsynaptic function – E-C coupling (sarcolemma excitability/channel activity)</td>
</tr>
<tr>
<td></td>
<td>- Ca2+ cycling (release and reuptake)</td>
</tr>
<tr>
<td></td>
<td>- myosin ATPase activity (amount and speed of ATP use)</td>
</tr>
<tr>
<td>Rise time (time to contract)</td>
<td>+ AUC = E-C coupling and/or cross-bridge activity (activity of Ca2+ release processes - DHPR/Ryr; mATPase function)</td>
</tr>
<tr>
<td>Fall time (time to relax)</td>
<td>+ AUC = Ca2+ buffering and reuptake processes; membrane repolarisation</td>
</tr>
</tbody>
</table>

4.2.4 Pharmacological Agents

Adenosine 5’-(β,γ-imido)triphosphate (tetralithium hydrate; β,γ-ATP; Sigma Aldrich, St Louis, MO, USA) was used as a competitive inhibitor of ATP. Stock was frozen in aliquots of 10^{-3}M, used at a final concentration of 30µM. Initial trials of ATP inhibitors also included the ATP antagonist, oxidized ATP (OxATP; Adenosine 5’-triphosphate, periodate oxidized sodium salt; Sigma Aldrich). This specifically inhibits presynaptic P2X7 receptors (ATP-gated ion channels), which have a role in neurotransmission; however no discernible effect on muscle contraction was observed with OxATP, thus subsequent experiments used β,γ-ATP only. Due to the competitive nature of this drug, muscle contraction still takes place, albeit it in a lower capacity due to remaining intracellular ATP supply (Subramanian and Gelles, 2007). The resulting accumulation of endogenous ATP slows normal processes associated with anoxia-related ATP.
depletion, and allows observation of NMJ functioning properties by contrasting these results with those of just the anoxic contraction. Nitric oxide synthesis was inhibited using a competitive arginine analogue, $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma Aldrich) to block NOS activity. L-NAME stock was frozen in aliquots of $10^{-2}$ M and used at a final concentration of 10 µM. Calcitonin gene-related peptide was inhibited with the peptide CGRP$_{8-37}$ (Auspep, Parkville, Vic, Australia). Stock of the peptide was frozen in aliquots of $10^{-2}$ M and used at a final concentration of 30 µM. Preliminary experiments (data not provided) were undertaken examining muscle contraction in the presence of each inhibitor under both oxygenated and anoxic conditions to ensure proper activity of the inhibitors.

### 4.2.5 Immunohistochemistry

Immunohistochemical protocols were modified from those of Godchild et al., (2001) and Powers-Martin et al., (2008). Briefly, muscle strips were dissected and fixed in 4% formaldehyde for 4 hours at 4°C, then placed in a 30% sucrose solution overnight at 4°C for sectioning on a cryostat. Tissue was cut into 12 µm longitudinal sections and mounted onto silinated slides and stored at -20°C until required. For processing, sections were washed in phosphate buffered saline (PBS, 0.1 M; pH 7.4), incubated for 30 minutes in 50% ethanol, washed in 0.1 M PBS containing 10 mM Tris (TPBS, 2 x 15 min), and then pretreated with TPBS containing 0.1% Triton-X100 and 0.3% sodium azide (TPBS-Tx-NaN$_3$; 2 x 15 min). Sections were then incubated with blocking solution [10% donkey serum (Abcam, Cambridge, MA, USA) in TPBS-Tx-NaN$_3$, 1 x 60 min, room temperature] before being incubated (3 days, 4°C) in primary antibody diluted in same solution (see Table 4.2 for antibody details). Sections serving as a negative control were not treated with primary antibody, but remained incubated in
blocking serum until secondary antibody treatment. After washing with TPBS (3 x 30 min), sections were treated with secondary antibody diluted in TPBS-Tx-Na$_3$ plus 5% donkey serum and incubated overnight at 4°C. Secondary antibodies used are also described in Table 4.2. Sections were then washed (3 x 30 min TPBS) and covered with ProLong Gold Antifade (Molecular Probes, Eugene, OR, USA) to limit fading of the fluorescent marker. After 24 hours to allow mounting media to seal, coverslips were sealed with nail polish.

### Table 4.2. Primary and Secondary Antibodies.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X$_7$;</td>
<td>Polyclonal anti rabbit P2X7; 1:500, Alomone Labs (Jerusalem, Israel)</td>
<td>Donkey anti rabbit FITC 1:500; Jackson ImmunoResearch (USA)</td>
</tr>
<tr>
<td>VACHT</td>
<td>Polyclonal anti rabbit VACHT; 1:500, Sigma Aldrich (USA)</td>
<td>Donkey anti rabbit Cy 3; 1:500; Jackson ImmunoResearch (USA)</td>
</tr>
<tr>
<td>nNOS</td>
<td>Monoclonal anti mouse nNOS; 1:500, BD Transduction Labs (San Jose, CA, USA)</td>
<td>Donkey anti rabbit FITC 1:500; Jackson ImmunoResearch (USA)</td>
</tr>
<tr>
<td>CGRP</td>
<td>Polyclonal anti rabbit CGRP 1:200, Sigma (USA)</td>
<td>Donkey anti rabbit FITC 1:500; Jackson ImmunoResearch (USA)</td>
</tr>
</tbody>
</table>

*P2X$_7$; purine receptor P2X$_7$; VACHT: Vesicular acetylcholine transporter; nNOS: neuronal nitric oxide synthase; CGRP: calcitonin gene related peptide.*

#### 4.2.6 Fibre typing

To examine the fibre type proportions in sheep SM and ST, an adapted version of the calcium-based method to visualize myosin ATPase differences was used (Brooke and Kaiser, 1970). Four muscles of each type were used for fibre typing, with 10 slides of each muscle examined microscopically. Pre-incubation of the tissue in various pH solutions inactivates different myosin ATPase isoforms. The remaining active ATPase
enzymes attach to calcium atoms, which are replaced by cobalt and precipitated as a black insoluble compound by ammonium sulfide, thus differentiating between fibre types. Table 4.3 outlines muscle appearance after preincubations with solutions of either pH 10.2 or pH 4.53. Fresh frozen tissue was cut into 12µm transverse sections and mounted onto silinates slides and stored at -80°C until required. The slides were dried at room temperature for 30-60 min then pre-incubated in a pH 10.2 solution for 15 min [20 mM Sodium Barbital (Sodium 5,5-diethylbarbiturate, Sigma Aldrich), 36 mM CaCl₂ adjusted to pH 10.2 with NaOH] or 5 min in a pH 4.53 solution (36 mM Sodium Barbital, 75 mM NaOAc adjusted to pH 4.53 with HCl). Tissue sections were then rinsed with ddH₂O before incubation (15 min for pH 10.2; 25 min for pH 4.53) in an ATP-containing solution (1.5 mM ATP disodium salt, 27 mM Sodium Barbital, 18 mM CaCl₂ adjusted to pH 9.4 with NaOH), washed with 1% CaCl₂ (3 x 3 min), incubated in 2% CoCl₂ (10 min), then rinsed with 5 mM Sodium Barbital and ddH₂O. Tissue sections were then incubated in 2% (NH₄)₂S for 20-30 s (or until darkened), rinsed in ddH₂O, dehydrated in ethanol and xylene, and mounted in Ultramount (Dako, Glostrup, Denmark) for viewing.

Table 4.3. The appearance of fibre types when pre-incubated at pH 10.2 or pH 4.53

<table>
<thead>
<tr>
<th>Pre-incubation pH</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX/IIB</th>
<th>Type IIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.2</td>
<td>light</td>
<td>dark</td>
<td>dark</td>
<td>dark</td>
</tr>
<tr>
<td>4.53</td>
<td>dark</td>
<td>light</td>
<td>intermediate</td>
<td>intermediate</td>
</tr>
</tbody>
</table>

4.2.7 Statistical Analysis

Contractile data are reported as mean ± standard error of mean (SEM), expressed as a percentage relative to control (control response = 100%), with the control differing between experiments as follows: for experiments investigating the effect of anoxia on
contractile properties, measurements made during anoxia (T_0) were calculated relative to an oxygenated control response in the same muscle strip (T_{20}; Figure 4.1); for experiments investigating the effect of each neuromodulator on contractile properties under anoxia (T_{20}), the measurements were calculated relative to the anoxic control from the same muscle strip (T_0; Figure 4.1). The raw data from which these calculations were made is provided in Supplementary Tables 4.1-4.4. A two-way ANOVA was performed to compare the main effects of muscle type and frequency on each muscle characteristic measured. A one-sample T-test was used to determine if the effects of either anoxia or the respective drugs was significantly different to the control stimulus (p<0.05; setting the control value to 100%; Vickers, 2001). A Kolmogorov-Smirnov normality test was performed to ensure the data remained normally distributed. To compare the effect of stimuli frequency on anoxic/drug-inhibited muscle, a one-way ANOVA was used on the percentage data (i.e., response relative to control; p<0.05). All data was analysed and visualized using GraphPad Prism (version 5.03, GraphPad Software, Inc.).
4.3 Results

4.3.1 Experiment 1: Response of post-mortem muscle to nerve-mediated electrical stimulation

Table 4.4 contains the main and interaction effects of muscle type and frequency on each muscle characteristic measured. There appeared to be no consistent influence of either variable on each of the measurements recorded within each drug group.

Post-mortem SM muscle contraction under anoxic conditions showed a diminished response when compared to its oxygenated control ($p$ values on Figure 4.2). This was particularly evident in the peak tension and AUC produced by SM muscle, regardless of frequency (all $p<0.026$). SM muscle showed a significant difference in response to low (2 Hz) vs. high (80 Hz) stimulation ($p<0.05$) for all measured properties under anoxic conditions. Interestingly, the muscle retained an increasing contractile response in correlation with increasing stimuli frequency, a physiological characteristic generally observed in oxygenated muscle (Supplementary Table S4.1, page 154). The rise time (time to contract) was approximately 20% less than oxygenated controls across all frequencies ($p<0.005$), but no significant difference was observed between the anoxic and control fall time (time to relax), despite a trend for an increase at 30Hz ($p=0.071$).

Muscle contraction of post-mortem ST also revealed a reduction in the size of the contractile response under anoxic conditions, being significantly reduced at all frequencies compared to the oxygenated control for both the tension and AUC (Figure 4.2; $p<0.05$). However in contrast to the SM muscle, differences in response to the varying stimulation frequencies was observed between low (2 Hz) vs. high (80 Hz) frequencies for peak tension measurements only. For the AUC measurements, there was...
no significant difference in the change under anoxic conditions between the frequencies, with all showing a ≥ 50% reduction in the response. For the rise time, a significant change (decrease) under anoxic conditions was only seen for the 2Hz stimulation, while for the fall time, the lower frequencies (2 Hz and 30 Hz) were significantly less than their oxygenated controls, correlating with a faster rise and fall time for these frequencies.

Table 4.4. Results of two-way ANOVA: Influence of muscle type and frequency on each muscle measurement.

<table>
<thead>
<tr>
<th></th>
<th>Peak</th>
<th>AUC</th>
<th>Rise</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anoxic Muscle</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Frequency</td>
<td>***</td>
<td>*</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>Interaction</td>
<td>ns</td>
<td>*</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>β_{1,7}-ATP</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>Frequency</td>
<td>***</td>
<td>**</td>
<td>ns</td>
<td>***</td>
</tr>
<tr>
<td>Interaction</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>L-NAME Muscle</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Frequency</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>Interaction</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CGRP_{8-37}</td>
<td>***</td>
<td>**</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Frequency</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>***</td>
</tr>
<tr>
<td>Interaction</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
</tbody>
</table>

Data represents the p value of each variable and the interaction. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001.
A Physiological Understanding of Post-mortem Muscle Stimulation

Figure 4.2. Effect of frequency on anoxic SM and ST muscle contraction characteristics

Data represents the percentage change in anoxic SM and ST muscle response relative to a control (oxygenated) muscle response. Adjoining lines and asterisks above columns indicate statistical differences between frequencies using a one-way ANOVA (*, p<0.05; **, p<0.01). P values in tables represent statistical differences between oxygenated control and anoxic response as determined by one-sample t-test (p<0.05). n=3-4 animals, averaging the response of at least 4 muscle strips/animal.
4.3.2 Experiment 2: The effect of inhibiting neuromodulators in nerve-mediated muscle contraction

We next examined the role of different neuromodulatory agents in post-mortem muscle contraction. All contraction properties were measured relative to the anoxic muscle response.

Effect of ATP inhibition in post-mortem nerve-stimulated sheep muscle

Inhibition of ATP hydrolysis in anoxic SM muscle using $\beta,\gamma$-ATP revealed a decline in tension and AUC compared to anoxic control by approximately 50% for both the lower frequencies (both 2 Hz and 30 Hz; $p<0.05$), while the response to high frequency showed no difference to the anoxic control (Figure 4.3). Application of $\beta,\gamma$-ATP resulted in significantly smaller rise time compared to control at 30 Hz only, but a significantly larger fall time for 30 Hz and 80 Hz.

In ST, muscle tension and AUC decreased after ATP inhibition with $\beta,\gamma$-ATP, with responses at all frequencies significantly decreased compared to their respective anoxic control (Figure 4.3). There were significant differences between the responses at the different frequencies for tension, AUC and rise time, suggesting muscle was responsive to different nerve stimuli. The reduction in peak tension was greatest at low (2 Hz and 30 Hz) frequency, while for AUC the response at 30 Hz decreased the most, suggesting that the submaximal stimulation (30 Hz) of anoxic ST was producing an odd response. The rise and fall times did not differ significantly from anoxic control, with the exception of the change in fall time produced by $\beta,\gamma$-ATP at 30 Hz ($p=0.019$).
Figure 4.3. Effect of frequency on ATP-inhibited, anoxic SM and ST muscle contraction characteristics

Data represents the percentage change in anoxic SM and ST muscle response relative to a control response (anoxic muscle response prior to drug incubation) after incubation with β,γ-ATP for 20min. Adjoining lines and asterisks above columns indicate statistical differences between frequencies using a one-way ANOVA (*, p<0.05; **, p<0.01; ***, p<0.001). p values in tables represent statistical differences between oxygenated control and anoxic response as determined by one-sample t-test (p<0.05). n=3-4 animals, averaging the response of at least 4 muscle strips/animal.
Effect of NO inhibition on post-mortem nerve-stimulated sheep muscle

Inhibiting NO in anoxic SM muscle produced a significant decrease in the tension at all frequencies (Figure 4.4), with the response to L-NAME at both 2 Hz and 30 Hz producing a greater relative decrease than at the high frequency (80 Hz). L-NAME also significantly reduced the AUC produced across all frequencies, by comparable amounts (approximately 30-40% less than the anoxic control). In the presence of L-NAME, the rise time was significantly less when stimulated with 30 Hz, and trended towards significance at 2 Hz \( (p=0.066) \); the relative change in fall time only trended towards a significant increase at 30 Hz \( (p=0.052) \) whilst actually increasing at 80 Hz.

Inhibiting NO in anoxic ST significantly reduced the tension response and AUC by more than half at both lower frequencies (Figure 4.4). Interestingly, NO inhibition had no effect on any of the responses to high frequency stimulation (80 Hz). L-NAME did not alter the rise time but did have a significant effect on fall time (reduction) when the muscle was stimulated at 2 Hz.
Figure 4.4. Effect of frequency on NO-inhibited anoxic SM and ST muscle contraction characteristics

Data represents the percentage change in anoxic SM and ST muscle response relative to a control response (anoxic muscle response prior to drug incubation) after incubation with L-NAME for 20min. Adjoining lines and asterisks above columns indicate statistical differences between frequencies using a one-way ANOVA (*, p<0.05; **, p<0.01). p values in tables represent statistical differences between oxygenated control and anoxic response as determined by one-sample t-test (p<0.05). n =3-4 animals, averaging the response of at least 4 muscle strips/animal
Effect of CGRP inhibition on post-mortem nerve-stimulated sheep muscle

Inhibiting CGRP function in post-mortem SM muscle significantly decreased muscle contraction at the lower frequencies compared to the anoxic control (in both tension and AUC; Figure 4.5). The high frequency response was unaltered compared to control. Use of CGRP₈₋₃⁷ reduced rise time when the tissue was stimulated at 30 Hz and 80 Hz albeit only by a small amount (approximately 10%). CGRP inhibition significantly reduced fall time under 2 Hz stimulation, yet increased it under 30 Hz, and there was no significant effect at 80 Hz.

Due to a technical error in the course of these studies, only 2 Hz and 80 Hz were used to induce contraction in CGRP-inhibited ST muscle (Figure 4.5). At 2 Hz, CGRP₈₋₃⁷ significantly reduced peak tension and AUC in anoxic ST muscle, but there was no significant effect on either of these variables at 80 Hz. CGRP inhibition reduced rise time by comparable levels under both 2 Hz and 80 Hz but increased fall time when the tissue was stimulated at 80 Hz only.
Figure 4.5. Effect of frequency on CGRP-inhibited anoxic post-mortem SM and ST muscle contraction characteristics

Data represents the percentage change in anoxic SM and ST muscle response relative to a control response (anoxic muscle response prior to drug incubation) after incubation with CGRP$_{8-37}$ for 20min. Adjoining lines and asterisks above columns indicate statistical differences between frequencies using a one-way ANOVA (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). $p$ values in tables represent statistical differences between oxygenated control and anoxic response as determined by one-sample t-test ($p<0.05$). $n=3-4$ animals, averaging the response of at least 4 muscle strips/animal.
4.3.3 Immunohistochemistry

Immunohistochemistry was used to determine the existence and location of neuromodulators in sheep SM and ST muscle tissue, using vesicular acetylcholine transporter (VACHT; to identify NMJ), nNOS (site of NO production), CGRP (distribution of CGRP peptide within muscle tissue), and P2X7 receptor (presynaptic ATP receptor involved in contraction; also identifies NMJ). Figure 4.6 shows representative images of staining in ST.

Staining for VACHT showed very distinct structures generally localized to the edges of both muscle types (ST Figure 4.6A, SM Figure 4.7A). This transporter is known to be located only in the presynaptic terminal (Weihe et al., 1996) and highlights NMJs present in both muscle types. It also appeared as diffuse intracytoplasmic granular staining in SM (Figure 4.7A). Staining for nNOS and CGRP was similar in both muscle types, with both showing less definition of the staining pattern compared to VACHT (ST Figure 4.6B, SM Figure 4.7B). nNOS is known to be expressed in both the sarcolemma and within the cytosol of muscle fibres, but is concentrated at synaptic sites (Capanni et al., 1998, Ribera et al., 1998). It appears to localise in the vicinity of the NMJ with VACHT (Figure 4.6B). Staining with CGRP produced a similar expression profile, with localisation between VACHT and CGRP in the NMJ area (ST Figure 4.6C, SM 4.7C). Staining for P2X7 was observed in ST muscle, where it appeared to overlap, but not necessarily colocalise, with VACHT at the sarcolemmal edges (Figure 4.6D). Successful immunohistochemistry for P2X7 in SM was not obtained.
Figure 4.6. Immunofluorescence staining of ST muscle for neuromodulators

Panel (A) shows VACChT only staining, arrows highlighting individual NMJs; panel (B) shows VACChT (red)/nNOS (green) staining, with arrows indicating colocalisation of VACChT and nNOS; panel C shows VACChT (red)/CGRP (green) staining, with arrowheads indicating colocalised VACChT and CGRP and arrows highlighting fine CGRP positive (sensory) fibres between individual muscle fibres; panel (D) shows VACChT (red)/P2X7 (green) staining, with arrowheads indicating close overlapping but not actual colocalisation of VACChT and P2X7 along edge of muscle fibre, arrows showing VACChT-stained NMJs. Scale bar = 20um.
Figure 4.7. Immunofluorescence staining of SM muscle for neuromodulators

Panel (A) shows VACHT staining of individual NMJs (arrows) and along edge of muscle fibres (asterisk); panel (B) shows nNOS staining from a side (arrows) and front on view (arrowhead); panel (C) shows CGRP staining (arrows). Scale bar = 20um.
4.3.4 Fibre typing

Figure 4.8 shows representative images of fibre typing in post-mortem SM and ST muscle. With this staining protocol we were able to definitively determine quantitative differences between type I and type II (includes A, X and B) fibre types. Qualitatively, we observed slightly more type I fibres per fascicle in SM (dark spots at pH 4.53). Both type IIA and IIX/B fibres stain dark at pH 10.2, but type IIA presents as light in pH 4.53 whilst type IIX/B appears as intermediate shade. These latter fibres were clearly observed in ST muscle, but indistinguishable in SM muscle limiting our capacity to compare relative proportions of type IIA between muscle types.

Figure 4.8. Fibre typing of sheep SM and ST

Representative images of SM and ST muscle pre-incubated in pH 4.53 and pH 10.2 to identify different fibre types. Type I fibres stained dark at pH 4.53 and light at pH 10.2. Type IIA fibres stained light at pH 4.53 and dark at pH 10.2. Type IIX/B at stained intermediate at pH 4.53 (blue arrows) and dark at pH 10.2. Images taken on a light microscope; scale bar = 500µm
4.4 Discussion

The present chapter examined if, and to what extent, nerve-mediated electrical stimulation could induce muscle contraction in post-mortem sheep muscle, which was suggested by the findings in Chapter 3. We showed that muscle contraction could still be induced via this type of electrical stimulus, but that the contractile response in both ST and SM muscle was significantly diminished compared to oxygenated controls. Overall, post-mortem ST muscle contraction was more adversely affected in the anoxic conditions than SM activity. The type of muscle also influenced the contractile response to nerve stimulation produced by the varying physiological frequencies, with SM muscle producing a distinguishing, and increasing, response to each increase in frequency; a distinction that was mostly lost in ST muscle. It is likely this muscle-specific response arises from differences in both the neuromuscular and metabolic properties of each muscle type, properties previously associated with regulating function in living muscle. Within this study we also examined the role of different neuromodulators in post-mortem muscle contraction, through pharmacological inhibition. Blocking each neuromodulator independently revealed contrasting results that were again muscle-specific, but also varied in terms of which muscle characteristic was affected, suggesting these neuromodulators impart a specific effect on particular elements of contractile process, even in anoxic muscle.
4.4.1 Post-mortem conditions emphasise differences in contractile activity between muscle types in response to nerve-mediated electrical stimulation

Previous post-mortem studies in sheep muscle have suggested that as a consequence of the nervous system failing, only direct electrical stimulation can elicit muscle contraction after death (Chrystall et al., 1980). There is, however, a substantial window during the post-mortem transition in which the nervous system remains responsive to stimuli, as shown in this study and others (England et al., 2013). Observations from our study suggest that processes that regulate nerve-induced muscle contraction in living muscle tissue appear to maintain their influence during the post-mortem transition period, particularly muscle-specific differences. Under normal functioning conditions in living skeletal muscle, the contractile activity that arises from nerve-mediated electrical stimulation is defined by the properties of the motor unit, as this dictates the innervating axon characteristics and the NMJ morphology. The axonal dimension and membrane properties determine if and how the muscle responds to different stimuli frequency, whilst the NMJ characteristics (i.e., number and type of receptors and ion channels, the amount of synaptic vesicles, the structure of the postsynaptic plate, etc) determine the efficiency of the neurotransmission (Prakash et al., 1996, Sieck and Prakash, 1997). The influence of fibre type-specific properties, including ATPase isoforms and Ca^{2+} dynamics that dictate the downstream contractile processes (i.e. E-C coupling and cross-bridge activity), also appear to remain during the transitional post-mortem period.
Post-mortem SM function: better suited to nerve stimulation?

The most notable observation of anoxic sheep muscle stimulated via nerves was that although there was an overall decrease in the magnitude of the peak tension produced during contraction from both muscle types (compared to oxygenated muscle), the pattern with which the muscle responded to the different frequencies was notably different. During nerve-mediated stimulation, the transmission of an electrical impulse is achieved by propagation along the nerves, thus the type of innervation defines the response to this stimuli. Motor units innervating SM muscle discharge at lower frequencies than ST (~40 Hz vs ~80 Hz; Hennig and Lomo, 1985) and it appears that remaining innervation points in the muscle are still responsive to their original discharging frequency in the post-mortem interval. Furthermore, oxidative-glycolytic muscle types are generally innervated by axons that have a lower excitation threshold than the larger axons of glycolytic muscle types, making them easier to excite at lower frequencies (Granit et al., 1957, Eccles et al., 1958); this suggests that lower frequencies remain more suitable for stimulating oxidative-glycolytic type muscles post-mortem (Kernell, 2003).

Our finding that post-mortem SM remains responsive to stimuli similar to its living discharge rate would suggest that ST would do so similarly at its preferred higher frequency (Kernell, 2003). However, we observed that reduction in the ST response under anoxic conditions was similar under the different frequencies. A disconnect between the stimuli frequency (i.e., number of APs) and amount of contraction (i.e. cross-bridge cycling) suggests that post-mortem ST may be more susceptible to aberrant NMJ functioning or crossbridge/metabolism issues than post-mortem SM under the induced anoxic conditions.
Aberrant post-mortem ST function: NMJ issues?

Early physiological studies showed that anoxia hinders nerve conductance by interfering with neurotransmitter release at the presynaptic cleft, which causes inappropriate depolarization and disrupted transmission (Ito and Oshima, 1964, Eccles et al., 1966, Hubbard and Loyning, 1966, Esau, 1994, Lipton, 1999). The degree to which synaptic transmission is disrupted appears to be dependent upon the fibre type (Fox and Kenmore, 1967, Hatzipantelis et al., 2001). This was succinctly demonstrated by Baxter et al. (2008), who showed that the nerve terminals on predominantly fast-twitch mouse muscle fibres were more susceptible to hypoxia than slow-twitch muscles. This fibre type-dependent susceptibility to anoxia likely arises from differences in the NMJ morphology between fibre types (which result from the type of innervation), with an overall increase in size and structural complexity from type I and IIA to type IIX and IIB fibres (Sieck and Prakash, 1997). Differences include size, undulation, length and spacing of junctional folds, transmitter release characteristics, and variation in the density of channels and receptors, all of which influence the efficacy of AP transmission and ultimately the amount of contraction produced by the muscle fibre (Prakash et al., 1996, Sieck and Prakash, 1997, Mantilla et al., 2004, Rowley et al., 2007). In particular, transmission failure (as reflected by a decrease in muscle tension) is more prevalent in type IIX/IIB fibres as a result of their decreased threshold for postsynaptic depolarization and diminished capacity for synaptic vesicle release and cycling (Mantilla et al., 2004, Ermilov et al., 2007). In addition to these properties resulting from the innervation pattern, a more recent study implicated a Ca$^{2+}$-dependent mechanism that detrimentally impacts on anoxic NMJ function in a fibre type-specific manner, with fast-twitch NMJ adversely affected by increased cytosolic Ca$^{2+}$-and subsequent activation of calpains (Talbot et al., 2012).
**Aberrant post-mortem ST function: cross-bridge and metabolism issues?**

Whilst this type of nerve-mediated stimulation does not directly impact upon the myosin ATPase characteristics of the fibres that comprise the examined muscle bundles, it does dictate how much contraction takes place by the speed at which it consumes and produces ATP (Barany, 1967). The rate of myosin ATPase activity in fast-twitch glycolytic fibres is much greater than that in other fibre types, resulting in a greater rate of ATP consumption (Han et al., 2003). This becomes detrimental when energy synthesis is limiting, as it has been reported that there is little difference between the metabolic reserve (maximal rate of ATP production) and the maximal rate of myosin ATPase (ATP usage) in these fibres, which results in a more rapid decline in ATP and total adenine pool (Han et al., 2001). Thus perturbations in the generation of ATP (such as under anoxic and post-mortem conditions) would have a serious impact on the mechanical function of fast-twitch glycolytic fibres, likely resulting in the same amount of contraction regardless of frequency, as observed here (Barany, 1967).

The consumption of ATP during muscle contraction is limited by its production, which during post-mortem is constrained. Ordinarily, skeletal muscle is one of the most hypoxia-resistant tissues as it frequently experiences low oxygen tension during normal exercise (Alberti, 1977). Yet oxygen doesn’t fall instantly after exsanguination, thus allowing muscle to utilize both aerobic and anaerobic processes as required (England et al., 2013). As such, the oxidative capacity of each fibre type likely contributes to its post-mortem function, in particular relating to the content and functioning capabilities of the mitochondria (Crow and Kushmerick, 1983, Schwerzmann et al., 1989, Jackman and Willis, 1996, Faucher et al., 2005, Hudson, 2012). Type I and IIA fibres have a
higher density of mitochondria than type IIX/B fibres, and thus have a greater oxygen capacity, particularly as mitochondria have been shown to retain up to 70% of initial functioning within the first 96 hours post-mortem, thus potentially contributing to SM muscle contraction under anoxic conditions (Planitzer et al., 2001, Gueguen et al., 2005b, Tang et al., 2005). There is also increasing evidence that mitochondrial specialization occurs across fibre type, in relation to respiratory proteins/energy production, ROS metabolism, and regulation by Ca\(^{2+}\) (Gueguen et al., 2005b, Picard et al., 2008). Mitochondria within type IIA fibres have been shown to have a better functional coupling between mitochondrial creatine kinase and oxidative phosphorylation, thus providing more efficient energy synthesis under oxygen-limiting conditions through increased respiration, and subsequent ATP turnover (Jackman and Willis, 1996, Schmidt and Herpin, 1997, Gueguen et al., 2005a, Gueguen et al., 2005b). Similar findings of decreased contractile activity in hypoxic glycolytic fibres were also reported in an in situ study examining rat hind limb muscle, with these findings attributed to the altered function of mitochondria during oxygen-limiting conditions (Howlett and Hogan, 2007). This may explain why we observed a more detrimental impact of anoxia on relative changes in ST muscle contraction, compared to those of the SM. Indeed, although initial fibre typing was done to estimate the percentages of fibre types within the muscle examined here proved inconclusive, a biochemical assay that is more definitive and economical than fibre typing was published after the end of this study, and confirms our rationale, with sheep SM containing nearly double the amount of isocitrate dehydrogenase activity (aerobic indicator, enzyme involved in mitochondrial citric acid cycle) than ST muscle (Gardner et al., 2007).
4.4.2 The role of neuromodulators in post-mortem muscle differs between muscle types

The neuromodulators examined here individually contribute to muscle contraction, yet their role in post-mortem muscle function has been previously unclear, as is how they influence muscle response to nerve-mediated stimulation under such conditions.

ATP: post-mortem ST more dependent on ATP-associated functions

The role of ATP in normal skeletal muscle contraction is well characterised, especially its critical role in maintaining the sliding actin and myosin filaments during the cross-bridge cycle and regulating intracellular Ca$^{2+}$ concentration (Barclay et al., 2007). Under anoxic conditions, ATP supply is limited and is likely to be preferentially targeted towards the biggest consumers of ATP under such conditions, namely myosin ATPase and F$_1$-ATPase in the mitochondria. We observed that inhibiting ATP hydrolysis by these enzymes resulted in the anoxic muscle distinguishing between the nerve-mediated stimuli of 2Hz, 30Hz and 80Hz’ (compared to its anoxic control). It is possible that by blocking the hydrolysis of ATP at these downstream points in the contraction process, a small pool of intracellular ATP accumulates which can then be used at the NMJ to potentiate neurotransmission-related activities, particularly ATP-dependent channels and receptors. This may contribute towards why we observed ST muscle being more receptive to the different frequencies during ATP inhibition [as seen in its differentiation between low (2 Hz and 30 Hz) and high (80 Hz) frequencies].

At the NMJ, one of the main ATP-modulated channels is the presynaptic P2X$_7$ ATP receptor (Moores et al., 2005), and the original experimental design included using both $\beta,\gamma$-ATP and OxATP to inhibit myosin ATPase and P2X$_7$ receptors, respectively. However, initial trials showed this latter agent had no effect in this anoxic set up,
suggesting that P2X7 receptors have little role in post-mortem muscle contraction. It does however indicate a more influential role for a different ATP-regulated channel, the postsynaptic ATP-sensitive K⁺ channel (K_{ATP}; (Noma, 1983). These channels are mainly located on the sarcolemmal membrane and T-tubules (Nielsen et al., 2003), and are associated with coupling the excitability of the muscle membrane to the metabolic state of the cells, as they are primarily activated by decreases in cellular ATP and pH during metabolic stress (Noma, 1983, Flagg et al., 2010, MacIntosh et al., 2012). Under physiological conditions where there is a consistent ATP supply, they remain closed. When ATP levels decline, they open, causing an increased K⁺ efflux that occurs during APs, that in turn results in reduced AP amplitude, decreased membrane excitability and Ca²⁺ release, and subsequently decreased force (Weselcouch et al., 1993, Duty and Allen, 1995, Gong et al., 2003, Cifelli et al., 2008). This decrease in Ca²⁺ leads to a decline in Ca²⁺-related process (both myosin ATPase activity and Ca²⁺ release/re-uptake dynamics), which in turn decreases contraction. This protective mechanism saves ATP when in short supply and avoids excessive fibre damage (MacIntosh et al., 2012). The role of K_{ATP} channels in post-mortem muscle contraction appears to be particularly relevant, for although there is little difference in the propagation of muscle fibre APs or in the E-C coupling between fibre types of normal oxygenated muscle tissue (Greising et al., 2012), multiple points along this process (i.e., the electrochemical process for initiating a muscle contraction) have been identified as being susceptible to failure or aberrant function under anoxic conditions, and this is likely to manifest in a fibre type-specific manner. As such, our muscle-specific observations are supported by several studies that have shown fibre type-dependent differences in the relative content and functioning dynamics of K_{ATP} (2.4 times higher content in mixed glycolytic muscle membrane compared to mixed oxidative muscles; Nielsen et al., 2003, Kristensen et al.,
2006, van Lunteren et al., 2006). In addition, $K_{\text{ATP}}$ channel function is influenced by stimuli frequency, with submaximal stimuli most detrimentally affected (Charter and Murrant, 2014), an observation we also noted. These results suggest that the stimulation of post-mortem ST muscle with nerve-mediated parameters may be attenuated due to preferential use of the limited ATP supply by muscle ATPases, with minimal remaining for neurotransmission-associated function (producing decreased membrane excitability).

**NO: likely influences upstream events in post-mortem ST, but downstream events in post-mortem SM**

The role of NO in post-mortem muscle is complicated as its specific action is dependent upon a number of conditions, including the type of stimulation, concentration of NO, fibre type, redox state, and even the presence of ATP (Eu et al., 1999, Marechal and Gailly, 1999, Hart and Dulhunty, 2000). We observed a subtle and contrasting difference in the way NO inhibition affected muscle contraction in the two muscle types, suggesting that NO affects different muscle properties in a muscle-specific manner.

Our results suggest that NO is involved in positively regulating the neurotransmitter synaptic release from nerve stimulation in post-mortem ST, as its inhibition reduced the tension and AUC significantly at both lower frequencies (2 Hz and 30 Hz). Under normal conditions, NO influences neurotransmission at the NMJ through inhibiting ACh release, thus dictating synaptic strength (i.e. number of activated myofibrils). Our data suggest that NO may act protectively to regulate vesicle release in ST muscle during post-mortem contraction, as type IIX muscle fibres are at
more risk of synaptic depression from vesicle depletion due to a bigger vesicle release per stimulation (Mantilla et al., 2004, Ermilov et al., 2007). This is particularly pertinent under such anoxic conditions, which in itself induces aberrant transmitter release (Lev-Tov and Fishman, 1986, Prakash et al., 1996, Reid et al., 1999, Thomas and Robitaille, 2001).

The role of NO in post-mortem SM muscle appears to be more important in downstream contractile processes, as its inhibition reduced AUC by comparable proportions at low and high frequency stimulation under anoxic conditions. In skeletal muscle, NO is known to regulate E-C coupling and contraction through modification of RYR and myosin ATPase (refer to section 1.2.4; Stamler, 1994, Meszaros et al., 1996, Klebl et al., 1998). These sites in SM muscle may be more dependent on NO modification than in ST, given the enrichment of nNOS in oxidative-glycolytic fibre types (Planitzer et al., 2001, Yu et al., 2008). The removal of NO-mediated RYR inhibition would increase Ca$^{2+}$ release in an unregulated manner, thus causing unrestrained Ca$^{2+}$ release in response to any stimuli. This is supported by the loss of difference in the relative change in fall time (time to relax) compared to the anoxic control, suggesting that the endogenous Ca$^{2+}$ reuptake mechanisms are unable to remove the excessive Ca$^{2+}$. The effect of NO on muscle contraction, however, is also dictated by the interaction between NO and ROS. Given that these molecules compete for the same redox-sensitive sites, it is likely that the increase in ROS (from both contractile activity and post-mortem conditions) and the inhibition of NO allows ROS modification at these sites that are normally preferentially NO-modified in SM muscle (Balon and Nadler, 1994, Reid, 1996, Reid et al., 1998, Hart and Dulhunty, 2000, Murrant and Reid, 2001, Anderson and Neufer, 2006).
CGRP is an important signaling peptide in skeletal muscle that is involved in the maintenance of excitability and contractility in nerve-stimulated muscle contraction (Macdonald et al., 2008). Although its role in anoxic muscle is unclear, our results suggest CGRP has a subtle effect in post-mortem muscle contraction, with more of a role in SM function at submaximal frequencies that may relate to aberrant membrane excitably. This is in line with a previously published role of CGRP, which is known to decrease the relative refractory period of nerve-stimulated muscle via increased activity of the \( \text{Na}^{+}/\text{K}^{+} \)-ATPase, which maintains the transmembrane \( \text{Na}^{+}/\text{K}^{+} \) gradients and membrane potential (Overgaard and Nielsen, 2001, Clausen, 2003, Macdonald et al., 2008). By counterbalancing the inhibitory effect of elevated \( \text{K}^{+} \) that arises during contraction and anoxia, it preserves membrane excitability during repetitive stimulation (Andersen and Clausen, 1993, Fleming et al., 1993, Nielsen et al., 1998). Interestingly, previous studies have shown glycolytic muscles have a higher density of \( \text{Na}^{+}/\text{K}^{+} \)-ATPase pumps, yet pump affinity for \( \text{Na}^{+} \) is greater in oxidative muscles, which correlates with findings that the activity of the \( \text{Na}^{+}/\text{K}^{+} \) pump is linked to the oxidative potential of a muscle (Everts and Clausen, 1992, Clausen, 2003, Fowles et al., 2004, Juel, 2009).

4.5 Conclusions

This study has shown that post-mortem sheep muscle does retain contractile activity in response to nerve-mediated electrical stimulation; however the response is dependent on the muscle type. Post-mortem SM contractile activity was less affected by anoxic conditions, and remained responsive and differentiating to different stimuli. In contrast,
the function of post-mortem ST showed aberrant activity that was likely the result of
dysfunction at several points in the contractile process. These results suggest that SM
may be more suited for nerve-mediated electrical stimulation, as not only is ST
neurotransmission seemingly more susceptible to transitional post-mortem conditions,
but that the inherent biochemical and metabolic properties of glycolytic fibres
detrimentally influence downstream contractile processes under such ATP-limiting
conditions (i.e., during post-mortem). The intrinsic activity of the neuromodulators in
both muscle types also appeared altered under anoxic conditions, with ATP and CGRP
playing slightly more dominating roles in anoxic ST and SM, respectively. The data
also suggests that NO function has more of an impact on neurotransmission in anoxic
ST neurotransmission, but on Ca\textsuperscript{2+} dynamics in anoxic SM.

**Acknowledgements**

Andrew Williams performed the fibre typing and assisted with the *in vitro* muscle
preparations of CGRP inhibition presented in this study.
Supplementary file for Chapter 4: raw data

Table S4.1. Raw data of post-mortem muscle: oxygenated (T-20) to anoxic (T0).

Muscle was stimulated at 0.07ms 30V with 2Hz, 30Hz or 80Hz. Data represent mean (+ SEM)

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<th>T-20</th>
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Table S4.2. Raw data of anoxic post-mortem muscle incubated with $\beta,\gamma$-ATP: pre-drug ($T_0$) to post-drug ($T_{20}$)

Muscle was stimulated at $0.07\text{ms}$ $30V$ with $2\text{Hz}$, $30\text{Hz}$ or $80\text{Hz}$. Data represent mean ($\pm$ SEM)

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Table S4.3. Raw data of anoxic post-mortem muscle incubated with L-NAME: pre-drug ($T_0$) to post-drug ($T_{20}$)

*Muscle was stimulated at 0.07ms 30V with 2Hz, 30Hz or 80Hz. Data represent mean ($\pm$ SEM)*

<table>
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<tr>
<th>Peak Tension (g.cm$^2$)</th>
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<td>438.6 (154.1)</td>
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Table S4.4. Raw data of anoxic post-mortem muscle incubated with CGRP<sub>8-37</sub> : pre-drug (T<sub>0</sub>) to post-drug (T<sub>20</sub>)

Muscle was stimulated at 0.07ms 30V with 2Hz, 30Hz or 80Hz. Data represent mean (± SEM)

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<td>11.4 (12.8)</td>
<td>-</td>
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<tr>
<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>207.7 (26.3)</td>
<td>191.6 (37.0)</td>
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<td>179.8 (58.1)</td>
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<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
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<td>0.60 (0.08)</td>
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<td>47.6 (5.53)</td>
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<td>45.6 (6.75)</td>
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<td>30Hz</td>
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<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>489.9 (134.9)</td>
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<td>-</td>
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<td>80Hz</td>
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<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>297.5 (112.7)</td>
<td>227.0 (72.0)</td>
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<td>T&lt;sub&gt;20&lt;/sub&gt;</td>
<td>375.6 (121.9)</td>
<td>425.6 (135.3)</td>
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Chapter 5.

General Discussion: Applying muscle research to meat science

Electrical stimulation of post-mortem skeletal muscle is a central tenet of current meat processing as a means of ensuring consistency in meat quality between individual carcasses. Refinement of this system has occurred over the last decade through advancement of electrical and computing equipment, resulting in medium voltage electrical stimulation (MVES) systems that produce the beneficial effects on meat quality as produced by high voltage systems, but with the safety and cost effectiveness of low voltage systems (Shaw et al., 2005, Hopkins et al., 2008). Yet even with these new units there remains a lack of scientific understanding relating to the specific interaction of the applied electrical inputs, and the resulting nerve and muscle response of the carcass. This undefined interaction likely contributes to the variation in carcass response to electrical stimulation that has notably remained, regardless of the stimulation system, and the subsequent variation observed in downstream processing applications and end meat products (Hollung et al., 2007, Hopkins and Toohey, 2008). Given that the use of electrical stimulation to enhance meat quality stems from its ability to engage muscle activity, this thesis examined the effect of MVES on contractile properties of post-mortem muscle as a means to identify how this type of system imparts its beneficial effects.
5.1 Do longer pulse widths and modulated frequency affect different aspects of muscle contraction?

MVES units have now been incorporated into many sheep processing facilities around Australia, and have significantly improved the percentage of sheep carcasses that comply with industry sheep meat guidelines (Pearce et al., 2010, Hopkins, 2011). The initial study of this thesis (Chapter 2) was undertaken at the same commercial sheep abattoir where one of the first post-dressing MVES systems was installed, and occurred concomitantly when its electrical parameters were being evaluated and optimised for widespread industry adoption (Pearce et al., 2006, Pearce et al., 2009).

Knowledge of how post-mortem contractile properties are influenced by newer electrical stimulation technologies has received little attention, with the exception of Simmons and colleagues (Simmons et al., 2008). By developing novel methodology to measure *in situ* carcass muscle tension in response to electrical stimulation, they showed it is possible to use indirect contractile properties of the whole carcass as a predictor of downstream meat quality. It is hard, however, to delineate specific responses of muscle to electrical stimulation in a whole carcass set up. The use of isolated nerve-muscle preparations, as performed in this thesis, provided novel information about the effect of MVES-like parameters on various characteristics of muscle contractions, whilst circumventing the issues associated with whole carcass studies (e.g., artifactual effects of fleece, bodily fluids, undefined electrical paths, etc; Chrystall and Devine, 1978). By examining muscle tension in conjunction with several other fundamental characteristics of a muscle contraction (AUC, time to contract and time to relax), we observed subtle differences in the way post-mortem muscle responded to different MVES-like parameters. There were particularly interesting responses from electrical inputs containing either 5 ms pulse width or a modulating
frequency increasing through 15/20/25 Hz, in that both produced the largest decrease in carcass pH (Chapter 2) and also produced the greatest muscle tension (Chapter 3). In a meat context, the degree of muscle tension generated is associated with myofibrillar breakage (Hwang et al., 2003); this action alone contributes to the overall tenderness, but the rupturing of fibres also releases Ca\textsuperscript{2+} which in turn activates degradative enzymes (e.g., calpains, cathepsins; Hollung et al., 2007) that degrade muscle proteins and enhances the tenderizing process. Our results suggest that both these stimulation parameters would generate similar fibre breakage; however the additional finding that the longer pulse width produced also more contraction than the modulated frequency (i.e., more crossbridge cycles), suggests an increased amount of fibre breakage and thus likely produces more tenderness overall (compared to 5ms pulse width), as evidenced by increased tenderness in organoleptic testing (Pearce et al., 2009).

Yet the observation that both stimuli produced similar ΔpH suggests that each causes similar rates of glycolysis (i.e., ATP use to produce similar levels of lactic acid; Gardner et al., 2005), indicating the modulated frequency parameter must engage other ATP-consuming processes/pumps in a non fibre-breaking dependent manner. We speculated that long pulse widths and modulated frequency might engage specific parts of contractile mechanisms differently, such as preferencing the activity of contractile and non-contractile ATPase activity, respectively. However the nature of the data presented in this thesis is not directly transferable to commercial meat research, as these findings highlight overall changes in the contractile response that result from alteration of a number of different processes. As such, further in vitro studies are required to firstly confirm these findings, and secondly delineate the exact mechanisms involved. Such studies would include examining microscopically the amount of fibre breakage in response to these electrical stimuli (e.g., electron microscopy; Hwang et al., 2003),
determining the associated Ca\(^{2+}\) and proteolytic enzyme activity (e.g. calpain: Kooohmarai and Geesink, 2006); calculating the glycogen and ATP usage (Ferguson et al., 2008); and examining the outcome of specific inhibition of different ATPase activities (Lytton et al., 1992, Clausen, 1996, Bartolommei et al., 2013). These physiological studies would have to simultaneously occur with studies on the downstream objective meat properties (such shear force, drip loss; Pearce et al., 2009) to ensure tenderness translation, which is the ultimate use of electrical stimulation. These findings do however highlight the possibility of engaging specific contractile elements of muscle that result in varying intensities of muscle contraction, which would result in different degrees of energy expenditure and consequently a range of ∆pH (Simmons et al., 2006). This flexibility would offer processors refinement of their stimulation settings based upon individual carcasses needs (in conjunction with refinement of the chilling rate, e.g., Toohey et al., 2013), thus avoiding over- and under-stimulation of carcasses and ultimately gaining greater control of the end product quality to meet required industry and market specific needs.

5.2 Muscle type dictates response to MVES-like stimulation

The second novel finding of this thesis was that the effect of the examined stimuli was distinguishable in only one muscle (ST muscle). This muscle-specific response to MVES-like stimuli is in agreement with previous suggestions that electrical stimulation works more effectively on muscle types predominantly comprised of glycolytic fibres (Scopes, 1974, Hunt and Hedrick, 1977, Klont et al., 1998, Gardner et al., 2006). This was thought to extend from the energy state of the muscle at the time of stimulation, as fast-twitch glycolytic fibres are more susceptible to post-mortem glycolysis, thus
resulting in a greater pH decline (Gardner et al., 2005). Our findings suggest that whilst the more oxidative fibre types do also contract in response to these electrical inputs, it may be that they are inappropriate for optimally generating contraction in this type of muscle. Specifically, muscles such as SM (fast-twitch glycolytic-oxidative fibre types) may respond better to stimuli that engages more of the nervous system, as our findings show that post-mortem SM contracts to MVES-like parameters using more nerve-mediated stimulation (Chapter 3). Furthermore, this muscle type produced a distinguishing response to solely nerve-mediated electrical stimulation than post-mortem ST (Chapter 4), suggesting that the parameters examined in this thesis (which were based on those used commercially) are over-stimulating these muscle types, and that more selective electrical inputs may be required to fully engage these muscles. Our data suggests that the innervation pattern of each muscle, including the type and the amount of innervation (features more strongly associated with dictating the function of ‘living’ muscle), also dictates the muscles response to electrical stimulation post-mortem; so although the neurons of the motor unit are removed approximately 20 min before stimulation takes place (animal heads are removed at the beginning of process chain), the axons and terminal branches of the unit retain considerable functionality throughout the carcass, and seemingly so too does their responsiveness to electrical stimuli.

Utilisation of the residual capacity for nerve activity in carcasses would strongly aid in reducing intra-animal variability between muscle groups, and a way to capitalize on differences in the innervation between muscle types would be through the use of specific stimulation parameters aimed at alternating excitation of large and small axons with different stimulation waveforms, as has been achieved in the rehabilitation of paralysed muscle (e.g., using quasitrapezodial-shaped vs. narrow rectangular-shaped
current pulses to activate smaller and larger axons, respectively; (Fang and Mortimer, 1991). Moreover, the varying degrees of direct and nerve-mediated stimulation produced in response to MVES-like parameters may also have implications for different processing procedures, particularly in tailoring electrical inputs for hot boned muscle cuts that would require muscle type-specific parameters.

Processing individual muscle groups may also need to take into account the activity of endogenous modulators, as our observation revealed their actions influence the response to nerve-mediated stimuli in a muscle type-specific manner (Chapter 4). The role of these agents in post-mortem muscle contraction has not previously been investigated, however their role in biochemical processes associated with meat has been. For example, the most examined neuromodulator is ATP, given its critical role in glycogen metabolism and pH development (e.g., (Jacob et al., 2005); several studies have examined the role of NO in meat tenderness, finding it affects both proteolysis and colouring (Cottrell et al., 2008; Cottrell et al., 2015); and whilst CGRP has not directly been linked to meat processes, it potentially influences lipid metabolism in skeletal muscle through induction of lipolysis by blocking insulin effects (Wang et al., 1995, Danaher et al., 2008). Whole carcass experiments under processing conditions are required to fully optimise any nerve-mediated electrical input at a suitable post-slaughter time point similar to when carcasses are commercially stimulated, which is usually 20-30 min post-slaughter.

5.3 Implications for current processing

These muscle-specific findings may explain an observation noted in our abattoir study (Chapter 2). We observed that variation in the LL response to MVES was associated
with hot carcass weight; however it may actually be due to the overall muscle composition of the carcass, i.e., if heavier carcasses are more glycolytic or oxidative based on predominating fibre types in muscles. Longer pulse widths and modulated frequency both produced the largest ΔpH in LL muscle of all carcasses, however only carcasses less than 23Kg (hot carcass weight) produced a ΔpH that distinguished between the varying MVES parameters. This association has not been noted in sheep before, and the responsiveness of the lighter carcasses may result from their particular muscle composition, as variations in the amount of muscle and fat produces differences in hot carcass weight. Over the past decade there has been an increase in genetic selection for muscling traits in sheep as a means of increasing meat yield whilst maintaining (or decreasing) fat levels (Hopkins and Fogarty, 1998, Hopkins et al., 2005, Warner et al., 2007, Ponnampalam et al., 2008, Prieto et al., 2010, Hopkins et al., 2011). This selection process alters the live animal properties and in turn influences the resulting structural composition of the carcass, thus producing variation in carcass weight (Kelman et al., 2014). This practice highlights a challenge facing the processing sector, as varying the proportions of muscle, fat and bone influences the penetration, distribution, and thus effectiveness, of the stimulation treatment. Yet whilst this genetic selection for muscle has a large impact on the overall efficacy of the applied electrical input, the integration of this information into downstream processing events has been limited. In particular, positive selection for muscling traits has been shown to result in an increased proportion of type II fibres in carcass muscles, with a corresponding decrease in oxidative fibres, and in turn is associated with reduced fat levels and subsequently reduced carcass weight (Gardner et al., 2006, Thompson et al., 2006). This suggests that lighter carcasses are leaner and have greater proportion of type II fibres, specifically an increase in type IIX fibre expression (Wegner et al., 2000, Pethick et al.,
Equally supportive, evidence has shown that an increase in hot carcass weight has been correlated with an increase in intramuscular fat (Pethick et al., 2004b, Greenwood et al., 2007), which in turn is associated with an increased percentage of oxidative fibres (type I/type IIA; Hawkins et al., 1985, Kadim et al., 1993, Sazili et al., 2005, Greenwood et al., 2007). A similar trend has also been observed in pigs and rabbits (Gondret et al., 1998, Lefaucheur and Gerrard, 2000). These carcass studies support our theory linking the variation observed in hot carcass weight to the variation in specific muscle response to stimulation. Although we were unable to attain information regarding the genotype of the lighter carcasses that were processed at the abattoir, recent studies support this theory by showing genetic selection of increased lean meat yield decreased the oxidative capacity of the LL, a traditionally oxidative-glycolytic muscle (Kelman et al., 2014, Pannier et al., 2014b), suggesting that in the lighter carcasses sampled this muscle was more representative of glycolytic muscle, and thus why these carcasses were more responsive to stimulation. In addition to selecting for muscle traits, a recent study has revealed animal age to be associated with muscle aerobicity, with lighter and leaner carcasses (i.e., more anaerobic) resulting from younger animals (Pannier et al., 2014a).

5.4 Conclusion

In order electrical stimulation technologies to move forward, a more intimate knowledge of muscle function, from the living animal through to the carcass, and the macro level through to molecular, is required to manipulate events to enhance both processor and consumer needs. The results presented in this thesis indicate that future carcass processing plans should account for phenotypic or genotypic traits of carcasses.
and stratify them accordingly, so as to apply targeted electrical stimulation that is specific for individual carcass properties (such as categorical groups based on hot carcass weight). The incorporation of nerve-mediated parameters into the processing plan would likely bode well for heavier, and more oxidative carcasses, in which direct stimulation may not only be limited due to the inherently non-conductive properties of increased fat levels associated with these types of carcasses, but also the possibly of over-stimulating the majority of muscles due to more oxidative-dominating muscle composition. The ability to engage remaining nerve activity may aid in generating a more homogenous carcass pH decline in combination with direct stimuli to induce robust muscle contraction. The varying degrees of direct and nerve-mediated stimulation induced by MVES parameters may also have implications for different processing procedures, particularly in tailoring electrical inputs for hot boned-muscle cuts that would require muscle type specific parameters. Overall, the results and observations presented in this thesis suggest that more nuanced information could be extracted from integrating muscle contraction information into the processing pipeline, which would augment the role of MVES systems in reducing variation in meat, particularly given the potential of MVES systems to take advantage of the muscles intrinsic architecture and individual muscle composition characteristics. Furthermore, this data highlights the need for further research to be undertaken from the physiological perspective in order to consolidate understanding of the interaction of electrical stimulation and post-mortem muscle response.
A Physiological Understanding of Post-mortem Muscle Stimulation

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