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Abstract

Following a century of major discoveries on the mechanisms determining meat colour and tenderness using traditional scientific methods, further research into complex and interactive factors contributing to variations in meat quality is increasingly being based on data-driven "omics" approaches such as proteomics. Using two recent meta-analyses of proteomics studies on beef colour and tenderness, this review examines how knowledge of the mechanisms and factors underlying variations in these meat qualities can be both confirmed and extended by data-driven approaches. While proteomics seems to overlook some sources of variations in beef toughness, it highlights the role of post-mortem energy metabolism in setting the conditions for development of meat colour and tenderness, and also points to the complex interplay of energy metabolism, calcium regulation and mitochondrial metabolism. In using proteomics as a future tool for explaining variations in meat quality, the need for confirmation by further hypothesis-driven experimental studies of post-hoc explanations of why certain proteins are biomarkers of beef quality in data-driven studies is emphasised.

Keywords: Beef; Colour; Tenderness; Proteomics; Meat quality; Muscle to meat conversion; Glycolysis; Mitochondria; Omics; Data integration

1. Introduction

In the twentieth century, most of the understanding of factors affecting basic qualities of meat (such as tenderness, colour and water-holding capacity), the molecular mechanisms underlying these qualities, and the biochemical pathways involved, was obtained by traditional scientific methods. These typically involved the formulation of a precise and testable hypothesis before the design and conduction of experiments, ideally under very controlled conditions where only one factor is allowed to vary at a time in a way that tests the hypothesis (Johnson 1945). This is also called the Hypothetico-Deductive (H-D) method of scientific investigation (Andersen & Hepburn 2016). This approach has been successful, but arguably the time has passed when experiments based on single effects will provide further significant insights into the sources of meat quality variation that remain to be understood. With the basic effects mapped, meat science is now concentrating on the more subtle effects and the myriad interactions between a number of complex events in order to make further progress in understanding and prediction of meat qualities. Fortunately, the rapid development of high-throughput techniques in genetics, protein analysis and metabolite analyses in the later 20th century has provided the tools to explore meat quality in ever greater detail, using "omics" techniques, where large quantities of data are obtained about the genome, transcriptome, proteome, metabolome, phosphorylome, lipidome or degradome and correlated to variations in meat quality (Munekata et al. 2021). The experimental approach typically applied in studies using these techniques has been described as data-driven, rather than hypothesis-driven research (Leonelli 2012; Strasser 2012). In relation to meat quality, the application of transcriptomics, proteomics and metabolomics has been reviewed by (Guo & Dalrymple 2017), (Picard et al. 2017) and (Bertram 2017), respectively. Genome-wide association studies (GWAS) also fit into this data-driven category, typically consisting of examinations of the whole genome (or as much as possible) for single nucleotide polymorphisms (SNPs) that are consistently associated with a particular trait or aspect of meat quality. Recent examples include GWAS studies on SNPs related to variations in tenderness (shear force) in pork (Zhang et al. 2016), or intramuscular fat (IMF) in pork (Duarte et al. 2018), and to meat colour, texture and marbling in beef (Bedhane et al. 2019). The amount of data provided by these techniques is large, and interpretation is often complex. While annotation and ontology (see below) are useful in interpreting this data, Yamada et al. (2021) remind us that these tools are less than perfect and can give misleading results. Further development of additional interpretive tools may remove the bottleneck that this step currently represents.

With the growing number of such omics studies (López-Pedrouso et al. 2020; Munekata et al. 2021), it is becoming possible to produce overviews of results on some specific meat quality traits, for example meat tenderness, or apply a meta-analysis and integrative studies to a number of comparable studies to further distil the information about genes, SNPs, proteins, metabolites or phosphorylation-driven signalling pathways that are repetitively flagged as markers of tenderness, water-holding capacity (WHC) or meat colour. Taking just meat tenderness as an example, overviews or meta-analyses have recently been provided by (Ouali et al. 2013), (Picard & Gagaoua 2020a) and (Gagaoua et al. 2021).

Two distinct motivations for omics studies of meat quality traits can be distinguished. The first is to formulate a consistent and reliable list of biomarkers for the quality trait with the aim of using this in a predictive model that can be used in quality control for the industry and the end-consumer. This goal does not intrinsically require knowledge of why the biomarker is related to the measured quality, only that its predictive ability is consistent across a wide range of conditions. The second motivation is to gain further insight into the molecular mechanisms and pathways underlying variations in meat quality,

and their interactions with a range of conditions. In pursuit of this second goal, the list of biomarkers is usually further analysed by gene ontology (GO) classification, which identifies the properties of gene products by molecular function, cellular location or compartment, and the biological process in which it is involved. This aids identification of proteins that are acting together in a common pathway. It is undeniable that omics techniques are at the forefront of developments for biomarkers and that they have greatly extended knowledge of underlying mechanisms, and a continuing increase in the use of these techniques in the future is to be expected.

Data-mining investigations using "omics" are very useful for finding hidden or surprising correlations. In the data-driven investigations, hypothesis building does not come before the design of the experiment and its conduction; rather, hypotheses and conclusions are generated post-hoc from the data. However, one criticism is that these post-hoc hypotheses are often not investigated further.

This review aims to understand the complimentary benefits of integrating knowledge from proteomics with the basic understanding of mechanisms underlying meat quality provided by traditional H-D investigations. We focus on the information given by two recent meta-analyses of proteomic investigations into beef colour (Gagaoua *et al.* 2020b) and beef tenderness (Gagaoua *et al.* 2021) and examine the extent to which it has been possible to interpret this information from information gained by traditional studies, and also the extent to which these proteomics studies are providing new information undiscovered so far by traditional methods.

2. Mechanisms underlying variations in tenderness and colour elucidated by traditional studies

It is not the intention to comprehensively review all knowledge in these two vast areas here, but to briefly highlight major mechanisms. Comprehensive reviews mentioned below provide extensive details of both of these aspects of meat quality.

2.1 Colour

The appearance of meat on the retail shelves is critical for consumer acceptance. In particular, colour of the lean tissue is an important quality indicator for the consumer. Furthermore, the aspects of colour on the shelves considered the most important are the paleness (measured by CIE-L* using a chromameter), the redness (measured by CIE-a* and calculated hue), and any browning present (measured by CIE b* and calculated hue). Paleness, or lightness, is related to the concentration of myoglobin as well as to light scattering, and both redness and brownness are related to the myoglobin concentration and the chemical state of the myoglobin. A brief discussion of light scattering and redox forms of myoglobin are given below and further detail is given in **Table 1**. Reviews on meat colour are available and the reader is referred to these for more in-depth discussion and influencing factors (Faustman & Cassens 1990; Young & West 2001; Bekhit & Faustman 2005; AMSA 2012; Jacob 2020; Mancini & Ramanathan 2020; Ramanathan *et al.* 2020a; Ramanathan *et al.* 2020c).

a. Redox forms of myoglobin and metmyoglobin formation in discolouration

Myoglobin is well-known to change in colour with different compounds bound at the sixth coordination site of the iron atom in the protoporphyrin ring structure formed by four pyrrole rings of the myoglobin molecule (Mancini & Hunt 2005). When oxygen is bound in the reduced state of the molecule, the oxymyoglobin red pigment occurs. When no ligand is attached at the 6th coordination site in the reduced state of the molecule, myoglobin is in the purple deoxymoglobin form and in the oxidised state we have

the brown metmyogobin form of the pigment. The balance of these three chemical states of the myoglobin molecule is critical in determining colour on the retail shelves and hence visual acceptability to the consumer; a predominance of the oxymyoglobin form is important to consumer acceptability.

Discolouration in fresh meat is predominantly associated with the formation of metmyglobin, the brown pigment where the iron is in the oxidized Fe^{3+} state. Any systems associated with oxidation, reduction, or indeed an imbalance between these, have the potential to be associated with the rate of discolouration (Suman & Joseph 2013) such as superoxide dismutase, catalase and glutathione peroxidase, metmyoglobin reductase and mitochondrial activity during which both H_2O_2 and O^{-2} are generated (Renerre & Labas 1987). There has long been an association between oxidation of lipids and oxidation of myoglobin and hydroxynonenal (HNE) has recently been found to be a product of lipid oxidation and directly binds to the myoglobin molecule (Faustman *et al.* 2010). Furthermore, HNE has also been shown to covalently bind to lactate dehydrogenase, and decrease NADH formation and metmyoglobin reduction (Ramanathan *et al.* 2014). The biological systems involved in defence against oxidation are many and varied, and are reviewed in (Suman & Joseph 2013). Feeding of Vitamin E to cattle was first shown to delay meat discolouration by (Faustman *et al.* 1989a) and (Faustman *et al.* 1989b), and this was a serendipitous discovery. Vitamin E feeding was then shown to reduce meat discolouration in other species (Monahan *et al.* 1990; Jose *et al.* 2016) and importantly, it was shown that cattle and sheep consuming green grass have high levels of muscle β-carotene and longer shelf-life (Yang *et al.* 2002a; Yang *et al.* 2002b; Ponnampalam *et al.* 2012; Ponnampalam *et al.* 2014). Oxygen consumption rate, oxygen penetration depth, and depth of colour change are important for determining the redox status and attachment of oxygen to the myoglobin molecule and these are variously influenced by temperature at blooming, ageing period and method, time post-mortem, pH and many other factors, some of which are listed in **Table 1**.

b. Structural proteins and denatured sarcoplasmic protein contributions to light scattering

The effect of light scattering on meat colour, particularly meat lightness, was first hypothesised by Macdougall (1970) who showed that the luminous reflectivity of fresh and processed pork was dependent on the change in light scattering that occurred with changes in pH. MacDougall (1982) speculated that light scatter from denatured proteins or myofibrillar structures at lower pH were responsible for increased paleness. Summarising his research and confirming previous opinion (Hamm 1961; Offer & Trinick 1983), Swatland (2004) stated that light scattering results from refractive indices mis-match between compartments such as the sarcoplasm and the myofibrils and pH-related scattering is thought to arise at boundaries between the sarcoplasm and myofibrils. Direct evidence for the increase in light scattering with reduced myofilament lattice spacing, lower ultimate pH, increased sarcoplasmic protein denaturation and increased transverse spacing of myofibrils, in muscle fibres, was recently shown using a light microscope in the reflectance mode (Hughes *et al.* 2017, 2018; Hughes *et al.* 2019). Any factors that contribute to the rate and extent of pH fall, which influence the myofilament lattice spacing, and myofibril spacing, as well as protein denaturation, will impact on the light scattering at a meat surface. If beef carcasses are graded too early, the meat surface appears dark, as the myofibrils are likely still swollen and close to each other and there are minimal gaps between muscle cells. In a study beef surface colour at grading, Hughes *et al.* (2014a) showed that delaying grading by 16 hrs resulted in a decrease in beef carcasses graded with unacceptable dark colour from 8% to 3 %, which has significant effects on a meat processors' return from a carcass.

c. Measurement

Surface colour is generally quantitated through instrumental measurements or through visual scoring (see reviews in (AMSA 2012; Warner 2014)). Instruments measure the CIE L*, a*, b* (see above) as well as the hue and saturation, measured using instruments such as the Minolta chromameter, the Hunterlab MiniScan or the less expensive but less accurate Nix color sensor. The Hunterlab MiniScan and some models of the Minolta chromameter are spectrophotometers which can be used to quantitate the reflectance at individual wavelengths and hence the change at the meat surface, as discolouration proceeds, from red oxymoglobin to brown metmyoglobin (Jacob *et al.* 2014). Importantly, the surface colour is influenced by many factors, some of which are listed in Table 1. Factors also include the time and temperature of blooming, and the configuration, specifications and geometry of the instrument (such as illuminant, observer angle, aperture size), making comparison between different instruments and studies problematic.

2.2 Tenderness

Five mechanisms dominating variations in tenderness are (a) collagen and cross-linking, (b) sarcomere length post-mortem, (c) muscle myofibril degradation by proteolysis during ageing, (d) intramuscular fat and (d) protein denaturation during cooking. A brief synopsis of these 5 mechanisms is given below. More details on various aspects of meat tenderness are provided in reviews (Koohmaraie 1988; Koohmaraie *et al.* 1991; Koohmaraie 1994, 1996; Purslow 2005, 2014; Ertbjerg & Puolanne 2017) (Warner *et al.* 2010a; Ouali *et al.* 2013; Purslow 2018) and a summary is provided in supplementary **Table S1**.

a. Collagen and cross-linking

In the context of tenderness, collagen is a heat-stable protein providing structure and support to the muscle. Importantly, the covalent cross-links between collagen fibrils increase with age of an animal, affecting its mechanical and thermal stability (McAnulty & Laurent 1987) and the connective tissue component of sensory or instrumental measures of toughness (Cross *et al.* 1973). The long residence time of collagen in the body is associated with the formation of stable trivalent cross-links, which are heat-stable. The main protease system involved in collagen breakdown is the matrix metallopeptidases (MMP's) (Christensen & Purslow 2016).

b. Sarcomere length

Post-mortem, during the pre-rigor period and before the acto-myosin bond is formed, the sarcomere can shorten, and this is prevalent during low temperatures less than 10-12°C when calcium is released due to membrane damage (Buege & Marsh 1975; Cornforth *et al.* 1980) and is called cold-shortening. Muscles frozen pre-rigor and thawed rapidly can also undergo considerable shortening (thaw-contracture; (Bendall 1973a)). Shortening also occurs at temperatures above 20-25°C, called heat-shortening or heat-toughening, and is less severe and frequent than the shortening occurring at cold temperatures (Locker & Hagyard 1963). Shorter sarcomeres, mostly below 1.5 μm, are associated with higher shear values in cooked meat (Dransfield & Rhodes 1976). Mechanisms underlying the contributions of muscle shortening and sarcomere length to cooked meat toughness are reviewed in (Ertbjerg & Puolanne 2017). As the actomyosin bond can only form once ATP levels have declined, the

rate of glycolytic metabolism, which generates ATP, is closely associated with cold-shortening and the final sarcomere length of a muscle (Chrystall & Devine 1978).

Shortening of muscle post-mortem is a highly variable process that is dependent on the degree of restraint but is essentially driven by the availability of ATP, hence the rate of glycolysis and associated pathways is important while the post-mortem temperature is still high (Chrystall & Devine 1978). Sarcomere length is driven by both glycolysis and muscle temperature, hence heavier carcasses, with thicker muscles and often with more fat, are less likely to exhibit cold-shortening than lighter carcasses with thinner muscles and often little fat cover (Lochner *et al.* 1980). This is particularly evident in some young animals (eg. veal) or small livestock (goats and sheep) which are more likely to exhibit cold-shortening (Savell *et al.* 2005). Temperature gradients can also occur in individual muscles, especially at fast chilling rates (Joseph 1996).

c. Myofibrillar degradation

The main contributors to myofibrillar degradation post-mortem are the proteases, predominantly the calpain/calpastatin protease system (Koohmaraie & Geesink 2006) but also the cathepsin/cystatin system (Ouali 1992). The contribution of other protease systems such as caspases/serpins, proteasomes and serine peptidases (Ouali *et al.* 2013) is still debated in the meat science literature. The contribution of proteases to meat tenderness is mainly regulated by the protease levels in the muscle at slaughter, duration of post-rigor ageing, the protease activity during ageing (Koohmaraie & Geesink 2006) and the level of the endogenous muscle inhibitors (Gagaoua *et al.* 2015a). The activity of these proteolytic enzymes is further regulated by the rate of pH and temperature decline during rigor development (Dransfield 1994a), ionic strength (Ouali 1984), oxidation of calpain post-slaughter (Rowe *et al.* 2004) and other factors (see **Table S1** for more details).

The effect of the calpain system on tenderisation post-slaughter relies on a balance between the rate of activation and activity, and the rate of inactivation or denaturation of the proteolytic enzymes (Dransfield 1994a). Specifically, the activity of calpains is mainly controlled by calcium ions, phospholipids and calpastatin, their specific inhibitor (Saido *et al.* 1994). The inactivation of the calpain system through denaturation occurs more rapidly when the pH-temperature conditions associated with heat-toughening occur (Dransfield 1994a, b). There has been considerable debate on the role of cathepsins in protein degradation but they have been shown to be important for myofibril protein degradation during extended ageing (Sentandreu *et al.* 2002), and have been found to be active much longer post-slaughter relative to calpains (O'Halloran *et al.* 1997) and also are active during cooking (see section below). Their activity is controlled by several factors including pH, redox potential, extent of precursor activation and specific endogenous inhibitors (Sentandreu *et al.* 2002).

d. Intramuscular fat

Intramuscular fat (IMF) has been shown to be related to both consumer scores for tenderness and to instrumental measures of tenderness such as Warner-Bratzler shear force (Warner *et al.* 2010b). The relationship between IMF and consumer scores could potentially be a 'halo' effect, as meat with a higher IMF in the range 3-7% is known to have highly acceptable flavour and juiciness (Savell & Cross 1988). But the relationship with objective measurement is likely related to the decrease in protein density, and also to the disruption in connective tissue that occurs in high IMF samples (Nishimura *et al.* 1999).

e. Protein denaturation on cooking

Cooking is the final step prior to consumption and has a significant effect on sensory qualities. The complex changes in the structure of proteins in meat brought about by cooking are reviewed by (Tornberg 2005). Heat denaturation causes changes in the structure and properties of the protein components which drive a series of changes in the shrinkage in the muscle tissue, which also contributes to changes in texture (Hughes *et al.* 2014b). In general, using Warner-Bratzler-shear force as the measure of tenderness, the tenderness decreases between 40 and 50 °C, increases between 50 and 60°C and then decreases between 60 and 90°C, and these changes are attributed to strength of perimysial connective tissue, and muscle fibres, respectively (Christensen *et al.* 2000).

f. Measurement

Tenderness can be measured either through sensory methods (consumer panels or trained panels) or through instrumental methods (see reviews in (Purchas 2014; AMSA 2016). Importantly, the values obtained with instrumental measurement of tenderness vary in an interactive way, with cooking duration, cooking temperature, rate of heating, time post-mortem muscle, size of sample, blade and deformation rate, to name a few, and of course with the factors detailed above. For sensory assessment, particularly when consumers are used to assess acceptability, the consumer response is influenced by many of these factors as well as the 'halo' effect where the tenderness scores can be influenced by the juiciness and flavour of the product. Hence although instrumental and sensory measures are consistent for comparison within a study, they become much less reliable for comparison across studies, and hence must be treated with caution.

3. How proteomics has provided new insights on mechanisms of tenderness and meat colour

It is beyond the scope of this paper to review the entire range of proteomics studies on meat quality and the meta-analyses (integromics) of these. So, for purposes of illustration, we will focus on two recent meta-analyses of proteomics papers in relation to (a) variations in the colour of raw beef muscles post-mortem (Gagaoua *et al.* 2020b), and (b) variations in the tenderness of beef muscles after cooking (as measured either by WBSF or sensory measures of tenderness; (Gagaoua *et al.* 2021). As these two studies are meta-analyses of several broadly comparable previous proteomics studies, they contain a large amount of detailed information and provide details of some of the variability between individual studies. However, by looking for consistent and significant similarities in the proteins reported to vary between extremes of (a) colour and (b) tenderness, these meta-analyses produced lists of the proteins which appear most frequently, and therefore can be considered central to the processes producing variability in these properties.

Fig. 1 represents a summary from (Gagaoua *et al.* 2020b) of some of the proteins which most consistently are associated with large differences in the various aspects of meat colour. The abbreviations in **Fig.1** refer to the gene names; the full names of each protein are given in **Table S2**.

It should be emphasised that post-mortem proteolysis of proteins of course reduces the concentration of the intact form of these proteins, and increases the concentration of smaller peptides. Thus, the variation in the amounts of the major structural proteins such as actin, myosin and alpha-actinins and cytoskeletal proteins such as titin reported in proteomics studies can reflect variations in the degree of

post-mortem proteolysis rather than large variations in the initial amounts of these structural proteins *in vivo*.

While the relevance of biomarkers identified by proteomics studies is often discussed in relation to the known properties and functions of each protein as identified by traditional studies, proteomics experiments have highlighted new findings and concepts. In relation to the development of meat tenderness by post-mortem proteolysis, the prevailing view has traditionally been that actin and myosin were not the main targets of proteolysis, and indeed this evidence was used to argue that the calpain system was central to post-mortem tenderization (Koohmaraie 1992; Geesink *et al.* 2006). Studies had shown that the most sensitive targets of calpains are cytoskeletal proteins such as desmin, filamin and vinculin as well as titin, nebulin, myosin binding protein C (MYBPC), tropomyosin, and troponin (Koohmaraie 1992; Huff-Lonergan *et al.* 1996). However, a proteomic study by (Lametsch *et al.* 2003) was able to show that there was some degradation in actin and myosin heavy chain, as later confirmed by the study of (Marino *et al.* 2013). Indeed, the relative amounts of intact actin and myosin show up in many lists of biomarkers for beef tenderness, as demonstrated by the results of the meta-analysis of (Gagaoua *et al.* 2021). The distribution of these potential tenderness biomarkers, according to the number of times they occur in the 28 studies included in the integromics meta-analysis, and their biological pathways, is given in **Fig. 2** (adapted from (Gagaoua *et al.* 2021). A full list of the proteins and their gene names is given in supplementary **Table S3.**

3.1 What new insights on factors/ pathways controlling beef colour and cooked beef toughness do these meta-analyses give us?

The meta-analysis of beef colour proteomics (Gagaoua *et al.* 2020b) reaffirmed several biological pathways which were previously known to be involved in meat colour development, including energy metabolism, myofibril structure, proteolysis, heat shock proteins, oxidative stress, and apoptosis. The glycolysis pathway and other associated energy metabolism mechanisms are the predominant functional pathways highlighted, and this reflects existing knowledge on the central role of energy metabolism in determining meat colour, as well as its role in determining post-mortem pH (as discussed above), and thereby water-holding capacity and the development of tenderness post-mortem. The novelty of this meta-analysis is the emphasis on oxidative stress, cell redox and contractile proteins, and on the interactions between them. While the predominant mechanism of colour changes in beef muscle post mortem are changes in oxygen distribution in the tissue and the different redox states of myoglobin (Ramanathan *et al.* 2020a), the role of pathways mitigating oxidative stress and redox homeostasis highlight the role of cellular mechanisms resisting post-mortem effects on this. As discussed above, the interaction of energy metabolism pathways and the integrity of contractile proteins also relate to structural mechanisms of light scatter between the myofilaments. pH-driven changes in myofilament lattice spacing and deposition of some denatured sarcoplasmic proteins onto the myofilaments in some pH and temperature conditions post-mortem are two major mechanisms of these changes in light scattering (Hughes *et al.* 2020).

The meta-analysis of beef tenderness proteomics studies (Gagaoua *et al.* 2021) highlighted the importance of the changing integrity of muscle contractile and structure proteins, energy metabolism enzymes, heat stress proteins and oxidative stress proteins in the determination of beef tenderness, in that order of importance. Most pathways and their corresponding proteins were highly interrelated, and indeed had very considerable overlap with the pathways highlighted from the colour proteomics meta-

analysis, particularly the dominating effects of the glycolysis and energy metabolism pathways in influencing the post-mortem conditions in which both properties (colour and tenderness) develop with time. It is interesting that the calpain system of enzymes and inhibitor are not consistently represented in the majority of proteomics studies on tenderness, despite the evidence from traditional biochemical studies of the importance of this system. However, it should be remembered that proteomics measures the abundance of these enzymes, and not their activity. It is also notable that the five major pathways identified in relation to tenderness can all be related directly or indirectly to apoptosis onset in post-mortem muscle, which may be initiated by mitochondrial degradation signals. Thus, one suggestion for future work is further investigation of the role of mitochondrial metabolism post-mortem on tenderness development, and how this is related to differences between muscles with different proportions of fibre types, where mitochondrial metabolism differs (England *et al.* 2018; Yu *et al.* 2020). Mitochondrial involvement is discussed in further detail below.

3.2 Different emphases between conventional and proteomic studies

Two of the five major mechanisms for variation in beef tenderness mentioned in section 2 above (intramuscular fat and cooking/protein denaturation effects) have shown no relation to proteomics studies of tenderness, likely because these factors have little relation to the relative abundance of proteins. Conversely, as proteolysis occurs during cooking (Vaskoska *et al.* 2021), proteomic studies using cooked meat samples may be beneficial (Gagaoua *et al.* 2019), even though cooked muscle is difficult to solubilise and new protocols for its analysis are worthy to develop. Initially, it is difficult to understand why proteomics would be related to sarcomere length, as the relation between toughness and sarcomere length is completely opposite in raw meat and cooked meat (Dransfield & Rhodes 1976), and both sarcomere length and proteomics studies are conducted on raw meat samples. As the delayed proteolysis in raw beef due to restricted access of proteolytic enzymes in shortened sarcomeres corresponds to increased toughness in meat cooked to 71°C (Weaver *et al.* 2008), it is difficult to see how proteomics would help resolve the sarcomere length effects that are revealed only after cooking. In contrast, the non-appearance of the protein collagen and any of the enzymes responsible for its initial crosslinking or proteolysis is more difficult to explain, until it is remembered that the protein solubilisation methods and gel electrophoresis techniques used in the majority of proteomics studies are not conducive to the study of collagen, and hence this contribution to cooked meat toughness is also under-represented in proteomics studies. The exact nature of cooking in the preparation of meat samples for sensory of instrumental measures of tenderness are obviously important, as Listrat *et al.* (2020) found, in an integration of three studies on beef tenderness, that the level of pyridinoline crosslinks in intramuscular collagen were consistently related to sensory measures of toughness when in beef samples were cooked to an internal temperature of 55°C. Biochemical studies, and proteomics studies, on raw meat obviously preclude the influence of many of the factors which interact with cooking time and temperature in their effect on meat toughness. As mentioned above, cooked meat samples are difficult to analyse but maybe future studies could include this in proteomic studies.

One difficulty in the juxtapositioning of traditional H-D method investigations and proteomics investigations is the very comprehensiveness of the proteomics approach, which inherently can detect numerous differences between individuals and their responses to given treatments. The sensitivity of the omics methods is the most likely explanation for the extensive diversity in the number of genes reported in meat quality transcriptomics (Guo & Dalrymple 2017) and the number of protein markers for meat quality in proteomics studies (Picard & Gagaoua 2017; Picard *et al.* 2017; Picard & Gagaoua

2020a); using a highly controlled group of animals under specific management conditions in one study may elicit a different gene of protein list from another study using even slightly different animals or conditions. There are very few omics studies that combine multiple breeds, multiple husbandry conditions or even multiple species in looking for genes, proteins, metabolites or phosphorylation pathways that may underpin variations in the properties of muscle in any animal.

A final note of caution concerns our interpretation of associations between proteins found to differ in quantity between beef of high and low toughness, or between beef meat samples with a different colour. As one strength of a data-led experiment such as a proteomics study is that it provides insight into mechanisms not previously considered, we should be cautious in framing hypotheses based on strong associations between proteins and a given phenotypic character of the meat. For example, in both the beef colour and tenderness meta-analyses, the enzymes involved in energy metabolism were emphasized. In forming a hypothesis to explain why this should be so for beef meat colour, it is natural to focus on the main function of these enzymes, and so the rational explanation given is that these enzymes affect the post-mortem metabolism and hence pH drop in the muscle, which has a strong influence on colour. However, as noted above, the precipitation of these enzymes onto the thin filaments of the sarcomere in some conditions of post-mortem temperature and pH also contribute to colour differences due to light scattering. The effect of these enzymes in driving post-mortem pH is the most obvious explanation of colour differences, but this second effect is not so obvious. When searching for the mechanisms underlying previously unrecognized associations between proteins and meat quality traits, we must therefore bear in mind that the conventional view of the proteins' functions should not restrict development of new hypotheses. Additionally, the inter-connectivity of many pathways implies that subtle secondary effects may also occur. It is important to note that any of the enzymes also have associations with variations in metmyoglobin reducing activity, and also NADH generated via reverse electron flow in beef mitochondria has been associated metmyoglobin reductase activity (Belskie *et al.* 2015). Thirty-six proteins identified by six different proteomics studies, based on the repertoire by Gagaoua *et al.* (2020b) to be correlated with metmyoglobin reducing activity (MRA) in bovine muscles are summarized in Table S3.

4. The importance of post-mortem metabolism conditions in both traditional and proteomic studies

4.1. Glycolysis and energy metabolism

Post-mortem metabolism in muscle foods has long been considered of paramount importance and intense peri-mortal activity has been suggested as the main reason for accelerated onset of rigor (Bate-Smith & Bendall 1949). The decrease in ATP during the post-mortem period triggers, among other pathways, glycolysis, in an effort to produce more ATP and an attempt to maintain muscle in a relaxed state. ATP production is well known to have a pivotal role in the execution of apoptosis processes, which include myriad energy-dependent steps (Elmore 2007; Ouali *et al.* 2013). Furthermore, the importance of post-mortem energy metabolism has been generally related to the rate of subsequent pH decline due to the failure of the electron transport chain. The production of energy in the core of the muscle fibres is a consequence of 1) glucose conversion and breakdown of glycogen and 2) metabolism of lipids, including triglycerides, ketone bodies, free fatty acids, and volatile fatty acids (Picard & Gagaoua 2020b). The two proteomic meta-analyses on beef tenderness and colour (Gagaoua *et al.* 2020b; Gagaoua *et al.* 2021) both highlight the pivotal role of enzymes from the energy-yielding phase of glycolysis, whose

enzymes ensure the conversion of triose-phosphate to pyruvate and anaerobically form lactate to produce net 2 ATP and one NADH.

Jia and co-workers identified the major proteome changes during the rigor development in beef and reported that a large number of proteins from the energy metabolism pathway were involved in these dynamic changes over different post-mortem times (Jia *et al.* 2007). The main metabolic pathways highlighted by this study are summarised in **Fig. 3**, based on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, GO Biological Processes, and Reactome gene sets. Comparison of muscle biopsies taken 4 days before slaughter and muscle samples taken approximately 1h post-mortem have identified increased aerobic energy metabolism immediately post-mortem, presumably with the aim to replenish the ATP levels in the muscle (Jia *et al.* 2006). Zhai *et al.* (2020) found that among the differentially abundant proteins, those involved in oxidative phosphorylation, ATP-related transport, Krebs cycle, fatty acid degradation, oxygen transport, NADPH regeneration, and degradation of the extracellular matrix were primarily more abundant in Psoas major at different post-mortem periods when compared to *Longissimus lumborum* muscle. These differences were in line with previous findings in a study comparing oxidative and glycolytic muscles (Picard *et al.* 2014).

4.2. Importance of calcium release, proteolysis and associated pathways

Translocation of calcium ions from the sarcoplasmic reticulum (SR) to the cytosol occurs rapidly post-mortem, and calcium has been found in the sarcoplasm and in the inter-myofibrillar space of beef muscle at approximately 4 h post-mortem (Vignon *et al.* 1989). The release of calcium contributes to the regulation of the energy metabolism pathways including glycolysis by affecting the speed and activity of crucial metabolic enzymes, mostly in a positive way (Bendall 1973b; Carafoli 2002; Ouali *et al.* 2013; Picard & Gagaoua 2017). In addition to its prime role in regulating muscle contraction, calcium also regulates the activity of calpains, which are involved in the major tenderization mechanism of proteolysis as explained above. Calpastatin inhibits both μ- (CAPN1) and m-calpain (CAPN2) in a calcium-dependent manner under concentrations reported to be close to or below those required to activate calpain (Goll *et al.* 2003). Both CAPN1 and CAPN2 interact with several proteins of different pathways, such as calcium homeostasis, myofibril turnover and proteolysis, glucose metabolism, mitochondrial activity, response to stress, and apoptosis (Brulé *et al.* 2010; Picard & Gagaoua 2017; Gagaoua *et al.* 2021).

Sophisticated regulation of calcium storage, uptake and release in skeletal muscle cells is achieved by the means of concerted action of three major classes of SR calcium-regulatory proteins: (i) calsequestrin, junctate, calmodulin and sarcalumenin for calcium storage; (ii) SR calcium release channels such as ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptor (ITPR1) for calcium release, and (iii) SR calcium-ATPase (SERCA) pumps for calcium reuptake (Berchtold *et al.* 2000). Calcium-binding proteins such as calsequestrin, sarcalumenin and calmodulin (CaM, calcium-modulated protein) were identified in several bovine proteomic studies as potential biomarkers of beef tenderness (Picard & Gagaoua 2020a; Gagaoua *et al.* 2021). These proteins are involved in calcium signaling modulation in the living animal (Berchtold *et al.* 2000; Picard *et al.* 2016), and after animal death they may contribute to lowering the levels of free calcium, thus lowering the activity of calcium-dependent proteases such as calpains and other metabolic and glycolytic enzymes. The association between calcium-regulating proteins and calcium-dependent calpains suggests a complex interaction of the mechanisms governing post-mortem proteolysis and thus meat tenderness.

4.3. Heat shock proteins

Heat shock proteins (HSPs) are a family of ubiquitous and evolutionarily conserved chaperone proteins classified into 5 subfamilies based on their molecular weight (MW), e.g., HSP60, HSP70, HSP90, and HSP100 and the small HSPs (MW 12–43 kDa). In most proteomics studies and whatever the muscle and species, they were correlated with several meat quality traits (Lomiwes *et al.* 2014; Picard & Gagaoua 2017; Malheiros *et al.* 2019; Gagaoua *et al.* 2020b; Picard & Gagaoua 2020a; Gagaoua *et al.* 2021). The consistent identification of HSPs was thought to be related to their role in maintaining cellular homeostasis as the production of HSPs is generally increased in cells undergoing stress and post-mortem events disturb the ante-mortem homeostatic set points. HSP were the third most important pathway impacting beef tenderness, and only two sub-families (small HSPs and large 70 kDa proteins) were identified (Gagaoua *et al.* 2021). For beef color, HSP60 was further identified in addition to the small and large HSP70 proteins (Gagaoua *et al.* 2020b). Furthermore, all HSPs were correlated negatively with lightness (*L**).

Several functions have been proposed for HSPs in post-mortem muscle. Ouali *et al.* (2006) first hypothesized their anti-apoptotic function role in muscle to meat conversion.HSP27 (the most interesting HSP for both tenderness and color of beef) has been shown to inhibit apoptosis by blocking the proteolytic activation of caspase 3, to prevent Bid from translocating to mitochondria and to delay the release of cytochrome C (Arrigo *et al.* 2002). Differences in the expression of different isoforms of HSP27 and other members of the HSP family vary according to metabolic and contractile properties of the investigated muscle (Neufer & Benjamin 1996; Picard *et al.* 2014; Gagaoua *et al.* 2017a). As regulators of apoptosis onset, HSPs proteins were proposed to affect post-mortem proteolysis and thus the level of breakdown of cytoskeletal proteins (Lomiwes *et al.* 2014; Picard *et al.* 2014; Gagaoua *et al.* 2015b; Ma & Kim 2020).

A second role for HSP is to protect against reactive oxidative species (ROS generated due to the rapid increase in energy metabolism enzymes early post-mortem and to avoid damage of waste metabolites. (Gagaoua *et al.* 2020b) proposed that, thanks to their pivotal role in protection against stress-induced denaturation of sarcoplasmic proteins and myosin, HSPs would protect myoglobin and affect reflectance, and light scattering, thereby influencing all color coordinates. Thirdly, and as discussed above, important changes occur in the mitochondrial proteome post-mortem, and large HSPs have been shown to help maintain the integrity of the mitochondrial membrane and potential its ATP levels, thus participating in energy production machinery (Daugaard *et al.* 2007).

 Overall, the causal effects between the biological and protective roles of HSPs and the development of meat qualities are still not fully understood, as stress status of the muscle or animals (as well as the notion of proteoforms) are not controlled or considered in many proteomic studies. These important unknowns are worthy of investigation in the future, to understand to which extent this protein superfamily may drive the final meat quality.

4.4 An increasing focus on mitochondrial function post-mortem

Interplay between apoptosis, mitochondria and energy metabolism pathways is evident in post-mortem muscle and the consequences for final eating qualities were discussed in several emerging studies

(Longo *et al.* 2015; Jiang *et al.* 2019; Ma & Kim 2020; Zhai *et al.* 2020). Very recent data support the importance of these pathways and more specifically that of mitochondria to modulate the rate of post-mortem metabolism through competing, for example, with lactate dehydrogenase for pyruvate (Matarneh *et al.* 2021). Indeed, mitochondria have been proposed to play an important role in the conversion of muscle into meat (Hudson 2012; England *et al.* 2013; Ouali *et al.* 2013; Sierra & Olivan 2013), which is supported by the large number of proteins from this pathway identified by proteomics studies (Jiang *et al.* 2019; Gagaoua *et al.* 2020b; Zhai *et al.* 2020; Gagaoua *et al.* 2021; Zhu *et al.* 2021). This agrees with the finding of England *et al.* (2018) that mitochondria in muscle function to some capacity up to 24-48 hours post-mortem. This supports the hypothesis of (Jia *et al.* 2006) that residual mitochondrial functionality exists in post-rigor muscle. Some mechanisms by which the proteins identified through proteomics could trigger the release of calcium from SR into mitochondria or the cytosol were proposed in several studies (Mammucari *et al.* 2011; Ouali *et al.* 2013; Gagaoua *et al.* 2015b; Gagaoua *et al.* 2015c; Rodrigues *et al.* 2017; Picard & Gagaoua 2020a). Several of the studies which demonstrated significant relationships between some of the proteins and several quality traits in both beef and fish warrant discussion in more detail. In a study by Gagaoua *et al.* (2015b), the stress 75 kDa glucose-regulated protein GRP-75 (known also as HSPA9) was found to be related to several pathways, including the proteolytic pathway involving μ-calpain. The authors suggested that this unique mitochondrial chaperone is of interest in the conversion of muscle to meat (Gagaoua *et al.* 2017b), especially through calcium homeostasis (for details, see (Gagaoua *et al.* 2021)). Indeed, HSPA9 is the only large heat-shock protein (HSP) regulated by calcium flux, perturbation of glycolysis or glucose deprivation (Mayer 2013). Therefore, HSPA9 may enhance calcium trafficking from the SR by linking the Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), an intracellular gated calcium release channel, to the mitochondrial voltage-dependent anion channels (VDACs) (Malli & Graier 2010; Ouali *et al.* 2013). Both ITPR1 and VDACs proteins were reported as biomarkers of beef tenderness (Gagaoua *et al.* 2021).

Post-mortem energy metabolism and pH decline rate could be affected by mitochondria through calcium homeostasis. Mitochondria have the ability to store calcium in their matrix which consequently affects post-mortem metabolism (Jiang *et al.* 2020; Matarneh *et al.* 2021). To exemplify this, the activity of dehydrogenases enzymes, such as PDHB (pyruvate dehydrogenase E1 component β) and MDH1 (malate dehydrogenase 1), both identified as biomarkers of several meat qualities (Ouali *et al.* 2013; Picard & Gagaoua 2017; Gagaoua *et al.* 2020b; Gagaoua *et al.* 2021), especially of colour and tenderness of beef, are dependent on mitochondrial calcium amounts (Gehlert *et al.* 2015). Moreover, recent investigations using an *in vitro* system to evaluate the contribution of mitochondria to post-mortem metabolism (Matarneh *et al.* 2017) showed that intact mitochondria lowered the rate of pH decline during the first 30 minutes post-mortem by stabilizing ATP levels and reducing glycolytic flux. Furthermore, after 2 h post-mortem, the mitochondria enhanced ATP hydrolysis, glycogen degradation, lactate accumulation, and pH decline. Following other experiments, the authors revealed that the causative agent was a mitochondrial F1-ATPase protein (Matarneh *et al.* 2018). However, the polymorphism and complexity of ATP synthase should be considered in a hypothesis-driven manner to better understand its real role on early post-mortem energy metabolism. On another hand, under elevated calcium flux, the integrity of mitochondria would be destroyed, leading to the release of cytochrome C and other pro-apoptotic factors, which would trigger apoptosis (Berchtold *et al.* 2000; Ouali *et al.* 2013; Sierra & Olivan 2013).

The elegant work of (Glancy & Balaban 2012) demonstrated the a link between mitochondrial and cytosolic calcium concentrations, providing evidence for sophisticated transport mechanisms as well as the closeness of mitochondria to calcium release sites, which together support the notion that calcium can be also an important signalling molecule in the energy metabolism interplay of the cytosol with the mitochondria.

Considering the above, an interesting but little investigated, the role that calcium may further play in post-mortem muscle, with impact on the eating qualities of meat, is its role in triggering the onset of apoptosis (Pinton *et al.* 2008; Ouali *et al.* 2013). Calcium overloading in the mitochondria could also lead to depolarization of the inner mitochondrial permeability transition pore (MPTP), hence triggering apoptosis (Malli & Graier 2010). MPTP is responsible for the release of mitochondrial products, playing a critical role in mitochondrion-initiated apoptosis, thereby impacting beef tenderness (Wang *et al.* 2017). For further roles of mitochondria and apoptosis in post-mortem muscle, we refer the reader to recent reviews on the topic (Demaurex & Distelhorst 2003; Ouali *et al.* 2013 Sierra & Olivan 2013; Lana & Zolla 2015; Longo *et al.* 2015).

In line with the mechanisms described above, mitochondrial function is known to impact on meat color (for review: (Ramanathan & Mancini 2018; Ramanathan *et al.* 2019)) and tenderness (for review: (England *et al.* 2013; Sierra & Olivan 2013; Dang *et al.* 2020)). According to Ramanathan *et al.* (2010), the diffusion of oxygen into the meat surface as well as its consumption by muscle is one of the pivotal factors that lead to the formation of bright-red color which are mostly influenced by mitochondrial functionality. This is known to be a consequence of the interplay between mitochondria and myoglobin (Mb) (Mitacek *et al.* 2019), as this later serves like a reservoir of oxygen in muscle, therefore allow producing energy or for the oxidative phosphorylation pathway from which myriad proteins were revealed by proteomics (Gagaoua *et al.* 2020; Kiyimba *et al.* 2020; Wu *et al.* 2020; Gagaoua *et al.* 2021). Wu *et al.* (2020) focused on the underlying mechanisms of dark-cutting beef and investigated the mitochondrial proteome that allowed identifying 28 proteins that differ in their abundance. A total of 75% of those proteins were involved in oxidative phosphorylation, generation of reducing equivalent, tricarboxylic acid cycle (TCA) and response to stress. Ramanathan *et al.* (2020b) revealed that TCA substrates (most likely malic and fumaric acids) were more abundant in dark-cutting beef compared to normal pH and color beef. For more details on the role of mitochondria and postulated mechanisms, the reader should refer to reviews on color (Ramanathan & Mancini 2018; Ramanathan *et al.* 2019; Gagaoua *et al.* 2020b; Ramanathan *et al.* 2020c) or tenderness (England *et al.* 2013; Ouali *et al.* 2013; Sierra & Olivan 2013; Wang *et al.* 2017; Gagaoua *et al.* 2021).

4.5. Oxidation/nitrosylation

The dynamic changes that occur in early post-mortem muscle are accompanied by an increase in the activity of enzymes associated with the glycolytic and Krebs cycles, which are at the origin of the accumulation of several waste metabolites including reactive oxygen species (ROS) and oxidative compounds such as nitric oxide (NO) (Warner *et al.* 2005; Lana & Zolla 2015). ROS, which are sourced from and target the mitochondria, can react with both proteins and lipids and often have deleterious effects, contributing to the onset of several processes including apoptosis and autophagy (Lana & Zolla 2015). Furthermore, peroxidation in mitochondria caused by ROS can induce harmful damage in the mitochondrial metabolism, the uptake of calcium, and the overall integrity of mitochondria (Bekhit *et al.*

2013), thus impacting the rate and extent of the pathways driving the conversion into meat. Proteomics and interactomics studies have identified several endogenous scavenger proteins involved in the oxidative stress pathway, such as peroxiredoxins members, superoxide dismutase isoforms, protein/nucleic acid deglycase DJ-1 (known as PARK7) and thioredoxin system among others, to interact in concert with heat shock proteins to scavenge ROS or to protect proteins from the energy metabolism and proteolysis pathways (Malheiros *et al.* 2019; Gagaoua *et al.* 2020b; Gagaoua *et al.* 2021).

Nitric oxide (NO), which is a free radical as well as a known effector of apoptosis (Brüne *et al.* 1999), is constantly produced or released throughout diverse tissues and is known to influence proteolytic activity in skeletal muscle as well as being involved in regulation of calcium homeostasis (Liu *et al.* 2018). Thus, both oxidation and nitrosylation play a role in post-mortem metabolism and determination of meat qualities. The common feature of both ROS and NO is that they can impact calcium flux, and thereby all the pathways that require calcium, as discussed above. For further details on these important post-mortem processes and their effect on the triptych of mitochondria, apoptosis, and post-mortem metabolism and their consequences of the development of meat quality, we refer the reader to excellent recent reviews and research papers in this area (Warner *et al.* 2005; Bekhit *et al.* 2013; Lana & Zolla 2015; Liu *et al.* 2018; Hou *et al.* 2019; Zhang *et al.* 2019; Hou *et al.* 2020a; Hou *et al.* 2020b). Reactive nitrogen species (RNS) are also known to play a role in physiological regulation and to interact with ROS (Martinez & Andriantsitohaina 2009; Domínguez *et al.* 2019).

5. Conclusions and suggestions for future integrations of traditional and proteomic methods

The burgeoning growth in proteomics studies of meat quality in the last two decades has produced large amounts of data. Some of the studies have been relatively comparable in the proteins they identified, and these meta-analyses have enabled identification of which protein biomarkers of tenderness or meat colour arise most consistently. These meta-analyses have also shown that studies on the same aspects of meat quality in the same breed and muscle studied can also be quite variable. Sources of this variation likely arise in the proteomics techniques used, the post-mortem conditions of the samples studied, the methods used to take phenotypic measure, and the cut-offs employed to decide on "high" versus "low" values of tenderness or colour.

The use of biomarkers for consistent prediction of tenderness and colour quality traits in the meat industry still remains a challenge, due in part to the variability in the proteins identified. Gagaoua *et al.* (2018) note that the complex processes involved in the post-mortem tenderisation of meat depends on a large number of factors along the continuum of the supply chain, both pre-and post-mortem. Due to the complexity in this process, Starkey *et al.* (2016) concluded that single protein biomarkers were unlikely to provide an adequate prediction of tenderness that could be used in the industry. In relation to how to move forward with industry-implementation of biomarkers, (Gagaoua *et al.* 2020a) propose a six-stage process of (i) protein discovery/identification, followed by (ii) qualification, (iii) verification, (iv) research assay optimization, (v) industrial validation and finally (vi) commercialization. At present, the value of proteomics studies and their meta-analyses principally lies in expanding our understanding of the molecular mechanisms and cellular pathways underlying the development of meat quality post-mortem.

In future, it is increasingly likely that omics techniques will be used to discover more of the complex interactions between pathways that combine to determine the qualities of meat after ageing and cooking that are appreciated by consumers. By examining as much as practically possible of the

proteome of meat samples showing variations in tenderness, WHC or colour, proteomics offers the possibility to identify unconsidered pathways affecting these qualities. However, discussion of how and why these proteins contribute to meat quality has sometimes been limited to speculation about the functions of each protein, drawing on conventional knowledge. Drawing potential conclusions from empirical correlations within a large data set is an insufficient endpoint, and the traditional H-D approach is invaluable in testing whether the associations between protein abundance and a given phenotypic quality could be causal rather than merely correlative. Winkler (2016) starts his article proposing strategies to develop omics data into resilient models by stating that "Omics techniques produce information, but not necessarily scientific knowledge" and states that the construction of predictive models from omics data is most useful only if they have predictive power outside the confines of the set of conditions in which the data were empirically collected. The construction of models must be on the basis of some logical mechanism and should be both testable and predictive. Winkler (2016) goes on to propose a scheme for "the scientific method" using omics, where the initial omics data provide not just descriptive or empirical models, but is also the basis for predictive models that are based on testable hypotheses. In relation to testable, predictive models, multivariate statistical techniques such as partial least squares (PLS) models and deep learning are one avenue. These can be purely descriptive, i.e. based entirely on statistical associations within the large dataset collected. However, another approach may be to use predictive computational modelling of known biochemical pathways. This latter approach is being developed by Vetharaniam and colleagues (Vetharaniam *et al.* 2010; Vetharaniam *et al.* 2018) in relation to meat tenderness development post-mortem.

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Declaration of Conflicting Interest

The authors declare that there is no conflict of interest.

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Conflict of Interest statement

All authors declare that there are no conflicts of interest.

Figure legends

Fig. 1. A simplified overview by biological pathway of 79 meat colour putative protein biomarkers identified across the 5 bovine muscles in relation to the different colour parameters. The distribution of the 79 proteins among the main 6 biological pathways are shown in brackets for each pathway. From the supplementary online material of (Gagaoua *et al.* 2020b), with permission. The full names of each protein are given in Table S2 (supplementary information).

Fig 2. Ranking of the 124 putative protein biomarkers of beef tenderness by the number of occurrences across the 28 proteomic-based studies assessed in the meta-analysis of (Gagaoua *et al.* 2021). The proteins are categorized and highlighted by different colours according to their functional pathways (see Table S2); blue = muscle contraction, structure and associated proteins, orange = energy metabolism proteins; red = heat shock proteins; green = oxidative stress proteins; purple = proteolysis; and black/grey= regulation of cellular processes, binding, apoptosis and transport proteins. The distribution of the shortlisted robust biomarkers (33 proteins, identified ≥ 4 times across all studies) between these pathways is depicted by the pie chart in the top right, following the colour scheme detailed above. The full protein names are given in the supplementary Table S3. Adapted from (Gagaoua *et al.* 2021), with permission.

Fig. 3. Enriched ontology clusters based on Gene Ontology, KEGG and Reactome Gene Sets prepared from the list of proteins (n = 27) identified by Jia *et al.* 2007). **A)** The bar graphs highlight all the enriched terms (functional clusters = 9) across the protein lists changing early post-mortem with the importance of metabolic process and ATP metabolic process and apoptosis/autophagy processes coloured according to -Log P-values: terms with a P-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5. **B)** The top-level Gene Ontology biological processes. Aggrephagy, identified as an important pathway, is a term for the selective degradation of protein aggregates by macroautophagy.

Fig. 1.

Number of identification	Number of proteins	Proteins
>15 times	2	ACTA1 HSPB1
≥10 times	6	MYH1 MYL1 TNNT3 CKM ENO3 HSPB6
9 times	1	HSPA1A
8 times	3	GAPDH CRYAB PARK7
7 times	1	MYH7
6 times	6	MYBPH ENO1 PGM1 TPI1 HSPA8 HSPA1B
5 times	6	FHL1 MYLPF TNNT1 HSPA9 SOD1 PRDX6
4 times	8	CAPZB TNNC1 TNNI2 MDH1 ALDOA PKM YWHAE CA3
3 times	12	TPM3 MYH2 MYL2 MYL6B MYL3 DES LDHA PGAM2 PYGM MSRA ALB HBB
2 times	19	ACTN3 CSRP3 KLHL41 MYOZ1 IDH1 GPD1 ALDH1A1 AK1 ATP5B UQCRC1 NDUFS1 GSTP1 HINT1 EEF1A2 VDAC2 HIST2H2AA3 LGALS1 TRIM72 TP53
1 time	60	ACTB CAPZA3 ACTN2 FLNC WDR1 MYOZ3 PDLIM7 PDLIM1 SMTNL1 TMOD1 VCL COL1A1 COL1A2 GPI SDHA PDHB AKR1B1 DLST GOT1 ACBD6 NNT OGDH SLC25A11 HSPA2 STIP1 CCT8 SOD2 PRDX1 PRDX2 PDIA3 SH3BGRL3 CSN2 ANKRD2 GDI2 RABGGTA AIMP1 NUTF2 VDAC1 TF HPX CAVIN1 ADSS1 STBD1 KCNJ15 CALM2 FABP3 SHC4 RAB21 PARP1 ZNF197 ZFHX4 HIP1R MPHOSPH9 POR MB ANXA6 AHCY ITPR1 PSMB2 PSMC2
Total proteins	124	

Fig. 2.

A)

GO:0042743: hydrogen peroxide metabolic process
R-HSA-9646399: Aggrephagy
GO:2001243: negative regulation of intrinsic apoptotic signaling pathway
GO:0046034: ATP metabolic process
GO:0042398: cellular modified amino acid biosynthetic process
R-HSA-2454202: Fc epsilon receptor (FCERI) signaling
GO:0006936: muscle contraction
GO:0046434: organophosphate catabolic process
GO:0010638: positive regulation of organelle organization

B)

GO:0008152: metabolic process
GO:0098754: detoxification
GO:0050896: response to stimulus
GO:0048519: negative regulation of biological process
GO:0048518: positive regulation of biological process
GO:0023052: signaling
GO:0050789: regulation of biological process
GO:0065007: biological regulation
GO:0032501: multicellular organismal process
GO:0051704: multi-organism process
GO:0071840: cellular component organization or biogenesis
GO:0022610: biological adhesion

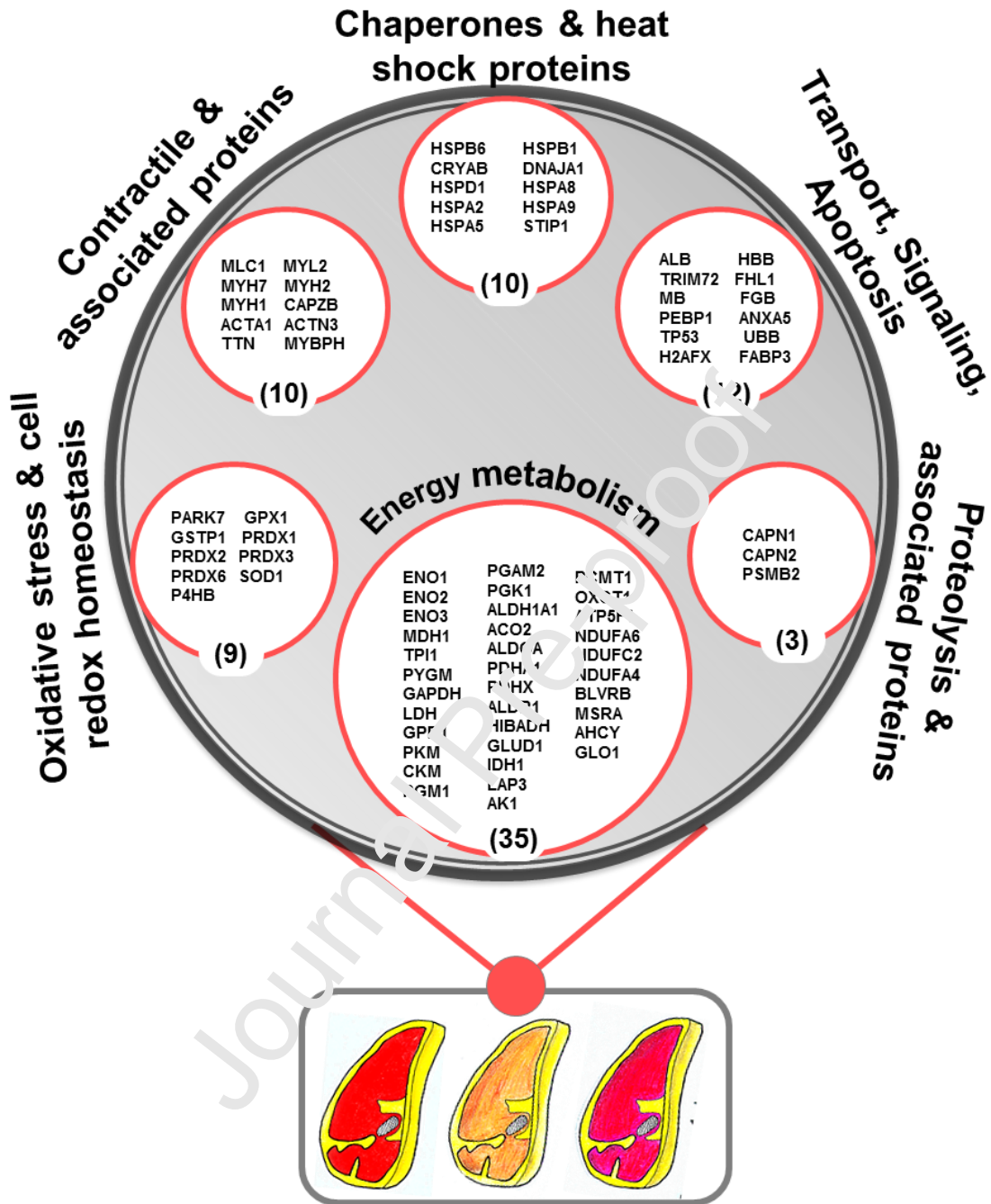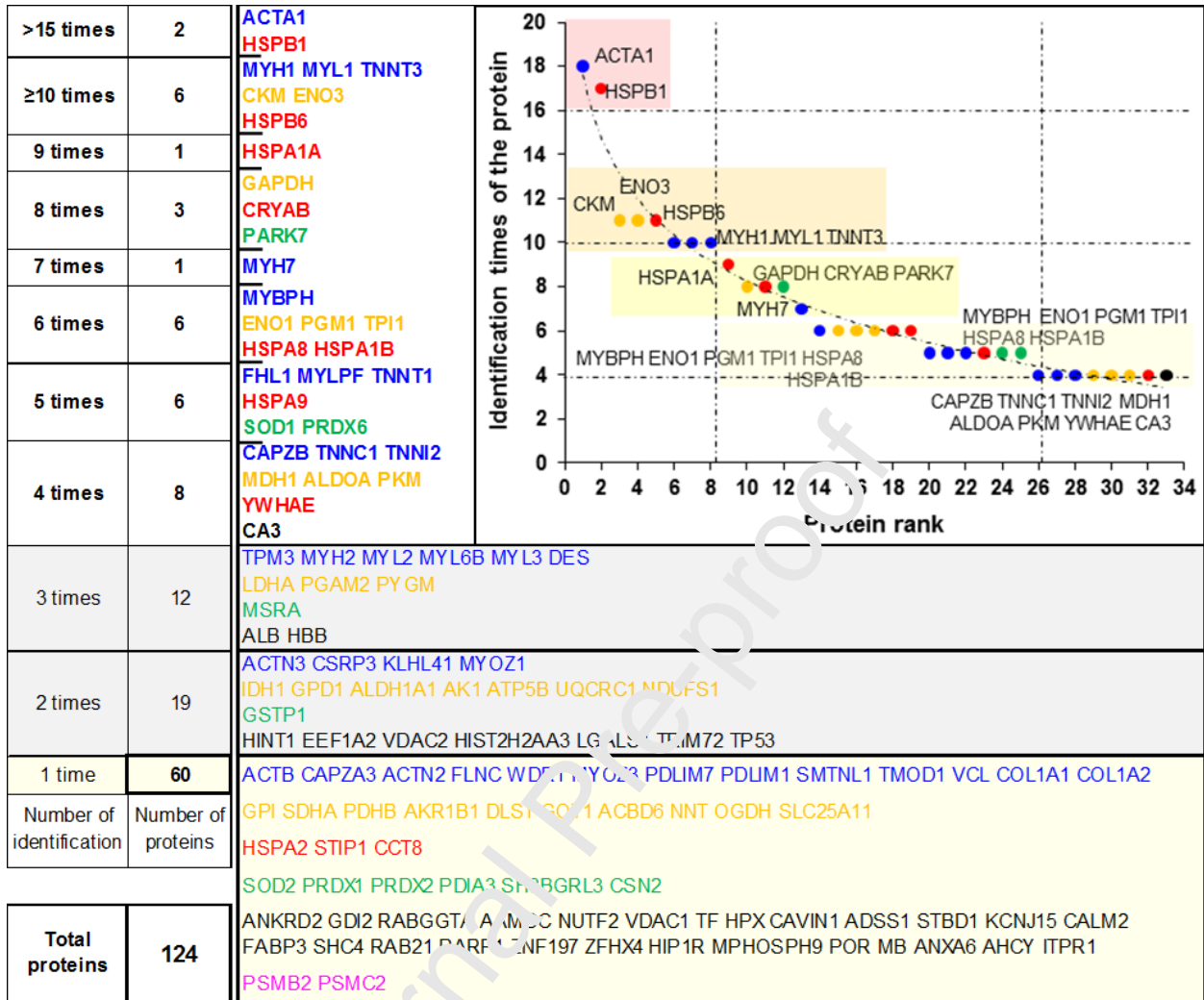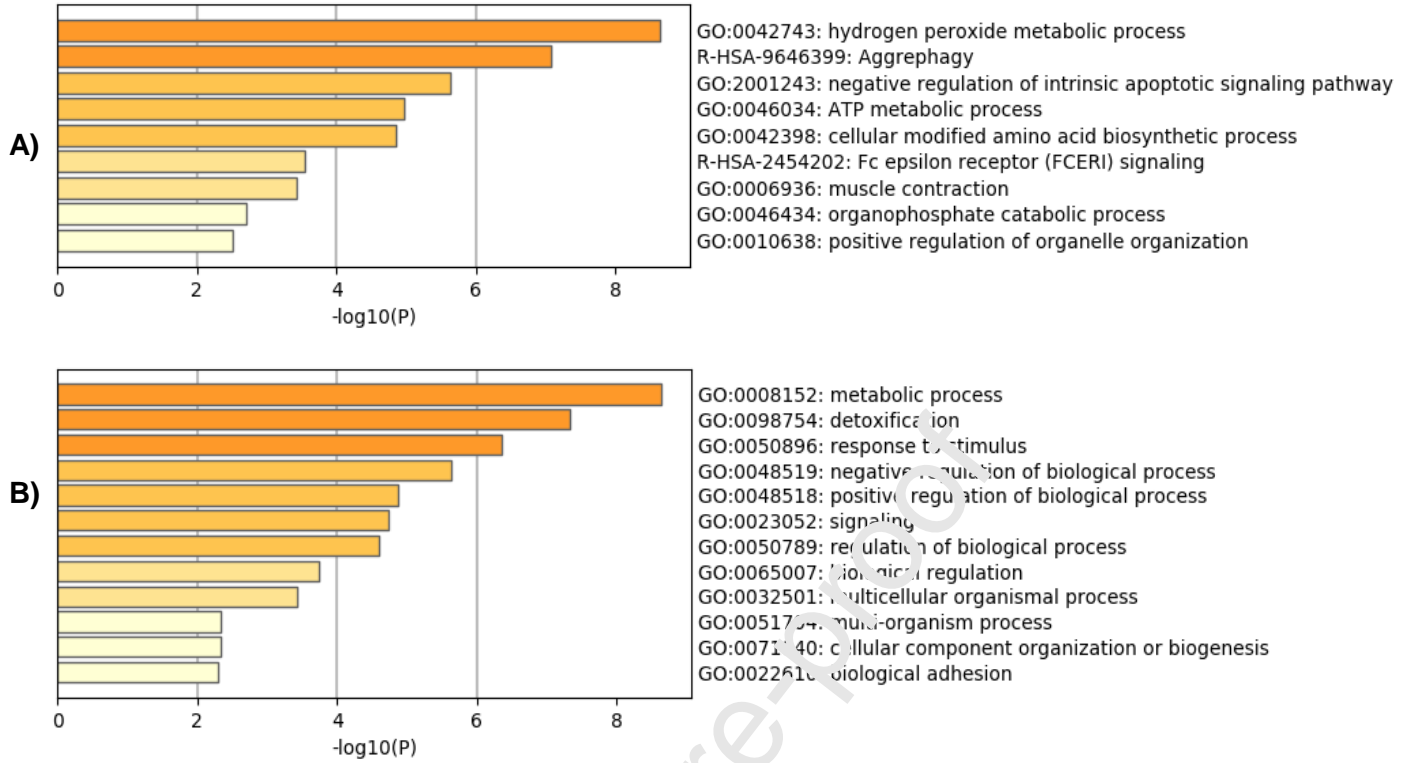**Fig. 3.**

Table 1. Summary of major mechanisms affecting beef colour, some influencing factors, chemistry/measurements and major references.

This summary does not include detailed mechanisms affecting the oxidation/reduction of myoglobin, such as enzymatic, non-enzymic and mitochondria-mediated metmyoglobin reduction, mitochondrial oxygen consumption and reversal of electron flow in mitochondria, or effects of primary and secondary lipid oxidation products.   Please refer to Ramanathan, Suman & Faustman (2020) for a discussion of these.

Mechanism	Aspects	Some influencing factors	Chemistry/measurements
Content of myoglobin [1,2]	Molecular structure of myoglobin [3]	Age of animal Muscle Fibre type Species Portion of muscle Double muscling	Total haem Total iron Concentration of myoglobin
Chemical state of myoglobin [1,4,5,6]	Rate of blooming Rate of browning	Temperature Ageing OCR O_2 depth Concentration/activity of mitochondria Metmyoglobin reductase Denaturation of myoglobin Oxygen partial pressure Ultimate pH	Metmyoglobin reductase activity NADH production Oxygen consumption Etc.
Light scattering [7-12]	Myofilament spacing & denatured proteins	Rate of glycolysis Ultimate pH Mitochondrial distribution Sarcoplasmic protein denaturation Myosin denaturation Myofilament spacing	Glycolytic enzymes X-ray diffraction Light microscopy Sarcoplasmic and myofibrillar protein solubility Myosin ATPase activity

[1] Young & West (2001); [2] Hunt and Hedrick (1977); [3] Faustman et al. (2010); [4] Jacob (2020); [5] Renerre (1984); [6] Mancini &Hunt (2005); [7]Swatland (2004); [8] Offer et al., (1989); [9] Macdougall (1970); [10]Hughes et al. (2019); [11] Hughes et al. (2018); [12] Hughes et al.(2017).

Highlights

- Insights from both conventional studies and proteomics into beef meat colour and tenderness are reviewed
- Proteomics meta-analysis of variations in beef colour highlights energy metabolism pathways
- Energy metabolism and proteolysis emphasised in proteomics of beef tenderness, together with calcium regulation, oxidation and nitrosylation pathways
- Increasing evidence points to a strong role of post-mortem mitochondrial metabolism in meat quality
- Potential mechanisms suggested by data-driven experiments must be checked by hypothesis-driven studies