Non-invasive prediction of flavour, tenderness and juiciness for individual animals at point of slaughter – Stage 2

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1.0 EXECUTIVE SUMMARY

The meat industry needs technologies capable of predicting meat quality and managing animal variation to optimise quality and consistency. Preliminary modelling presented in the previous Stage 1 MS3 Milestone Report showed good qualitative comparison with previously published data for energy metabolites; demonstrated the ability of the model to simulate the effects of electrical stimulation; and produced predictions for calpain activity, protein degradation and water holding capacity that are sensible.

The study presented herein is an extension of this model and measures the time-course of energy and flavour metabolites, proteases and other factors for one muscle, without electrical stimulation, from a single animal held at 5 °C. Following slaughter, the meat was characterised using a combination of analytical techniques to assess changes in the key biochemical and biophysical properties, such as the levels of energy metabolites, proteases, structural proteins and water-holding capacity during the conversion of muscle to meat and subsequent ageing, as well as measure important flavour precursors such as intramuscular fat and free amino acids. The measured values obtained from slaughter to final tenderisation were then compared with the predicted values from the model.

The relationships between the predicted and measured values indicates that the model qualitatively describes the dynamic changes in most of the metabolites. The model performed well for most metabolites, with creatine phosphate, adenosine triphosphate (ATP), lactate, pH, hypoxanthine and inosine having an R^2 ranging from 0.64 – 0.92; although the model showed lower performance predicting adenosine diphosphate (ADP), inosine monophosphate (IMP) and adenosine monophosphate (AMP), the breakdown products of energy metabolism, with an R^2 of less than 0.4.

The activities of calpain I and calpastatin released and muscle myofibrillar fragmentation index have been predicted well with an R^2 between 0.66 and 0.94. This system is responsible for tenderisation resulting in the release of free amino acids and peptides. The degradation of the myofibrillar structure is expressed as myofibrillar fragmentation index and the degradation of the proteins during tenderisation affects their tertiary structure thus affecting the drip loss expressed as water-holding capacity of the meat. The drip loss was not well predicted by the model (R^2 of 0.32). The model predicted drip loss to commence later than what was observed under the present experimental conditions and as such the model will likely need to be refined.

The advantage of using a mathematical model to predict meat quality attributes is that easily measured parameters such as pH, temperature or an near infrared spectra can provide the basis for the model outputs, making the model more readily available to commercial plants due to the lower operating cost and technical difficulty compared with other more complex measurement systems.

To conclude, in the energy metabolism system, protease system and meat quality attributes, the model predictions have been shown to have a good degree of fit with the experimental data for most of the key variables, under the experimental conditions used. Further testing will be required to assess the behaviour of the model under a broader range of scenarios and additional optimisation of the model will require data from a large number of animals, representing a wide variety of breed, feed and pre-slaughter handling and with known post-slaughter handling conditions.

Based upon the outcomes of this project the following recommendations are made:

1. Determine model performance for single muscles for a broader range of experimental conditions including muscles low in initial glycogen (high pH), subjected to electrical stimulation and an array of chilling and ageing temperatures using the biochemical, biophysical

and meat quality assays performed here. This will identify which key factors can be consistently modelled well across a range of probable real-world conditions. These key factors will form the basis of any model refinement and provide the relevant reference measures needed for validation.

Once key factors have been proven well-modelled across a range of conditioning temperatures:

2. Develop a non-invasive system capable of measuring the key factors in real-time.
3. Calibrate the non-invasive system for meat eating quality using a trained sensory panel, and specific measurable meat quality attributes.
4. Test and validate a prototype in a commercial processing plant for predicting consumer liking and meat quality attributes

2.0 INTRODUCTION

This project aims to provide meat processors with a non-invasive tool that reliably predicts tenderness, flavour and juiciness outcomes for individual animals/carcasses at or around the time of slaughter through modelling of key biochemical components. Such a tool would allow meat processors to tailor and stream post-slaughter processing operations to account for variation in the individual animals/carcasses and increase earnings from the meat industry through improved quality and consistency of products.

Initially a survey of factors and mechanisms involved in the biochemical and biophysical changes occurring throughout rigor mortis and tenderisation were compiled based on their influence on tenderness, juiciness and flavour outcomes. These key factors became the basis for what would then be modelled and measured; reported in the Project Code 2018-1083 Stage 1 MS2 Milestone Report.

Preliminary mechanistic model equations were developed and subsequently refined to predict tenderness, juiciness and energy-metabolism-related flavour metabolites. The modelling approach used was to simulate changes at the level of a single muscle fibre, allowing the muscle to be simulated as a collection of the fibres with variation in their metabolite concentrations, temperature profiles over time, and reaction rates.

Preliminary modelling showed good qualitative comparison with previously published data for energy metabolites, demonstrated the ability of the model to simulate the effects of electrical stimulation, and produced predictions for calpain activity, protein degradation and water holding capacity that are sensible; reported in the Project Code 2018-1083 Stage 1 MS3 Milestone Report.

The purpose of Stage 2 (Milestones 5, 6 & 7) has been to assess how the model prediction compares with data acquired from a single muscle in a controlled post-slaughter environment. The experimental design was carried out using four striploins (*M. longissimus thoracis et lumborum*) excised from two heifer cows immediately post-slaughter, with time-course sampling carried out throughout rigor mortis and ageing. The experimental design is detailed in Project Code 2018-1083 Stage 2 MS5 Milestone Report.

Each striploin was subjected to a different experimental condition and sampling timeframe:

1. electrically stimulated (5 ^{o}C), 14 days
2. non-stimulated (5 ^{o}C), 14 days

3. non-stimulated (15 °C), 7 days
4. non-stimulated (5 °C) repeat, 14 days

Throughout the rigor and ageing process sub-samples were taken, flash frozen in liquid nitrogen and stored until analysis. Only striploin 2 was characterised (no electrical stimulation, held at 5 °C for 14 days) for comparison with the model. Compositional analysis included: pH, nucleotides/nucleosides, phosphate, lactate, calpain I and calpastatin activities, titin protein (qualitative), glycogen, intramuscular fat, minerals, free and total amino acids, hydrogen ion buffers and thiamine. Meat quality was assessed by drip loss, MIRINZ shear force and myofibrillar fragmentation index. Empirical results were reported in the Project Code 2018-1083 - Stage 2 MS6 Milestone Report.

The primary constraint of the research undertaken in Stage 2 was the requirement for method development for some of the proposed analytes. In the case of quantifying calpain I, calpastatin and titin the assays are not routinely carried out. It was found during method development that components within the samples or solvents used to prepare the meat samples for analysis led to interferences in the measurements using the commercial ELISA kits, and meant the method was unable to be validated. This has meant that quantitative results could not be obtained for the protease system and therefore the model was unable to be compared to the data to determine the goodness of fit. Instead, the model has been compared qualitatively against the results for calpain I and calpastatin activity, with qualitative assessment of titin providing a basis for discussion on protease activity and water holding capacity.

Similarly, quantification of the energy metabolites also posed challenges due to the properties of the analytes and their interaction with the column and buffers used to elute them. The majority of the energy metabolites were able to be accurately quantified, but there was some degree of co-elution of creatine phosphate and creatine with an unknown compound, as well as back-to-back elution of IMP and ADP with a small degree of peak overlap; which means that the quantification of these four analytes may not be fully accurate. This may result in a sub-optimal model fit for these analytes, where it cannot be discerned whether the poor fit is a result of a poor model prediction, or a result of under/over- estimates in the measured analyte.

Finally, Stage 2 experimental work evaluating a range of muscle biochemicals and their comparison to the model predictions has been done at one temperature, for one animal for providing proof-of-concept for the proposed model. As such, the study can only indicate the best suite of approaches needed. For further evaluation of the model applicability, or further optimisation, the experimental work must be repeated for many animals and at many temperatures to capture the natural variation in the animals and their interaction with process conditions.

3.0 PROJECT OBJECTIVES

This project is spread over several different development stages, each relating to a research objective.

Stage 1: Mathematical modelling to identify key variables underlying meat quality attribute pathways.

Milestone 1 –Agreement of project plan and signing of contract.

Milestone 2 – Model variables identified and preliminary models developed (Project Code 2018-1083 Non-invasive prediction of flavour, tenderness and juiciness for individual animals at point of slaughter - Stage 1 MS2 Milestone Report).

Milestone 3 – Preliminary models refined after scrutiny of model behaviour by research team, model description and implications presented in a written report and presented at ICOMST 2018, Project Code 2018-1083 Non-invasive prediction of flavour, tenderness and juiciness for individual animals at point of slaughter - Stages 1 & 2 MS3 Milestone Report.

Milestone 4 – Major Milestone Review by AMPC, with Stop/Go decision.

Stage 2: Measurement of muscle-to-meat factors and sensory indicators and physical attributes in at least one muscle

Time-course measurement of energy and flavour metabolites, proteases and other factors for at least one muscle. Animals will be slaughtered and key factors (metabolites, proteases, and factors regulating them) that are identified in Stage I will be measured during the conversion of muscle to meat using a combination of analytical techniques, for example wet chemistry, LCMS/MS, gas chromatography, and novel proteomic and metabolomics approaches developed in-house in AgResearch. Additionally, standard measures such as dimension, weight and intramuscular fat content will be measured.

Milestone 5 – Collect meat, time-course sampling, sample preparation for laboratory analysis, purchase of consumables (solvents, columns, gels) and dispatch of samples to laboratories. Reported as Project Code 2018-1083 Non-invasive prediction of flavour, tenderness and juiciness for individual animals at point of slaughter - Stage 2 MS5 Milestone Report.

Milestone 6 – Sample analysis. Results collated in spreadsheet. Reported as Project Code 2018-1083 Non-invasive prediction of flavour, tenderness and juiciness for individual animals at point of slaughter - Stage 2 MS6 Milestone Report.

Milestone 7 – Data analysis. Raw data analysed and results interpreted in the context of meat quality and Stage 1 modelling results (summarised in this report).

Further stages are proposed but not yet contracted:

Proposed Stage 3: Technology for non-invasive measurement of key identified factors, calibrated for eating quality using a trained sensory panel.

Proposed Stage 4: Development and calibration of a prototype tool for non-invasive prediction of meat quality.

Proposed Stage 5 Testing and validation of a prototype in a commercial processing plant environment for predicting consumer responses.

4.0 METHODOLOGY

4.1 Collection, Sub-sampling and Chemical Analysis

Four striploins (*M. longissimus thoracis et lumborum*) were excised from the two beef carcasses immediately post-mortem (within 11 minutes of slaughter), with one striploin being electrically stimulated (from the first kill only). The fat cap was removed from each striploin and frozen at -18 ^{o}C. The pH was measured at the anterior of the loin, and two sub-samples were removed from the

posterior of the striploin: approx. 30 g for measuring myofibrillar fragmentation index (MFI), and the remaining sub-sample (approx. 135 g) immediately frozen in liquid nitrogen for further analysis.

The whole striploin samples were then wrapped in seven layers of cling wrap to prevent cold shortening. The wrapped striploins were then placed in a weighted oxygen impermeable bag and held at different rigor temperatures in regulated water baths (5 °C and 15 °C) for 7 or 14 days depending on the treatment type:

1. electrically stimulated (5 °C), 14 days
2. non-stimulated (5 °C), 14 days
3. non-stimulated (15 °C), 7 days
4. non-stimulated (5 °C) repeat, 14 days

Each water bath was regulated separately via a heat exchanger, the 5 °C water bath was maintained by water recirculated through a Julabo F32-HE, and the 15 °C water recirculated through a Julabo F34-HE.

4.1.1 Sub-sampling and Analytes of Interest

Throughout the rigor and ageing process, a total of 30 sub-samples were taken and immediately frozen in liquid nitrogen to minimise further biochemical changes to the sample and stored at -80 °C for subsequent freeze-grinding. The sub-sampling schedule for the non-stimulated, 5 °C treatment which was prepared for further analysis and reported in the Project Code 2018-1083 - Stage 2 MS6 Milestone Report.

Sub-samples obtained from striploin 2, (non-stimulated, 5 °C) were subjected to analyses, the remaining sub-samples from the three untested striploins remained frozen at -80 °C, one of which was subjected to the same rigor and ageing protocol as the striploin analysed here (non-stimulated, 5 °C) for testing in future stages in the event of available budget.

Sub-samples were collected for the following two suites of tests:

Test Suite 1 (Sub-samples collected every timepoint):

- pH
- Nucleotides/nucleosides
- Phosphate
- Lactate (until pH drop has completed)

Test Suite 2 (Sub-samples collected every timepoint post-rigor):

- Proteases (calpain I and calpastatin)
- Cytoskeletal protein (titin)
- Glycogen (two timepoints early post-slaughter, one at end of ageing)
- Intramuscular fatty acid profile (one timepoint early post-slaughter)
- Minerals (one timepoint early post-slaughter)
- Free amino acids (one timepoint early post-slaughter, one at end of ageing)
- Total amino acids (one timepoint early post-slaughter)
- Thiamine (one timepoint early post-slaughter)
- Hydrogen ion buffers including phosphate, carnosine, ornithine (one timepoint early post-slaughter)

4.1.2 Methods for Chemical Analysis

The majority of the analytes of interest were able to be quantified using standard methods either internally, or through external laboratories (Table 1). Of the analytes noted above, quantitative results were unable to be obtained for calpain I, calpastatin and titin. Full methodology for chemical analysis is provided in Appendix 1: Methodology.

Table 1: Methods employed for chemical analysis.

Analyte	Method
pH	pH meat probe
Nucleotides	HPLC
Phosphate	Commercial method, Gribbles Veterinary
Lactate	Commercial method, Gribbles Veterinary
Glycogen	Spectroscopic (UV-VIS)
Intramuscular fat profile	GC-FID
Free & total amino acids and phosphate buffers	HPLC
Minerals	Commercial method, Massey Nutrition Laboratory
Thiamine	Commercial method, Massey Nutrition Laboratory
Calpain and calpastatin activity	Ion exchange chromatography, casein proteolysis
Titin (qualitative analysis)	SDS-PAGE gel electrophoresis
Calpain, calpastatin and titin quantitation	ELISA kit

4.2 Model Development and Comparison of Model Fit

Preliminary mathematical models were developed to represent the evolution of dynamical variables of interest by adapting existing models from the literature or by writing new equations based on first principles. The models were implemented in the open source software package GNU Octave 4.40 (available online at https://www.gnu.org/software/octave). The models were refined using the method of "expert parameterisation", in which members of the research team used their experience to identify deficiencies in qualitative behaviour of the model predictions, reported in Project Code 2018-1083 Stage 2 MS3 Milestone Report.

Model simulations were conducted for a 5˚C non-stimulated fibre using the model parameters and initial conditions established previously, with no further optimisation was undertaken. The model outputs were compared to the empirical data obtained from the chemical analysis (Project Code 2018-1083 Stage 2 MS6 Milestone Report) and statistics describing the relationship between the model simulation and dataset have been determined from the coefficient of determination (R^2) and the standard deviation of the residuals, reported as the root mean square error (RMSE). The measurement error for analytes was determined from the technical replicates carried out in the chemical analysis where applicable.

5.0 PROJECT OUTCOMES

Results obtained for the chemical analysis and meat quality measures are comparable with those typically reported for beef *M. longissimus thoracis et lumborum* produced under similar conditions (no electrical input and similar ageing regime).

Model simulations were conducted for a 5˚C non-stimulated fibre using the model parameters and initial conditions established prior to the experiment. The comparison between the model predicted and the measured fibre variables from the independent experiment are shown in Figures 1 – 11.

5.1 Baseline measures

Four baseline measures associated with the meat quality and flavour development were carried out in this report including intramuscular fatty acid profile, total amino acid profile, mineral content and thiamine.

The fatty acid profile observed for the lean portion of the striploin is shown in Table 2. Comparable fatty acid levels have been reported previously by Knight et al. (2003) in the *M. longissimus lumborum* from New Zealand heifers. Intramuscular fat has been suggested to play an important role in affecting juiciness and production of volatile compounds which contribute to some characteristic cooked meat aroma (Thompson, 2004, Wood et al., 2004). Fatty acid composition of meat is influenced by mostly pre-slaughter factors such as diets, feeding regimes and breeds (Wood et al., 2004).

Table 2: Overview of fatty acid profile and ratios of tested striploin and reference values for New Zealand cows and heifers (Knight et al., 2003).

	Project 2018-1083 Results	**Literature Values**	
Fatty Acid Concentration (mg/100g)		**Heifer Knight *et al* (2003)**	**Cow Knight *et al* (2003)**
Total fatty acids	2440.27	2895	1645
Saturated fatty acids (SATFA)	1052.79	828	793
trans-monounsaturated fatty acids	34.55	49	25
cis-monounsaturated fatty acids	835.84	728	822
Total monounsaturated fatty acids (MUFA)	1016.05	776	847
n-6 polyunsaturated fatty acids	111.95	103	81
n-3 polyunsaturated fatty acids	67.68	78	66
Total polyunsaturated fatty acids (PUFA)	212.22	197	162
Ratio of n-6:n-3	1.65	1.34	1.23
Ratio of PUFA:SATFA	0.20	0.28	0.24
Ratio of MUFA:SATFA	0.97	0.91	1.03

The total amino acid profile for the tested striploin is provided in Table 3. Sixteen common amino acids were detected with comparable concentrations to the amino acid profile reported by the USDA (U.S. Department of Agriculture, 2019). Amino acids are able to contribute directly to the meat flavour in their free forms, and/or participate later in flavour development during the cooking process (Schiffman and Dackis, 1975).

Table 3: Total amino acid profile of striploin measured at 30 minutes post-mortem, compared with reference data from the USDA (U.S. Department of Agriculture, 2019).

Amino Acid Concentration (mg/g)	Project 2018-1083 Results	Literature Values USDA (2019)
Alanine	11.3	10.9
Arginine	12.3	13.8
Aspartic Acid	18.7	-
Cystine	2.6	-
Glutamic Acid	30.7	29.2
Glycine	8.4	11.4
Histidine	8.3	7.5
Isoleucine	9.0	8.9
Leucine	16.0	15.8
Lysine	17.9	16.6
Methionine	6.3	6.9
Phenylalanine	7.9	8.1
Proline	7.3	6.7
Serine	8.0	7.4
Threonine	9.8	7.6
Tyrosine	6.8	7.0
Valine	9.8	9.2

In this project, the amino acid profile of the striploin contained a high percentage of aspartic acid, glutamic acid, alanine, leucine, lysine and arginine. Aspartic acid may contribute to the buttery flavour whereas glutamic acid has a combination of sour and umami taste. Alanine on the other hand provides a combination of sweet and umami taste. Leucine provides malty and fruity flavour. Although the high percentage of lysine and arginine will contribute to a bitter taste, arginine could balance the bitter taste with a slight sweet sensation. The tyrosine and phenylalanine detected in the sample could react with carnosine to provide savoury and umami flavour profiles in the cooked meat (Van Ba et al., 2012).

The nutritional mineral profile was obtained via an accredited ICP-MS method, and is reported in Table 4. The mineral content of the tested striploin was found to be comparable to that of raw New Zealand beef striploin (separable lean only) reported by the USDA (U.S. Department of Agriculture, 2019).

Table 4: Mineral profile of striploin measured at 14 days post-mortem compared with reference data from the USDA (U.S. Department of Agriculture, 2019)

Mineral (mg/kg)	Project 2018-1083 Results	Literature Value USDA (2019)
Calcium	29	40
Copper	0.60	0.55
Iron	24.0	16.1
Magnesium	204	200
Manganese	0.13	0.06
Phosphorus	1905	1770
Potassium	3670	3090
Selenium	0.04	0.03
Sodium	435	470
Zinc	40.0	27.6

The tested striploin was also determined to have 0.04 mg/100 g thiamine, which is comparable to that of raw New Zealand beef striploin (separable lean only) found to contain 0.048 mg/100 g thiamine (U.S. Department of Agriculture, 2019). Thiamine is a vitamin which acts as an important flavour precursor. Thermal degradation of thiamine results in the formation of transient and final volatile compounds, and consequently contributes to the development of meat aroma compounds, such as thiazoles, thiophenes and furans (Dwivedi et al., 1973).

5.2 Energy metabolism

5.2.1 pH and lactate

As shown in Figure 1a, the pH values of the tested striploin linearly decreased post-slaughter from 6.97 to 5.48, which were in line with the predicted changes using the current prototype model with some fluctuation observed in the measured results. The lactate accumulated rapidly during the pre-rigor stage and plateaued after the rigor set in (approx. 22 hours post-mortem) with the final concentration of 91.2 mMol/kg, which is comparable with that observed by Lewis *et al* (89 mMol/kg) (Lewis et al., 1963).

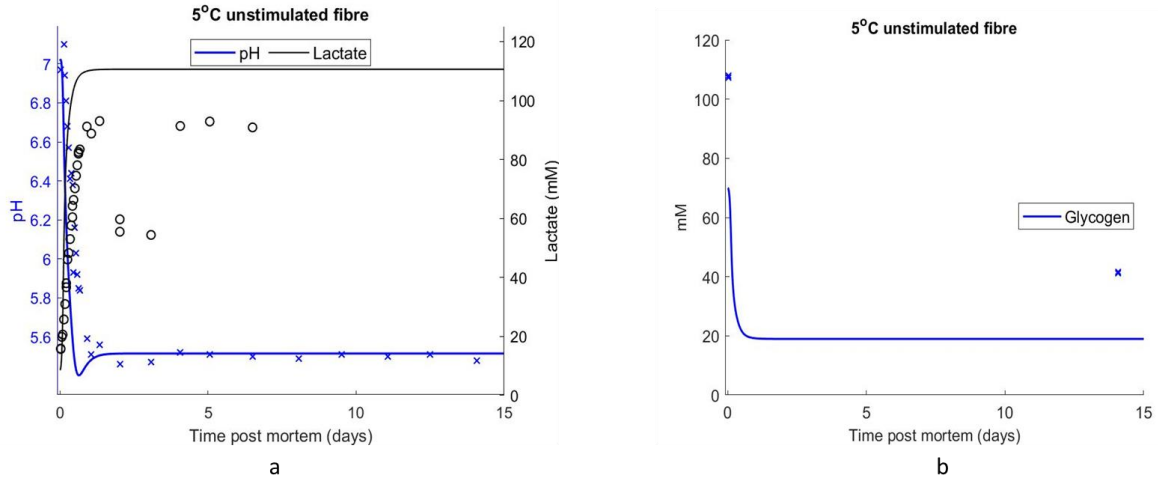

Figure 1: Observed and predicted changes in (a) pH and lactate, (b) glycogen with simulated ageing for 14 days at 5 °C. Solid lines denote the model output.

5.2.2 Glycogen

The depletion of energy during post-mortem ageing resulted in the decrease of glycogen stores from 107.9 mMol/kg to 41.7 mMol/kg shown in Figure 1b, and as expected from the model prediction. The initial glycogen level at slaughter and the residual level following 14 days of simulated ageing process at 5 °C were higher than predicted levels.

Initial and residual glycogen is strongly dependent on the animal's glycogen status prior to slaughter and is influenced by pre-slaughter stresses. The residual glycogen observed here (41.7 mMol/kg) after 14 days of ageing is much higher than that observed by Lewis *et al* (3.3 mMol/kg), likely owing to differences in transportation and lairage conditions prior to slaughter.

5.2.3 Phosphates

Phosphate can be found in organic (phosphoprotein, nucleosides, nucleotides) and inorganic forms (free phosphate, PO_4^{3-}). In general, free inorganic phosphorus represents approximately 50 - 60 % of

the total phosphorus in the *longissimus dorsi* muscle (Lewis et al., 1963). In this study, the total phosphate level detected in the sample after rigor set in was 61.50 mMol/kg (Figure 2) consisting of 33.5 mMol/kg free phosphate (Figure 3a).

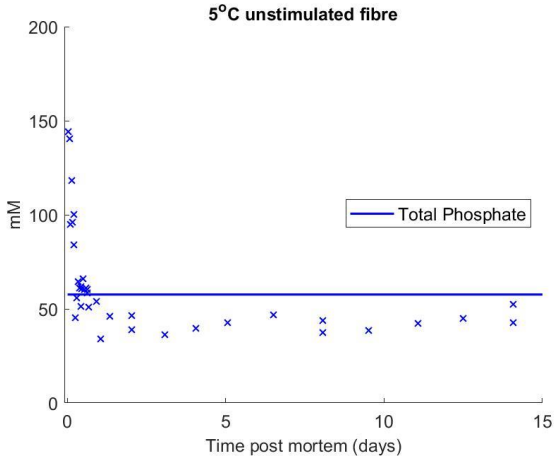

Figure 2: Observed and predicted changes of the concentration of total phosphate with simulated ageing for 14 days at 5 °C. Solid line denotes the model output. Total phosphate (P_{tot}) is defined in the equation for inosine monophosphate (IMP) on p. 11 of Milestone 2 report for Stage 1, July 2018 where P_{tot} = Free phosphate+AMP+2ADP+3ATP+IMP+CP.

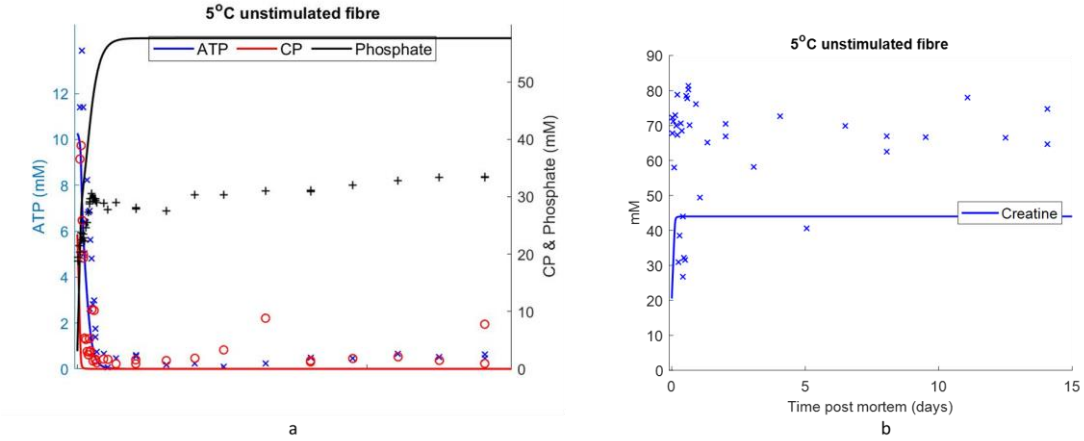

Figure 3: Observed and predicted changes of the concentration of phosphates: (a) adenosine triphosphate (ATP), creatine phosphate (CP) and free phosphate (b) creatine. Solid lines denote the model output.

The decrease of total phosphate concentration during the early stage of rigor mortis was detected and plateaued out during the extended simulated ageing process with minor changes. This was not expected from the model prediction, which was mainly attributed to the significantly reduced levels of creatine phosphate and ATP as shown in Figure 3a. The metabolism of these two metabolites agreed with the model predictions except for the higher initial levels observed. Free phosphate content progressively increased with rigor mortis as predicted and plateaued after about 12 hours of conditioning. As shown in Figure 3b, there was no clear pattern observed for creatine content during first 12 hours of rigor mortis which differed with the model. Some fluctuations of creatine content ranging between 60 - 80 mM was detected, with considerably higher levels compared to model predictions.

The metabolism of nucleotides with simulated ageing process was shown in Figure 4 and Figure 5. The concentration of total nucleotides (A_{tot}) was calculated by the equation as reported previously in

Milestone 2 for Stage 1 in 2018, A_{tot} = ATP+ADP+AMP+IMP+inosine+hypoxanthine. Total nucleotides changed widely during rigor mortis with no clear pattern observed and plateaued out during the extended ageing period, as shown in Figure 4.

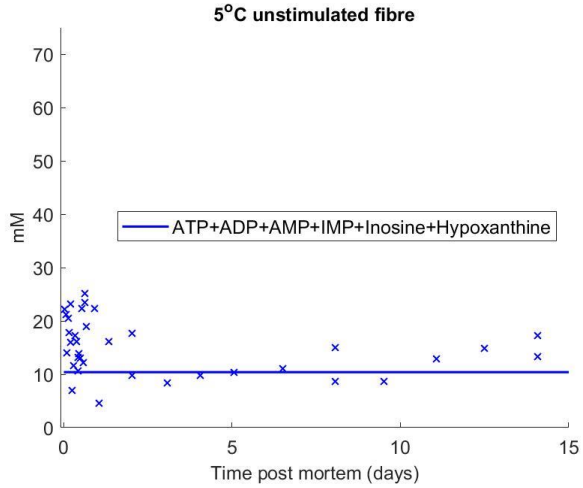

Figure 4: Observed and predicted concentration of total nucleotides. Solid line denotes the model output.Total nucleotides (A_{tot}) is defined in the equation for inosine on p. 11 of the Milestone 2 report for Stage 1 July 2018; A_{tot} = ATP+ADP+AMP+IMP+inosine+hypoxanthine and the definition of hypoxanthine on p. 30 of the Milestone 3 report for Stage 1, September 2018.

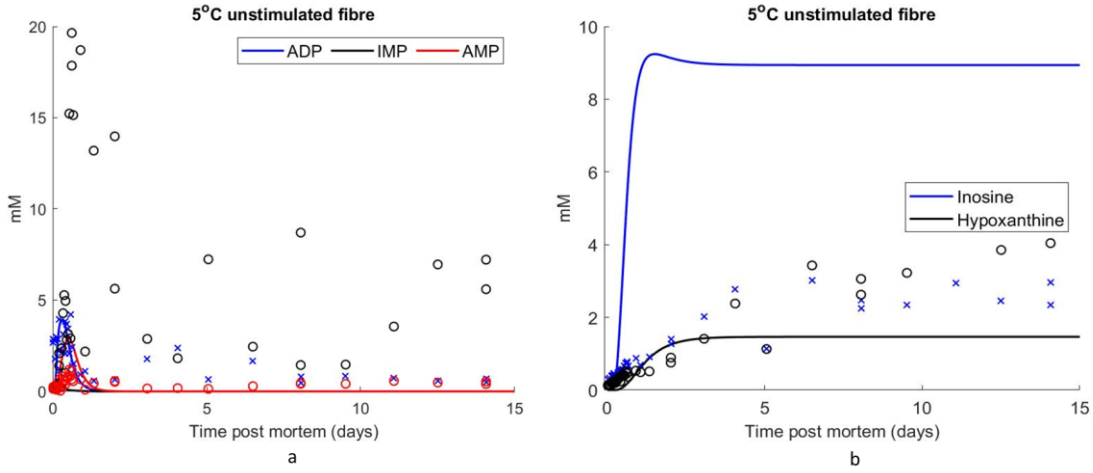

Figure 5: Observed and predicted changes of the concentration of nucleotides: (a) adenosine diphosphate (ADP), inosine monophosphate (IMP), adenosine monophosphate (AMP); (b) inosine and hypoxanthine. Solid lines denote the model output.

The overall metabolic pattern of ADP was paired with the model prediction with some variations during the extended ageing period (Figure 5a). A lower level of AMP was detected during the rigor mortis in comparison with the predicted values. IMP changed widely throughout the ageing process with no clear pattern observed. Inosine and hypoxanthine generally accumulated at a slower rate compared to the model predictions during the early stage of ageing within five days, then plateaued out as expected (Figure 5b). While the peak levels differed from the predicted values.

The variations observed in the present report could be attributed to the chromatography undertaken here resulted in some compounds being sub-optimally resolved. For example, creatine phosphate and creatine co-elute with an unknown compound near the very start of chromatograph (just after the void volume of the column). Overall, ATP, hypoxanthine and inosine were well separated, however IMP and ADP eluted back-to-back on the column with a small degree of peak overlap. Nucleotides are known to be challenging to separate and quantify. In this experiment, there are differences in replicate values for IMP and ADP in some timepoints post-rigor (48, 193 hours), as well as IMP, ATP and Creatine at timepoint 10 hours. The controls showed large levels of variation between all analytes – nevertheless the replicate samples were deemed good enough to indicate 'repeatability' of the extraction and quantification.

5.3 Protease system

5.3.1 Protease Activity

The model prediction was established based on the quantitative changes of calpain and calpastatin in different forms (activated or bound) present in the muscle fibres. Total concentrations of both enzymes decreased with ageing time with a faster autolysis observed in calpain during the early stage of ageing, shown in Figure 6.

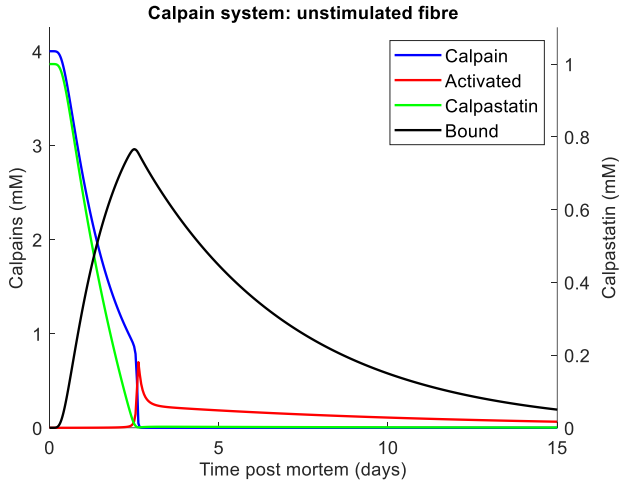

Figure 6: Model simulated concentrations of calpain, activated calpain, calpastatin and bound calpastatin.

In this study, the enzyme activities of extractable calpain I and calpastatin were measured as qualitative estimates of the real concentration. Both enzymes activities reduced progressively with simulated ageing time for 14 days (Figure 7). The extractable calpain I activity reduced faster than calpastatin within 48 hours post-mortem to about 60 % of the at-death activity, meanwhile about 70 % of at-death activity was observed for calpastatin. Both enzymes reached similar levels around 30 % of the at-death activity following 14 days of simulated ageing. The overall changes of the enzyme activity throughout ageing period agreed with the model prediction on the changes of enzyme concentration, suggesting the potential to establish correlation between two features.

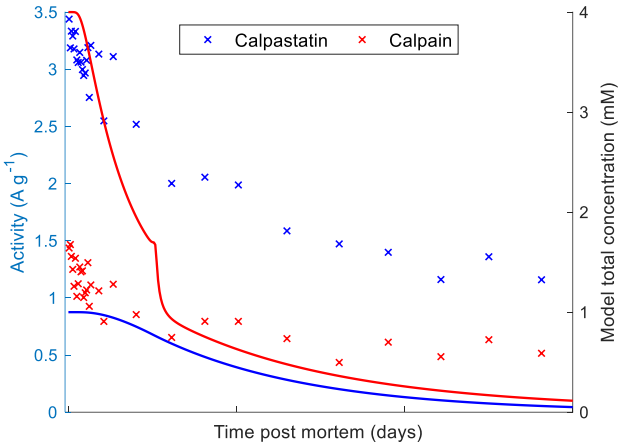

Figure 7: Measured activity of calpain I and calpastatin along with predicted total concentrations of calpain (inactive calpain + activated + bound in Fig. 6) and calpastatin (active calpastatin + bound in Fig. 6). Solid lines denote the model output.

5.3.2 Protein degradation

The myofibrillar fragmentation index of current striploin was measured to correlate with model prediction of protein degradation. As shown in Figure 8, myofibrillar fragmentation progressively increased from 26.7 at 30 minutes to the peak value of 57.9 at 300 hours post-mortem suggesting the occurrence of protein degradation during the simulated ageing process. The general trend of the changes in myofibrillar fragmentation index agreed with the prediction of protein degradation with some discrepancies observed during the early stage of ageing. Based on the current prototype model, protein degradation was expected to initiate after 48 hours post-mortem, at a rate dependent on total calpain activity as indicated in Figure 6. To further refine the model, active calpain I will need to be accurately quantified in future.

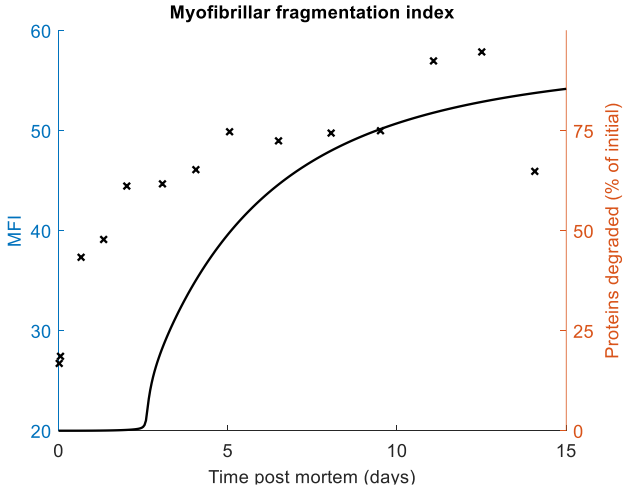

Figure 8: Measured MFI and the model predicted dynamics of the protein degradation during the simulated ageing for 14 days at 5 °C. Solid line denotes the model output.

The degradation of high molecular weight (> 460 kDa) proteins including titin and nebulin was determined using gel electrophoresis as shown in Figure 9. The degradation of titin 1 occurred within the first hour post-slaughter resulting in the appearance of its proteolytic fragments which degraded

further with the post-mortem ageing. The cleavage of nebulin was also observed throughout the ageing period especially during the early stage of ageing.

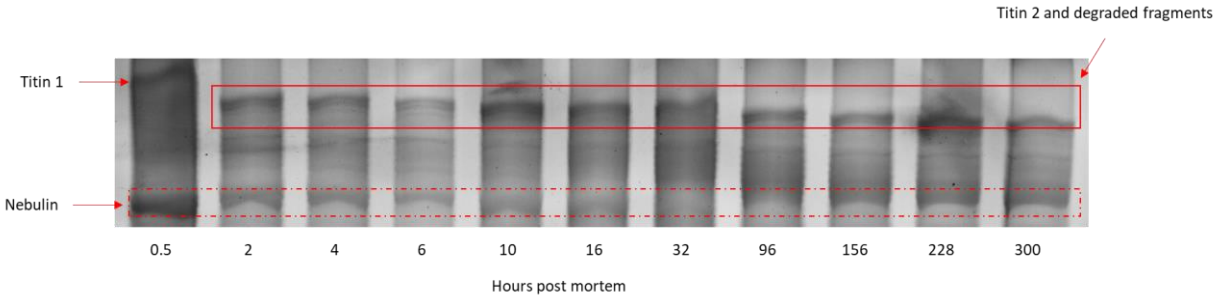

Figure 9: Representative gel electrophoresis of high molecular weight protein (> 460 kDa) throughout the simulated ageing period.

The concentration of free amino acids and dipeptides (anserine and carnosine) at slaughter and post 14 days of simulated ageing were determined using an accredited HPLC method, with results shown in Table 5. The release of free amino acids after ageing was observed as expected, indicated by an approximate two-fold increase in the total free amino acid content compared with the initial level. Four amino acids were observed to decrease after ageing, including β-alanine, glutamine, ornithine and taurine. The greatest increases were observed for leucine, methionine and phenylalanine. Alanine, glutamine and glutamic acid were the dominant free amino acids observed at both sampling time points.

Anserine and carnosine are major dipeptides naturally present in skeletal muscles. A slight decrease of anserine was observed following the ageing process, along with a considerable increase in carnosine. The concentration of free amino acids and dipeptides (anserine and carnosine) for the tested striploin, both pre-rigor and upon 14 days ageing are comparable with results reported in literature as shown in Table 5 (Dashmaa et al., 2013, Polak et al., 2007, Watanabe et al., 2004).

Table 5: Release of free amino acid and dipeptides (µMol/g) at 30 minutes post-mortem and ageing for 14 days at 5 °C. Literature references provide for comparison after 1, 7 or 14 days of ageing (Dashmaa et al., 2013, Polak et al., 2007, Watanabe et al., 2004).

	Project 2018-1083 Results			Literature Values		
	30 minutes post-mortem	**14 days post-mortem**	**Fold change**	**1 day Watanabe *et al* (2004)**	**7 days Dashmaa *et al* (2013)**	**14 days Polak *et al* (2007)**
Amino acid						
Alanine	2.85	5.48	1.92	3.74	4.06	6.56
β-Alanine	0.15	0.13	0.87	-	-	-
Arginine	0.30	0.76	2.53	0.50	0.18	0.95
Asparagine	0.08	0.35	4.38	0.24	0.82	1.33
Aspartic acid	0.08	0.22	2.75	0.58	ND	0.33
Cystine	0.04	-	-	-	0.27	-
Glutamic acid	1.48	2.03	1.37	0.64	0.75	2.4
Glutamine	3.94	2.95	0.75	3.12	4.2	5.42
Glycine	0.57	1.26	2.21	1.26	0.81	1.67
Histidine	0.06	0.31	5.17	0.28	-	0.36
Hydroxyproline	-	-	-	-	-	0.22
Isoleucine	0.10	0.86	8.60	0.46	0.98	0.87
Leucine	0.13	1.51	11.62	0.82	1.56	1.66
Lysine	0.19	0.69	3.63	0.57	1.01	1.13
Methionine	0.04	0.70	17.50	0.28	1.05	0.64
Ornithine	0.25	0.10	0.40	-	-	-
Phenylalanine	0.04	0.71	17.75	0.36	1.57	1.22
Proline	0.11	0.34	3.09	-	0.44	0.59
Serine	0.31	1.36	4.39	0.73	0.29	1.47
Taurine	1.29	0.56	0.43	1.17	-	-
Threonine	0.12	0.73	6.08	0.48	0.93	1.06
Tryptophan	0.12	0.16	1.33	-	-	-
Tyrosine	0.40	0.66	1.65	0.35	0.07	0.6
Valine	0.29	1.33	4.59	0.69	2.12	1.44
Total free amino acids	12.94	23.20	1.79	16.27	21.11	29.92
Dipeptides						
Anserine	1.43	1.15	0.80	3.09	-	-
Carnosine	3.17	17.66	5.57	21.41	-	-

Degradation of proteins during post-mortem ageing also contributed to the tenderisation of meat. The MIRINZ shear force of striploin after 14 days of simulated ageing for 14 days at 5 °C was 11 kgF (107.8 N) which is considered to be the threshold for acceptable tenderness for a consumer. The moderate shear force level observed here could be associated with a low level of cold shortening that might have happened due to conditioning at 5 °C.

5.4 Water-holding capacity

Water holding capacity was estimated by measuring the level of centrifugal drip during the simulated ageing process. Increase in drip loss from the muscle suggests a reduced capacity of the protein to bind and retain the *de novo* water.

De novo water production was modelled proportionally to the extent of protein degradation, with water holding capacity (WHC) modelled to reflect changes occurring as a result of rigor contraction, pH and the effect of protease activity on the cytoskeletal protein (Figure 10).

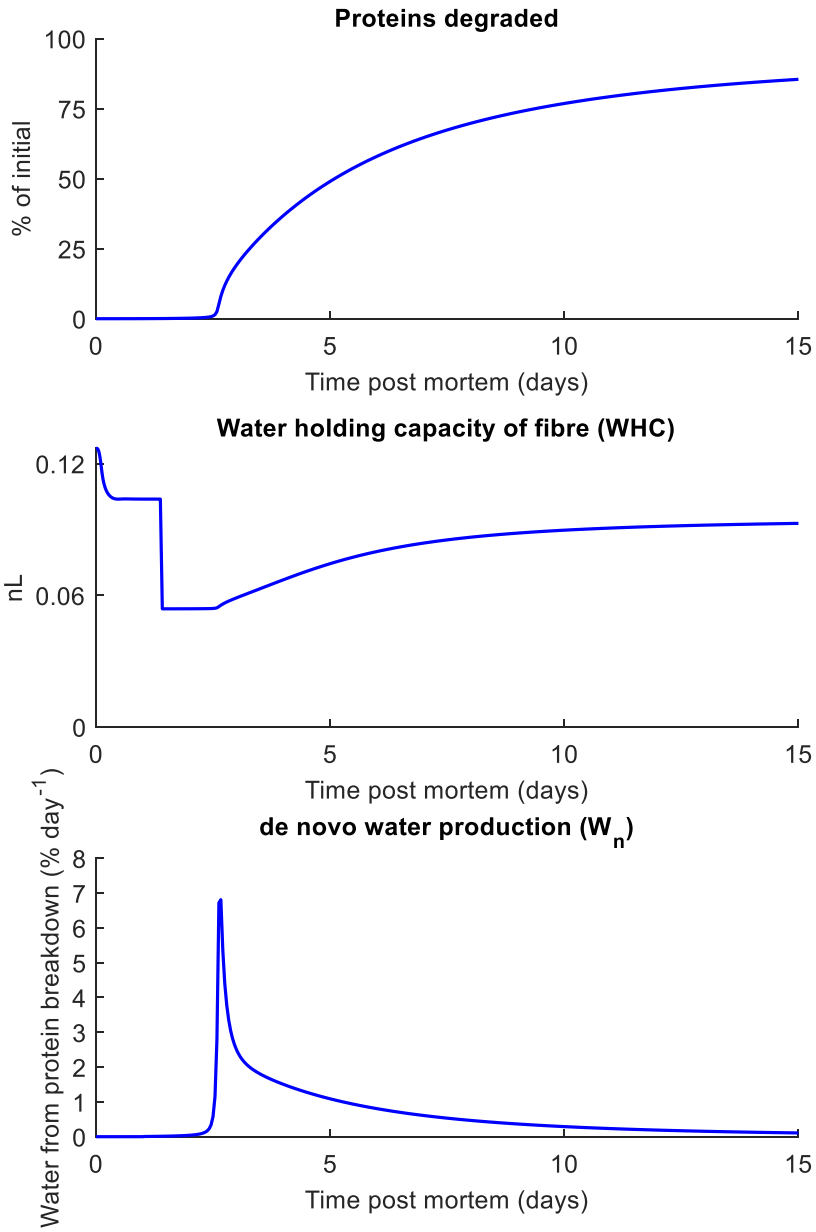

Figure 10: Model simulated dynamics in proteins degraded, water holding capacity (WHC) and de novo water production.

As shown in Figure 11, the drip loss observed experimentally generally remained unchanged during rigor mortis then increased considerably during the early days of ageing; suggesting a decrease in the water-holding capacity associated with the protein degradation as described above. The peak level was observed after 4 days of simulated ageing and decreased afterwards. There is a discrepancy observed during the early post-mortem period (days 1 - 2) between the measured and predicted values.

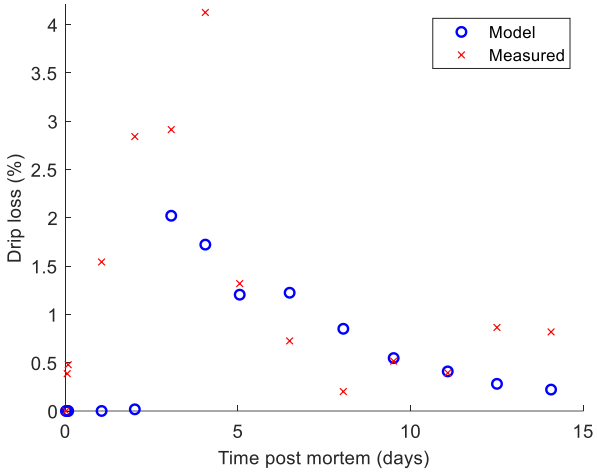

Figure 11: Measured and model prediction of drip loss. The model rate of drip loss is assumed to be proportional to the rate of de novo water (Wn) production.

5.5 Model performance

At a qualitative level, the model compares well with the data as shown in Figures 1 – 11. The relationships between the predicted and measured variables (e.g. Figure 12 for pH) have R^2 up to 0.92 (Table 6), which indicates that the model qualitatively describes the dynamic change in most of the metabolites. However, the R^2 values for ADP, IMP and AMP were all less than 0.5 indicating deficiency in this area. Metabolites with an R^2 significantly different from unity (specifically unity minus the ratio of the measurement error variance divided by the variance in the metabolite over the experiment) suggest a qualitative difference between the measurements and the model simulation.

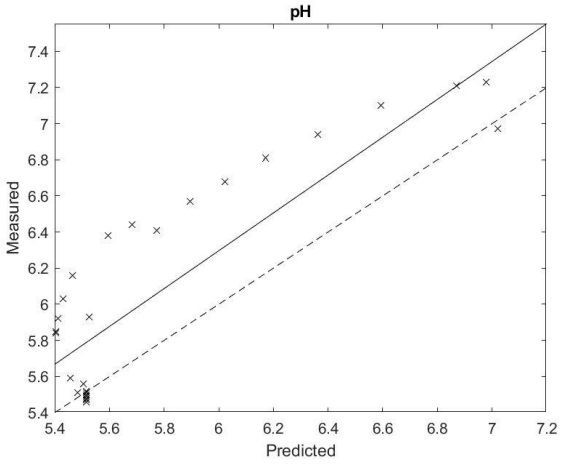

Figure 12: *Relationship between the model predicted and the measured pH during the experiment. The solid line denotes the regression and the dotted line denotes the 1 to 1 line. For this sample the model decrease in pH is greater than in the experiment, although the model initial and final pH are both similar to their respective measurements.*

The measurement error for each assay was estimated from seven technical replicate measurements collected during the experiment (Table 6, based on a normally distributed error model). All assays demonstrate repeatability, although the measurement errors for ADP, IMP, AMP are relatively larger (Table 6 and Figure 3a) and form a greater proportion of the RMSE for the slope/intercept adjusted model predictions (Table 6).

Some of the model assumptions about combinations of variables (total phosphate and total nucleotides) being constant during the experiment appear approximate (Figures 2 and 4), and these assumptions require further testing on a larger set of replicate samples. Further, no attempt was made to adjust the model parameters or initial conditions. The performance of the model on a wider range of samples is required for ongoing calibration and validation purposes.

Table 6: *Table of statistics describing the relationship between the model simulation and the measurements. Standard errors for R^2 for were calculated via bootstrap. RMSE denotes the error for slope/intercept adjusted model (solid line in Figure 15).*

Variable	R^2	RMSE	Intercept	Slope	Bias	Measurement error[c]
Creatine phosphate	0.80 ± 0.10[a]	8.1 mM	5.94 ± 1.41 mM	3.26 ± 0.28	-9.6 mM	2.14 mM
ATP	0.92 ± 0.04	1.82 mM	0.86 ± 0.37 mM	1.70 ± 0.09	-2.6 mM	1.67 mM
Phosphate	0.81 ± 0.06	1.77 mM	16.8 ± 0.9 mM	0.24 ± 0.02	17 mM	0.31 mM
pH	0.74 ± 0.06	0.32	0.009 ± 0.61	1.05 ± 0.10	-0.29	0.05 pH units
Lactate	0.73 ± 0.06	13.3 mM	0.40 ± 7.2 mM	0.67 ± 0.08	29.3 mM	1.87 mM
Inosine	0.64 ± 0.08	0.57 mM	0.20 ± 0.15 mM	0.19 ± 0.03	3.8 mM	0.19 mM
Hypoxanthine	0.69 ± 0.08	0.89 mM	0.12 ± 0.18 mM	1.85 ± 0.21	-0.61 mM	0.33 mM
ADP	0.40 ± 0.12	0.89 mM	1.27 ± 0.20 mM	0.47 ± 0.10	-0.53 mM	0.71 mM
IMP	0.04 ± 0.04	5.81 mM	6.4 ± 1.2 mM	-11.3 ±10.7	-5.6 mM	3.47 mM
AMP	0.42 ± 0.11	0.22 mM	0.31 ± 0.05 mM	0.16 ± 0.03	0.53 mM	0.18 mM
Glycogen	1.0[b]	0.48 mM	16.6 ± 0.48 mM	1.31 ± 0.01	-30.2 mM	0.48 mM
Calpain activity	0.80	0.13 A g^{-1}	0.54 ± 0.05 A g^{-1}	0.17 ± 0.02 A g^{-1} mM^{-1}	0	NA
Calpastatin activity	0.94	0.19 A g^{-1}	0.98 ± 0.09 A g^{-1}	2.1 ± 0.1 A g^{-1} mM^{-1}	0	NA
MFI	0.66	5.6	36 ± 3	0.21 ± 0.05[*]	0	NA
Drip loss	0.32	1.06 %	0.62 ± 0.38 %	1.0 ± 0.43	0	NA

[*] % of initial proteins degraded

[a] R^2 assumes the errors are homoscedastic and does not provide information about whether the prediction lags measurement.

[b] Glycogen was measured at the start and end of the experiment so R^2 is expected to be unity.

[c] Measurement error was determined from seven technical replicate measurements during the experiment (based on a normally distributed error model).

NA denotes no technical replicate measurements.

6.0 DISCUSSION

The ability to predict the quality of conditioned meat very early post-mortem, determined from a prediction equation or measured non-invasively early during the rigor process, will be a great advantage to the meat industry. In countries such as Australia and New Zealand, the commercial reality is that a number of key export markets are a long distance from the producer, and as a result some of their exported meat is held chilled for long periods of time before it reaches the ultimate consumer or processor. For such countries, the ability to use easily controlled factors such as the rate of chilling, muscle composition and/or metabolites early pre-rigor to predict the quality of the meat after storage will enable suppliers in these countries to supply a more consistent product (Farouk and Lovatt, 2000).

Several studies have been published in which muscle pre-rigor process conditions and/or muscle biochemical or biophysical states were used to develop models for predicting meat quality attributes later post-rigor (Dransfield et al., 1992, Ledward, 1985, Offer, 1991, Farouk and Lovatt, 2000, Young et al., 1999, Vetharaniam et al., 2010, Simmons et al., 1996). To our knowledge, there has been no study that has attempted to use the number of muscle and meat metabolites used in the present study to predict meat quality post-mortem modelled from a single muscle fibre. The current project assessed the performance of the prototype models using a single beef *M. longissimus thoracis et lumborum* muscle with no electrical stimulation followed by conditioning for 14 days at 5 °C. The time-course measurements of some key biochemical parameters started from 30 minutes after slaughter to 14 days of simulated ageing. The collected data was directly compared with the model predictions for the dynamic biochemical changes in the muscle.

During the conversion of muscle to meat, the depletion of residual energy results in a series of biochemical changes associated with the changes in pH values, lactate, glycogen and phosphates as determined in the present project. The high correlation in the present study between measured and predicted products of muscle biochemical and biophysical processes suggest that within the parameters of this study (non-stimulated single muscle fibre held at 5 °C), that some of these products or metabolites could be measured early pre-rigor and be used to predict the quality of meat after ageing. For example, some of the metabolites such as creatine phosphate, ATP, phosphate, pH, lactate, inosine and hypoxanthine levels could be used due to their higher degree of predictability ($R^2 > 0.6$) compared to those with a lower degree of predictability like ADP, IMP and AMP.

A decrease in the concentration of total phosphate and nucleotides during the early stage of rigor mortis has been previously reported (Calkins et al., 1982) and is also observed within this study. This contrasts with the current model prediction, which indicates that constant levels should be expected throughout the simulated ageing. There may be some other factors, such as drip loss or other chemical reactions involving phosphate containing energy metabolites which result in phosphate end-products, that have not been accounted for which result in the observed differences between the experimental results and model predictions.

The activities of calpain and calpastatin and muscle myofibrillar fragmentation index have been predicted well ($R^2 > 0.6$) in the present study (Table 6). Enzymes such as calpains are responsible for breaking the highly organised muscle myofibrillar structure and the degradation of key proteins resulting in the release of free amino acids and peptides. The degradation of the myofibrillar structure is expressed as myofibrillar fragmentation index. The state of the muscle myofibrillar structure combined with the biochemical states of the meat proteins directly affects the water-holding capacity of the meat (Farouk et al., 2012).

The calpain I and calpastatin activity determined in this project were comparable with the levels reported by Ferguson et al. (2000). The changes in the activity of calpain I and calpastatin with post-mortem ageing qualitatively correlated (R^2 = 0.80 and 0.94, respectively) with the dynamic changes in enzyme concentration. The initially high activity of calpain I and its rapid decrease during the first two days post-mortem is consistent with what was observed during the conversion of bovine muscles of low, intermediate and high ultimate pHs (Lomiwes et al., 2013). The decreasing activity of calpain I early post-mortem may be interpreted as an indicator of calpain I activation and autolysis. Although some lower level of activity of calpain I is detected later than 48 hours post-mortem in the present study, Lomiwes et al. (2014) posited that due to the relatively rapid autolysis of calpain I to the inactive 76 kDa isoform in post-mortem cells, it is highly likely that their role in meat tenderness is restricted to the first 48 hours after slaughter.

Protein degradation was assessed through myofibrillar fragmentation index, and further explored using gel electrophoresis and free amino acids content. Large protein molecules such as titin and nebulin degraded quickly after slaughter, with only a slight increase in myofibrillar fragmentation observed. The rapid breakdown of titin and other large molecular weight proteins such as nebulin and filamin was previously reported (Wu et al., 2014a, Wu et al., 2014b, Lomiwes et al., 2013, Lomiwes et al., 2014). The rapid breakdown of titin is due to the early activation of calpain I, which is optimally active at physiological pH. The breakdown in titin is not expected to impact significantly on myofibrillar fragmentation because it is mostly associated with the fragmentation of intra-myofibrillar linkages in the I-band rather than the breakdown of the myofibrils *per se* (Lomiwes et al., 2013).

Myofibrillar protein degradation increased significantly after rigor mortis, then progressively plateaued with ageing time. Concomitant with this increase was the increased level of free amino acids and fragmentation of myofibrils. Myofibrillar fragmentation index has been widely used to predict protein degradation and correlates well with sensory tenderness. Discrepancy was observed in model prediction at the early stage of post-mortem from myofibrillar fragmentation results. Calibration of the model may be necessary if using myofibrillar fragmentation index as a measure of protein degradation.

The water-holding capacity measured as drip loss increased initially and then decreased with time post-mortem (Figure 11). The observed change in the drip mirrored the predicted initial decrease in water-holding capacity and its subsequent increase with time; and the degradation of protein and the initial increase and subsequent decrease in the *de novo* water production (Figure 10). A weak correlation was observed between measured drip loss with the model prediction, probably due to *de novo* water being assumed to remain unchanged during the first 2 - 3 days post-mortem. The initial increase and subsequent decrease in drip loss observed in the present study has been consistently observed in intact bovine, ovine and cervine muscles/meat held chilled for extended periods (Farouk et al., 2007, Farouk, 2007, Farouk et al., 2009).

The underlying mechanisms responsible for changes in water holding capacity during ageing are not yet fully elucidated. Farouk et al. (2012) summarised several hypotheses put forward to explain the improvement in water-holding of meat with long term ageing:

1. Offer and Trinick (1983) hypothesised that the gain or loss of water in meat is due to the swelling or shrinkage of myofibrils as a result of the expansion or shrinkage of the filament lattice
2. Joo et al. (1999) and Van Moeseke and De Smet (1999) attributed the improvement in water-holding capacity in pork to the loss of water early post-mortem in the form of drip or

evaporation resulting in less water being lost at a later time post-mortem (the "leaking out" hypothesis)

3. Kristensen and Purslow (2001a) discounted the "leaking out" hypothesis when they observed improvement in the water-holding capacity of pork without any changes in the total water content of the samples, and in turn, they hypothesised that the improvement in water-holding capacity they observed in pork was due to the degradation of cytoskeletal proteins during ageing, which reduces the rigor-induced lateral shrinkage of myofibrils, associated with the formation of drip and also enable the inflow of previously expelled water, thereby improving the water-holding capacity.
4. Farouk et al. (2012) proposed the improvement in water-holding capacity post-mortem results from the structural breakdown of meat, resulting in the disruption of the channels through which drip from meat is lost in the form of exudates by forming a "sponge like effect", slowing the flow of the drip to the cut surface of meat. The extent of this muscle/meat structural breakdown and the disruption caused to drip channels could determine the degree of improvement in water-holding capacity with ageing and the higher water holding of high pH compared to low pH meats.

The validation of the current model would provide a valuable research tool to investigate these hypotheses which would support the development of better predictive models in future.

As meat quality is determined by the interactions of a number of biochemical and biophysical processes, it is likely that a number of these metabolites and products (rather than any single one of them) will be required to obtain a robust prediction of overall meat quality post-mortem. More specifically, the relationship between each of the metabolites and products, and their subsequent relationship with meat quality attributes. For example, the amount of glycogen at slaughter is a good indicator of the ultimate pH and lactate levels in meat, and ultimate pH is a good indicator of meat quality due to its relationship with meat colour and water-holding capacity.

This study followed one muscle from one non-stimulated animal only. Thus, one important determinant of meat quality such as that influenced by preslaughter stress was not considered, although the measurement tools were used in this study. In particular, the rigor process affects subsequent proteolytic activity and hence tenderisation. The use of one animal also does not consider that there are procedures such as electrical stimulation that are transformative in the positive effect of tenderisation.

7.0 CONCLUSIONS/RECOMMENDATIONS

Meat quality is determined by the interactions of a number of biochemical and biophysical processes occurring post-slaughter which are impacted by the pre- and post-slaughter environments. Therefore, obtaining a robust prediction of overall meat quality post-mortem will require a number of these metabolites and products (rather than any single one of them) to be measured. The advantage of using a mathematical model to predict meat quality attributes is that easily measured parameters such as glycogen, pH, temperature or a near infrared spectra can provide the basis for the model outputs, making the model more readily available to commercial plants due to the lower operating cost and technical difficulty compared with alternative or *ad hoc* measurement systems such as the use of sensory panels.

The model performed well for most metabolites, with creatine phosphate, ATP, lactate, pH, hypoxanthine and inosine having an R^2 ranging from 0.64 - 0.92; although the model showed lower

performance predicting ADP, IMP and AMP (the breakdown products of energy metabolism) with an R^2 of less than 0.4.

In addition, the model showed a good degree of fit for protease activities and protein degradation, with an R^2 between 0.66 and 0.94. However, some refinement of the model may be required for predicting water holding capacity which showed a lag in the timeline for drip loss, evidenced by an R^2 of 0.32.

In both cases, it has been shown that the model predictions have a good degree of fit with the experimental data for most of the key variables, under the experimental conditions used. However, as the reaction rates for both energy metabolism and proteolysis change with temperature – in a non-uniform way. It is not possible to assume that the model will also predict these variables equally well for meat handled using a different set of experimental conditions.

The model developed here is in a preliminary phase and requires considerably more work to test the behaviour of the model under a range of scenarios, and to identify where it can be improved and extended if necessary. Further optimisation of the model will require data from a large number of animals, representing a wide variety of breed, feed and pre-slaughter handling and with known post-slaughter handling conditions.

Based upon the outcomes of this project the following recommendations are made:

1. Determine model performance for single muscles for a broader range of experimental conditions including muscles low in initial glycogen (high pH), subjected to electrical stimulation and an array of chilling and ageing temperatures using the biochemical, biophysical and meat quality assays performed here. This will identify which key factors can be consistently modelled well across a range of probable real-world conditions. These key factors will form the basis of any model refinement and provide the relevant reference measures needed for validation.

Once key factors have been proven well-modelled across a range of conditioning temperatures:

2. Develop a non-invasive system capable of measuring the key factors in real-time.
3. Calibrate the non-invasive system for meat eating quality using a trained sensory panel, and specific measurable meat quality attributes.
4. Test and validate a prototype in a commercial processing plant for predicting consumer liking and meat quality attributes.

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9.0 APPENDIX 1

9.1 Sampling and cryogenic grinding

Throughout the rigor mortis and ageing process, 20 - 60 g sub-samples were sliced from the posterior end of the striploin and immediately frozen in liquid nitrogen. Sampling was carried out typically every hour until rigor set in and every 24 - 26 hours thereafter. Frozen sub-samples were pulverised with liquid nitrogen using a freezer mill (Freezer/Mill, 6970EFM, SPEX SamplePrep) and divided up for analysis. All sub-samples were stored at -80 °C at all times until further analysis.

9.2 Suite 1 Tests

9.2.1 pH measurement

The pH of the meat at each sampling point was measured with a portable pH meter (HANNA® HI99163 Portable Meat pH Meter, Mediray, New Zealand) with automatic temperature compensation. The pH meter was calibrated at pH 7.0 and 4.0 with buffers (Hanna Calibration Solution HI7007L and HI7004L, Hanna Instruments, Mediray, New Zealand) stored at room temperature (20 °C). Duplicate measurements were performed.

9.2.2 Lactate, phosphate and nucleosides

Reagents

Perchloric acid, AR grade 70.0% purity, Ajax Finechem
Extraction buffer: 0.6 M perchloric acid

Perchloric acid (0.6 M) solution was prepared by pipetting 52 mL of 70% perchloric acid into a 1000 mL volumetric flask and diluted to volume with distilled water.

Sample preparation for lactate, phosphate and nucleosides

The analysis of glycogen, lactate, phosphate and energy metabolites (nucleosides and nucleotides) was carried out on the pulverised sub-samples. Briefly, frozen meat sub-samples (approx. 1.0 g) were homogenised in 0.6 M perchloric acid to precipitate the proteins present followed by centrifugation. An aliquot of the supernatant was kept for glycogen measurement (Suite 1 Tests), while the remaining supernatant was neutralised and for physiological phosphate, lactate and nucleoside measurements.

Frozen sub-samples of approx. 1g (exact weight recorded using 4 decimal place balance) were prepared by weighing directly into a centrifuge tube (12 mL), immediately adding ice cold 0.6 M perchloric acid (9 mL) and homogenising (Ultra-turrax, IKA) for 30 - 40 seconds.

The homogenate was stored on ice for 30 minutes before centrifuging at 17000 x *g* (10000 rpm) for 15 minutes at 4°C (Sorvall Lynx 4000, Thermo Scientific). The supernatant was then transferred into a clean tube and re-centrifuged to obtained clear supernatant.

Glycogen

An aliquot of supernatant (1.5 mL) was transferred into a 2 mL microcentrifuge tube and stored at -80 °C until analysis (Section 9.3.1).

Lactate, physiological phosphate and nucleotides

An aliquot of supernatant (4 mL) was neutralised using potassium carbonate (5 M) and kept cool in an ice bath while adding the potassium carbonate solution in 0.1 mL increments using a pipette until the mixture is was pH neutral. Regular vortexing and gas release were also performed. The pH was determined using pH strips and the exact volume of potassium carbonate solution was recorded. The neutralised mixtures were kept in an ice bath for 30 minutes before centrifuged at 3000 x *g* for 5 minutes.

Aliquots of 1.5 mL neutralised supernatants were transferred to 2 mL microcentrifuge tubes and frozen at -80 °C for lactate and physiological phosphate analysis (Gribbles Veterinary, Hamilton NZ).

Aliquots of 200 µL of the neutralised supernatant were transferred into 2 mL HPLC vials with inserts (Vial screw top TPX with glass 200uL insert, Thermo Scientific, NZ) for nucleoside/nucleotide analysis.

Physiological phosphate and lactate analysis

Physiological phosphate and lactate analysis were carried out on aliquots of neutralised supernatant by a commercial lab (Gribbles Veterinary Pathology Limited, Mosgiel, New Zealand). The in-house standard procedures for determining serum phosphate (trace) and lactate were used. Results are expressed as concentration in mMol per kilogram of meat sample after correcting for the dilution factor.

Nucleoside analysis (energy metabolites)

Reference samples

A reference sample (approx. 200 g) of *M. longissimus thoracis et lumborum* muscle from hot-boned bull beef was collected from a local abattoir within the first hour of slaughter. The sample was halved into two sub-samples as pre-rigor and rigor reference samples. Pre-rigor sample was prepared following the procedure outlined in Section 9.1 straight after collection. The remaining half was vacuum packed and stored at 10 °C for 3 days until rigor set in before further processed as described in Section 9.1. Both reference samples were prepared for the analysis of nucleosides and nucleotides following the same methods of meat samples as described above. The two reference samples were used to establish whether the HPLC-UV method was able to detect differences in nucleoside concentration owing to changes in the muscle pre- and post-rigor.

HPLC-UV Method

Chromatographic separation of nucleotides and nucleosides was carried out according to the method developed by Qiu *et al* (Qiu et al., 2016). In brief, the neutralised supernatant consisting of nucleotides and nucleosides were load on a Shiseido Capcell Pak C18 column, 4.6 mm x 250 mm, 5 µm (Phenomenex, New Zealand) with mobile phases (A) 20 mMol/L KH_2P0_4:20 K_2HPO_4 (V/V 1:1) adjusted to pH 5.8 with phosphoric acid; (B) Methanol. The injection volume of 10 µL with a flow rate of 0.8 mL/minute were used. Column temperature was held at 30 °C. Detection wavelengths of 210 nm for creatine, creatine and phosphate and 250 nm for nucleosides (DAD 205 nm to 300 nm collected) were used. Results used for comparison with model.

Buffer gradient:

10.00 minutes Pumps Solvent B Conc. 0
20.00 minutes Pumps Solvent B Conc. 30
21.00 minutes Pumps Solvent B Conc. 0
27.00 minutes Controller Stop

LCMS/MS Method Development

An LCMS method was trialled using a Phenomenx Gemini column with a pH 10 ammonium acetate buffer. Initial indications were that this method worked well for separating nucleosides from the control pieces of meat used in the HPLC-UV method.

However, sample analysis could not be completed due to a loss of sensitivity (ion suppression) in the MS owing to potential residual contamination from other samples analysed by another MS user; and the ion suppression was unable to resolved by in-house maintenance within the project timeframe.

9.3 Suite 2 Tests

9.3.1 Glycogen

Reagents

0.6 M perchloric acid, prepared from Perchloric acid, AR grade 70.0% purity, Ajax Finechem
Glycogen, AR grade 85.0% purity, Sigma-Aldrich
Iodine, Fisher Scientific
Potassium iodide, AR grade 99.0% purity, Thermo Fisher Scientific
Calcium chloride, AR grade 99.0% purity, Ajax Finechem

Sample preparation for glycogen

Freeze-ground glycogen sub-samples were prepared using the method for the preparation of lactate, phosphate and energy metabolites (nucleosides and nucleotides) outlined in the Suite 1 tests (Section 9.2.2).

Glycogen analysis

An aliquot of the supernatant is treated with iodine reagent and the resulting colour is measured spectrophotometrically.

Reagents

Iodine-iodide solution was prepared by dissolving 0.26 g iodine and 2.6 g potassium iodide into 10 mL of distilled water. This solution was stored at 4 °C in a brown reagent bottle. A saturated solution of calcium chloride was prepared the day before use and kept at room temperature. Iodine reagent was prepared freshly by combining 130 mL of saturated calcium chloride solution with 0.5 mL iodine-iodide solution each day of use.

Preparation of the standard curve

A glycogen stock solution was prepared by addition of 0.15 g of glycogen to 10 mL distilled water in a volumetric flask (15 mg/mL). Working standards were prepared by diluting the stock solution 1:9 in perchloric acid solution (0.6 M). Standards were prepared as follows:

#	µL working standard	µL 0.6 M Perchloric	Conc. mg total in 3 mL	Expected absorbances
1	0	400	0.0	0.318
2	25	375	0.0375	0.486

3	50	350	0.075	0.629
4	100	300	0.15	0.980
5	200	200	0.3	1.603
6	300	100	0.45	2.182
7	400	0	0.60	2.719

Add 2.6 mL of iodine reagent to each tube and mix by vortexing and allow to stand for at least 10 minutes. Absorbance was read at 460 nm at 2 hours. Zero the spectrophotometer using the 0.6 M perchloric acid solution and include the blank as 0 mg/mL on the standard curve.

Calculations

The concentration of the samples (in mg), is calculated from the sample absorbance using linear regression as calculated from the standard solutions. Results are expressed as concentration in mMol per kilogram of meat sample after correcting for the dilution factor and converting using the molar mass of glycogen.

$$Glycogen\left(\frac{mg}{g}\right) = \frac{Glycogen\ (mg)}{Mass\ sample\ (g)} \times \frac{Dilution}{Aliquot}$$

9.3.2 Free amino acids, total amino acids and hydrogen ion buffers

Sample preparation for free amino acids, total amino acids and hydrogen ion buffers

Freeze-ground sub-samples prepared in Section 9.1 were used for all subsequent sample preparation outlined below.

Free amino acids, total amino acids and hydrogen ion buffers analysis

Analyses of free amino acids, total amino acids and hydrogen ion buffers were carried out at AgResearch Limited (Palmerston North, New Zealand). Total amino acids were determined for the meat sample using an IANZ accredited procedure for acid-stable amino acids by acid hydrolysis (AOAC 994.12 modified) and sulphur amino acid by performic acid oxidation (AOAC 994.12 modified). Free amino acids were determined for the two meat samples using an in-house pico tag reverse phase chromatography procedure. In addition to standard amino acids, β-alanine, carnosine, anserine and ornithine (hydrogen ion buffers) and taurine were also quantified.

9.3.3 Fatty acid profile

Sample preparation for fatty acid profile

Meat samples for intramuscular fatty acid profiling was freeze-dried using a pilot scale Cuddon FD80 freeze dryer (Cuddon Freeze Dry, Blenheim, NZ). The freeze dryer was programmed with a temperature gradient with − 30 °C for 3 hours with a vacuum applied before stepping to − 10 °C for 4 hours and 0 °C for the final 72 hours. The weights of samples prior and post drying were recorded using a calibrated 3 decimal place balance. Freeze-dried samples were pulverised using a commercial coffee grinder then sealed into a ziplock bag and stored at -20 °C until analysis.

The fat cap was pulverised using the procedure described in Section 9.1 prior to analysis.

Fatty acid profile analysis

A single meat sample and fat cap sample were analysed in duplicate at AgResearch Limited (Palmerston North, New Zealand). A direct trans-methylation method was employed for fatty acid analysis, which combined the extraction and esterification of lipids in a single step to generate fatty acid methyl esters (FAME) as described by Craigie *et al.* (Craigie et al., 2017).

9.3.4 Minerals

The mineral profile was measured by a commercial lab (Analytica Laboratories Limited, Hamilton, New Zealand) from freezer-ground meat sub-samples was described in Section 9.1. Meat samples were analysed as received using an IANZ accredited ICP-MS method following an in-house standard procedure for acid digestion. The mineral results are expressed as concentration per gram of meat.

9.3.5 Thiamine

Analysis of thiamine was carried out at Massey University Nutrition Laboratory (Palmerston North, New Zealand). An IANZ accredited procedure was used for determining vitamin B1 (thiamine hydrochloride): EN14122:2003 and the result expressed as concentration per gram of meat.

9.3.6 Water holding capacity

Centrifugal drip loss

Centrifugal drip loss was carried out according to Kristensen & Purslow (Kristensen and Purslow, 2001b). From a slice of the striploin, small samples were cut parallel to the fibre direction. The samples were approximately 1 cm long, 0.4 × 0.4 cm in cross-sectional area and weighed 0.3 - 0.5 g. Meat samples were accurately weighed and transferred to open-ended tubes (Mobicols with the filters removed from MoBiTec, Göttingen, Germany). To prevent surface drying, the Mobicols were closed with a screw-on cap. The Mobicols were put in 2 mL Eppendorf tubes and centrifuged at 40 x *g* for 1 hour at 4 °C (Eppendorf 5810-R Centrifuge). Centrifugation loss of the meat was calculated as the difference in weight before and after centrifugation and expressed as a percentage of the original sample weight, shown as below.

$$\%\ Centrifugal\ Drip\ Loss\ = \frac{m(meat) - m(centrifuged\ meat)}{m(meat)} \times 100\%$$

Drip loss

The Honikel Bag method (Honikel, 1998) was used. Samples were prepared free of skin, fat and sinew (approx. 30 g) from the striploin, weighed, placed in plastic netting and then suspended in inflated plastic bags that were subsequently sealed. The sealed bags were hung in a chiller (4 °C) for 48 hours before samples were removed, blotted dry and re-weighed. Drip loss was calculated as the weight difference expressed as a percentage of the initial sample weight, shown as below.

$$\%\ Drip\ Loss\ = \frac{m(meat) - m(blotted\ meat)}{m(meat)} \times 100\%$$

Thaw loss and cook loss

Thaw loss

A steak sample (2 cm thickness) was taken at the end of the ageing process, vacuum packaged and stored at -20 °C to determine thaw and cook loss. The frozen steak was thawed at 4 °C overnight, blotted and weighed in the bag. The mass of the meat after blotting dry was recorded, as was the mass of the dry bag. Thaw loss was calculated as the difference between the total meat and thaw, and the final blotted dry meat. Thaw loss was is expressed as a percentage of the original sample weight, shown as below.

$$\%\ Thaw\ Loss = \frac{m(meat + exudate + bag) - m(dry\ bag) - m(blotted\ meat)}{m(meat + exudate + bag) - m(dry\ bag)} \times 100\%$$

Cook loss

The blotted dry sample from above was weighed then cooked in a sous vide bag to an internal temperature of 75 °C using a thermocouple inserted into the centre of the sample (Digi-Sense 12 Channel Scanning Data Logging Benchtop Thermocouple Thermometer, John Morris Scientific). The cooked sample (with bag) was immediately immersed in an ice slurry after the exudate was drained. The steak sample was blotted dry after chilled down to 4 °C and weighed again. The cooking loss was calculated as the amount of weight lost and expressed as a percentage of the original sample weight, shown as below:

$$\%\ Cook\ Loss = \frac{m(raw\ meat) - m(cooked\ meat)}{m(raw\ meat)} \times 100\%$$

9.3.7 Tenderness

Fourteen cross-section slices (1 cm × 1 cm bites) were prepared from the cooked sample as above (4 °C) with the muscle fibres running longitudinally along the slice. Each sample was then sheared with the long axis of the fibres running perpendicular to the blade, using a MIRINZ tenderometer (Macfarlane and Marer, 1966). The results were converted to shear force values (kgF) by applying the calibration equation for the equipment to the pressure obtained to cut through a 1 x 1 cm cross-section and are expressed as an average of the fourteen bites. The calibration equation is shown as below:

$$Shear\ force\ = 0.2016(Pressure) - 1.7232$$

9.3.8 Myofibrillar fragmentation index

Reagents

Potassium Phosphate, Monobasic (K_2HPO_4): 3246-01, J.T. Baker®, Avantor™
Potassium Phosphate, Dibasic (KH_2PO_4): 3252-01, J.T. Baker®, Avantor™
Potassium chloride (KCl): P/4280/60, Fisher scientific.
Ethylenediaminetetraacetic acid (EDTA): 104245S, AnalaR®, BDH.
Biuret Regent

Preparation of Biuret reagent

5 g of sodium potassium tartrate (AnalaR®, BDH) was dissolved in approx. 500 mL distilled water, then 30 mL of 10 N potassium hydroxide (Potassium hydroxide, AnalaR NORMAPUR®) and 60 mL 4% $CuSO_4$ (Copper (II) Sulphate, 5-hydrate, Ajax chemicals, Univar®) were added and mixed thoroughly. The solution was made up to a final volume of 2 L with distilled water and store in an amber bottle in dark at 4 °C.

Sample preparation for myofibrillar fragmentation index

Myofibrillar fragmentation index was determined using a modified version of the procedure of Culler *et al* (Culler et al., 1978) which measures turbidity of a sample extract of known protein concentration. Approximately 20 g of lean tissue were diced (from frozen) into small pieces then minced using a handheld food processor (Jamie Oliver HR1680/00, Philips) for 30 seconds until homogenised mince was obtained. Approximately 1 g of mince was further homogenised using an ultra-turrax (Ultra-turrax, IKA) for 30 seconds (twice) in 10 mL (1:10, w/v) of 20 mM potassium phosphate isolating medium (20 mM K2HPO4/KH2PO4, pH=7, 100 mM KCl and 1 mM EDTA). The homogenate was sedimented at 1000 x *g* for 15 minutes and the supernatant decanted. The sediment was resuspended in 10 mL of isolating medium using a horizontal shaker (KS250 basic shaker, IKA labortechnic) at 400 rpm for 10 minutes, then sedimented at 1000 x *g* for 15 minutes to recover the sediment, and this step repeated. After the second wash, the sediment was resuspended in 10 mL of isolating medium and passed through a stainless strainer (18 mesh) to remove connective tissue and debris. Additional 5 mL (×2) of isolating medium was used to facilitate passage of myofibrils through the strainer. The total volume of final myofibril suspension was 20 mL.

Myofibrillar fragmentation index analysis

The protein concentration of each myofibril suspension was determined by the biuret method of Gornall *et al (Gornall et al., 1949)*. Briefly, 0.20 mL of myofibril suspension or isolating medium (blank) was transferred into a glass tube with 2 mL biuret reagent added and vortexed. Glass tubes were placed at room temperature (22 ^{o}C) for 1 hour before the absorbance was read at 550 nm. Bovine serum albumin (BSA) was used to establish a standard curve from 0 to 10 mg/mL. An aliquot of the myofibril suspension was diluted in isolating medium to a protein concentration of 0.5 ± 0.05 mg/mL. The diluted myofibril suspension vortexed and measured immediately at 540 nm. Absorbance was multiplied by 200 to give the MFI result.

9.3.9 Calpains and calpastatin activities

Calpain I and calpastatin activities were measured after extraction and subsequent ion exchange separation of the proteases. In addition, to obtain concentrations of calpain I and calpastatin, ELISA assays were carried out (Section 9.3.10).

Calpain I and calpastatin activities

Reagents

Tris and Phenylmethylsulfonyl fluoride (PMSF), AR grade 99.9% purity (Appli Chem)
Ethylenediaminetetraacetic acid (EDTA), AR grade 99.0% (BDH)
Phenylmethylsulfonyl fluoride (PMSF), Appli Chem
Leupeptin, 90% purity (Sigma Aldrich)
2-mercaptoethanol, AR grade, 99.0% purity (Sigma Aldrich)
Casein, AR grade, 95.0% purity (Merck)
Calcium chloride, AR grade 99.0% purity (Ajax Finechem)

Buffer preparation
Pre-rigor extraction buffer (4°C)

50mM Tris/HCl, pH 8.3
10 mM EDTA
0.05% 2-mercaptoethanol (MCE)
100 mg/L ovomucoid
2mM PMSF

6 mg/L leupeptin

Post-rigor extraction buffer (4°C)

100mM Tris/HCl, pH 8.3
10 mM EDTA
0.05% 2-mercaptoethanol (MCE)
100 mg/L ovomucoid
2mM PMSF
6 mg/L leupeptin

Dialysis buffer (4°C)

40mM Tris/HCl, pH 7.35
5 mM EDTA
0.05% 2-mercaptoethanol (MCE)

Washing buffer ion-exchange chromatography (4°C)

40mM Tris/HCl, pH 7.35
0.5 mM EDTA
0.05% 2-mercaptoethanol (MCE)

Elution buffer A

200mM NaCl

Elution buffer B

400mM NaCl

Calpain I and calpastatin activities analysis

Calpain protease activity was determined using casein as a substrate, with the quantity of liberated peptides indicative of protease activity.

Sample extract preparation

Beef samples collected at all the time points (prepared as described in Section 9.1) were used for analysis. The procedure to extract, separate and assay calpains and calpastatin activities was a modification of the method described by Koohmaraie (Koohmaraie, 1990). For dispersion, 15 mL of pre-rigor extraction buffer (for pre-rigor samples) or post-rigor extraction buffer (for post-rigor samples) was added to 5 g of each freeze-ground meat sub-sample and homogenised for 30 seconds at 19,000 rpm (IKA Ultra-turrex, T25).

The extracts were centrifuged at 30,000 x *g* for 20 minutes at 4 °C, the supernatant was then filtered through glass wool. The final volume of the filtrate was measured by measuring cylinder. The filtrate was then added to dialyzing tubing (12 kDa membranes) and dialyzed overnight against the dialysis buffer in a 4 °C chiller. After dialysis the extract was centrifuged at 30,000 x *g* for 20 minutes at 4 °C.

Ion exchange chromatography

Thirty glass columns (ø2.8 x 20 cm) packed with DEAE-Sephacel (4.5 cm height) by gravity were set up. The columns were washed with Milli-Q water (20 mL) followed by 10 mL of washing buffer. Then the dialysed extract (volume equivalent to 5 g of meat) was loaded onto the column. Each column

was washed with 25 mL of washing buffer to remove un-adsorbed protein components. Thereafter, it was eluted with 25 mL of elution buffer A (200 mM NaCl). This eluent was collected in tube, and contains both calpastatin and calpain I. The column then was eluted with 25 mL of elution buffer B (400 mM NaCl), the eluent was collected in separate tube and contains calpain II.

Preparation of purified calpain II

In order to measure calpastatin activity, calpain II was extracted and purified from beef spleen. Beef spleen was collected freshly (slaughter 30 min) from a local abattoir and calpain II was purified using the process as above.

Calpain II activity analysis

The activity of calpain II was determined using a method established by Geesink & Koohmaraie (Geesink and Koohmaraie, 1999). This method determines the extent of proteolysis by measuring the amount of peptides released from casein by Ca^{2+} dependent protease (CDP), i.e. calpain.

Reagents

Reaction mixture A

100mM Tris/HCl, pH 8.3
10 mM 2-mercaptoethanol
5 mg/mL casein
10 mM $CaCl_2$ adjust pH to 7.5 at 25 ^oC with 6M HCl

Reaction mixture B

100mM Tris/HCl, pH 8.3
10 mM 2-mercaptoethanol
5 mg/mL casein
10 mM EDTA adjust pH to 7.5 at 25 ^oC with 6M HCl

Method

A 0.5 mL aliquot of each sample from the buffer B eluent (400 mM NaCl) was added into 0.5 mL of reaction mixture A (100mM Tris-HCl, pH 8.3, 10 mM MCE, 5 mg/mL casein, 10 mM $CaCl_2$, pH 7.5), and 0.5 mL of reaction mixture B (100mM Tris-HCl, pH 8.3, 10 mM MCE, 5 mg/mL casein, 10 mM EDTA, pH 7.5), respectively. The reaction was initiated by adding the fraction containing Ca^{2+} dependent protease (calpain II) and stopped by adding an equal volume (1 mL) of 5% trichloroacetic acid (TCA). After centrifugation at 3000 x *g* for 10 minutes, the absorbance at 278 nm of the supernatant was determined.

Calpastatin activity analysis

The inhibitor activity (calpastatin) was determined by incubating appropriate amounts of inhibitor and the Ca^{2+} dependent protease (calpain) before adding 1.0 mL of reaction mixture A containing $CaCl_2$ to start the reaction. Similarly, the reaction was stopped with 5% trichloroacetic acid (1 mL) and centrifuged as before. Three tubes were necessary to carry out the assay of the inhibitor:

1) Positive control: Protease alone (calpain I or calpain II)
2) Sample: Protease + inhibitor fraction in reaction mixture with $CaCl_2$
3) Blank: Inhibitor alone in reaction mixture with EDTA

Tube 1 was a positive control in which 0.3 mL of 200 mM NaCl buffer and 0.2 mL of calpain II eluent was added with 0.5 mL of reaction mixture A (100mM Tris-HCl, pH 8.3, 10 mM MCE, 5 mg/mL casein, 10 mM CaCl2, pH adjusted to 7.5).

Tube 2 was the analysis sample in which 0.3 mL of the inhibitor (calpastatin) eluent fraction and 0.2 mL of calpain II was added with reaction mixture A.

Tube 3 is the sample blank and contains 0.3 mL of the inhibitor (calpastatin) eluent fraction and 0.2 mL of 200mM NaCl buffer with 0.5 mL of reaction mixture B (100mM Tris-HCl, pH 8.3, 10 mM MCE, 5 mg/mL casein, 10 mM EDTA, pH adjusted to 7.5).

All three tubes were vortexed and incubated at 25 °C for 1 hour. After incubation 1 mL of 5% trichloroacetic acid was added and tubes vortexed to stop the reaction. The tubes were centrifuged at 3000 x *g* for 10 min. The absorbance of the supernatant was recorded at 278 nm using glass cuvettes. Total inhibitor activity was calculated according to the following formula:

$$Calpastatin\ Activity = A_1 - (A_2 - A_3) \times Dilution\ Factor$$

One unit of inhibitory activity is the amount of the calpastatin inhibitor that inhibits one unit of Ca^{2+} dependent protease (calpain II) activity.

Calpain I activity analysis

Calpain I activity was calculated based on calpastatin activity in the 200 mM NaCl fraction before and after heat treatment. Heat treatment is applied to inactivate the calpain I present in the fraction, which reduces each sample eluent fraction (200 mM NaCl), was heated at 95 °C for 15 minutes and samples analysed using the method as above.

$$Calpain\ I\ Activity = Calpastatin\ Activity_{(after\ heating)} - Calpastatin\ Activity_{(before\ heating)}$$

9.3.10 Calpains and calpastatin ELISA

Two ELISA assays were used to determine the concentration of calpain I and calpastatin in extracts obtained from freeze-ground beef samples 0.5 - 337.7 hours post-mortem.

Two validation experiments (recovery and linearity) were carried out on a limited number of samples, given the limitation of the amount of kit available to discern the validity of the results obtained from the assays. All sample extracts were assayed undiluted.

Calpain I analysis

Reagents

Bovine Calpain-1 catalytic subunit ELISA kit, 1 x 96 well pre-coated plate
Cat. No. ABIN1124977
Antibodies Online Inc, PO Box 5201, Limerick, PA 19468, USA

Sample preparation

Sub-sample myofibrillar protein extracts obtained from titin gel preparation were used to for analysis of calpain I using the ELISA kit (Section 9.3.11).

Assay recovery experiment

To determine if the sample matrix is influencing the recovery of Calpain in the assay, two samples (Strp#2/1 and Strp#2/30) were 'spiked' with equal volume of top standard (50 ng/mL).

The un-spiked and spiked samples were assayed according to the assay protocol and 'observed' vs 'expected' results were compared, to determine the % recovery. Where [ ] denotes 'concentration' of the analyte.

$$Expected\ Concentration = \frac{1}{2} \times [Unspiked] + \frac{1}{2} \times [Standard]$$

$$\%\ Recovery\ = \frac{[Observed]}{[Expected]} \times 100\ \%$$

Linearity experiment

To determine whether the sample matrix influences the measurement of calpain in the assay, one sample (Strp#2/17) was serially diluted.

These dilutions were assayed according to the assay protocol. When multiplied by the dilution factors the results should be similar. If this was the case and the coefficient of variation (CV%) of the dilutions was similar to the intra assay CV% (not determined), then it can be assumed that the sample matrix is not interfering in the measurement of calpain in the assay.

Calpastatin analysis

Reagents

Bovine CAST/Calpastatin ELISA Kit, 1 x 96 well pre-coated plate
Cat. No. 000-26213, Vendor Cat. No. LS-F16388-1
Sapphire Bioscience Pty Ltd, Unit G1, 44-70 Rosehill St, Redfern NSW 2016, Australia

Sample preparation

Sub-sample myofibrillar protein extracts obtained from titin gel preparation (Section 9.3.11) were used to for analysis of calpastatin using the ELISA kit.

Assay recovery experiment

To determine if the sample matrix is influencing the recovery of calpastatin in the assay, two samples (Strp#2/1 and Strp#2/30) were 'spiked' with equal volume of top standard (10 ng/mL).

The un-spiked and spiked samples were assayed according to the assay protocol and 'observed' vs 'expected' results were compared, to determine the % recovery. Where [ ] denotes 'concentration' of the analyte.

$$Expected\ Concentration = \frac{1}{2} \times [Unspiked] + \frac{1}{2} \times [Standard]$$

$$\%\ Recovery\ = \frac{[Observed]}{[Expected]} \times 100\%$$

Calpain I and calpastatin recovery using saline extract

Sample extracts obtained from ion exchange (Section 9.3.9) containing 200mM NaCl and tested un-spiked and spiked for calpain I and calpastatin to determine % recovery in this matrix using the same procedure above.

9.3.11 Titin

Reagents

Buffer A

50 mM Tris-HCl, 100 mM KCl and 5 mM EDTA, adjusted pH to 7.5

Buffer B

50 mM Tris-HCl, pH 5.8, 10% glycerol, 2% SDS and 2% 2-mercaptoethanol
RC-DC Assay Method Bio-Rad Assay kit

Preparation of titin extract

1 g of the pulverised powder from each sampling point was weighed into a 15 mL centrifuge tube. To the tube, 10 mL of cold buffer A (50 mM Tris-HCl, 100 mM KCl and 5 mM EDTA, adjusted pH to 7.5) was added into each sample and homogenised using Ultra-turrex at 12,000 rpm for 30 seconds. The homogenates were filtered through wet cheese cloth and then centrifuged at 2,000 x *g* for 10 minutes at 4 °C. The supernatant (containing sarcoplasmic proteins) was discarded and the precipitate was homogenised again with 10 mL of the cold buffer and centrifuged using the same procedure. The recovered precipitate (mainly containing myofibrillar proteins) was washed (to remove remaining soluble sarcoplasmic) with 10 mL of the cold buffer by vortexing for 30 seconds, then centrifuged again. The obtained precipitate (containing myofibrillar proteins) was extracted with 5 mL of cold extraction buffer B (50 mM Tris-HCl, pH 5.8, 10% glycerol, 2% SDS and 2% MCE) using Ultra-turrex at 19,000 rpm for 30 seconds twice. After Centrifugation at 10,000 x *g* for 10 minutes at 4 °C, the supernatant was collected for analysis. These extract samples were stored at -80 °C for further titin analysis.

Protein concentration determination

The protein concentration of the extracts was measured by RC-DC assay method using Bio-Rad Assay kit.

Titin analysis

For SDS-PAGE, each muscle extract obtained (0.5 mL) was mixed with 0.5 mL of 2-fold reducing sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 10% 2-mercaptoethanol (2-MCE) and 0.02% bromophenol blue, to achieve a concentration of approximately 10 mg/mL protein. These solutions were well mixed and heated for 5 minutes in a 95 ˚C water bath. Following centrifugation at 10,000 *x g* for 5 minutes, supernatants were loaded onto gels. 4 - 12% and 6% Tris-glycine separating gels (Invitrogen precast gels, Life Technology) were used for the analysis of titin and other high molecular weight proteins. Each sample mixture with equivalent to 80 µg protein was loaded on each well to obtain bands with higher intensity. The electrophoresis was conducted for 16.5 hrs at a constant current of 10 mA/2 gels in a cold room at 4 °C. Novex Tris Glycine SDS Running buffer (Life Technology NZ Ltd) was used in both the upper and lower buffer chambers of the Criterion Cell system. HiMark™ Pre-stained higher molecular weight protein standards (Invitrogen, Lot no. 735084) were used (8 µL per well) to assist interpretation of protein molecular masses. Gels were stained using Simply Blue™ Safe Stain solution (Invitrogen). After three washes with Milli-Q water, the gels were scanned using a GS900 Calibrated Densitometer Scanner (Bio-Rad).

Semi-quantitative analysis of titin

Interpretation and semi-quantitative analyses of titin protein bands on the SDS PAGE gels were performed using Image lab (version 5.2.1, Bio-Rad®). The results are presented as relative quantity (%) to the total bands in each lane.

9.3.12 Titin ELISA

Titin analysis

Reagents

Bovine Titin (TTN) ELISA Kit, 1 x 96 well pre-coated plate
Antibodies Online ABIN996338, Schloss Rahe Strasse 15, 52072 Aachen, Germany. Actual supplier Blue Gene Bovine E11T0552.

Sample preparation

Sub-sample myofibrillar protein extracts obtained from titin gel preparation were used to for analysis of titin using the ELISA kit (Section 9.3.11).

Titin detection experiment

To determine if the titin could be detected in the sample extract samples (Strp#2/1, Strp#2/2, Strp#2/8 and Strp#2/17 - Strp#2/30) were assayed. No titin was detected in any sample, most likely caused by interference with the 2-mercaptoethanol.