School of Animal and Veterinary Sciences

Identifying Storage Thresholds in Frozen and Chilled Lamb Meat

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“Academics don’t retire.. they die” – B.W.B. Holman

“Don’t go into academia, son, follow the money. Although the aim of education is to make YOUR hour worth more than the next bloke’s” – J.H.B. Coombs (1944-2016)

Coincidentally this thesis is submitted on the date of my parents’ 27th wedding anniversary, and the first without my father. This thesis is dedicated primarily to him.
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Certificate of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis.

Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this thesis be accessible for the purpose of study and research in accordance with the normal conditions established by the Executive Director, Library Services or nominee, for the care, loan and reproduction of theses.
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List of Publications:

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**Conference papers**


loading following chilled-then-frozen storage. *Proceedings: 62nd ICoMST. Bangkok Thailand* (p. 67).


**Other presentations:**

HDR Seminar June 2015 at CSU Wagga – Identifying Storage Thresholds in Frozen and Chilled Red Meat – A Proposal

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ASAP Symposium at University of Sydney, Camden Campus (February 2016) – Effect of chilled storage (up to 8 weeks) on lamb meat quality traits

HDR Seminar June 2016 at CSU Wagga – Examining the relationship between colorimetric measurements and microbial loading of beef meat – this poster won a Special Acknowledgement by the Executive Dean

LambEx Young Guns (Albury, NSW) finalist July 2016 - Identifying Storage Thresholds in Frozen and Chilled Lamb
List of Abbreviations present in the manuscript

AMSA: American Meat Science Association
asreml: Analysis software: Restricted maximum likelihood
$\text{a}_w$: water activity
CFU: colony forming units
CIE: Commission International l’Eclairage
CL: cooking loss
CoA: coenzyme A
DHA: docosahexaenoic acid
DMb: deoxymyoglobin
EPA: eicosapentaenoic acid
FAME: fatty acid methyl ester
FAP: fatty acid profile
Fig.: Figure
GC: gas chromatography
$\text{H}_2\text{O}_2$: hydrogen peroxide
$\text{H}_2\text{SO}_4$: sulfuric acid
IMF: intramuscular fat
KOH: potassium hydroxide
LAB: lactic acid bacteria
LL: m. longissimus lumborum
$\log_{10}$: logarithmic scale (base 10)
MDA: malondialdehyde
MLA: Meat and Livestock Australia
Mb: myoglobin
MC: moisture content
MMb: metmyoglobin
MRS: de Man, Rogosa & Sharpe (agar)
MUFA: monounsaturated fatty acid
mV: millivolts
$n$: number (experimental unit)

N: newton

N: normality equivalent concentration (equivalent per litre)

NaCl: sodium chloride

NADH: nicotinamide adenine dinucleotide hydride (reduced form of NAD)

OMb: oxymyoglobin

ORP: oxidation-reduction potential

pHu: ultimate pH

PUFA: polyunsaturated fatty acid

R630/580: Reflectance ratio (Reflectance at 630 nm divided by reflectance at 580 nm)

RIPA: radioimmunoprecipitation assay

SC: synthetic complete

SD: standard deviation

SE: standard error

SF: shear force

SFA: saturated fatty acid

SL: sarcomere length

STAA: streptomycin-thallous acetate-actidione

TBARS: 2-thiobarbituric acid reactive substances

U: amount of peroxidase (in nanomoles) to reduce 1 micromole of hydrogen peroxide (expressed per gram of meat at 37 °C)

UFA: unsaturated fatty acid (MUFA + PUFA)

VFA: volatile fatty acid

VRBG: vile red bile glucose

WHC: water holding capacity

$\omega_3$: omega-3 fatty acid (final carbon-carbon double bond 3$^{rd}$ from methyl side)

$\omega_6$: omega-6 fatty acid (final carbon-carbon double bond 6$^{th}$ from methyl side)
ABSTRACT

The experiment on which this thesis is based tested the effects of chilled, frozen and chilled-then-frozen storage of lamb at various durations. It was hypothesised that prolonged chilled storage (up to 8 weeks) would lead to rancidity, compromised sensory and nutritional qualities and spoilage due to proliferation of specific microbes. Likewise, it was hypothesised that following chilled storage, frozen storage as per industry practice would preserve quality regardless of duration. Following prescribed chilled-then-frozen durations however, it was hypothesised that meat frozen for longer durations (up to 52 weeks) would exhibit a faster loss of quality upon retail display post-thawing.

A total of 360 lamb m. longissimus lumborum (LL) were collected from a collaborating export abattoir and allocated to storage durations of 0, 2, 4, 6 and 8 weeks chilled storage and 0, 4, 8, 12, 24 and 52 weeks frozen storage (n = 360). Frozen samples were held at two different frozen storage temperatures (-12 and -18 °C). Upon completion of each chilled-then-frozen storage duration treatment, samples were sub-sampled for instrumental analyses of meat quality and safety traits and one sub-section was placed under simulated retail display conditions for 3 days (approximately 72 hours) where instrumental colour measures were taken daily.

Results from this experiment indicated that chilled-then-frozen storage influenced sensory quality by decreasing shear force and colour stability and altering lipid oxidation. Nutritional quality, based upon proportions of health-claimable fatty acids, and food safety, were maintained following maximum chilled and frozen storage periods in this experiment, although
spoilage microbes (lactic acid bacteria and *Brochothrix thermosphacta*) were close to proliferating above spoilage thresholds following 8 weeks chilled-only storage. Following this experiment, storage thresholds (chilled, frozen and chilled-then-frozen) could be formed based upon previously determined consumer thresholds for selected meat quality and safety traits.
General Introduction

Red meat (beef and lamb; for the purpose of this thesis, with particular focus on the lamb sector) is a valuable international commodity, with Australia exporting a majority of its red meat to overseas nations including Russia, Papua New Guinea, South Africa, China, Japan, South Korea, the USA and the Middle East. A decline in live animal export in recent years has led to the extensive use of preserved meat product in chilled (-1 to 5 °C) or frozen (below -10 °C) forms being exported to these and other nations through means of air or sea freight. Fifty-six percent of Australian lamb is exported overseas and the lamb export industry is valued at $1.78 billion (MLA, 2016). Chilled export predominates due to market access, with more than 50% of Australian lamb being exported to the Middle East and the United States of America (MLA, 2016), where chilled product is generally preferred in comparison to frozen. China is an emerging market, and is the fastest growing export market for Australian lamb (MLA, 2016), providing opportunity for frozen meat trade.

Australian export meat needs to maintain its high standing within the international community – based on sensory (eating and visual) and nutritional (lipid and protein) qualities, as well as food safety (low microbial loading, free from spoilage). Coupled with this is the rising cost of transportation, with an opportunity to slow this transportation (slow steaming) offering the scope to optimise fuel use and allow for bulk shipments of Australian product to overseas markets while still ensuring quality and safety of red meat over long-term storage of one year or more using frozen storage temperatures. Frozen storage temperatures are
traditionally -18 °C (0 °F) for international export based upon regulations (Food Science Australia, 2005; USDA, 2013), although -12 °C has been mentioned as the minimum temperature for acceptable safety of meat entering the European Union (UK P & I Club, 2006). The lack of testing of frozen storage temperature and its effects on quality and safety of red meat have led to the use in this study of two temperatures (-12 and -18 °C).

Prior research, examined in the literature review (Chapter one), has determined that anaerobic chilled storage or ‘wet ageing’ of red meat in vacuum packs, can improve its qualities, particularly tenderness and flavour (Wood et al., 1999; Colle et al., 2015), which are considered the primary drivers of lamb quality (Muela et al., 2012; MLA, 2015). Additionally, provided that freezing rate is sufficiently fast and storage temperature remains consistent, the freezing process and frozen storage duration will not lead to quality deterioration (Muela et al., 2012; Bueno et al., 2013). This has also been proven to occur when meat is frozen following chilled storage, however, the frozen storage durations in this situation have not exceeded 2-3 months (Shanks et al., 2002; Lagerstedt et al., 2008; Vieira et al., 2009; Kim et al., 2011; Kim et al., 2013). What is missing is any effect of longer frozen storage periods which are commonly used in industry. Such extended storage helps maintain market access of Australian lamb worldwide by evening out its supply. However, sensory quality ramifications of extended frozen storage periods beyond one year have been identified, particularly following thawing and aerobic chilled storage post-thawing (Muela et al., 2015; Muela et al., 2016). Frozen storage durations of 2 years or longer have been proposed by Winger (1984), with minimal quality deterioration or
compromise of safety, provided chilled storage prior to freezing is kept to a minimum.

It is apparent based on the literature that there is a dearth of studies looking at chilled ageing followed by long-term freezing (for the purpose of this thesis, termed **chilled-then-frozen storage**), and this could be the missing link to long-term preservation at low costs to improve trade links and facilitate market access of Australian red meat products. There is also an absence of storage thresholds for either chilled or frozen product in the literature, and the establishment of these to ensure meat exported is safe and of optimal nutritional and eating quality, is paramount to fulfilling this paucity. The majority of recent studies on long-term preservation (> 9 months) have used only frozen storage (Fernandes et al., 2013; Muela et al., 2015; Muela et al., 2016), or have included packaging technologies manipulating the atmospheric gas composition, freezing and thawing pressure alterations and the addition of anti-freeze proteins or brine/salt to meat (Zhou et al., 2010; Leygonie et al., 2012). As these pose consumer health and safety risks at varying degrees, and increase the cost of meat processing, the study described in this thesis used vacuum packaging to restrict oxygen, followed by chilled ageing and then freezing under these anaerobic conditions. Storage temperatures (chilled and frozen) are controlled and recorded to prevent the quality and safety risks associated with accidental freezing and thawing of meat, and in doing so pose a safe, healthy and cost effective solution to long-term preservation of Australian lamb.

The following research questions were posed:
1. What are the current practices within global red meat industries and future potential options regarding long-term preservation of red meat?

2. How does short- and long-term preservation, particularly chilling and freezing combinations, affect the quality of lamb?

3. How does short- and long-term preservation affect the nutritional value of lamb?

4. Can short- and long-term preservation of lamb compromise food safety beyond currently established market thresholds?

5. Where do these quality and safety thresholds exist for chilled-then-frozen lamb meat and can current market thresholds be extended based on this research?

For this purpose, a comprehensive study focusing on several aspects of meat quality is essential to more thoroughly understand the effects of long-term chilled-then-frozen storage. The experiment on which this thesis is based was designed to contribute to overcoming an absence of scientifically supported storage thresholds. With this information, the international lamb trade can be improved beyond its current boundaries, with the purpose of increasing the safe storage duration of a high-quality product to allow for longer exportation distances at slower speeds to save costs and provide product when demanded instead of being restricted to seasonal availability.

**Chapter one** comprehensively reviews the literature relating to the topic on both beef and lamb, and identifies the key paucities which justify why this study was undertaken. The review encapsulates the effects of chilled, frozen and the combination of chilled and frozen storage on the meat quality...
(tenderness, juiciness, flavour and colour) and food safety (microbial profile) of beef and lamb.

Chapter two evaluates the effect of chilled-then-frozen storage at different storage durations on the meat quality and safety traits of lamb *m. longissimus lumborum* (LL). The hypothesis was that the quality traits related to tenderness (shear force) will be improved by chilled storage and then preserved by subsequent frozen storage, and that meat will remain microbiologically safe for up to one year frozen, and up to eight weeks chilled provided temperature is sufficiently controlled. Colour parameters will allow for post-thawing display thresholds to be developed across a range of chilled-then-frozen storage durations, with colour being the main quality factor limiting meat purchasing decisions.

Chapter three evaluates the effects of chilled-then-frozen storage on the parameters of lipid oxidation, and the fatty acid profile, in lamb LL, as this is what primarily affects flavour (taste and aroma) and contributes to sensory rejection of the product. It was anticipated that lipid peroxidation biomarkers would increase with chilled and frozen storage, albeit at different rates, though remain below the consumer rejection threshold levels for the duration of the experiment. Additionally, health-claimable fatty acids were measured to determine the effect of chilled-then-frozen storage upon the nutritional value of lamb.

Chapter four presents a holistic summary of the findings of the experiment, drawing general conclusions from Chapters two and three, and outlining the paucities identified from previous literature findings in Chapter one. This chapter covers the implications of the findings, with practical
application, and also identifies new paucities from this research for future investigation, integrating the previous chapters to provide the reader with sufficient information on the outcomes of the research detailed in this thesis.

A completed list of references for the thesis is provided following the conclusion of Chapter four, comprising literature cited in Chapters one through four.
Chapter 1. Literature review (based on publication in Meat Science)

1.1 Introduction

Beef and lamb, collectively termed as red meat, are valuable sources of high-quality protein (Williams, 2007), and their preservation throughout export as anoxic vacuum-packaged product in either chilled or frozen form and, at sufficiently cold temperatures, is fundamental for successful international trade (Bell & Garout, 1994; Deards et al., 2014; Eustace et al., 2014). However, over long-term chilled and frozen storage durations, meat structure and biochemistry undergoes significant changes, particularly in the case of accidentally frozen or thawed meat due to poor temperature control (Bell & Garout, 1994), contributing to losses in quality and perceived value (Leygonie et al., 2012; Eustace et al., 2014). The impact of long-term chilled storage for export purposes has been investigated previously in lamb (Sumner & Jenson, 2011; Kiermeier et al., 2013) and beef (Bell & Garout, 1994; Small et al., 2012). Furthermore, the process of frozen storage has been reviewed (Leygonie et al., 2012), while storage durations have been extended for more than one year in some studies (Muela et al., 2015; 2016). Despite these advances, improvement of preservation technologies within the current export cold chain remains an issue, especially at the industrial level, with potential advances including superchilling, ionising radiation, biopreservation and high hydrostatic pressure (Zhou et al., 2010). Therefore, the scope of this review is to compare and contrast the effects of chilled and frozen storage, comprehensively review their combination, and examine differences in storage duration and temperature upon meat quality and
spoilage parameters, defined as tenderness, juiciness, flavour, colour and microbial profile, respectively.

1.2 Chilled storage effects on meat quality and spoilage parameters

Chilled storage (-1.5 to 5 °C) is routinely used post-slaughter, whether in the form of whole carcase hanging (pre-boning) or vacuum-packaged primals (post-boning). Chilled storage generally takes place from between 24 hours to several weeks post-mortem and often varies in duration and temperature based upon species, market and processor nuances. Chilled storage preserves meat from microbial spoilage through low temperatures and in an anaerobic environment, while it can also improve meat quality through proteolysis before, during and after rigor mortis (Hopkins & Thompson, 2002). It also allows the meat to be held for longer periods aiding market distribution and smoothing the supply pattern to consumers.

1.2.1. Tenderness

Tenderness is defined as the ease of mastication by a consumer and has been identified as the primary driver influencing perceived quality, particularly in the case of beef (Miller et al., 2001). It is most reliably and easily measured instrumentally using shear force (SF) as a proxy for consumer sensory panels which cannot always be undertaken (Hopkins et al., 2006; Hopkins et al., 2013), although SF in and of itself does not provide a complete tenderness profile (Honikel, 1998; Hopkins et al., 2013). Such variation can be explained in part by sarcomere length, connective
tissue content and myofibrillar proteolysis (Koohmaraie et al., 2002; Starkey et al., 2015).

The tenderising process begins in meat following rigor mortis, which can occur between 3 to 24 hours post-mortem depending on the carcase (Koohmaraie et al., 2002). Calcium release from the sarcoplasmic reticulum and mitochondria leads to the activation of calcium-dependent proteases, known as the calpains (1 and 2) (Dransfield, 1994; Hopkins & Thompson, 2002). Also implicated in post-mortem proteolysis are the cathepsin enzymes (Ouali, 1992), proteasomes (Huff-Lonergan et al., 2010) and caspases (Kemp & Parr, 2012), although their exact role, if any, in tenderisation is still a matter of debate, whereas there is ample evidence for the role of the calpains regardless of species, breed or muscle type (Hopkins & Thompson, 2002). Continued chilled storage results in calpains progressively degrading myofibrillar proteins (titin, nebulin, desmin and troponin-T) in a process known as proteolysis (Koohmaraie et al., 2002; Huff-Lonergan et al., 2010). As proteolysis occurs, the meat becomes tenderer and SF decreases (Hopkins & Thompson, 2002). Electrical stimulation and higher rigor temperatures can also inhibit cold shortening, resulting in longer sarcomeres and therefore improved tenderness of beef and lamb (Smulders et al., 1990; Hopkins & Thompson, 2002) in the absence of chilled storage (Starkey et al., 2015).

Despite these positive effects on tenderness from chilled storage, other studies reported that prolonged chilled storage durations do not pose any additional improvement on SF or sensory tenderness (Table 1.1). From this, chilled storage duration thresholds for optimal tenderness can be
recommended; namely 7 to 10 days in lamb at 1-4 °C (Dransfield, 1994; Starkey et al., 2015) and 2 to 3 weeks in beef at 1-2 °C (Dransfield, 1994; Shanks et al., 2002). Optimal beef tenderness may, however, not be reached until a longer chilled storage duration (12 weeks at -1 °C) according to a recent study (Hughes et al., 2015), and as a result this chilled storage threshold should be re-examined with particular focus on chilled storage temperature. It has been suggested in other recent studies that tenderisation continues for longer durations in beef at low temperatures (e.g. 0 °C) (Colle et al., 2015) and optimal SF of lamb is attained at shorter durations of 8 days when chilled storage temperatures are 3-7 °C compared to 14 days at -1.5 °C (Choe et al., 2016) due to higher calpain-1 activity at these temperatures (Camou et al., 2007).

1.2.2. Fluid losses and juiciness

Juiciness refers to the amount of fluid released from meat, in addition to the salivation stimulated upon mastication. A partial insight into juiciness can be made through measuring water holding capacity (WHC) as a percentage of liquid losses during storage (purge or drip) and cooking. However, the relationship between WHC and sensory juiciness is inconsistent across chilled storage durations due to the many factors influencing mouthfeel (Honikel & Hamm, 1994; Winger & Hargyard, 1994; Honikel, 1998; Kim et al., 2014) including the content of intramuscular fat (IMF) (Savell & Cross, 1988; Hopkins et al., 2006). However, Hopkins et al. (2006) reported an improvement in overall acceptability rather than juiciness alone as cooking loss decreased, while juiciness could be estimated by increased fat on salivary flow (Winger & Hargyard, 1994); therefore
WHC and IMF are not suitable proxies for sensory juiciness (Honikel & Hamm, 1994).

The effects of chilled storage upon juiciness parameters are shown in Table 1.1, from which it is evident that chilled storage rarely inferred a negative effect upon sensory juiciness (Lagerstedt et al., 2008; Vieira et al., 2009; Colle et al., 2015), despite decreases in WHC resulting from increased chilled storage duration. In particular, purge losses significantly increased at shorter durations of 0 to 2 weeks (Lagertstedt et al., 2008; Vieira et al., 2009) and cooking losses from 3 to 5 weeks (Shanks et al., 2002; Colle et al., 2015) in chilled beef, compared to fresh meat, dependent on the muscle (Colle et al., 2015). Shanks et al. (2002) suggested a mechanism for these results; namely damage to cell membranes and proteolysis prompting increased purge and cooking losses, while the lack of clear effects on sensory juiciness can be explained by confounding with improvements in tenderness (Watson et al., 2008), denoted as halo effects (Shorthose & Harris, 1991). Furthermore, juiciness perception may have been affected by the melting of meat fat due to cooking, which may have accounted for the lack of effect on sensory juiciness despite liquid losses (Vieira et al., 2009).
1.2.3. Flavour

Flavour is a sensation of the mouth provoked by fats and other precursors such as sugars and amino acids from within a meat product which impacts upon consumer taste (Khan et al., 2015). In lamb, flavour is often denoted as the most important quality trait (Muela et al., 2012; MLA, 2015). The process of chilled storage tends to increase flavour with storage duration with the oxidation of volatile fatty acids (VFAs) and amino acids (Wood et al., 1999). However, once this duration and associated oxidation has breached an acceptability threshold, it can infer rancidity via its association with a rancid flavour by consumers (Campo et al., 2006; Bueno et al., 2013; Corbin et al., 2015). A key marker for lipid oxidation has been identified as malondialdehyde (MDA), measured using the 2-thiobarbituric acid reactive substances (TBARS) method (Tarladgis et al., 1960).

Despite the widespread use of TBARS, its relationship with sensory perception of rancidity is still quite low (Greene & Cumuze, 1981), while thresholds based on MDA content correlated with consumer acceptance and rejection often vary between studies. For instance, in one study beef could be considered “unacceptable” based on TBARS (Campo et al., 2006) while in another at the same storage duration, TBARS was not measured, but flavour perception by consumers continued to improve with further chilled storage (Hughes et al., 2015). This variation likely occurred due to differences in packaging (McMillin, 2008) and chilled storage temperature (Jeremiah & Gibson, 2001; Zhou et al., 2010), while MDA measurement methods also commonly vary between studies, providing variation at similar chilled storage durations. Furthermore, a large spectrum of chilled storage
duration thresholds have been derived based upon an absence of confinement odour for up to 12 weeks in lamb at -0.5 to -2.4 °C (Sumner & Jenson, 2011) and up to 24 weeks in beef at -0.5°C (Small et al., 2012) and on consumer acceptability for up to 12 weeks in lamb at -0.3 °C (Kiermeier et al., 2013) and for up to 26 weeks in beef at -0.5 to -1 °C (Small et al., 2012; Hughes et al., 2015). No significant differences in flavour or excessive TBARS were found following 9 weeks storage of beef at 0 °C (Colle et al., 2015), though in other studies, TBARS values were higher at these durations due to methodological differences (Ponnampalam et al., 2014a).

Confinement odour occurs due to oxygen restriction in vacuum-packaged meat and is a major limiting factor, even though it does not indicate spoilage (Reis et al., 2016) and, if meat is unspoiled, the odour usually dissipates within 30 minutes of opening of the vacuum pack (Spoonser, 1988). Hughes et al. (2015) reported flavour to be more preferable in beef stored chilled at -1 °C for 20 weeks compared to 2 weeks. The evaluation of TBARS concurrent with consumer flavour acceptance is scarce in the literature; as a result the need for further research has been suggested in a recent study (Ponnampalam et al., 2014a). In doing so, TBARS (based upon a standardised measurement) could potentially be established as a proxy for off-odour and flavour formation from prolonged chilled storage.

The quantification of fatty acids to provide similar predictive information to TBARS would also prove valuable, particularly the changes in the fatty acid profile relating to storage (Fisher et al., 2000), due to the
effects of the profile on meat flavour (Calkins & Hodgen, 2007) and susceptibility to lipid oxidation (Cifuni et al., 2000). Of particular interest is the volatile aldehyde hexanal, which is linked to a fatty, grassy odour and a lack of consumer acceptability (Calkins & Hodgen, 2007; Callejas-Cárdenas et al., 2014). In addition, increased IMF content (Hopkins et al., 2006; Corbin et al., 2015) and lack of anaerobic microbial proliferation (Bell & Garout, 1994; Kiermeier et al., 2013) are strongly related to improved meat flavour.

The contents of both MDA and hexanal can decrease as chilled storage duration increases due to the formation of secondary lipid oxidation products; as a result further measures should be taken in addition to these in order to form consumer acceptability thresholds and prevent confusion associated with prior studies.

1.2.4. Colour

Meat colour is an important parameter at the retail level due to its perceived indication of freshness and quality to consumers (Risvik, 1994). Subjective definitions of colour vary, although consumer preference for bright cherry-red beef and brick-red lamb has been reported (Mancini & Hunt, 2005; AMSA, 2012). The use of objective colorimetric measures is common, with research recording Commission International l’Eclairage (CIE) colour scale values: L* denoting lightness to darkness, a* denoting redness to green and b* denoting yellowness to blue (CIE, 1978). Of these, a* was found to be most influential in both lamb (Khliji et al., 2010) and beef (Holman et al., 2017) consumer appraisal.
Oxidative changes to myoglobin (Mb) occur as chilled storage increases, which is reflected in instrumental colour measurement variation (L*, a*, b*) over anaerobic chilled storage durations due to the formation of deoxymyoglobin (DMb) with increased storage duration contributing to a purple colour and oxymyoglobin (OMb) formation upon package opening giving a bright red colour (Mancini & Hunt, 2005). Prolonged chilled storage in an aerobic environment (e.g. retail display) leads to increased oxidation of Mb to metmyoglobin (MMb) which contributes to a brown colour. Similarly, increased chilled storage temperatures can also decrease colour stability (from -1 to 2 °C - Rosenvold & Wiklund, 2011; from -1.5 to 7 °C – Choe et al., 2016).

Increased MMb formation has been shown to negatively affect consumer scoring, with acceptability thresholds set for beef at 40-50% MMb (Kropf et al., 1986; Jeremiah & Gibson, 2001) and 23% MMb for lamb (Khliji et al., 2010). However, the proportion of MMb in these studies was calculated via instrumental colorimetric measurements using the percentage of light reflected at 630 nm divided by the percentage of light reflected at 580 nm (R630/580) (Jacob et al., 2007; AMSA, 2012). As such, R630/580 does not directly measure MMb and instead is used as a proxy of Mb oxidation and therefore an indication of colour deterioration or ‘brownness’ (AMSA, 2012). A standard protocol for Mb quantification in its different forms has been developed using the isobestic point or absorbance at 525 nm, following its extraction from haem pigment (AMSA, 2012).
Using a visual panel, beef stored chilled and anaerobically was found to be acceptable for up to 24 weeks following a 20 minute bloom period; longer chilled storage durations resulted in unacceptable scores (Small et al., 2012). In lamb, colour acceptability decreased following 3 weeks chilled storage plus 3 to 14 days of retail display when compared to fresh meat (Callejas-Cárdenas et al., 2014). When based upon MMb content, beef was ruled unacceptable following 8 weeks chilled storage plus 6 to 8 days of retail display (Liu et al., 1996). Differing results reported in the literature (Table 1.1) appear to occur due to differences in methodological measurement of colour acceptability (MMb, visual panel or CIE values), storage temperature, muscle type and oxygen exposure, all of which have been shown to influence colour (Mancini & Hunt, 2005).

It can be concluded that increased anaerobic chilled storage duration results in more rapid discolouration (decreased $a^*$ and $R_{630/580}$) upon exposure to oxygen, which has been found to occur concurrent to lipid oxidation (Ponnampalam et al., 2001; Kim et al., 2011; Callejas-Cárdenas et al., 2014; Colle et al., 2015). It has been determined that Mb and lipid oxidation can both act as precursors for each other (Faustman et al., 2010).

1.2.5. Microbial profile

The microbial profile, particularly spoilage bacteria including lactic acid bacteria (LAB), Enterobacteriaceae sp., Brochothrix thermosphacta and psychrotolerant Clostridium sp. (e.g. C. perfringens, have been found to increase as chilled storage duration increases (Gram et al., 2002; Gribble et al., 2014). These species primarily originate from abattoir contamination (Dainty & Mackey, 1992), and with the exception of LAB and clostridia are
limited to proliferate only with oxygen availability; other risk factors involved include chilled storage temperature, pH, water activity ($a_w$) and packaging conditions (Egan et al., 1988; Gram et al., 2002; Mills et al., 2014). Often, the result of microbial proliferation and the increase in spoilage levels is the development of off-flavours described as cheesy and dairy (Egan et al., 1988), discolouration (Li et al., 2015) and reduced product safety (Gram et al., 2002; Mills et al., 2014).

Anaerobic chilled storage using vacuum packaging generally results in proliferation of LAB, which has a spoilage potential of $10^7$ colony forming units (CFU) per cm$^2$ and results in off-odour detection (Sumner & Jenson, 2011; Small et al., 2012). LAB proliferation leads to sour flavours and greening or discolouration from the production of hydrogen sulphide reacting with Mb to form sulphmyoglobin (Egan et al., 1988; Mills et al., 2014). Prolonged anaerobic chilled storage also increases availability of water from the meat substrate (increased $a_w$) (Mills et al., 2014) and increases pH due to muscle tissue breakdown, particularly of lamb meat stored for 6 weeks or more (Moore & Gill, 1987; Kim et al., 2011; Kiermeier et al., 2013) and beef meat stored for 12 weeks or more (Hughes et al., 2015). Both of these traits ($a_w$ and pH) can provide insight into microbial spoilage potential, such that if $a_w$ exceeds 0.93 (Egan et al., 1988) and pH exceeds 5.8 (Gribble et al., 2014) this will result in more rapid proliferation of specific spoilage microbes. However, storage temperature has the greatest impact on microbial loading of chilled-stored meat (Bell & Garout, 1994; Giannuzzi et al., 1998) and ideally should be between 0 and -1.5 °C, although in practice this is not always achieved (Bell & Garout, 1994; Eustace et al., 2014; Mills et al., 2014).
Chilled storage duration thresholds based on the spoilage thresholds for LAB (10^7 CFU/cm²) and aerobic spoilage bacteria (10^5-10^7 CFU/cm²) have been proposed in the literature, though these differ based on species and nation (Bell & Garout, 1994; Hinton et al., 1998; Gribble et al., 2014). Microbial proliferation spoilage thresholds are often reported as their maximum level of proliferation (Gribble et al., 2014), and as a result the loading alone is often insufficient to rule on the occurrence of sensory spoilage. Variations in safe storage durations can be attributed to high pH, temperature fluctuations, improper packaging, high a_w and muscle glycogen reserves (Dainty & Mackey, 1992; Gribble et al., 2014), the latter especially as it provides a favourable substrate for microbes prior to their metabolism of amino acids (Gill & Newton, 1981; Newton & Gill, 1981). Temperature logging is only included spasmodically in chilled storage studies, and temperature abuse (> 5 °C) and accidental freezing (< -2 °C) can lead to more rapid microbial spoilage and product rejection by consumers (Bell & Garout, 1994; Eustace et al., 2014). However, psychrotolerant Clostridium sp. causing pack blowing (hydrogen and carbon dioxide gas production) within 2 to 6 weeks can proliferate below 0 °C and as low as -5 °C and also present a cheesy odour upon pack opening and meat greening (Broda et al., 1996; Adam et al., 2010). This highlights the need for good supply chain management - especially at processor and transportation levels (Clemens et al., 2010).
Table 1.1. Summary of the effects of vacuum-packed chilled storage on the shear force, tenderness, fluid levels, flavour and colour of beef and lamb.

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration</th>
<th>Temperature</th>
<th>Effects on selected traits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>35 days</td>
<td>2°C</td>
<td>Peak tenderisation (SF around 39 N) reached around 2 to 3 weeks</td>
<td>Shanks et al. (2002)</td>
</tr>
<tr>
<td>Beef</td>
<td>14 days</td>
<td>4°C</td>
<td>Gradual decrease in SF between 2, 7 and 14 days (66 to 56 to 42 N)</td>
<td>Lagerstedt et al. (2008)</td>
</tr>
<tr>
<td>Beef</td>
<td>3 to 10 days</td>
<td>4°C</td>
<td>Decreased SF (73 to 50 N) and improved tenderness (2.8 to 3.6)</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>7 weeks</td>
<td>-1.5 to 2°C</td>
<td>Decreased SF due to chilled storage though but not temperature (to 56 N)</td>
<td>Rosenvold &amp; Wiklund (2011)</td>
</tr>
<tr>
<td>Beef</td>
<td>20 weeks</td>
<td>-1°C</td>
<td>Tenderness improved between 2 and 12 weeks (2.0 to 2.8) – not 12 and 20</td>
<td>Hughes et al. (2015)</td>
</tr>
<tr>
<td>Lamb</td>
<td>2 weeks</td>
<td>3-4°C</td>
<td>SF improved between 1 and 7 days (36 to 25 N) – not 7 and 14</td>
<td>Starkey et al. (2015)</td>
</tr>
<tr>
<td>Beef</td>
<td>63 days</td>
<td>0°C</td>
<td>Continued SF decrease over 63 days chilled storage (33.5 to 22.2 N)</td>
<td>Colle et al. (2015)</td>
</tr>
</tbody>
</table>

**Fluids**

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration</th>
<th>Temperature</th>
<th>Effects on selected traits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>35 days</td>
<td>2°C</td>
<td>Cooking losses do not increase prior to 35 days storage (23% to 26%)</td>
<td>Shanks et al. (2002)</td>
</tr>
<tr>
<td>Beef</td>
<td>14 days</td>
<td>4°C</td>
<td>Peak liquid losses at 7 days (16.3% to 24.6%)</td>
<td>Lagerstedt et al. (2008)</td>
</tr>
<tr>
<td>Beef</td>
<td>10 days</td>
<td>4°C</td>
<td>No effect on juiciness between 3 and 10 days (score 2.6 and 2.7)</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>7 weeks</td>
<td>-1.5 to 2°C</td>
<td>No difference in purge losses due to temperature after 7 weeks (4.2 to 4.9%)</td>
<td>Rosenvold &amp; Wiklund (2011)</td>
</tr>
<tr>
<td>Beef</td>
<td>63 days</td>
<td>0°C</td>
<td>Cook loss increases between 14 and 21 days (24.5 to 27.9%)</td>
<td>Colle et al. (2015)</td>
</tr>
</tbody>
</table>

**Flavour**

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration</th>
<th>Temperature</th>
<th>Effects on selected traits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>13 days</td>
<td>1°C</td>
<td>More species-specific flavour after 13 days, decreased with 4 days display (TBARS &gt; 2.28)</td>
<td>Campo et al. (2006)</td>
</tr>
<tr>
<td>Lamb</td>
<td>5 days</td>
<td>0-4°C</td>
<td>More species-specific flavour after 6 days (sensory score 3.16)</td>
<td>Hopkins et al. (2006)</td>
</tr>
<tr>
<td>Lamb</td>
<td>90 days</td>
<td>0 to -2.5°C</td>
<td>85 days storage acceptable (-0.5°C), unacceptable at 0°C after 90 days</td>
<td>Sumner &amp; Jenson (2011)</td>
</tr>
<tr>
<td>Beef</td>
<td>30 weeks</td>
<td>-0.5°C</td>
<td>24 weeks storage retaining acceptable flavour</td>
<td>Small et al. (2012)</td>
</tr>
</tbody>
</table>

**Colour**
| Beef | 56 days | 4°C | 56 days storage led to acceptable colour (MMb < 50%) for 6 days display | Liu et al. (1996) |
| Beef | 24 weeks | -1.5 to 5°C | Reduced 24 week storage temperature (-1.5°C) improves a* (from 8 to 11) | Jeremiah & Gibson (2001) |
| Lamb | 4 weeks | 0°C | 4 weeks vacuum storage results in more rapid a* deterioration over 6 days | Ponnampalam et al. (2001) |
| Beef | 10 days | 4°C | Decreased a* and L* between 3 and 10 days | Vieira et al. (2009) |
| Lamb | 7 weeks | -1.5 to 2°C | Decreased a* and increased L* at increased chill temperature | Rosenvold & Wiklund (2011) |
| Beef | 30 weeks | -0.5°C | “Fresh” appearance until 26 weeks – “marginal” at 28 and 30 weeks | Small et al. (2012) |

All sensory panel scores for different traits given on a scale from 0 to 5. All shear force values converted to newtons (N) by the equation 1 kgF = 9.80665 N. SF = shear force; TBARS = 2-thiobarbituric reactive substances (mg malondialdehyde per kg meat); MMb = metmyoglobin; a* = colorimetric measure of redness.
1.3. Frozen storage effects on meat quality and spoilage parameters

The objective of frozen storage is to preserve quality and, through the use of comparatively longer safe storage durations, even out the supply variations (Shanks et al., 2002). Several studies have investigated the effects of frozen storage temperature and freezing rate on meat quality, however frozen storage duration appears to be the crucial factor in terms of maintaining meat quality and preventing spoilage for export purposes (Leygonie et al., 2012). For example, the routinely applied export storage temperature (-18 °C) has been found to maintain acceptable quality and prevent spoilage in lamb for more than one year (Fernandes et al., 2013; Muela et al., 2015; 2016). In fact, no further improvements in sensory quality or storage duration have been noted when frozen storage temperatures are lower than -18 °C (Hagyard et al., 1993; Farouk et al., 2003; Estévez, 2011). Frozen storage temperatures above -18 °C have been rarely reported in literature, though based on sensory quality, -15 °C yielded longer acceptable frozen storage durations than -10 °C and -5 °C, although temperature variation was not reported in this study (Hagyard et al., 1993). One study reported that, provided frozen storage temperature remained below -10 °C and freezing rate was controlled, lamb could be stored safely without deleterious effects on quality for two years or longer (Winger, 1984). This was thought to be due to sufficiently low $a_w$ (0.9) preventing microbial growth and low temperatures preventing oxidative flavour changes.
1.3.1. Tenderness

When meat is frozen, the rate of proteolysis is halted due to suppression of calpain activity, but not destruction of calpains (Koohmaraie, 1992; Dransfield, 1994). Furthermore, proteolysis will be restored upon thawing, due to the re-activation of the calcium-dependent proteases (Dransfield, 1994), and normally at a faster rate than prior to freezing (Crouse & Koohmaraie, 1990). This proteolysis rate increase can be attributed to the inhibition of calpastatin by frozen storage allowing for more rapid tenderisation upon thawing (Crouse & Koohmaraie, 1990; Koohmaraie, 1992). For this reason, results for the effect of frozen storage on tenderness and SF (as shown in Table 1.2) are often dependent on when (whether cooked from frozen or thawed) and how (by instrumental SF or a sensory panel) tenderness is measured (Holman et al., 2016a). Honikel (1998) mentioned that cooking from frozen is ideal for SF determination, despite a majority of studies reporting cooking from thawed (Holman et al., 2016a).

Trained sensory panels often present conflicting results when reporting tenderness differences between fresh and frozen meat (Table 1.2) – for instance, Lagerstedt et al. (2008) reported decreased tenderness in beef frozen (24 hours) post-chill (7 days at 4 °C) compared to chilled beef, though other studies on lamb have reported no difference between fresh (unaged) and frozen meat (Muela et al., 2012; Bueno et al., 2013; Fernandes et al., 2013; Muela et al., 2016), although for the most part, untrained sensory panels have been unable to distinguish between fresh and frozen meat (Lagerstedt et al., 2008; Muela et al., 2012) unless the frozen storage duration was excessively long, although storage post-thaw may have had
some effect on tissue damage (Muela et al., 2016). Results suggest that
tenderness and SF were more affected by chilled storage prior to (Vieira et
al., 2009) or following freezing (Muela et al., 2015) than frozen storage
itself.

Two recent studies on lamb which did not apply chilled storage before
freezing observed the lack of any effect from 8-9 months frozen storage on
SF, although at longer durations SF was significantly higher than their fresh
(unaged) counterparts (Fernandes et al., 2013; Muela et al., 2015). Neither
study, however, was able to associate SF increases with reduced tenderness
scores (Fernandes et al., 2013; Muela et al., 2016). Slaughter day variation
has been shown to confound SF results due to handling, weather and stress
factors (Warner et al., 2010). Display post-thawing (3 to 6 days) resulted in
decreased SF (Muela et al., 2015), and a lack of change in sensory
tenderness compared to unfrozen meat (Muela et al., 2016). These are
thought to be the basis of the observed results in the aforementioned studies.
Thawing and storage at chilled temperatures post-thaw could potentially
initiate proteolysis and reduce SF (Crouse & Koohmaraie, 1990). The
adoption of a protocol of cooking frozen meat without thawing first for SF
(Honikel, 1998; Hopkins & Thompson, 2001) and particle size analysis to
measure protein degradation (Starkey et al., 2015) could provide insight into
the true effect of freezing on the proteolysis and tenderness of meat.

Frozen storage temperatures below -18 °C have been linked to decreased
myofibrillar protein solubility (Farouk et al., 2003), while slower freezing
rates or high freezing temperatures above -10 °C may accelerate protein
oxidation, measurable as carbonyl content (Xiong, 2000; Huff-Lonergan et
al., 2010) and cause excessive damage to muscle cell ultrastructure, decreasing myofibrillar protein solubility (Petrović et al., 1993). Protein oxidation correlates with tougher meat due to the formation of cross-linkages (Xia et al., 2009) inhibiting proteolysis due to the inactivation of calpain-1 (Rowe et al., 2004). Another study associated lamb frozen at slower rates with decreased sensory quality (Muela et al., 2012). Protein solubility and carbonyl content measurements can explain variations in SF and sensory tenderness associated with frozen storage, particularly when SF results are unexpected (Estévez, 2011).

1.3.2. Fluid losses and juiciness

The process of frozen storage generally results in diminished WHC compared to fresh meat and chilled-stored meat due to liquid losses upon thawing (Añón & Calvelo, 1980; Winger & Hagyard, 1994; Vieira et al., 2009; Kim et al., 2015) resulting from disruption to muscle fibres during the freezing process (Rahelić et al., 1985). WHC has also decreased concurrently with frozen storage duration due to thawing losses (Farouk et al., 2003; Vieira et al., 2009; Muela et al., 2010; Fernandes et al., 2013). Rapid freezing has been reported to result in more, smaller and intracellular ice crystals, which therefore contribute to reduced thaw losses (Petrović et al., 1993; Zhou et al., 2010), although frozen storage temperatures have shown little to no effect on WHC (Farouk et al., 2003).

Despite the effects on WHC, few studies have reported a reduction in sensory juiciness due to the process of frozen storage (Lagerstedt et al., 2008; Bueno et al., 2013), thus highlighting the lack of support for WHC as an indicator for juiciness (Winger & Hagyard, 1994). Meanwhile,
alterations in $a_w$ and consequent losses of the odorant furaneol due to freezing have been mentioned to potentially reduce juiciness (Bueno et al., 2013), although other studies have found little to no effect of the freezing process, rate, duration or storage temperature on juiciness (Vieira et al., 2009; Muela et al., 2012).

1.3.3. Flavour

The effects of frozen storage on flavour parameters is not entirely clear – this is supposed to be due to a lack of flavour detection measures for frozen meat (Leygonie et al., 2012). This paucity was investigated for frozen lamb, and in spite of the lack of storage effects on sensory flavour, the absence of furaneol was found to be a marker of frozen-stored meat (Bueno et al., 2013). The general consensus is that if meat is frozen at or below -15 °C, thawed and cooked immediately upon thawing, its flavour will be acceptable regardless of frozen storage duration (Winger, 1984; see also Table 1.2). In fact, lamb frozen for 15 months could be displayed for 3 days post-thawing and still be considered acceptable in sensory quality; however this was not true for lamb kept frozen for 21 months (Muela et al., 2016). Frozen storage temperature posed a greater effect on sensory flavour than storage duration (Hagyard et al., 1993), even though a prior study found lamb to be of acceptable quality following 2 years frozen storage at -10 °C (Winger, 1984), although these results could have been affected by variations in temperature, chilled storage prior to freezing and sensory panel protocol and demographics. These factors and lack of reported methodology from earlier sensory panels restricts the interpretation of these results.
Following nine months frozen storage, TBARS levels peaked (Muela et al., 2010) and then continued to increase post-thawing when on retail display over 6 days (Muela et al., 2015). Despite these high levels of MDA, an oxidised perception of flavour was not detected in corresponding sensory panel studies following these durations (Muela et al., 2012; 2016). Decreases in TBARS following longer frozen storage and retail display durations can be explained by the formation of secondary products of lipid oxidation (Leygonie et al., 2012; Muela et al., 2015), particularly as consumer acceptability decreases with frozen storage duration (Muela et al., 2016). Further investigation into these secondary products comprising aldehydes, ketones and hydrocarbons (Bueno et al., 2013; Khan et al., 2015), along with a re-examination of the TBARS rancidity threshold, are necessary for a more complete understanding of frozen storage effects on meat flavour.

1.3.4. Colour

Freezing has proven to affect colour stability of beef – this is evident in the literature and is manifested as a decrease in instrumental colour parameters (L*, a*, b*) as frozen storage durations increase (Table 1.2; Farouk & Swan, 1998; Ben Abdallah et al., 1999; Vieira et al., 2009). Meanwhile, lamb stored frozen up to 6 months exhibited increased a* and b*, though when placed under retail display both values decreased and L* increased, and negative effects on a* and b* were more severe when frozen storage duration was greater (Muela et al., 2010; Muela et al., 2015). The basis for this colour deterioration could be the inherent muscle fibre damage contributions and thus, thaw losses and ultimate leaching of MMb reducing
enzymes (NADH cytochrome and β-hydroxyacyl CoA-dehydrogenase). This is supported by results which demonstrated that MMb is greater in frozen meat compared to fresh (unaged) meat (Farouk & Swan, 1998) or beef aged for 9-11 days at 5 °C (Ben Abdallah et al., 1999). The effect on $L^*$ was noteworthy when freezing rates were faster due to the reduced thaw losses and decreased light reflectance potential (Farouk et al., 2003; Muela et al., 2010). Similarly, decreases in $a^*$ were apparent in both beef and lamb with display duration (Table 1.2), likely because of the increased susceptibility of Mb to oxidation as a result of the globin moiety denaturation due to freezing (Calvelo, 1981). Bloom time and colour measurement schedule can influence CIE values and these are not always reported in literature (Tapp et al., 2011), which, along with temperature of the meat at measurement (Honikel, 1998), can compromise any interpretation of reported effects of frozen storage on instrumental colour.

Long-term frozen storage duration can produce varied effects on colour parameters. For instance, an increase in beef $L^*$ was reported following 6-12 months frozen storage compared to fresh meat or meat frozen for 3 months and this was thought to be due to increased protein denaturation, or even lipid oxidation, at longer storage durations (Farouk et al., 2003). Although the CIE values were not tested across retail display in this study, little change in $L^*$ was found in lamb following long-term frozen storage (Fernandes et al., 2013) and when followed by 6 days retail display (Muela et al., 2015), although both studies reported increased $b^*$, but not $a^*$ on day 0 when frozen storage duration exceeded 9 months. The ramifications of this are highlighted by research that identified a positive relationship between consumer acceptability of beef colour and $L^*$ values (Holman et
al., 2016b), whereas in lamb this association with consumer acceptance was not evident (Khliji et al., 2010). Following 6 days display of frozen-thawed lamb, $b^*$ increased and $a^*$ decreased (Muela et al., 2015); this can be related to similar increases in TBARS with display duration and the lipid oxidation level being associated with yellowness of the red meat via contributions to Mb oxidation (Faustman et al., 2010; Estévez, 2011).

1.3.5. Microbial profile

Generally, frozen storage (below -5 °C) inhibits microbial proliferation (Adam et al., 2010), although similarly to what is observed for protease enzymes, microbial activity will resume and often accelerate upon thawing (Lowry & Gill, 1985; Löndahl & Nilaaon, 1993; Vieira et al., 2009). A reason for this is the decrease in $a_w$ during frozen storage (Nollet, 2012). Increases to $a_w$ upon thawing and, consequently, values above 0.94 can result in the proliferation of spoilage microbes (Egan et al., 1988; Nollet, 2012). In terms of storage duration, one study on beef (Hinton et al., 1998) and two on lamb (Winger, 1984; Fernandes et al., 2013) reported that meat could be stored frozen for one year and remain safe (unspoiled) based on domestically and internationally recognised bacterial enumeration thresholds.

Despite these findings, it is apparent that there are a lack of studies investigating microbial profiles and $a_w$ of frozen meat following retail display, although reports exist of more rapid spoilage of frozen-thawed meat compared to fresh meat (Vieira et al., 2009), due to the reactivation of microbial activity upon thawing coupled with cellular damage resulting from frozen storage (Farouk et al., 2003; Leygonie et al., 2012). Also,
frozen storage temperatures have not been tested for their effects on bacterial proliferation potential and $a_w$, despite -18 °C being reported and applied as the maximum frozen storage temperature for growth inhibition (Ben Abdallah et al., 1999; Fernandes et al., 2013; Eustace et al., 2014).
Table 1.2. Summary of the effects of frozen storage on the shear force, tenderness, fluid levels, flavour and colour of beef and lamb.

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td><strong>Shear force and tenderness</strong></td>
<td></td>
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</tr>
<tr>
<td>Lamb</td>
<td>42 days</td>
<td>-20°C</td>
<td>SF improved up to 8 days and maintained in callipyge lamb (59 to 34 N)</td>
<td>Duckett et al. (1998b)</td>
</tr>
<tr>
<td>Beef</td>
<td>12 months</td>
<td>-18 to -75°C</td>
<td>SF decreased when stored frozen (particularly 9 months – 70 to 57 N)</td>
<td>Farouk et al. (2003)</td>
</tr>
<tr>
<td>Beef</td>
<td>90 days</td>
<td>-20 to -80°C</td>
<td>SF decreased after 75 days storage (73 to 66 N), no effect on sensory</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>6 months</td>
<td>-18°C</td>
<td>No difference in preference or tenderness between fresh and frozen meat</td>
<td>Muela et al. (2012)</td>
</tr>
<tr>
<td>Lamb</td>
<td>10 months</td>
<td>-18°C</td>
<td>No effect on tenderness (3.1 to 2.7)</td>
<td>Bueno et al. (2013)</td>
</tr>
<tr>
<td>Lamb</td>
<td>12 months</td>
<td>-18°C</td>
<td>No effect on tenderness after 8 months – SF increased between 4 and 12 months (48 to 78 N)</td>
<td>Fernandes et al. (2013)</td>
</tr>
<tr>
<td>Lamb</td>
<td>21 months</td>
<td>-18°C</td>
<td>No difference in preference between fresh and frozen meat</td>
<td>Muela et al. (2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Storage beyond 9 months increased SF (68 to 91 N)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased storage duration to 9 months improved tenderness (2.9 to 3.5)</td>
<td>Muela et al. (2016)</td>
</tr>
<tr>
<td><strong>Fluids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb</td>
<td>12 months</td>
<td>-18°C</td>
<td>Purge loss increased between 20 and 42 days (9.2 to 12.9%), no effects on cook loss or juiciness</td>
<td>Duckett et al. (1998b)</td>
</tr>
<tr>
<td>Beef</td>
<td>42 days</td>
<td>-20°C</td>
<td>Decreased WHC over 12 months storage (69.9 to 59.1%)</td>
<td>Farouk et al. (2003)</td>
</tr>
<tr>
<td>Beef</td>
<td>12 months</td>
<td>-18 to -75°C</td>
<td>Decreased WHC over 90 days storage (64.1 to 51.1%), no effect on juiciness</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>90 days</td>
<td>-20 to -80°C</td>
<td>1 and 10 months storage decreased juiciness (2.9 to 2.3)</td>
<td>Bueno et al. (2013)</td>
</tr>
<tr>
<td>Lamb</td>
<td>10 months</td>
<td>-18°C</td>
<td>Increased cook loss until 8 months storage (13 to 26%)</td>
<td>Fernandes et al. (2013)</td>
</tr>
<tr>
<td><strong>Flavour</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb</td>
<td>1 year</td>
<td>-15 to -35°C</td>
<td>No rancid flavours detected following up to 1 year storage</td>
<td>Hagyard et al. (1993)</td>
</tr>
<tr>
<td>Lamb</td>
<td>42 days</td>
<td>-20°C</td>
<td>No effect on flavour</td>
<td>Duckett et al. (1998b)</td>
</tr>
<tr>
<td>Beef</td>
<td>90 days</td>
<td>-20 to -80°C</td>
<td>No effect on flavour</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>10 months</td>
<td>-18°C</td>
<td>No effect on flavour</td>
<td>Bueno et al. (2013)</td>
</tr>
<tr>
<td>Lamb</td>
<td>21 months</td>
<td>-18°C</td>
<td>TBARS below 0.2 for more than 21 months if meat not displayed</td>
<td>Muela et al. (2015)</td>
</tr>
<tr>
<td><strong>Colour</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

47
<table>
<thead>
<tr>
<th>Meat</th>
<th>Storage Duration</th>
<th>Storage Temperature</th>
<th>Observed Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>3 days</td>
<td>-18°C</td>
<td>Increased MMb (24.8% compared to 3 days chilled) due to thaw losses</td>
<td>Ben Abdallah et al. (1999)</td>
</tr>
<tr>
<td>Beef</td>
<td>90 days</td>
<td>-20 to -8°C</td>
<td>All CIE parameters (L*, a*, b*) decreased with storage duration</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>6 months</td>
<td>-18°C</td>
<td>Increased b* and decreased a* with display due to freezing</td>
<td>Muela et al. (2010)</td>
</tr>
<tr>
<td>Lamb</td>
<td>12 months</td>
<td>-18°C</td>
<td>Rapid discolouration (a*) following storage durations of 9 months or more</td>
<td>Muela et al. (2015)</td>
</tr>
<tr>
<td>Lamb</td>
<td>12 months</td>
<td>-18°C</td>
<td>Frozen meat appeared more discoloured at all times – particularly after 9 months</td>
<td>Muela et al. (2016)</td>
</tr>
</tbody>
</table>

All sensory panel scores for different traits given on a scale from 0 to 5. All shear force values converted to newtons (N) by the equation 1 kgF = 9.80665 N. SF = shear force; WHC = water holding capacity; TBARS = 2-thiobarbituric reactive substances (mg malondialdehyde per kg meat); MMb = metmyoglobin; a* = colorimetric measure of redness.
1.4. Effects of combining chilled and frozen storage on meat quality and spoilage parameters

To date, the majority of studies combining chilled and frozen storage have investigated either altered storage temperatures (Hagyard et al., 1993; Bell & Garout, 1994) or frozen-then-chilled storage (Crouse & Koohmaraie, 1990; Duckett et al., 1998a; Duckett et al., 1998b; Muela et al., 2015). By contrast, few studies have examined the effects of chilled-then-frozen storage – being the process which benefits from the advantages of chilled storage (Table 1.1) which are then preserved using frozen storage until utilisation (Table 1.2). Common to these studies were the comparisons between chilled only storage treatments followed by frozen storage (Shanks et al., 2002; Lagerstedt et al., 2008) or the use of total storage durations that were less than four months (Vieira et al., 2009; Kim et al., 2011; Kim et al., 2013; Kim et al., 2015; Choe et al., 2016). From these, it is possible that several studies which have examined frozen storage for much longer durations (Table 1.2) may not have gained the maximum benefits of chilled storage – for example, using storage durations of only 10 days at 4°C for beef (Vieira et al., 2009). Recent studies on lamb have found chilled storage (2-3 weeks at -1.5 °C)-then-frozen storage (6-7 weeks) to yield similar quality to 9 weeks chilled storage without the associated increases in oxidation and to be superior to 9 weeks frozen storage (Kim et al., 2011; 2013). This highlights the knowledge gap identified in this review, namely whether chilled-then-frozen storage can be extended beyond its current parameters (Table 1.3) with minimal negative effects on quality or spoilage. In particular, the extension of the frozen storage component is warranted
given the general lack of detrimental effects resulting from frozen storage duration (Table 1.2) based on consumer and instrumental quality thresholds.

1.4.1. Tenderness

The effects of both chilled-then-frozen and frozen-then-chilled storage treatments on SF and sensory tenderness are summarised in Table 1.3. The use of frozen-then-chilled storage promoted a greater effect on SF in beef compared to beef stored chilled only for the same period (Crouse & Koohmaraie, 1990). Interestingly for lamb, this phenomenon only occurred when the callipyge gene was present (Duckett et al., 1998a, b). Prolonging frozen storage duration between 9 and 21 months was found to result in significant SF decreases in lamb (Muela et al., 2015), as well as improved sensory tenderness (Muela et al., 2016) when these parameters were measured following 4-6 days chilled storage in modified atmospheric packaging post-thaw. This did not occur in frozen-only (not chilled prior) meat, in fact SF increased (Muela et al., 2015). This could have resulted from a reduction in calpastatin activity due to frozen storage and a corresponding increase in proteolysis rate via calpains during chilled storage post-freezing (Crouse & Koohmaraie, 1990; Dransfield, 1994; Duckett et al., 1998a, b), and this effect is reflected in SF results comparing chilled and frozen storage combinations (Kim et al., 2011; 2013) regardless of whether frozen or chilled storage was applied before the other (Table 1.3).

Previously, frozen storage has been suggested as a method for preserving tenderness following chilled storage, although studies often did not report frozen storage duration or any effects of the frozen storage (Dransfield, 1994). Shanks et al. (2002) investigated chilled-then-frozen
storage in beef and reported decreased SF to arise from chilled-then-frozen storage compared to chilled storage alone, albeit when the chilled storage durations were less than 3 weeks at 2 °C. These results suggest that frozen storage may have improved tenderness due to its physical disruption to muscle cells via intracellular ice crystal formation, however these effects may have been confounded by the frozen meat being thawed for 24 hours prior to cooking and therefore providing additional chilled storage (Shanks et al., 2002) and thus opportunity for proteolysis and thus tenderisation. Instead, the meat should have been cooked from frozen to overcome this potential confounding effect (Honikel 1998). Similar results were found for thawing prior to cooking when chilled storage (4 °C) was 7 days, but not 14 days (Lagerstedt et al., 2008). However, a reduced SF effect resulting from freezing and thawing of beef chilled for 4 weeks (-1.5 °C) was not explained by desmin degradation, instead attributed to structural weakening of myofibrils from the thawing process (Kim et al., 2015).

Lamb stored chilled (3 weeks at -1.5 °C)-then-frozen (6 weeks) had comparable similar SF results to lamb stored chilled only for 9 weeks (Kim et al., 2011). Further research found chilled (2 weeks at -1.5 °C)-then-frozen (7 weeks) storage of lamb to also present comparable results (Kim et al., 2013). Similarly based on SF, Choe et al. (2016) found that 2 weeks chilled storage at -1.5 °C could be shortened to 8 days if chilled storage temperatures were increased to 3 or 7 °C; with no SF decrease occurring due to freezing following this duration. Peak tenderness of lamb was attained at 7 to 10 days at 1-4 °C (Dransfield, 1994; Starkey et al., 2015), consequently, once this is achieved, frozen storage would pose no further effect on tenderisation. Similarly for beef, peak tenderness is reported to
occur following 2 to 3 weeks chilled storage at 1-2 °C (Morgan et al., 1991; Dransfield, 1994), meaning that frozen storage following longer chilled storage durations will not reduce SF (Shanks et al., 2002; Farouk et al., 2009a).

These conflicting results suggest that the effect of chilled-then-frozen storage upon proteolysis and SF requires further investigation, similar to that of the work of Starkey et al. (2015). Furthermore, results investigating sensory tenderness of chilled-then-frozen meat have been scarce and inconsistent, likely due to consumer panel and cooking method variations (Lagerstedt et al., 2008; Vieira et al., 2009; Wiklund et al., 2009a; Wiklund et al., 2009b). These factors should be investigated in relation to meat stored chilled-then-frozen to provide better understanding and management.

1.4.2. Fluid losses and juiciness

The effects of chilled-then-frozen storage on juiciness and WHC are varied, though there is a general trend toward a reduction in both (Table 1.3). Particular attention is to be paid towards the effect of chilled and frozen storage periods upon cooking losses, as two studies on beef found no effect of frozen storage when it was preceded by chilled storage of 21 days or longer (Shanks et al., 2002; Farouk et al., 2009a). One later study in fact found cook losses from chilled beef (4 weeks) to be decreased by freezing (2 weeks) after the chilled storage period (Kim et al., 2015). When comparing chilled (2-3 weeks)-then-frozen (6-7 weeks) storage of lamb, no effect on cooking losses was found; instead, rigor temperature and electrical stimulation were mentioned as factors (Kim et al., 2013).
Purge losses are increased by increased durations of both chilled and frozen storage, though more so by frozen storage due to thaw loss (Vieira et al., 2009). Kim et al. (2015) found that while freezing and thawing increased purge losses, chilling increased cook losses.

Regarding sensory juiciness, one study found a decrease following chilled (7 days)-then-frozen (24 hours) storage (Lagerstedt et al., 2008), while another did not find effects following chilled (3-10 days)-then-frozen (30-90 days) storage (Vieira et al., 2009). The lack of studies examining sensory juiciness following chilled-then-frozen storage highlights the need for a more comprehensive study.

1.4.3. Flavour

Results from chilled-then-frozen storage studies have reported some negative effects on flavour compared to chilled storage alone. For example, when chilled storage duration of beef prior to freezing is one week consumer flavour scores have decreased (Table 1.3; Lagerstedt et al., 2008; Wiklund et al., 2009b) and when long-term frozen storage temperatures of lamb are above -15 °C before the temperatures are then lessened, increased flavour intensity has been reported (Hagyard et al., 1993). Other studies have reported no effect on flavour of chilled (10 days at 4 °C)-then-frozen (90 days at -20 or -80 °C) beef (Vieira et al., 2009) or frozen (42 days at -20°C)-then-chilled (14 days at 2 °C) lamb (Duckett et al., 1998b). Effects upon TBARS can provide insight into these results as the chilled storage component (duration and temperature) is the main factor leading to lipid oxidation (Kim et al., 2011), and consequently, flavour perception as rancid (Campo et al., 2006). Excessively high frozen storage temperatures or
accidental thawing of frozen meat may increase the oxidative flavour (Hagyard et al., 1993; Bell & Garout, 1994), and along with prior chilled storage likely had greater effect on negative flavour development than any frozen storage duration, and thus decreased meat shelf life (Winger, 1984).

Further studies may also examine accidental freezing of chilled product, or the use of chilled-then-frozen storage, and its effects on flavour, as there have been accidental freezing in chilled export product (Leygonie et al., 2012). Another potential area for further study is the relationship between TBARS and sensory flavour for chilled-then-frozen meat (Table 1.3). Decreases in flavour scores of chilled-then-frozen meat have not always resulted in a lack of consumer acceptability or preference difference between such meat and unfrozen meat (Lagerstedt et al., 2008; Muela et al., 2016).

1.4.4. Colour

Studies reporting on colorimetric measures for meat stored chilled-then-frozen are scarce, and even more so are those investigating colour stability over retail display. Beef stored chilled (4 °C, 10 days)-then-frozen (-20/-80 °C, 90 days) was found to have the majority of its colour deterioration caused by the frozen storage phase rather than the chilled storage phase (Vieira et al., 2009; Kim et al., 2015). Another study meanwhile found that L* and a* increased in beef due to chilled storage (3 weeks at -1.5 °C) prior to frozen storage (6 weeks) (Farouk et al., 2009b); similar results were found for lamb L* and b* following chilled (2-3 weeks at -1.5 °C)-then frozen (6-7 weeks at -18 °C) storage remaining higher than those for frozen stored (9 weeks at -18 °C) meat (Kim et al., 2011). Chilled (2-4 weeks at -
1.5 °C)-then-frozen (5-7 weeks at -18 °C) storage was found to preserve lamb redness (a*) better over 5-8 days display compared to chilled meat (9 weeks at -1.5 °C) (Wiklund et al., 2009a; Kim et al., 2011; 2013). Similar results were found for colour stability, measured by a lower hue angle with display duration compared to chilled only meat, as chilled storage duration depletes metmyoglobin reducing activity and enzyme respiratory activity (Kim et al., 2011; 2013). This was not seen in beef (Kim et al., 2015), although the beef was only chilled for 4 weeks rather than 9 weeks. However, chilled (3-4 weeks)-then-frozen (2 weeks) beef presented a hue angle more similar to that of frozen (2 weeks) compared to chilled beef (4 weeks at -1.5 °C) (Kim et al., 2015). These results are consistent with results from chilled and frozen storage effects upon colour stability, wherein chilled storage increases the potential for lipid and Mb oxidation, although it also improves colour bloom on exposure to oxygen, compared to fresh or frozen meat. The lack of studies reporting on colour stability of chilled-then-frozen meat, however, prompts the need for further investigation.

1.4.5. Microbial profile

Vieira et al. (2009) reported that Enterobacteriaceae sp. were not detected in beef stored chilled (10 days at 4 °C)-then-frozen (90 days), although psychrotrophic bacteria proliferated regardless of frozen storage temperature (-20°C or -80 °C), though more so during the chilled storage period. However, all microbial loadings were measured as being below a threshold for frozen beef safety set at $10^5$ CFU/cm² (Hinton et al., 1998). Increased pH due to chilled storage may have facilitated microbial proliferation (Moore & Gill, 1987; Kim et al., 2011), though no change
occurred during the frozen storage period (Kim et al., 2013). Bacterial proliferation is halted in frozen meat provided temperatures remain sufficiently low (Winger, 1984; Bell & Garout, 1994; Eustace et al., 2014).

The microbial profile has not been thoroughly investigated in chilled-then-frozen meat, particularly in relation to long-term frozen storage following chilled storage. This is alarming as it constitutes a potential failure in the primary measure of food safety (Wood et al., 1999), which could undermine the safe export and long-term storage of product, should microbial loading be above safety thresholds (Bell & Garout, 1994; Hinton et al., 1998; Mills et al., 2014). However, previous studies have shown that frozen storage for 2 years or more at -10 °C or below has been ruled safe provided no chilled storage was used prior (Winger, 1984), and that chilled stored lamb (12 weeks below -0.5 °C; Sumner & Jenson, 2011) and beef (24 weeks below -0.5 °C; Small et al., 2012) have also been ruled safe depending on storage temperature. The combination of these findings may provide a basis for the use of safe chilled-then-frozen storage of red meat for export purposes, particularly given the associated benefits on meat quality (Table 1.3), but further study is merited for verification.
Table 1.3. Summary of the effects of combined chilled and frozen storage on the shear force, tenderness, fluid levels, flavour and colour of beef and lamb.

<table>
<thead>
<tr>
<th>Species</th>
<th>Chill</th>
<th>Freeze</th>
<th>First</th>
<th>Effects on selected traits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>7 days at 2°C</td>
<td>27 days at -30°C</td>
<td>Freezing</td>
<td>SF decreased further when freezing used first (56 to 50 N) Chilling had greater effect on SF (74 to 53 N)</td>
<td>Crouse &amp; Koohmaraie (1990)</td>
</tr>
<tr>
<td>Lamb</td>
<td>24 days at 2°C</td>
<td>42 days at -20°C</td>
<td>Freezing</td>
<td>Frozen callipyge lamb had lower SF (38 to 27 N) SF lower after 12 days chilling if callipyge lamb frozen prior Freezing had no effect on normal lamb SF (around 27 N)</td>
<td>Duckett et al. (1998a)</td>
</tr>
<tr>
<td>Beef</td>
<td>35 days at 2°C</td>
<td>2 months at -16°C</td>
<td>Chilling</td>
<td>When chilling was less than 21 days, SF decreased and cook losses increased (22.3 to 25.2%) due to freezing At 35 days, SF 38 N chilled, 30 N chilled-then-frozen</td>
<td>Shanks et al. (2002)</td>
</tr>
<tr>
<td>Beef</td>
<td>14 days at 4°C</td>
<td>2 months at -20°C</td>
<td>Chilling</td>
<td>When chilling was 0-7 days, freezing reduced SF (61 to 51 N) When chilling was 0-7 days, freezing reduced WHC (79.5 to 74.3%) No preference for chilled-then-frozen compared to chilled, though this method decreased tenderness (2.9 to 2.2), juiciness (2.3 to 1.7) and flavour (2.0 to 1.7)</td>
<td>Lagerstedt et al. (2008)</td>
</tr>
<tr>
<td>Beef</td>
<td>10 days at 4°C</td>
<td>90 days at -20°C/-80°C</td>
<td>Chilling</td>
<td>When chilling was 3 days, freezing improved tenderness (2.8 to 3.4) Similarly SF (73 to 52 N at 3 days; 52 to 47 N at 10 d) Freezing increased cook losses (18.1 to 23.5%) No effect of either on flavour (2.4 to 2.9) or juiciness (2.4 to 2.7) Freezing increased TBARS (0.89 to 1.93 mg MDA/kg) No effect of frozen storage temperature on any parameters</td>
<td>Vieira et al. (2009)</td>
</tr>
<tr>
<td>Lamb</td>
<td>2-3 weeks at -1.5°C</td>
<td>6-7 weeks at -18°C</td>
<td>Chilling</td>
<td>Chilling decreased SF more than freezing (28 N and 48 N, respectively, after 9 weeks) Chilling increased TBARS more than freezing (all below 0.05 mg MDA/kg)</td>
<td>Kim et al. (2011)</td>
</tr>
<tr>
<td>Animal</td>
<td>Chilling/Freezing Details</td>
<td>Storage Details</td>
<td>Storage Type</td>
<td>Notes</td>
<td></td>
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</tr>
<tr>
<td>Lamb</td>
<td>2-3 weeks at -1.5°C 6-7 weeks at -18°C</td>
<td>Chilling</td>
<td>Chilling</td>
<td>Chilling decreased SF (50 to 28 N) from 0 to 2 weeks Lack of influence of longer chilled storage (2-9 weeks) on SF Freezing increased drip losses Chilling (2-3 weeks)-then-freezing (6-7 weeks) retained a* better than 9 weeks either chilling or freezing</td>
<td></td>
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<tr>
<td>Kim et al. (2013)</td>
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</tr>
<tr>
<td>Beef</td>
<td>3-4 weeks at -1.5°C 2 weeks at -18°C</td>
<td>Chilling</td>
<td>Chilling</td>
<td>Chilled-then-frozen storage decreased SF (58 N) compared to chilled only (74 N) or frozen only (93 N) Freezing increased purge losses more so than chilling Longer chilled storage increased cook losses Frozen meat had lower L* and a* values than chilled meat Chilled-then-frozen meat had intermediate L* and a* values Hue angle higher in frozen meat if chilled prior to freezing</td>
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<tr>
<td>Kim et al. (2015)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lamb</td>
<td>6 days at 2-4°C 21 months at -18°C</td>
<td>Freezing</td>
<td>Freezing</td>
<td>Freezing increased SF after 15 months (66 to 88 N) Decreased SF due to chilling post-thaw (91 to 40 N after 21 months) SF decreased more on display (3-6 days) if freezing duration longer</td>
<td></td>
</tr>
<tr>
<td>Muela et al. (2015)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lamb</td>
<td>8 days at 3°C/7°C 14 days at -1.5°C</td>
<td>Chilling</td>
<td>Chilling</td>
<td>Neither chilling temperature nor freezing affected SF (around 40 N) Water loses unaffected by freezing (6.6 to 7.6%) – though higher chilling temperatures increased combined water losses (7.6 to 10.7%) Cook loss improved by increased chill temperatures (27.4 compared to 31.2%) Freezing post-chill decreased L* and b*, though higher chill temperatures better preserved it over 7 days display Lower chill temperature improved a* over display, though chilled-then-frozen a* was lower than chilled only</td>
<td></td>
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<tr>
<td>Choe et al. (2016)</td>
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</tr>
<tr>
<td>Lamb</td>
<td>4 days at 2-4°C 21 months at -18°C</td>
<td>Freezing</td>
<td>Freezing</td>
<td>Sensory panel used Acceptable up to and including 15 months frozen storage (3.4 to 3.1) Acceptability lower at 21 months (2.4)</td>
<td></td>
</tr>
<tr>
<td>Muela et al. (2016)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tbody>
</table>
All sensory panel scores for different traits given on a scale from 0 to 5. All shear force values converted to newtons (N) by the equation 1 kgF = 9.80665 N. SF = shear force; WHC = water holding capacity; TBARS = 2-thiobarbituric reactive substances (mg malondialdehyde per kg meat); MMb = metmyoglobin; a* = colorimetric measure of redness.
1.5. Conclusion

From this review, it is clear that there are few studies focusing on combined chilled and frozen storage practices, particularly regarding long-term chilled-then-frozen storage beyond 3-4 months total duration. Effects on quality parameters have mostly related to tenderness, of which encouraging results have been found, indicating the potential benefits of this combined storage approach for export purposes. However, it is apparent that there is a lack of reliable instrumental proxies for measuring other sensory quality traits, namely juiciness and flavour. Additionally, instrumental colour and microbial profile investigations have been scarce in chilled-then-frozen storage studies. As these investigations are highly important for consumer approval and safety, respectively, the need for a large-scale, comprehensive investigation of the quality traits identified herein over long-term chilled-then-frozen storage durations is apparent. Such a study could build on existing knowledge and enhance our understanding of chilled and frozen storage of red meat and develop quality and safety thresholds, assist in the delivery of a high quality meat product between nations and therefore improve global market access of meat, thus addressing the paucities present within this review.
Aims and objectives of this research

As limitations of current research were clearly stated in the literature review, it can be seen that the chilled-then-frozen storage combination poses an alternative to conventional chilled or frozen storage; however, further work is required on extending the storage duration beyond 3-4 months.

The aim of the proposed research was to use chilled storage to improve lamb meat quality in the short term (up to eight weeks), and determine a threshold upon which no further benefits are attained and quality deterioration has begun to occur. Similarly, the proposed research aimed to use frozen storage following chilled storage to allow for preservation of quality over long-term durations up to one year; where again storage thresholds based on consumer-derived quality and safety thresholds can be developed. Shear force (tenderness), display colour, microbial loading, health-claimable fatty acids and lipid oxidation are key traits which have been previously explored in literature and are known to change based on storage. This proposed study provides a comprehensive, large-scale investigation of the meat quality and food safety parameters previously absent from literature. It is within the bounds of this study that this safe, easy, cost-effective practice can be used to preserve export lamb for long periods of time, and can be applied in a similar way to beef meat.
Chapter 2: Effect of chilled-then-frozen storage (up to 52 weeks) on lamb *m. longissimus lumborum* quality and safety parameters (based on publication in Meat Science)

Abstract

This study evaluated the effect of chilled followed by frozen storage on lamb quality and safety parameters. Experimental (*n* = 360) *m. longissimus lumborum* (LL) were randomly sampled from the boning room of a commercial Australian abattoir, at 24 h *post-mortem*, and assigned to five chilled storage periods (0, 2, 4, 6 and 8 weeks) and six subsequent frozen storage periods (0, 4, 8, 12, 24 and 52 weeks). Upon completion of each storage treatment combination, corresponding LL were sub-sectioned and analysed for colour stability (0, 1, 2 and 3 days), shear force, fluid losses (purge, thaw and cooking losses), intramuscular fat content, sarcomere length, water activity and microbial load (lactic acid bacteria, *Enterobacteriaceae* sp., *Brochothrix thermosphacta*, *Clostridium perfringens* and *Escherichia coli*). LL stored chilled for 2–4 weeks prior to freezing up to one year presented superior results for shear force (below 40 N; *P* < 0.001), display colour (increased a* and R630/580; *P* < 0.001) and low levels of spoilage microbes (*P* < 0.001) compared to longer durations, correlating with good eating quality and safety following more than one year of frozen storage. Furthermore, frozen storage decreased the load of *B. thermosphacta* (*P* < 0.001) and *Enterobacteriaceae* sp. (*P* < 0.05) and slowed the rate of proliferation of lactic acid bacteria.

*Keywords:* Lamb, Preservation, Shelf life, Eating quality, Red meat
2.1. Introduction

Chilled or frozen storage are routinely used to preserve lamb meat over extended periods (Zhou et al., 2010; Leygonie et al., 2012; Bellés et al., 2017), however the sequential combination of these approaches may prove a better alternative (Coombs et al. (2017a); Chapter 1). For example, past research has found chilled-then-frozen storage resulted in improved tenderness (as shear force, SF), for lamb stored chilled (2-3 weeks)-then-frozen (6-7 weeks) compared to that kept frozen-only for 9 weeks (Kim et al., 2011). Furthermore, chilled-then-frozen lamb had equivalent colour stability to that kept chilled-only for the same duration (Kim et al., 2011). Other studies have examined different chilled storage temperatures and chilling rates prior to storage and observed only limited variation in meat quality across the subsequent relatively short frozen storage period (Kim et al., 2013; Choe et al., 2016). These studies did not examine chilled-then-frozen storage across long-term periods – a practice important to lamb processors who use freezing to smooth supply. Lamb kept under frozen-only storage for such ‘long term’ periods (9 months to 2 years) resulted in only minimal effects on eating quality (Winger, 1984; Muela et al., 2010; Fernandes et al., 2013; Muela et al., 2015). From this, it may be hypothesised that frozen storage could preserve the improvement in lamb eating quality inferred during the chilled storage period.

In examination of the effects of frozen storage on lamb preservation, frozen storage temperature effects have received minimal attention, though the general consensus was that any temperature below -15 °C would not pose any detrimental effect on meat sensory quality (Hagyard et al., 1993;
Farouk et al., 2003; Vieira et al., 2009). One study reported that storing meat at temperatures as high as -10 °C could still result in good quality (Winger, 1984), despite most studies using the internationally recognised temperature of -18 °C (Eustace et al., 2014; Coombs et al. (2017a); Chapter 1). Storing meat at warmer frozen storage temperatures, combined with increased frozen storage periods, could allow for bulk shipments of frozen product at slower ship speeds, with additional cost benefit from an increased storage temperature (Sumner, 2016).

Also apparent from Coombs et al. (2017a) (Chapter 1) was the relative scarcity of chilled-then-frozen storage studies that considered microbial analyses. This is an important oversight, as specific microbial loadings can advance the spoilage of meat and compromise product safety. Studies which examined microbial profiles of lamb kept under chilled-only (Sumner & Jenson, 2011; Kiermeier et al., 2013) or frozen-only storage periods (Fernandes et al., 2013) reported that microbial proliferation is a function of storage temperature and duration. This is relevant to spoilage and pathogenic bacteria common for anaerobically stored meat, such as; lactic acid bacteria (LAB), Enterobacteriaceae sp., Brochothrix thermosphacta, Clostridium perfringens and Escherichia coli (Borch et al., 1996; Mills et al., 2014).

This study therefore aimed to evaluate the effect of chilled-then-frozen storage combinations (maximum 8 weeks and 52 weeks, respectively) on the quality and safety of lamb, and simultaneously tested the independent effects of chilled- and frozen-only storage, the latter with two frozen storage temperatures (-12 and -18 °C).
2.2. Materials and Methods

2.2.1. Sampling, sectioning and purge loss assessment

On a single day, at 24 h post-mortem, a total of 360 lamb *m. longissimus lumborum* (LL) were randomly sampled from the boning room of a commercial Australian abattoir. These were assigned to five chilled storage periods (0, 2, 4, 6 and 8 weeks) and six ensuing frozen storage periods (0, 4, 8, 12, 24 and 52 weeks), set at two frozen storage temperatures (-12 and -18 °C). All LL were individually weighed, vacuum-packaged and stored onsite as per commercial practice for the duration of their assigned chilled storage period (mean temperature 0.6 ± 1.8 °C). LL assigned to frozen storage were frozen onsite using a commercial plate freezer and then transported to the Centre for Red Meat and Sheep Development (NSW Department of Primary Industries, Cowra, New South Wales, Australia) where they were allocated to four freezers set as two replicates per frozen storage temperature. Chilled-then-frozen temperature profiles were recorded by temperature loggers and are presented per treatment combination in Table 2.1.
Table 2.1. Mean (± SD) temperature (°C) of each storage treatment.

<table>
<thead>
<tr>
<th>Frozen storage (weeks)</th>
<th>0</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chilled storage (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.88</td>
<td>-12.73 ± 3.58</td>
<td>-15.88 ± 3.73</td>
</tr>
<tr>
<td>2</td>
<td>1.27 ± 2.40</td>
<td>-12.36 ± 3.01</td>
<td>-15.21 ± 3.64</td>
</tr>
<tr>
<td>4</td>
<td>0.69 ± 1.89</td>
<td>-11.98 ± 2.63</td>
<td>-15.07 ± 3.62</td>
</tr>
<tr>
<td>6</td>
<td>0.29 ± 1.89</td>
<td>-11.77 ± 2.83</td>
<td>-15.02 ± 3.86</td>
</tr>
<tr>
<td>8</td>
<td>0.59 ± 1.77</td>
<td>-12.83 ± 3.72</td>
<td>-15.99 ± 3.98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frozen storage (weeks)</th>
<th>12</th>
<th>24</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-11.19 ± 0.25</td>
<td>-17.84 ± 0.28</td>
<td>-10.66 ± 0.64</td>
</tr>
<tr>
<td>12</td>
<td>-11.35 ± 0.34</td>
<td>-18.00 ± 0.17</td>
<td>-10.64 ± 0.60</td>
</tr>
<tr>
<td>12</td>
<td>-10.86 ± 0.72</td>
<td>-17.86 ± 0.49</td>
<td>-10.89 ± 0.67</td>
</tr>
<tr>
<td>12</td>
<td>-10.74 ± 0.64</td>
<td>-17.85 ± 0.51</td>
<td>-11.00 ± 0.75</td>
</tr>
<tr>
<td>12</td>
<td>-10.50 ± 0.63</td>
<td>-17.78 ± 0.45</td>
<td>-11.38 ± 0.73</td>
</tr>
</tbody>
</table>

Chilled storage preceded frozen storage. Each frozen storage treatment is a continuation from the previous treatment.
Upon completion of each storage treatment (chilled-only, frozen-only or chilled-then-frozen), each corresponding LL was removed from its vacuum packaging; excess moisture removed with a paper towel; weighed; and the change in weight between pre- and post-treatment used to calculate purge loss as a percentage (Honikel, 1998). These LL were then sub-sectioned as per Fig. 2.1, with care taken to maintain status (i.e. frozen LL were not allowed to thaw) and aseptic conditions. Portions assigned for colour stability and shear force testing were immediately tested and all other portions stored frozen at -80 °C until evaluation.

**Fig. 2.1.** Diagram of experimental lamb LL at sub-sectioning. M: microbial profile (~100 g); P: peroxidase activity; S: shear force (~ 65 g); I: intramuscular fat; C: colour stability; F: fatty acid profile; W: water activity (also includes sarcomere length and ultimate pH); T: TBARS; G: glycogen content; O: oxidation-reduction potential.

### 2.2.2. Colour stability and thaw loss

Colour stability was evaluated as per Holman *et al.* (2016b), whereby portions used were 3-4 cm thick slices individually placed on Styrofoam trays (13.5 cm x 13.5 cm) so that the muscle fibres were orientated perpendicular to the measured surface. These were then over-wrapped with PVC food film (thickness: 15 µm) and permitted to bloom in a chiller (mean temperature: 2.8 °C; mean relative humidity: 81.6%) under
continuous fluorescent lighting (NEC Tubes 58 W; mean light intensity: 866 lx as per handheld lux meter readings) for approximately 1 h post-slicing prior to the first colour measurement (day 0). This blooming time meant slices from the frozen treatments were thawed before measurement. A spectrophotometer (MiniScan Model 45/0-L, Series No. 7237, HunterLab Associates Laboratory, Inc., Hong Kong, China) with 25 mm aperture size and calibrated using Illuminant D-65 and 10° observer settings calibrated on black and white standard tiles was used to measure reflectance at 400-700 nm and CIE colorimetrics (L*, a* and b*; CIE, 1978). Duplicate measures were recorded with care taken to avoid fat and connective tissue, and this was repeated over the following 3 days (0-3). Hue, chroma and the ratio of absorbance at 630 nm and 580 nm were then calculated as per AMSA (2012).

These same portions were used to determine thaw loss; being the percentage change in weight of each slice between day 0 (frozen) and day 1 (thawed).

2.2.3. Cooking loss and shear force

Sub-sections (mean ± SD: 62.6 ± 9.6 g) for shear force (SF) testing were prepared, weighed and cooked for 35 min in a 71 °C water bath from chilled or frozen depending on whether they had been stored chilled or frozen. Immediately after cooking they were immersed in cold water for 30 min to ensure the cooking process was halted. Cooked samples had excess moisture removed with paper towel and were weighed so that cooking loss (CL) could be calculated as the percentage weight difference between pre- and post-cooking (Honikel, 1998).
Cooked SF portions were refrigerated overnight (4-5 °C) before being sectioned into six cuboidal strips (cross-sectional area: 1 cm x 1 cm) parallel to muscle fibre direction. These were then cut perpendicular to the fibre direction using a Warner-Bratzler vee-shaped blade with 200 mm/min crosshead speed and attached to a Lloyd texture analyser (Model LRX, Lloyd Instruments, Hampshire, United Kingdom) to measure peak force (in newtons, N). Care was taken to avoid connective and fatty tissue, and SF was calculated as the average of six technical replicates (Holman et al., 2015).

2.2.4. Sarcomere length

Sarcomere length (SL) measurements were taken using thin slices (< 1 mm thickness) parallel to the muscle fibres from each sample. These were analysed using a laser light diffraction unit (5 technical replicates) following the method of Bouton et al. (1973).

2.2.5. Ultimate pH

Ultimate pH (pHu) was quantified using a pH meter (smartCHEMC-CP, TPS Pty Ltd., Queensland, Australia) with a polypropylene spear-type gel electrode (IJ-44, Ionode™, Queensland, Australia) calibrated using pH 4.0 and 6.8 standards (at 20 °C), as the average of duplicate measures as per De Brito et al. (2016).

2.2.6. Water activity

Water activity (a_w) was measured using a specialised handheld probe (Model HC2-AW: HygroPalm23-AW, Rotronic Instruments Corp., New York, USA) that capped a 14 mm sample cup containing ~1.0 g still frozen
and diced samples. This remained in place for approximately 30 min until 
a_w stabilised and this and temperature values were then recorded. Each 
sample was measured as technical duplicates with their results averaged.

2.2.7. Moisture content and intramuscular fat

Moisture content and intramuscular fat (IMF) determinations were 
made using approximately 40.0 g of diced LL freeze-dried at -50 °C 
(ScanVac CoolSafe™, LaboGene ApS., Lynge, Denmark) and ground in a 
FOSS sample mill (Model 1095, KnifeTech™, FOSS Pacific, New South 
Wales, Australia) as per Hopkins et al. (2014). Moisture content was 
determined as the percentage difference between sample weight pre- and 
post-freeze drying.

IMF was then calculated using the adapted Soxhlet method (Smith et 
al., 2017), where 3.0 g freeze-dried sample was extracted with 85.0 mL 
hexane for 80 min within a Soxtec machine and the residual dried for 30 
min at 105 °C and weighed. The difference in weight before and after 
extraction constituted the IMF, which was then expressed as a percentage of 
fresh (wet) sample weight.

2.2.8. Glycogen content

Glycogen content was determined using 1.0 g sample incubated with 
10.0 mL Milli-Q water in boiling water (100 °C) for 5 min, homogenised at 
22,000 rpm (Series X10/25, Ystral™, Germany) and centrifuged (Model 
5810R, Eppendorf Pty. Ltd., Hamburg, Germany) at 2,465 g and 4 °C for 
15 minutes as per De Brito et al. (2016). The supernatant was then 
compared against a glycogen standard using the colorimetric protocol
outlined in the Glycogen Assay Kit (no. MAK016, Sigma-Aldrich Pty. Ltd., Missouri, United States of America) technical bulletin (Sigma-Aldrich, 2016), with absorbance at 570 nm measured using a micro-plate reader (FLUOStar OPTIMA™, BMG Labtechnologies, Victoria, Australia). Results were the average of technical duplicates and were expressed as mmol per kg fresh (wet) weight of meat.

2.2.9. Microbial loading

The analyses for microbial loading were undertaken at a commercial laboratory where, under aseptic conditions, 10.0 g of each sample was individually homogenised with 90.0 mL 0.1% peptone salt solution for 60 s using a laboratory paddle blender. From these, microbial loads, determined as colony forming units (CFU) per g fresh (wet) meat, were tested as:

*Brochothrix thermosphacta* samples (0.1 mL) were serially diluted using 0.1% peptone salt solution and spread on duplicate streptomycin-thallous acetate-acidione (STAA) plates which were then incubated for 48 ± 4 h at 22-25 °C. Suspect colonies (shiny, off-white and round) were verified using an oxidase test (oxidase negative) before being counted. Results of technical duplicates were averaged.

*Clostridium perfringens* samples (1.0 mL) were combined with 10.0-15.0 mL synthetic complete (SC) agar with added supplement on a petri dish, mixed well, and incubated at 44-47 °C in a water bath until solidification. This was then overlaid with 10.0 mL of the same SC agar and streaked with a reference agar. Control blank plates were also made and evaluated. All plates were then incubated anaerobically for 20 ± 2 h at 37 ±
1 °C before the number of black colonies was counted, with successive dilutions employed when necessary (< 150 colonies).

*Escherichia coli* samples (1.0 mL) were plated onto the centre of individual *E. coli* petrifilm count bases; upon which the petrifilm tops were then replaced and the sample was spread across the plate. These were allowed to solidify before incubation for 20 ± 2 h at 37 ± 2 °C before the number of blue colonies, with evident bubble formations, was counted.

*Enterobacteriaceae sp.* samples (1.0 mL) were pour plated with violet red bile glucose (VRBG) agar as the medium; once solidified, these were then overlaid with additional VRBG agar and incubated for 21 ± 3 h at 25-27 °C. Presumptive colonies (verified as dark red or purple with 0.5-2 mm diameter) were counted.

Lactic acid bacteria (LAB) samples were decimally diluted (0.1 mL) and spread on MRS agar to be then incubated for 72 ± 2 h at 30 ± 1 °C under anaerobic conditions – facilitated using a Campygen modified atmosphere gas generator. Typical colonies were counted once verified using both catalase (catalase negative) and Gram stain testing (Gram positive cocci, coccobacilli or rods).

2.2.10. Statistical analysis

To fit smooth non-linear effects of chilled and frozen storage periods, the linear mixed model smoothing spline framework was used as described by Verbyla et al. (1999) and was implemented in the statistical package *asreml* (Butler et al., 2009) under R (R Core Team, 2015). The full model for each response incorporated all the terms required to fit a two
dimensional smoothing spline for chilled storage period by frozen storage period, as well as accounting for the experimental design.

Fixed effects included were linear chilling period, linear freezing period, temperature and all interactions; random effects were spline chilled and frozen storage periods, as well as their interactions with linear chilled and frozen storage periods, temperature and each other. Remaining terms in the model accounted for design effects and included non-smooth (factor) effects of chilled and frozen storage period, freezer effects and interactions of freezer with chilled and frozen storage periods. Of the measured traits only data for purge required transformation (square root). Additional covariates were added to the above model as fixed linear effects and a random spline effect; these included pHu on all traits, SL on SF, and glycogen content on microbial loading.

For microbial data, random data was imputed as data above or below the upper or lower limits of detection, which was generated as random uniform data on a logarithmic (log10) scale. In this case, the imputed data was given a reduced weight of 0.5 in the analysis.

Following the approach in Verbyla et al. (1999), the statistical significance of fixed and random effects were examined separately in asreml (Butler et al., 2009) in R (R Core Team, 2015), with a Wald-type test with Kenward-Roger adjustment (Kenward & Roger, 1997) and likelihood ratio comparisons used to examine significance of temperature-related and spline components, respectively. The level of significance was set at $P < 0.05$. 
2.3. Results

2.3.1. Fluid losses

Purge losses of frozen-only and chilled-then-frozen samples were lower than those of chilled-only samples ($P < 0.001$; Fig. 2.2). Within chilled-only and chilled-then-frozen samples, purge losses increased with increasing chilled storage period ($P < 0.001$; Fig. 2.2), though this tended to decrease within chilled-then-frozen samples as the frozen storage period increased ($P = 0.086$; Fig. 2.2). No treatment effects were observed for thaw losses ($P > 0.05$), although it is worth noting that only LL exposed to frozen storage periods exceeding 0 weeks were tested for thaw loss assessment. Cooking loss increased with corresponding increases in chilled-only storage period ($P = 0.013$), with this effect also evident across chilled-then-frozen storage periods wherein frozen-only LL demonstrated no variation in cooking loss regardless of period ($P > 0.05$). Freezer temperature and interaction effects were not significant ($P > 0.05$).
Fig. 2.2. Predicted mean (± SE) purge loss (square root transformed) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. For frozen storage, purge losses were measured on frozen LL without thawing.

2.3.2. Colour stability

Chilled-only storage resulted in decreased colour stability over the display period (3 d) for all CIE traits except b* ($P < 0.05$; Fig. 2.5). The variation and the difference due to chilled-only storage (frozen = 0 weeks) between L* ($P = 0.046$; Fig. 2.3) and hue angle ($P = 0.006$; Fig. 2.8) on day 0 and L* ($P < 0.001$; Fig. 2.3), a* ($P < 0.001$; Fig. 2.4), R630/580 ($P < 0.001$; Fig. 2.6), chroma ($P = 0.003$; Fig. 2.7) and hue angle ($P < 0.001$; Fig. 2.8) on day 3 are illustrated. L* was not influenced by frozen storage period, whether frozen-only or in combination with chilled (chilled-then-frozen; $P > 0.05$; Fig. 2.3), unlike a*, b*, R630/580 and chroma ($P < 0.05$), which all decreased as frozen storage period increased (Figs. 2.4-2.7), or hue angle...
which increased \((P < 0.01; \text{Fig. 2.8})\). This shift was especially obvious following 52 weeks frozen storage (whether frozen-only or chilled-then-frozen). The longer periods of frozen storage (24 and 52 weeks) led to the greatest decline in colour stability and for traits like \(a^*\) and R630/580 there was a notable effect of freezing compared to chilled meat (Fig. 2.4; Fig. 2.6). No chilled-then-frozen storage period combination or frozen storage temperature effects were observed \((P > 0.05)\).
Fig. 2.3. Predicted mean (± SE) lightness score (CIE L*) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line.
**Fig. 2.4.** Predicted mean (± SE) redness score (CIE $a^*$) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line. The dotted horizontal lines indicate the minimum $a^*$ threshold for 50% consumer acceptability (Khliji et al., 2010), with any data below the line indicative of unacceptability.
Fig. 2.5. Predicted mean (± SE) yellowness score (CIE b*\textsuperscript{a}) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line.
Fig. 2.6. Predicted mean (± SE) reflectance ratio (R630/580) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line. The dotted horizontal lines indicate the minimum R630/580 threshold for 50% consumer acceptability (Khliji et al., 2010).
**Fig. 2.7.** Predicted mean (± SE) colour intensity (chroma) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line.
**Fig. 2.8.** Predicted mean (± SE) hue angle of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line.
2.3.3. Shear force

Shear force was reduced with increased chilled-only \((P < 0.001)\) and frozen-only \((P = 0.028)\) storage. There was a noticeable decrease in SF after only 2 weeks of chilled storage, and the relationship between storage period and SF is non-linear (Fig. 2.9) for both chilled and frozen storage, where SF declines were evident between 0 and 8 weeks frozen storage before levelling out at longer storage periods \((P > 0.05)\). Chilled-then-frozen storage period combination, pHu and frozen storage temperature did not have any observed effect on SF \((P > 0.05)\). There was also no effect \((P > 0.05)\) of SL on SF.

![Graph showing predicted mean shear force (SF) for chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line. The horizontal dotted line indicates the maximum SF threshold for 50% consumer acceptability (Hopkins et al., 2006).](image)

**Fig. 2.9.** Predicted mean (± SE) shear force (SF) of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line. The horizontal dotted line indicates the maximum SF threshold for 50% consumer acceptability (Hopkins et al., 2006).
2.3.4 Sarcomere length

No storage type, period or temperature effects were observed for SL (mean ± standard error (SE): 1.71 ± 0.03 µm; \( P > 0.05 \)).

2.3.5 Ultimate pH

No storage type, period or temperature effects were observed for pHu (mean ± SE: 5.70 ± 0.04; range: 5.30 – 6.57; \( P < 0.05 \)). However, pHu was found to exhibit a significant negative relationship with glycogen content, SL, fluid losses and colour on day 0 (\( P < 0.001 \)) and a positive relationship with a* and R630/580 on days 1-3 and chroma on day 1-2 (\( P < 0.05 \)), but this did not change the significance of the fixed terms in the model (e.g. storage type, storage period). The coefficients for pHu effects upon these traits are shown in Table 2.2.
Table 2.2. Interaction of ultimate pH (pHu) with selected meat quality traits. Traits were considered for this table if they were significantly affected by pHu ($P < 0.05$).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Coefficient (± SE)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>-26.6 (6.65)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SL</td>
<td>-0.14 (0.04)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TL</td>
<td>-6.96 (1.08)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PL</td>
<td>-0.53 (0.12)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CL</td>
<td>-6.78 (1.00)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>L* (days 0, 1, 2, 3)</td>
<td>-4.08 (1.47); -5.90 (0.99); -5.66 (0.94); -5.73 (0.91)</td>
<td>0.006; &lt; 0.001; &lt; 0.001; &lt; 0.001</td>
</tr>
<tr>
<td>a* (days 0, 1, 2, 3)</td>
<td>-2.20 (0.84); 3.57 (0.55); 4.16 (0.58); 2.61 (0.70)</td>
<td>0.010; &lt; 0.001; 0.003; &lt; 0.001</td>
</tr>
<tr>
<td>b* (days 0, 1, 2, 3)</td>
<td>-3.25 (0.78); -0.83 (0.62); -1.43 (0.54); -1.92 (0.44)</td>
<td>&lt; 0.001; 0.208; 0.045; &lt; 0.001</td>
</tr>
<tr>
<td>R630/580 (days 0, 1, 2, 3)</td>
<td>0.15 (0.36); 2.83 (0.30); 2.18 (0.22); 1.61 (0.15)</td>
<td>0.681; &lt; 0.001; &lt; 0.001; &lt; 0.001</td>
</tr>
<tr>
<td>Chroma (days 0, 1, 2, 3)</td>
<td>-3.65 (1.09); 1.78 (0.68); 1.82 (0.65); -0.01 (0.82)</td>
<td>&lt; 0.001; 0.009; 0.006; 0.995</td>
</tr>
<tr>
<td>Hue (days 0, 1, 2, 3)</td>
<td>-0.04 (0.02); -0.13 (0.02); -0.18 (0.02); -0.19 (0.02)</td>
<td>0.041; &lt; 0.001; &lt; 0.001; &lt; 0.001</td>
</tr>
</tbody>
</table>

2.3.6 Water activity

No effects of chilled or frozen storage period or temperature were observed for $a_w$ (mean ± SE: 0.94 ± 0.01; $P > 0.05$).

2.3.7 Moisture and intramuscular fat contents

Moisture content (MC) and IMF tended to decrease ($P = 0.090$) and increase ($P = 0.052$), respectively, with increased chilled-only storage period (Table 2.3). The effect of the chilled storage period was more noticeable in both MC and IMF when LL were chilled, then frozen for 52 weeks (Table 2.3). Reflecting this trend, moisture content decreased as frozen-only storage period increased ($P < 0.001$), unlike IMF which did not vary ($P > 0.05$) (Table 2.3). IMF did increase due to chilled-then-frozen storage interactions with frozen storage temperature ($P = 0.013$), with IMF higher at -18 °C at shorter chilled-then-frozen periods than -12 °C counterparts, which, in turn, exhibited higher IMF at longer storage periods.
Table 2.3. Predicted means (± SE) for the effect of chilled-then-frozen storage on the proportions of moisture content (MC) and intramuscular fat (IMF). Both are expressed as percentages.

<table>
<thead>
<tr>
<th>Chilled Storage (weeks)</th>
<th>Frozen Storage (weeks)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC</td>
<td>53.3 (0.8)^a</td>
<td>52.8 (0.7)^ab</td>
<td>52.4 (0.6)^ab</td>
<td>52.0 (0.7)^b</td>
<td>51.0 (0.7)^bc</td>
<td>50.0 (1.3)^c</td>
</tr>
<tr>
<td></td>
<td>IMF</td>
<td>4.80 (0.3)</td>
<td>4.78 (0.3)</td>
<td>4.77 (0.3)</td>
<td>4.75 (0.2)</td>
<td>4.69 (0.2)</td>
<td>4.56 (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>MC</td>
<td>52.7 (0.6)^a</td>
<td>52.3 (0.5)^a</td>
<td>51.9 (0.5)^ab</td>
<td>51.6 (0.4)^ab</td>
<td>50.4 (0.6)^b</td>
<td>47.9 (1.0)^c</td>
</tr>
<tr>
<td></td>
<td>IMF</td>
<td>4.86 (0.2)</td>
<td>4.86 (0.2)</td>
<td>4.86 (0.2)</td>
<td>4.85 (0.2)</td>
<td>4.85 (0.2)</td>
<td>4.83 (0.4)</td>
</tr>
<tr>
<td>4</td>
<td>MC</td>
<td>52.4 (0.6)^a</td>
<td>52.1 (0.5)^ab</td>
<td>51.7 (0.4)^ab</td>
<td>51.3 (0.5)^ab</td>
<td>50.3 (0.7)^b</td>
<td>48.1 (1.0)^c</td>
</tr>
<tr>
<td></td>
<td>IMF</td>
<td>4.92 (0.2)</td>
<td>4.94 (0.2)</td>
<td>4.95 (0.1)</td>
<td>4.96 (0.1)</td>
<td>5.00 (0.1)</td>
<td>5.10 (0.3)</td>
</tr>
<tr>
<td>6</td>
<td>MC</td>
<td>52.4 (0.6)^a</td>
<td>51.8 (0.5)^a</td>
<td>51.3 (0.5)^ab</td>
<td>50.9 (0.5)^ab</td>
<td>49.8 (0.7)^bc</td>
<td>49.0 (1.0)^c</td>
</tr>
<tr>
<td></td>
<td>IMF</td>
<td>4.98 (0.2)</td>
<td>5.01 (0.2)</td>
<td>5.04 (0.2)</td>
<td>5.07 (0.2)</td>
<td>5.16 (0.2)</td>
<td>5.37 (0.4)</td>
</tr>
<tr>
<td>8</td>
<td>MC</td>
<td>52.5 (0.9)^a</td>
<td>51.7 (0.7)^ab</td>
<td>51.0 (0.6)^abc</td>
<td>50.3 (0.7)^bc</td>
<td>49.2 (0.9)^c</td>
<td>50.5 (1.3)^bc</td>
</tr>
<tr>
<td></td>
<td>IMF</td>
<td>5.04 (0.3)</td>
<td>5.09 (0.3)</td>
<td>5.14 (0.3)</td>
<td>5.18 (0.2)</td>
<td>5.32 (0.2)</td>
<td>5.64 (0.5)</td>
</tr>
</tbody>
</table>

abc: Different letters in the same row reflect a significant effect of frozen storage upon MC ($P < 0.05$).
2.3.8. Glycogen content

Glycogen content was unaffected by chilled-only storage periods ($P > 0.05$). Frozen-only storage period did increase glycogen content ($P < 0.001$), particularly at 52 weeks (Fig. 2.10). Chilled-then-frozen storage was influential when frozen storage temperature was -12 °C, resulting in greater glycogen content increases compared to -18 °C – a difference more apparent as chilled storage periods increased ($P = 0.05$).

![Graph showing glycogen content over time](image)

**Fig. 2.10.** Predicted mean (± SE) glycogen content of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line.
2.3.9. Microbial loading

Brochothrix thermosphacta, LAB, and Enterobacteriaceae sp. were all detected (limit of detection = 1 log$\text{_{10}}$ CFU/g) across each storage treatment combination. Clostridium perfringens and E. coli were not detected. Increased chilled-only storage periods resulted in parallel increases to the loading of these detected microbial types ($P < 0.001$; Fig. 2.11). LAB generally increased with increasing frozen-only storage periods ($P < 0.001$), albeit a decrease was evident at 24 weeks frozen storage, but this trend was recovered by 52 weeks (Fig. 2.11a). B. thermosphacta increased between 12 and 52 weeks frozen storage when LL were frozen-only ($P < 0.001$; Fig. 2.11b). Furthermore, B. thermosphacta was observed to decrease within the chilled-then-frozen storage context ($P = 0.004$; Fig. 2.11b), such that as the period of frozen storage increased, the growth of B. thermosphacta was reduced, particularly in LL chilled for between 4-8 weeks. Frozen-only storage had no effect on Enterobacteriaceae sp. loadings ($P > 0.05$, Fig. 2.11c). Likewise, frozen storage temperature had no significant effect on microbial load for any tested species ($P > 0.05$). Increased muscle glycogen content prevented microbial proliferation of all three detected species in this study ($P < 0.05$), particularly Enterobacteriaceae sp. ($P < 0.001$; Table 2.4).
Fig. 2.11. Predicted mean (± SE) microbial loadings of A) *lactic acid bacteria*; B) *Brochothrix thermosphacta*; and C) *Enterobacteriaceae sp.* of chilled-then-frozen lamb LL stored at two frozen storage temperatures (-12 and -18 °C), with predicted means for each temperature averaged. Frozen-only data has been included as a grey line. The y-axes are the logarithmic count of each microbial species. CFU = colony forming units. No detections of *Clostridium perfringens* or *Escherichia coli* were found in any LL.
Table 2.4. Effect of glycogen content upon microbial loading of chilled-then-frozen meat. Microbial species were selected based on loading being above the limit of detection.

<table>
<thead>
<tr>
<th>Species</th>
<th>Coefficient (± SE)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid bacteria</td>
<td>-0.01 (0.01)</td>
<td>0.017</td>
</tr>
<tr>
<td><em>Brochothrix thermosphacta</em></td>
<td>-0.01 (0.01)</td>
<td>0.048</td>
</tr>
<tr>
<td><em>Enterobacteriaceae sp.</em></td>
<td>-0.03 (0.01)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
2.4. Discussion

Both chilled and frozen storage affected the meat quality of lamb LL in this study. The relative absence of any interaction effect from chilled-then-frozen storage on meat quality was interesting. Additionally, frozen storage temperature only had negligible effects on meat quality parameters and no effect on microbial loading.

The use of the warmer frozen storage temperature (-12 °C) compared to the internationally recognised temperature (-18 °C; Eustace et al., 2014) tested the potential to maintain lamb LL quality and safety at different frozen storage temperatures. Differences in quality due to temperature were minimal, and combined with no difference in the load of spoilage microbes suggests -12 °C could be used to provide comparable safe frozen storage to the -18 °C temperature for the maintenance of the quality of conventionally chilled meat. It is also noted that variability in temperature existed at different storage periods, however this can be attributed to the position of the temperature logger within samples and within the wider context of the study, this was unlikely to have any effect upon the results. A basis for this observation could be the use of a commercial freezer/freezing rate, which prevented excess damage to muscle ultrastructure (Grujić et al., 1993; Petrović et al., 1993; Ngapo et al., 1999; Fernández et al., 2007) and maintained a uniform ice crystal size (Grujić et al., 1993; Ngapo et al., 1999). This was also evident in the lack of frozen storage period effects upon thaw losses in the present study. Similarly, most prior frozen storage studies have found that freezing method and rate, not storage period or temperature; have resulted in alterations to meat quality
(Muela et al., 2010; Muela et al., 2012; Bueno et al., 2013). This result means that there is potential to reduce the costs associated with maintaining freezers at lower temperatures, and even higher frozen storage temperatures such as -10 °C could be tested for use, provided good temperature control is used, which can fulfil paucities arising from prior studies examining eating quality of lamb stored at -10 °C (Winger, 1984; Hagyard et al., 1993). It would, however, be advantageous to validate this result further through consumer studies focusing on odour and flavour (a complementary sensory study did take place but was limited by its design; Coombs et al., 2016) or through measurement of ice crystal size.

In terms of eating quality type traits, the decreased SF and increased IMF proportion showed the positive effects of both chilled- and frozen-only storage periods. The increased proportion of IMF may be related to the proportional moisture loss from experimental LL, as purge and thaw losses, and the corresponding effect of ‘concentrating’ IMF levels. This could, in turn, result in improved juiciness and flavour traits (Hopkins et al., 2006; Pannier et al., 2014), but in practice may not be perceived by consumers, whom identify IMF > 4.5% as preferable in terms of juiciness (Pannier et al., 2014); mean values were all higher than this in the present study regardless of treatment. This observation is supported with juiciness found to rarely increase across frozen storage periods (Muela et al., 2012; Fernandes et al., 2013; Muela et al., 2016) and even decrease in some instances (Bueno et al., 2013).

Tenderness increased due to increased chilled storage periods, based on the decreased SF in this study showing consistency with prior studies on
chilled lamb (Bellés et al., 2017). The decrease was most significant following 2 weeks chilled storage, which is consistent with prior studies suggesting tenderisation occurs within 7-10 d from slaughter in lamb (Dransfield, 1994; Thompson et al., 2005; Starkey et al., 2015). Frozen lamb has rarely increased in sensory tenderness (Muela et al., 2012; Bueno et al., 2013; Fernandes et al., 2013; Muela et al., 2016) despite decreases in SF in the short term (Muela et al., 2015). Nevertheless, SF results in the current study remained under the consumer acceptability threshold (40 N) based on the modelling of Hopkins et al. (2006) if chilled for more than 4 weeks, irrespective of the period of frozen storage. By contrast the unchilled lamb was not acceptable based on the threshold, and two weeks chilling provided a significant improvement. It is believed that under the extended storage periods, sufficient proteolysis had occurred to tenderise the meat via calpains in the case of chilled storage (Dransfield, 1994; Huff-Lonergan et al., 1996; Hopkins & Thompson, 2002) and tissue damage in the case of frozen storage (Leygonie et al., 2012). It was likely; however, that tenderisation via proteolysis did also occur in frozen-only meat, albeit at a slower rate due to diminished calpain activity at frozen temperatures (Crouse & Koohmaraie, 1990; Dransfield, 1994; Duckett et al., 1998b).

Upon display, both chilled and frozen storage periods increased the rate of discolouration compared to fresh meat, reflected by a decrease in $a^*$, R630/580 and chroma values, which is consistent with prior studies examining chilled (Ponnampalam et al., 2001) and frozen (Muela et al., 2015) storage of lamb. Denaturation of myoglobin from cellular disruption during chilled and frozen storage results in its more rapid oxidation to metmyoglobin and accumulation on the meat surface upon exposure to
oxygen, resulting in a brown colour (Leygonie et al., 2012; Muela et al., 2015) – frozen storage additionally reduces stability due to the metmyoglobin reducing system being compromised which thereby affects the ability of these anaerobically stored samples to bloom upon display (Ledward, 1985). Chilled-only storage did, however, result in higher a* values on day 1 of display after periods of 2-6 weeks compared to zero weeks (Fig. 2.4), which likely presented as a stabilised bloomed red colour due to peak oxygenation from myoglobin to oxymyoglobin, consistent with Ponnampalam et al. (2001). This oxygenation effect from the chilled storage was preserved when the lamb was then frozen for 4 weeks, having increased redness compared to frozen-only storage (Fig. 2.4) as per Kim et al. (2011), however this effect did not continue with increased frozen storage period.

The presence of spoilage microbial species increased as the chilled storage period lengthened, although all microbial species were below safety thresholds even after 8 weeks; 8 log CFU/g for LAB (Bell, 2001; Kiermeier et al., 2013) and 6 log CFU/g for B. thermosphacta and Enterobacteriaceae sp. (Gill, 2004; Gribble et al., 2014). B. thermosphacta decreased with subsequent frozen storage, particularly following 12 weeks (Fig. 2.11b), which indicates one advantage of freezing. Similarly, proliferation of Enterobacteriaceae sp. decreased and proliferation of LAB increased at a slower rate as applied frozen storage periods increased beyond 12 weeks (Fig. 2.11a). Microbial profiles in this study indicate good management practices (initial load < 3 log CFU/g; Gill, 2004), safety (no E. coli; Mills et al., 2014) and good temperature control (maintained safety), even though mean chilled storage temperatures (Table 2.1) were above the ideal temperature for export safety of around -1.5 °C (Bell, 2001; Sumner &
This good management may have contributed to the observation that frozen-stored LL (regardless of prior chilled storage period) exhibited low microbial loading that remained below spoilage thresholds for up to 52 weeks, which is consistent with prior frozen storage studies (Hinton et al., 1998; Fernandes et al., 2013). The implications from this suggest a maintenance of meat quality, as microbial spoilage can result in greening (Egan et al., 1988), discoulouration (Li et al., 2015) and off-odours and flavours (Gill, 2004; Gribble et al., 2014; Mills et al., 2014). Even so, the observed discolouration (decreased a* and R630/580, increased hue angle) for 8 weeks chilled storage may be attributable to this microbial proliferation despite safety being assured. Increased glycogen content was associated with decreased microbial proliferation in this study due to it presenting a favourable substrate for spoilage microbes and thus preserving chilled-then-frozen lamb LL from spoilage, where amino acids become consumed by bacteria resulting in off-odours (Coombs et al., (2017); Chapter 1). This result should however be interpreted with caution due to the sharp increase in glycogen content observed between 24 and 52 weeks frozen storage, and the steady decrease in \textit{B. thermosphacta} and \textit{Enterobacteriaceae sp.} at the same period. Meanwhile, glycolysis produces lactic acid as a by-product (Pethick et al., 1995), as does LAB, and the results do not show an inverse relationship between LAB and glycogen at 24 to 52 weeks frozen storage.

Glycogen content, water holding capacity (decreased water losses) and colour stability (increased a* and R630/580 and decreased hue on all days of display) all exhibited strong positive relationships with increased pHu. Higher pHu was associated with a dark red colour, particularly on days
1-2 of display (decreased L* and b*, increased a* and chroma), which is consistent with low glycogen levels at slaughter resulting in lower lactic acid and pH not declining (Pethick et al., 1995). Increased pHu was also associated with shorter sarcomeres, which likely occurred due to pre-rigor shortening (Starkey et al., 2015), although this did not translate into any effect on SF from either pHu or SL, despite prior findings suggesting lower pHu (5.5 – 5.8) to result in increased tenderness (Devine et al., 1993). The wide ranges of pHu and glycogen content encountered in this study may have occurred due to random sampling of LL, and different LL being assigned to different storage period combinations. This may also explain the marked increase of glycogen content in the LL sampled at 52 weeks, given the influence of pre-slaughter factors (Warner et al., 2010); however such an increase as reported in the present study is not biologically plausible. It would be beneficial to validate this observation in future studies through measurement of glycogen content in frozen meat using different techniques and gaining further insight into this mechanism.

In comparison to existing thresholds for meat quality measures and microbial levels we can suggest that the temperatures and periods for chilled storage (Table 2.1) and frozen storage (-12 °C) used in this study could be used in commercial chilled and frozen storage for export and lamb would remain below spoilage thresholds. Meat quality effects were mostly negligible, although decreased colour stability and increased water losses (purge and thaw) can affect the marketability of chilled and frozen lamb LL at a retail level, particularly when displayed aerobically. Colour acceptability was likely to be the most limiting factor towards adoption of these long-term chilled-then-frozen storage combinations with all
experimental LL considered as too discoloured following 3 d display based on R630/580 limits (Khliji et al., 2010), while at 1 d display LL were acceptable up to and including 2/52, 4/12, 6/4 and 8/0 (weeks chilled/frozen combinations). In terms of a* limits from this same study, chilled-only storage for 0-4 weeks would result in acceptable colour for 3 d and 2/52, 4/8, 6/4 and 8/0 (weeks chilled/frozen combinations) would result in acceptable colour for only 1 d.
2.5. Conclusion

It can be concluded that chilled storage prior to conventional frozen storage can improve and preserve the meat qualities of lamb, evident as tenderness (SF) and colour stability parameters. Furthermore, the optimal pre-freeze chilled storage duration was identified as 2 to 4 weeks – this facilitating the least negative effects in terms of promoting microbial spoilage. Chilled storage beyond 4 weeks is not recommended based on the results presented here as there was negligible improvement in shear force and increased development of discolouration (upon aerobic display) and spoilage microbial proliferation. Frozen storage, meanwhile, proved acceptable for up to one year at both -12 and -18 °C regardless of prior chilled storage period with minimal quality deterioration based upon the results of this study, although it would be beneficial if future studies investigated odour/flavour properties, retail storage and display conditions. Additional insight into the effects from similar chilled-then-frozen storage periods on protein and lipid oxidation would complement these recommendations and together could benefit product distribution in terms of guaranteeing product quality and value. Food service markets such as restaurants or additional processing would be suitable destinations for long-term chilled-then-frozen meat given excessive colour deterioration and thaw losses.
Chapter 3: Effects of chilled-then-frozen storage (up to 52 weeks) on the lamb $m. \text{longissimus lumborum}$ fatty acid profile and lipid oxidation biomarkers (based on submission to Meat Science)

Abstract

This study investigated the variations to the fatty acid profile and lipid oxidation parameters in lamb $m. \text{longissimus lumborum}$ (LL) kept under various chilled-then-frozen storage period combinations. These LL ($n = 360$) were randomly sampled at 24 h post-mortem from a commercial Australian abattoir and distributed over five chilled storage periods (0, 2, 4, 6 and 8 weeks) followed by six frozen storage periods (0, 4, 8, 12, 24 and 52 weeks) at either -12 °C or -18 °C. All LL were analysed for fatty acid profile and lipid oxidative parameters (TBARS, oxidation-reduction potential and peroxidase activity). Health-claimable polyunsaturated fatty acids (EPA and DHA) were unaffected by neither storage period nor temperature ($P > 0.05; 0.4-0.7 \text{ g/ 100 g lipid}$). TBARS levels did not exceed rancidity thresholds recommended in the literature (2.0 mg/kg meat), with these and other lipid peroxidation parameters showing broad increases in concentration with more ‘long-term’ chilled and frozen storage periods ($P < 0.05$). Combined, the results suggest nutritional and eating quality were maintained across various chilled-then-frozen storage periods applied in this study.

Keywords: Lamb; Preservation; Lipid peroxidation; Rancidity; Fatty acids; Nutritional quality; Health-claimable fatty acids.
3.1. Introduction

Lamb is an important international commodity, with countries like Australia and New Zealand being major exporters (MLA, 2016). Participation in the international market, and to benefit from its associated revenue depends on the delivery of high quality meat (Eustace et al., 2014). Such lamb has been characterised as having: 1) desirable sensory or eating quality, for traits such as flavour which underpin lamb’s desirability to consumers (Muela et al., 2012; MLA, 2015); and, 2) nutritional quality, with particular emphasis on health-claimable fatty acids (FA) eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3) because of their association to anti-inflammatory and anti-cancer functions (McAfee et al., 2010; Alvarenga et al., 2015). Already, effort has been made to improve EPA and DHA content of lamb meat, most commonly through managing animal feeds, and these have contributed to its classification as a source of these healthy FA (Alvarenga et al., 2015; Ponnampalam et al., 2016), in which a “source” is defined as 30 and 40 mg per 100 g and per 100 kcal and a “good source” as 60 and 80 mg per 100 g and per 100 kcal in Australia and New Zealand (FSANZ, 2014) and the European Union (Commission Regulation of European Union, 2010), respectively. In doing so, this may have inadvertently also increased its susceptibility to rancidity and associated detriments to flavour acceptability (Wood et al., 2003; Hopkins et al., 2014; Alvarenga et al., 2015; Ponnampalam et al., 2016). All polyunsaturated fatty acids (PUFA) are
somewhat susceptible to rancidity, which arises from the prevalence of their double carbon bonds to oxidise (Cifuni et al., 2000).

Chilled and frozen storage are routinely used to preserve meat against such oxidation, with recent studies reporting their potential combination (chilled-then-frozen storage) to deliver long-term preservation of lamb meat (Kim et al., 2011; Coombs et al. (2017a); Chapter 1; Coombs et al. (2017b); Chapter 2). These studies, however, did not examine this effect on nutritional quality, specifically whether chilled-then-frozen storage influences lamb fatty acid profile (FAP) or the extent of lipid oxidation in long-term chilled-then-frozen meat (Campo et al., 2006; Coombs et al. (2017a); Chapter 1).

Lipid oxidation (measured as TBARS) has been found to increase with increased chilled (Campo et al., 2006; Colle et al., 2015) and frozen storage periods (Muela et al., 2010; Muela et al., 2015). However, TBARS levels were observed to decrease when frozen storage periods exceed 6 months in some cases (Leygonie et al., 2012; Muela et al., 2015; Alonso et al., 2016). In fact, Kim et al. (2011) reported TBARS levels of chilled-then-frozen lamb meat to be intermediate of chilled and frozen lamb stored for the same period. This study did not include a fresh meat (0 weeks storage) control or chilled-then-frozen storage periods beyond 9 weeks, which could have added to its value. Furthermore, the study of Kim et al. (2011) and others investigating chilled and frozen storage conditions failed to investigate peroxidase activity, an important indicator of anti-oxidation in meat tissue due to its catalysis of hydrogen peroxide, thus it would be expected to increase concurrently with TBARS (Lee et al., 1996; Daun et
Such studies also did not measure oxidation-reduction potential (ORP) that similarly indicates potential for oxidation and which acts as a precursor to the increase in TBARS (Bekhit et al., 2013; Alonso et al., 2016). Understanding variations to these would provide insight into lipid oxidation as a result of anaerobic storage (both chilled and frozen).

Based on the literature, it was also apparent that characterisation of the effect of frozen storage temperature upon chilled-then-frozen lamb lipid profile is scarce (Coombs et al. (2017a); Chapter 1), although one previous study found flavour intensity towards rancidity to occur more rapidly at frozen storage temperatures of -10 °C compared to -15 °C (Hagyard et al., 1993), but they did not measure FAP or lipid peroxidation markers. This general lack of frozen storage temperature examination in the literature is unfortunate and suggests the generally internationally recognised frozen storage temperature of -18 °C (Food Science Australia, 2005; USDA, 2013) is arbitrary rather than scientifically founded (Coombs et al. (2017a); Chapter 1).

This study aimed to evaluate the FAP and biomarkers for lipid peroxidation (including TBARS, peroxidase activity and ORP) for lamb stored under chilled-then-frozen storage period combinations (up to 8 weeks and 52 weeks, respectively) and at two different frozen storage temperatures (-12 and -18 °C).
3.2. Materials and methods

3.2.1. Sample collection and storage treatments

Experimental lamb *m. longissimus lumborum* (LL) were collected (*n* = 360) as per Coombs *et al.* (2017b) (Chapter 2). Following this, LL were assigned to experimental treatment groups which comprised all combinations of five chilled storage periods (0, 2, 4, 6 and 8 weeks), six ensuing frozen storage periods (0, 4, 8, 12, 24 and 52 weeks) and two frozen storage temperatures (-12 and -18 °C). It should be noted that for the analysis, samples allocated to the two nominal groups for chilled-only (0 weeks frozen) storage at -12 and -18 °C were merged to form one group. Vacuum-packaged LL were stored chilled under commercial conditions (export abattoir), with LL assigned to frozen storage first frozen onsite using an industrial plate freezer and then transported to the Centre for Red Meat and Sheep Development (Cowra, New South Wales, Australia) where they were kept in duplicate freezers set at each frozen storage temperature (total: 4 freezers). Once the storage period (chilled-then-frozen) combinations were completed, each LL was sub-sectioned and care was taken to maintain their status (i.e. frozen LL were not permitted to thaw). All subsections not immediately tested were kept at -80 °C.

3.2.2. Fatty acid profile

The quantification of FAP used a protocol adapted from Ponnampalam *et al.* (2014b) and freeze-dried samples sourced as described
in Coombs et al. (2017b) (Chapter 2). As such, 50.0 mg of homogenised freeze-dried muscle was combined with 500.0 µg tridecanoic acid dissolved in methanol to act as an internal standard (C13:0, Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) and then hydrolysed with 700.0 µg 10 N potassium hydroxide (KOH, Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) and 5.3 mL methanol through 90 min incubation at 55 °C and agitation every 20 min. Solutions were cooled to room temperature and combined with 600.0 µg sulfuric acid (H₂SO₄, Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) in water and then incubated for 90 min at 55 °C. Solutions were cooled at room temperature, combined with 3.0 mL hexane and 1.0 mL standard sodium chloride solution (NaCl, Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia); vortex mixed for 5 min and aliquoted into auto-sampler vials for gas chromatography (GC) analysis.

FAP quantification column specifications were: 60.0 m x 0.25 mm, 70% cyanopropyl polysilphenylene-siloxane with 0.25 µm BPX-70 (SGE). Oven settings were: 30 s at 100 °C before 20 °C temperature increases per minute to 130 °C, at which it was isothermally held for 2 min, then 1 °C temperature increases per minute until 150 °C and isothermal holding for 3 min, then 3 °C temperature increases per minute until 220 °C and isothermal holding for 6 min. FAP were identified against reference standards (no. 47885-U Suplco® 37 Component FAME mix, and no. 47116 Menhaden fish oil, Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) and reported as g per 100 g total fatty acid and calculated in summation per FA category to form the FAP (Table 3.1).
3.2.3. Thiobarbituric acid reactive substances

TBARS content determination was adapted from Hopkins et al. (2014), where 100.0 mg sample was added to 500.0 µL RIPA buffer (no. 10010263, RIPA buffer concentrate, Cayman Chemicals, Michigan, United States of America) and homogenised using micro-tube pestles. These were then centrifuged at 6, 708 g (MiniSpin Plus, Eppendorf Pty. Ltd., Hamburg, Germany) for 10 min and the supernatant was then analysed as per the OXItok TBARS assay kit technical bulletin (ZeptoMetrix, 2011) and absorbance read at 532 nm on a bench top spectrophotometer. Results were expressed as mg malondialdehyde (MDA) per kg fresh meat.

3.2.4. Oxidation-reduction potential

Sample ORP by was measured through homogenising 1.0 g sample LL with 25.0 mL Milli-Q water using a homogeniser (series X10/25, Ystral™, Germany) at 22, 000 rpm and then the homogenate was centrifuged (Model 5810R, Eppendorf Pty. Ltd., Hamburg, Germany) for 15 min at 2, 465 g and 4 °C. Supernatant ORP and temperature values were then quantified using a calibrated ORP probe and benchtop monitor (ORP110-GS and HQ440d, Standard ORP Probe, HACH Pacific Ltd., Victoria, Australia) calibrated using ZoBell’s ORP standard solution (no. 2316949, HACH Pacific Ltd., Victoria, Australia). Results were the average of technical duplicates and expressed as millivolts (mV) per g fresh meat.
3.2.5. Peroxidase activity

Peroxidase activity was measured using approximately 25.0 mg of sample homogenised with 200.0 µL RIPA buffer (no. 10010263, RIPA buffer concentrate, Cayman Chemicals, Michigan, United States of America) using micro-tube pestles. These were then centrifuged at 6,708 g (MiniSpin Plus, Eppendorf Pty. Ltd., Hamburg, Germany) for 10 min and the supernatant analysed against peroxide (H$_2$O$_2$) standards using the colorimetric protocol detailed by the Peroxidase Activity Assay Kit (no. MAK092, Sigma Aldrich Pty. Ltd., Missouri, United States of America) technical bulletin (Sigma-Aldrich, 2015) using a micro-plate reader (FLUOStar OPTIMA™, BMG, Labtechnologies, Victoria, Australia) to measure absorbance at 570 nm. Peroxidase activity was expressed as the amount of peroxidase (n mole) that reduces 1.0 µmole H$_2$O$_2$ per min at 37 °C per sample (U/g) and was the average of technical duplicates.

3.2.6 Statistical analysis

The response of the measured parameters to the experimental factors (chilled storage period, frozen storage period and frozen storage temperature) was estimated by linear models. A response surface modelling framework was attempted given the quantitative nature of the treatment matrix, though exploration of the data revealed no systematic responses to
the treatments. Therefore, the experimental factor levels were taken as qualitative and a linear model was formed to estimate mean responses at every combination of chilled storage period, frozen storage period and frozen storage temperature. The nonsensical contrast of frozen storage temperature (-12 vs -18 °C) for chilled-only (unfrozen) samples was excluded from the model by construction of a 2 level control factor classifying the samples as frozen or unfrozen. Terms in the linear model were then formed by nesting the main effects and interactions between chilled storage period, frozen storage period and frozen storage temperature within the control factor levels. In the commonly accepted shorthand for linear statistical models, the treatment model was:

\[
\text{Control/(Frozen storage temperature*Chilled storage period*Frozen storage period)}
\]

Null hypothesis significance tests for the experimental factors were conducted by analysis of variance derived from the model. Mean responses under each treatment combination and standard errors of the means were also calculated. Pairwise comparison of means after rejection of the relevant null hypothesis was conducted by least significant difference calculated at 5% critical value. All data analysis was conducted in the R environment (R Core Team, 2016). The level of significance in this study was set at \( P < 0.05 \).

Because of the large number of individual FA evaluated in this study, these were simplified as summative values and ratios (Table 3.1) to be analysed, along with the major individual contributors for each FA type.
3.3. Results

3.1. Fatty acid profile

For the most part, saturated fatty acids (SFA) were found to be predominantly palmitic and stearic acids (Table 3.1). The total levels of SFA did not change throughout the entire storage period ($P > 0.05$; Table 3.2). Palmitic acid concentration decreased as chilled storage period increased from 0 to 2 weeks ($P = 0.03$).

Total monounsaturated fatty acids (MUFA) were primarily comprised of oleic acid (Table 3.1), with no changes in concentration due to increased storage periods ($P > 0.05$; Table 3.2).

In this study, PUFA was the summation of both omega-3 (n-3) and omega-6 (n-6) FA. Major n-3 PUFA contributors were α-linolenic acid (ALA) and docosapentaenoic acid (DPA). Major n-6 PUFA contributors were linoleic acid and arachidonic acid (Table 3.1). Total PUFA tended to increase in concentration across chilled storage periods ($P = 0.05$; Table 3.2), particularly n-6 PUFA between 0 and 8 weeks chilled-only storage ($P = 0.02$).
Table 3.1. Mean fatty acids found in this study based on fresh meat (time zero).

<table>
<thead>
<tr>
<th>Formula</th>
<th>Name</th>
<th>Content (g/100 g total fatty acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4:0</td>
<td>Butyric acid</td>
<td>0</td>
</tr>
<tr>
<td>C6:0</td>
<td>Caproic acid</td>
<td>0</td>
</tr>
<tr>
<td>C8:0</td>
<td>Caprylic acid</td>
<td>0</td>
</tr>
<tr>
<td>C10:0</td>
<td>Capric acid</td>
<td>0.15</td>
</tr>
<tr>
<td>C11:0</td>
<td>Undecylic acid</td>
<td>0</td>
</tr>
<tr>
<td>C12:0</td>
<td>Lauric acid</td>
<td>0.11</td>
</tr>
<tr>
<td>C13:0</td>
<td>Tridecylic acid</td>
<td>0</td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic acid</td>
<td>2.79</td>
</tr>
<tr>
<td>C15:0</td>
<td>Pentadecylic acid</td>
<td>0.33</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic acid</td>
<td>25.17</td>
</tr>
<tr>
<td>C17:0</td>
<td>Margaric acid</td>
<td>1.08</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic acid</td>
<td>16.12</td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic acid</td>
<td>0.64</td>
</tr>
<tr>
<td>C21:0</td>
<td>Heneicosylic acid</td>
<td>0.21</td>
</tr>
<tr>
<td>C22:0</td>
<td>Behenic acid</td>
<td>0</td>
</tr>
<tr>
<td>C23:0</td>
<td>Tricosylic acid</td>
<td>0</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric acid</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total SFA</strong></td>
<td></td>
<td><strong>46.60</strong></td>
</tr>
<tr>
<td>C14:1</td>
<td>Myristoleic acid</td>
<td>0.01</td>
</tr>
<tr>
<td>C15:1</td>
<td>Pentadecanoic acid</td>
<td>0</td>
</tr>
<tr>
<td>C16:1ω7</td>
<td>Palmitoleic acid</td>
<td>2.06</td>
</tr>
<tr>
<td>C17:1ω7</td>
<td>Heptadecanoic acid</td>
<td>0</td>
</tr>
<tr>
<td>C18:1iso</td>
<td>Iso-oleic acid</td>
<td>1.11</td>
</tr>
<tr>
<td>C18:1ω9</td>
<td>Oleic acid</td>
<td>41.67</td>
</tr>
<tr>
<td>C18:1ω9t</td>
<td>Elaidic acid</td>
<td>1.31</td>
</tr>
<tr>
<td>C20:1</td>
<td>Paullinic acid (ω7) or Gondoic acid (ω9)</td>
<td>0.04</td>
</tr>
<tr>
<td>C22:1ω9</td>
<td>Erucic acid</td>
<td>0</td>
</tr>
<tr>
<td>C24:1ω9</td>
<td>Nervonic acid</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total MUFA</strong></td>
<td></td>
<td><strong>46.21</strong></td>
</tr>
<tr>
<td>C18:2ω6</td>
<td>Linoleic acid</td>
<td>3.72</td>
</tr>
<tr>
<td>C18:2ω6t</td>
<td><em>trans</em>-Linoleic acid</td>
<td>0.11</td>
</tr>
<tr>
<td>C18:3ω6</td>
<td>γ-Linolenic acid</td>
<td>0.05</td>
</tr>
<tr>
<td>C20:2ω6</td>
<td>Eicosadienoic acid</td>
<td>0</td>
</tr>
<tr>
<td>C20:3ω6</td>
<td>Dihomo-γ-Linolenic acid</td>
<td>0.08</td>
</tr>
<tr>
<td>C20:4ω6</td>
<td>Arachidonic acid</td>
<td>1.07</td>
</tr>
<tr>
<td>C22:2ω6</td>
<td>Docosadienoic acid</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total n-6 PUFA</strong></td>
<td></td>
<td><strong>5.04</strong></td>
</tr>
<tr>
<td>C18:3ω3</td>
<td>α-Linolenic acid</td>
<td>1.03</td>
</tr>
<tr>
<td>C18:3ω3t</td>
<td><em>trans</em>-Linolenic acid</td>
<td>0.04</td>
</tr>
<tr>
<td>C20:3ω3</td>
<td>Eicosatrienoic acid</td>
<td>0</td>
</tr>
<tr>
<td>C20:4ω3</td>
<td>Eicosatetraenoic acid</td>
<td>0</td>
</tr>
<tr>
<td>C20:5ω3</td>
<td>Eicosapentaenoic acid (EPA)</td>
<td>0.41</td>
</tr>
<tr>
<td>C22:5ω3</td>
<td>Docosapentaenoic acid (DPA)</td>
<td>0.52</td>
</tr>
<tr>
<td>C22:6ω3</td>
<td>Docosahexaenoic acid (DHA)</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Total n-3 PUFA</strong></td>
<td></td>
<td><strong>2.15</strong></td>
</tr>
<tr>
<td>n-3/n-6 ratio</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Component</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Total PUFA</td>
<td>7.19</td>
<td></td>
</tr>
<tr>
<td>Total UFA (MUFA + PUFA)</td>
<td>53.40</td>
<td></td>
</tr>
<tr>
<td>Total (UFA + SFA)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>EPA + DHA</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>IMF (% fresh meat)</td>
<td>4.56</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Effect of chilled-then-frozen storage upon the proportions of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) (g per 100 g total FA) in lamb *m. longissimus lumborum* (LL).

<table>
<thead>
<tr>
<th>Chilled storage duration (weeks)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PUFA</td>
<td>MUFA</td>
<td>SFA</td>
<td>PUFA</td>
<td>MUFA</td>
<td>SFA</td>
</tr>
<tr>
<td>0</td>
<td>7.19</td>
<td>46.2</td>
<td>46.6</td>
<td>8.06</td>
<td>46.1</td>
<td>46.4</td>
</tr>
<tr>
<td>2</td>
<td>7.97</td>
<td>45.6</td>
<td>46.4</td>
<td>7.23</td>
<td>46.5</td>
<td>46.0</td>
</tr>
<tr>
<td>4</td>
<td>7.47</td>
<td>45.0</td>
<td>47.5</td>
<td>8.11</td>
<td>46.8</td>
<td>45.6</td>
</tr>
<tr>
<td>6</td>
<td>7.89</td>
<td>45.8</td>
<td>46.3</td>
<td>9.21</td>
<td>45.0</td>
<td>45.8</td>
</tr>
<tr>
<td>8</td>
<td>8.44</td>
<td>45.2</td>
<td>46.4</td>
<td>7.30</td>
<td>47.9</td>
<td>47.0</td>
</tr>
<tr>
<td>SEM</td>
<td>0.60</td>
<td>1.05</td>
<td>0.86</td>
<td>0.84</td>
<td>1.49</td>
<td>1.22</td>
</tr>
</tbody>
</table>

ab: Means with different superscripts are significantly different from other PUFA in the same column due to chilled storage ($P < 0.05$).
Health-claimable n-3 PUFAs (EPA + DHA) were summed and their concentrations at different chilled-then-frozen storage periods are shown in Table 3.3. It is worth noting that, while counted towards total PUFA, EPA and DHA were not the two greatest contributors to total PUFA concentration (Table 3.1). Chilled-only storage maintained the EPA + DHA concentration of fresh meat for 8 weeks, and this continued throughout the frozen storage period ($P > 0.05$; Table 3.3). Frozen storage period and temperature had no effect on EPA + DHA concentration ($P > 0.05$).

**Table 3.3.** Effect of chilled-then-frozen storage on the sum of health claimable fatty acids, eicosapentaenoic acid and docosahexaenoic acid (EPA + DHA) (g per 100 g total FA).

<table>
<thead>
<tr>
<th>Chilled storage duration (weeks)</th>
<th>Frozen storage duration (weeks)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.550</td>
<td>0.525</td>
<td>0.595</td>
<td>0.520</td>
<td>0.700&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.620</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.550</td>
<td>0.410</td>
<td>0.445</td>
<td>0.485</td>
<td>0.380&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.455</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.420</td>
<td>0.540</td>
<td>0.520</td>
<td>0.520</td>
<td>0.525&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.440</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.410</td>
<td>0.540</td>
<td>0.465</td>
<td>0.590</td>
<td>0.495&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.520</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.420</td>
<td>0.385</td>
<td>0.500</td>
<td>0.565</td>
<td>0.300&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.570</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.08</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

<sup>ab</sup>: Means with different superscripts are significantly different from others in the same column ($P < 0.001$).
3.3.2. TBARS

The TBARS levels increased with increasing chilled-only storage periods ($P < 0.001$), with a peak at 4 weeks chilled-only storage apparent (Fig. 3.1). Frozen-only storage was observed to increase TBARS levels as storage period increased to 8 weeks ($P < 0.001$; Fig. 3.1). Chilled-then-frozen storage interactions also increased TBARS, showing a general increase parallel with total storage period ($P < 0.001$). No other storage treatment combination, including frozen storage temperature, significantly influenced TBARS levels ($P > 0.05$).

**Fig. 3.1.** Predicted means (± SE) showing the effect of chilled-then-frozen storage period upon TBARS (2-thiobarbituric acid reactive substances) levels (measured as mg malondialdehyde per kg meat) of lamb LL. For simplicity of presentation, standard error has been included on the frozen-only (0 weeks chilled) line.
3.3.3. Oxidation-reduction potential

Chilled-only storage had significant effects on ORP ($P < 0.001$); where there was a decrease noted following 6 weeks storage (Fig. 3.2). ORP trends across increasing frozen-only and chilled-then-frozen storage periods were significant ($P < 0.001$); although it was difficult to ascertain a consistent trend as ORP levels were observed to decrease over frozen-only storage and increase with chilled-then-frozen storage, but only when the preceding chilled storage period was 8 weeks and this was followed by a frozen storage period of 8 to 52 weeks (Fig. 3.2). No frozen storage temperature or other storage effects were significant ($P > 0.05$).

Fig. 3.2. Predicted means ($\pm$ SE) showing the effect of chilled-then-frozen storage period upon oxidation-reduction potential (ORP) of lamb LL. For simplicity of presentation, standard error has been included on the frozen-only (0 weeks chilled) line.
3.3.4. Peroxidase activity

Peroxidase activity was affected by chilled-only storage ($P < 0.001$), though minimal variation between means was found between 0 and 6 weeks storage (Fig. 3.3). However, peroxidase activity increased in a quadratic manner with increasing frozen-only storage ($P < 0.001$), peaking at 8 weeks (Fig. 3.3). Similarly, peroxidase activity increased with increased chilled-then-frozen storage periods ($P < 0.001$), with particular increases due to 52 weeks frozen storage (Fig. 3.3). No frozen storage temperature or other storage effects were significant ($P > 0.05$).

![Graph showing peroxidase activity](image)

**Fig. 3.3.** Predicted means ($\pm$ SE) showing the effect of chilled-then-frozen storage period upon peroxidase activity of lamb LL. For simplicity of presentation, standard error has been included on the frozen-only (0 weeks chilled) line.
3.4. Discussion

In this study, chilled-only, frozen-only and chilled-then-frozen storage of lamb LL did not affect the FAP, although the storage regimes did increase biomarkers of lipid peroxidation. Frozen storage temperature had minimal effects on either FAP or lipid peroxidation biomarkers of lamb LL, which provides further evidence for the use of a warmer frozen storage temperature of -12 °C for the preservation of meat quality over long periods; this is corroborated by our previous work in meat quality (Coombs et al., 2017b; Chapter 2).

The summation of FA categories revealed comparative levels to that previously reported for supermarket lamb LL (Enser et al., 1996). The only significant point of PUFA increase and diversion from fresh meat FAP occurred at 24 weeks frozen-only storage, where PUFA increased compared to all other storage periods. One possible explanation for this outlier was the use of different LL for each chilled-then-frozen storage period as per Coombs et al. (2017b; Chapter 2), rather than continued sampling of the same LL. This was different to the study of Alonso et al. (2016), who found that frozen-only storage of pork for 6 months to 2 years resulted in PUFA oxidation to MUFA and SFA. Prior studies on frozen-only storage of lamb LL for 3-6 months have not reported changes in PUFA, MUFA or SFA concentrations (Samouris et al., 2011; Popova, 2014). Oxidation of PUFA has been suggested to precede formation of TBARS (Popova & Marinova, 2013), suggesting that lipid oxidation commencing in the chilled storage period continued into frozen storage, or that microbial agents may have contributed to rapid oxidation of frozen-only lamb LL (Coombs et al.,
Chilled-only storage has been found to increase PUFA concentration previously (Ponnampalam et al., 2014b), though decreases are more common and occur concurrent with increased TBARS (Wood et al., 1999).

The lack of change in FAP due to chilled and frozen storage similarly did not impact on the nutritional quality of lamb in terms of health-claimable n-3 FA (EPA + DHA) levels, however the levels of PUFA, including EPA + DHA, were not sufficient to be considered as a “source” of n-3 PUFA (Alvarenga et al., 2015). The nutritional quality was, however, reduced due to the increase in TBARS due to chilled-then-frozen storage, MDA known to have cytotoxic and mutagenic properties (Esterbauer et al., 1991). It is also noteworthy that lamb higher in PUFA through dietary measures (Ponnampalam et al., 2014b) will exhibit more rapid oxidation and will therefore have different nutritional and eating quality thresholds (Cifuni et al., 2000; Hopkins et al., 2014). Although this lamb LL may not be marketable as “healthy” or “source of long-chain omega-3 FA”, its nutritional value is unlikely to change from a marketing perspective (Commission Regulation of European Union, 2010; FSANZ, 2014). Previous research has found that EPA decreased in concentration in lamb LL frozen for 3-6 months at -20 °C, though DHA and shorter frozen storage periods were not measured (Popova, 2014).

Chilled-then-frozen storage effects upon lipid oxidation biomarkers (TBARS, ORP and peroxidase activity) were consistent with prior studies which conclude that anaerobic storage of red meat increases lipid peroxidation, though not to the extent of aerobic storage (Kim et al., 2011;
Bekhit et al., 2013; Popova & Marinova, 2013; Coombs et al. (2017a); Chapter 1). The observed inconsistencies in all these parameters likely arose from the use of different loins for each storage treatment combination. Meanwhile, the use of anaerobic storage in this study, and the sampling procedure occurring from the frozen state rather than thawed (Muela et al., 2015; Ponnampalam et al., 2017), likely prevented excess lipid oxidation from occurring in long-term chilled and frozen lamb. This lack of excess lipid oxidation is reflected in the TBARS levels not breaching the rancidity threshold of 2.0 mg MDA per kg of meat (Campo et al., 2006) at any chilled-then-frozen storage period. Despite this, TBARS exhibited a general increase due to both chilled and frozen storage in the present study, which is consistent with prior lamb studies finding TBARS to increase due to increased chilled-only storage periods (Berruga et al., 2005; Kim et al., 2011; Samouris et al., 2011; Fernandes et al., 2014). This increase in oxidation occurred more rapidly in chilled-stored meat compared to frozen meat most likely due to the increases of free PUFA and greater instability of lipid free radicals during chilled storage as previously determined (Igene et al., 1980). Frozen storage, meanwhile, has been found to result in slower, albeit continuing, lipid oxidation (Kanner, 1994), evidenced by increased TBARS following frozen-only storage periods for 3-9 months (Muela et al., 2010; Kim et al., 2011; Samouris et al., 2011; Popova & Marinova, 2013; Muela et al., 2015; Coombs et al. (2017a); Chapter 1). Beyond 9 months frozen storage, however, TBARS has been found to decrease or stabilise (Leygonie et al., 2012; Muela et al., 2015), which did not occur in this study. Results of these previous studies, however, should be interpreted with
caution when compared to the present study due to the TBARS measurement taking place on thawed and/or aerobically displayed meat.

One potential reason for TBARS stabilisation or decrease with increased frozen storage periods in prior studies is that MDA acted as a precursor to protein oxidation (Xiong, 2000), and this can be reflected by myoglobin oxidation, causing colour changes over a display period (Coombs et al., 2017b; Chapter 2). This area of integrative oxidative processes, particularly the effect of lipid oxidation facilitating myoglobin oxidation, and vice versa, has been extensively reviewed (Kanner, 1994; Min & Ahn, 2005; Faustman et al., 2010; Estévez, 2011), although the interaction has not been examined in chilled-then-frozen meat. Greater muscle myoglobin content can increase ORP and thus myoglobin oxidation (Min & Ahn, 2005), while increased peroxidase activity may infer a greater ORP of the muscle involved and therefore increase its susceptibility to increased TBARS (Daun & Åkesson, 2004).

Rancidity (Martínez-Cerezo et al., 2005) and confinement odour (Bell & Garout, 1994; Sumner & Jenson, 2011) have been noted in meat stored chilled-only for periods beyond 8 weeks. Although, short-term chilled-only storage periods (< 8 weeks) and associated minor increases in TBARS have been found to improve species-specific flavour (Wood et al., 2003), this may also be linked to increases in medium-chain SFA (6-12 carbon atoms) for sheep meat flavour (Rousset-Akrim et al., 1997; Young et al., 1997). In the present study, all TBARS levels remained below the rancidity threshold regardless of treatment period, which can be corroborated by an accompanying sensory study finding flavour and overall
liking acceptability following 8 weeks chilled-only storage, which in turn was significantly greater than lamb LL stored frozen-only for 8 weeks (Coombs et al., 2016). This threshold could potentially be greater than 2.0 mg/kg for lamb based on prior sensory results showing a majority of consumer odour acceptability at TBARS levels ranging from 2.0 to 4.2 mg/kg (Berruga et al., 2005). Regarding frozen-only meat, prior sensory studies have noted acceptability and a lack of difference between lamb meat frozen for one year compared to fresh meat (Hagyard et al., 1993; Ponnampalam et al., 2002; Fernandes et al., 2013; Muela et al., 2016). It is noteworthy that the majority of the aforementioned studies reported lower TBARS values than the present study at corresponding storage periods, which may have occurred due to the lack of a standardised TBARS procedure (Ponnampalam et al., 2014a). Meanwhile, resolving discrepancy in the literature between TBARS levels and consumer sensory panels, particularly for lamb, is an additional future investigation (Coombs et al. (2017a); Chapter 1).

Based on these results, it is worthwhile to test lipid peroxidation and FAP of lamb LL stored frozen for 2 years or longer as per Winger (1984). At such long frozen storage periods it is unlikely the FAP will change further (Alonso et al., 2016), although the health claimable FA did not significantly differ at shorter frozen storage periods in the present study. Aerobic display under retail conditions post-thawing should also be tested, as previous studies have reported increases to TBARS (Muela et al., 2015), ORP (Kim et al., 2002) and rancidity (Muela et al., 2016) to result from aerobic storage. Such increases were particularly significant following long-term (> 9 months) frozen storage.
3.5. Conclusion

It can be concluded that chilled-then-frozen storage of lamb LL did not affect the nutritional quality (health-claimable fatty acids EPA + DHA, nor total n-3 PUFA), meaning that LL can be stored for up to 8 weeks chilled-then-52 weeks frozen, and potentially even longer, and retain these fatty acids, although it is worth noting the meat in the present study did not constitute a “source” of n-3 PUFA and consequently, variation to initial levels could result in alternative responses to chilled-then-frozen storage treatments. Increases in lipid oxidation were observed due to increased storage periods; however TBARS levels remained below the rancidity threshold regardless of storage period. Furthermore, frozen storage temperatures of -18 °C and -12 °C did not result in significant differences in either lipid oxidation or FAP of chilled-then-frozen lamb. It is recommended that further research be undertaken to investigate the linkages between lipid oxidation and oxidation of myoglobin and other proteins, as well as the relationship between TBARS to rancidity and flavour determination of chilled-then-frozen lamb LL using consumer sensory panels.
Chapter 4: General Discussion and Conclusions

From the experimental chapters of this thesis, it can be concluded that both chilled and frozen storage periods independently affected meat quality and safety traits of lamb. Additionally, the frozen storage temperature posed negligible effects upon meat quality and safety parameters. In particular, shear force, colour stability and microbial loading (Chapter two); and lipid oxidation biomarkers (Chapter three) were all significantly affected by increased chilled-only storage period. Several of these effects were positive, particularly in the short term, leading to a more tender product (lower shear force for 2-8 weeks compared to 0-weeks; Chapter two) and a more nutritional product (slight increase in PUFA concentration for 2-8 weeks compared to 0 weeks; Chapter three).

However, chilled-only storage did increase microbial loading, as was seen in Chapter two, though at no point during the experimental period did it result in spoilage of meat based on previously defined spoilage thresholds (Mills et al., 2014). Previous studies have also observed that at chilled storage temperatures (particularly below 0 °C), lamb can be stored chilled for at least 8 weeks (and in some cases 12 weeks) with minimal spoilage (microbial load below threshold and absence of confinement odour, greening or off-flavour) (Sumner & Jenson, 2011; Kiermeier et al., 2013). Similarly, lipid oxidation rancidity thresholds defined in previous research (Berruga et al., 2005; Campo et al., 2006) were not breached (Chapter three); rancidity occurring more commonly following aerobic storage (Chapter one). This lack of effect occurred in spite of an increase in TBARS with chilled storage period (Chapter three).
Display colour was the most affected trait by chilled storage and subsequent frozen storage, as shown by a reduction of redness (a*) and increased browning (increased hue and decreased R630/580) (Chapter two) due to myoglobin oxidation, which occurred while lamb LL were kept under both chilled and frozen storage. While prior chilled storage did improve the colour of frozen meat upon display, most experimental LL slices would have been ruled unacceptable at a retail level even after just 1 day (24 hours) post-cutting, based upon previously defined consumer thresholds (Khliji et al., 2010). Additionally, thaw and purge losses from frozen and chilled storage, respectively (Chapter two), would cloud the consumer perception of visual quality, freshness and nutritional value. This information in turn leads to the question of how to market this safe, high quality product with a poor visual shelf life with two key markets identified: food service (i.e. restaurants) or vacuum storage of frozen product at display.

Sumner (2016) stated that meat export voyages rarely last more than eight weeks in total from processor to overseas consumer, due to shelf life regulations based upon microbial profiles (Mills et al., 2014). As the paper only examined chilled storage, the frozen storage component as investigated in this thesis could safely follow any of the prescribed chilled storage durations and continue the preservation of quality and safety for up to and, potentially beyond, one year. In terms of quality, it was found that frozen storage similarly decreased shear force, increased lipid oxidation and promoted increases in lactic acid bacteria, albeit at slower rates than those of chilled storage. In fact, frozen storage in the present study was found to reduce the concentrations of some microbial species that had proliferated during chilled storage, though shelf-life upon thawing was not tested.
Winger (1984) stated that frozen storage at -10 °C or lower could preserve meat quality and safety for 2 years or more, provided pre-freeze chilled storage was kept to a minimum. With these in mind, it can be added that 4 weeks chilled storage could constitute a good target for optimal quality prior to frozen storage, as it results in sufficient tenderness as per Hopkins et al. (2006), with SF not improved by further chilled storage, which in turn increases microbial load (Chapter two).

The examinations of frozen storage temperature (-12 and -18 °C) and chilled-frozen storage interactions in this study were novel. It was found that chilled and frozen storage rarely interacted, instead acted separately upon meat quality. Frozen storage temperature comparisons were re-examined due to the long duration between the present day and the study by Hagyard et al. (1993), which only examined consumer sensory quality at -10 °C and -15 °C. The finding of negligible differences in quality between -12 and -18 °C from the present thesis is significant as it poses an important outcome for the export industry, in that a temperature of -12 °C can be used to preserve quality and safety over frozen storage periods up to one year, in comparison to the conventional international frozen storage temperature of -18 °C, which can save funds related to freezer operating costs and allow for bulk shipments of product.

It is apparent that from this thesis, several further research questions relating to the topic have arisen:

- Can frozen storage thresholds of lamb be extended for more than one or two years with minimal additional effects on quality or safety (Winger, 1984; Muela et al., 2015)?
- How long is the safe shelf life of thawed product following these chilled-then-frozen storage periods, at which sensory and nutritional quality is maintained?

- Upon thawing, which qualities will be first to deteriorate and why?

- Could frozen storage temperature be increased to -10 °C (Winger, 1984), and provided good control is used, maintain meat quality and safety, which was not possible according to Hagyard et al. (1993)?

- What is an appropriate method to quantify meat flavour, a traditionally consumer-based trait, instrumentally?

- Is there an effect on proteins and their oxidation to carbonyls from chilled-then-frozen storage? This has been demonstrated previously in myoglobin (evidenced by colour change) in this study (Chapter two) and to be influenced by lipid oxidation (Faustman et al., 2010; Estévez, 2011).

- What governs the variation in thaw losses between samples, even following identical frozen storage periods?

- What is the best method to minimise thaw losses and promote a cherry red muscle colour to improve retail marketability without compromising eating quality or safety?

- Will other species, such as beef and pork, exhibit similar thresholds?

- Will other muscles than the loin exhibit similar thresholds?

Regardless of these outstanding questions, this study was able to determine storage thresholds for chilled-then-frozen lamb LL, based on several different quality and safety parameters. These were:
- Minimum of 4 weeks chilled-only storage for acceptable tenderness based on Australian consumer standards (Hopkins et al., 2006) – this period could be followed by frozen storage of 0-52 weeks and the tenderness preserved (Chapter two).

- Maximum of 2 weeks chilled-then-52 weeks frozen, 4 weeks chilled-then-8 weeks frozen, 6 weeks chilled-then-4 weeks frozen, and 8 weeks chilled-only storage to retain acceptable retail colour based on Australian consumer standards (a* and R630/580 as per Khliji et al., 2010) for 1 day post-thaw (Chapter two).

- Maximum of 4 weeks chilled-only storage with no ensuing frozen storage period for acceptable redness (a*; Khliji et al., 2010) for 3 days display period (Chapter two).

- Lamb LL could be stored for the entire experimental storage period (8 weeks chilled, 52 weeks frozen) and remain safe based on spoilage thresholds for lactic acid bacteria, Enterobacteriaceae sp. and Brochothrix thermosphacta (Mills et al., 2014), which were all detected in Chapter two.

- Lamb LL could be stored for the entire experimental storage period and not undergo significant PUFA oxidation, maintain acceptable nutritional quality by levels of EPA and DHA comparable to fresh meat, and TBARS levels below rancidity thresholds (Chapter three).

- Frozen storage reduced concentrations of Enterobacteriaceae sp. and B. thermosphacta, the latter of which was relatively high following 4-8 weeks chilled storage (Chapter two).
It can be concluded that the independent effects of chilled and frozen storage influenced lamb meat quality through mechanisms of proteolysis and oxidation of myoglobin and lipids. Additionally, the microbial profile was affected by both chilled and frozen storage. From this information, several thresholds were formed for the chilled, frozen and chilled-then-frozen storage of lamb LL for export purposes to result in the delivery of a safe, high-quality product. However, further information on proteins and their effect on shear force, post-thaw storage, aerobic and anaerobic product shelf life, and potential extension of the frozen storage period would be required for a more comprehensive insight into long-term preservation of lamb using chilled and frozen storage combinations.
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