Proteomic approach to characterize biochemistry of meat quality defects

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Abstract

Proteomics can be used to characterize quality defects including pale, soft, and exudative (PSE) meat (pork and poultry), woody broiler breast meat, reddish catfish fillets, meat toughness, and beef myoglobin oxidation. PSE broiler meat was characterized by 15 proteins that differed in abundance in comparison to normal broiler breast meat, and eight proteins were differentially expressed in woody breast meat in comparison to normal breast meat. Hemoglobin was the only protein that was differentially expressed between red and normal catfish fillets. However, inducing low oxygen and/or heat stress conditions to catfish fillets did not lead to the production of red fillets. Proteomic data provided information pertaining to the protein differences that exist in meat quality defects. However, these data need to be evaluated in conjunction with information pertaining to genetics, nutrition, environment of the live animal, muscle to meat conversion, meat quality analyses and sensory attributes to understand causality, protein biomarkers, and ultimately how to prevent quality defects.

Keywords: Proteomics, Meat quality, Woody breast, PSE (pale, soft, and exudative) meat, Red-catfish fillet, Myoglobin oxidation

1. Application of proteomics to meat quality

Proteomics is the study of the proteome (i.e. a set of proteins) which contains information on gene expression and protein translation (Petracci & Cavani, 2012). Two-dimensional electrophoresis (2-DE) in combination with mass spectrometry (MS) has been applied in meat quality research to understand growth and development, postmortem metabolism, calpain's role in tenderness, protein biomarkers related to tenderness, and water holding capacity (WHC) (Bendixen, 2005; Bouley, Chambon, & Picard, 2004; Carvalho et al., 2014; Desai et al., 2016; Gorg, Weiss, & Dunn, 2004; Phongpa-Ngan, Grider, Mulligan, Aggrey, & Wicker, 2011; Picard et al., 2015). In addition, proteomics is applied for elucidating protein modifications such as reversible phosphorylation, oxidation, degradation, and denaturation in postmortem meat (Huang & Lametsch, 2013). Several researchers have also focused on understanding early postmortem protein changes using proteomics (D'Alessandro, Marrocco, Zolla, D'Andrea, & Zolla, 2011; Jia et al., 2007; Lametsch et al., 2003; Promeyrat et al., 2011; Wu, Fu, Therkildsen, Li, & Dai, 2015). Proteomics is an important tool in determining quality biomarkers that are indicators of meat quality defects (Laville et al., 2009; Marcos & Mullen, 2014; Rodrigues et al., 2017; van de Wiel & Zhang, 2007).

Application of proteomics for investigating meat quality is a relatively new approach (less than 20 years old), but has been used to elucidate the relationship between the muscle proteome and the conversion of muscle to meat (Jia et al., 2007; Jia et al., 2006b; Lametsch et al., 2011; Morzel et al., 2004; Sayd et al., 2006), tenderness (Jia, Hollung, Therkildsen, Hildrum, & Bendixen, 2006a; Lametsch et al., 2003; Laville et al., 2009), color (Canto et al., 2015; Joseph, Suman, Rentfrow, Li, & Beach, 2012; Nair et al., 2016; Yu et al., 2017), and water holding capacity (van de Wiel & Zhang, 2007).

2. Application of proteomics to beef quality

2.1 Proteome analysis of bovine muscle in postmortem period

Bouley et al. (2004) studied the mapping of bovine skeletal muscle using two-dimensional gel electrophoresis and mass spectrometry. They identified 129 protein spots corresponding to metabolism, cell structure, cell defense, and contractile apparatus. Jia et al. (2006b) analyzed the changes in enzymes associated with energy metabolism during the early postmortem period in *longissimus thoracis* bovine muscle. Twenty four metabolic and heat shock proteins changed in samples collected at different postmortem times. Jia et al. (2006a) conducted proteome analysis of two bovine muscle types: *M. longissimus dorsi* and *M. semitendinosus*, and reported that five proteins (cofilin, lactoylglutathione lyase, substrate protein of mitochondrial ATP-dependent proteinase SP-22, HSP 27, and HSP20) were affected during early post-mortem storage in both muscles. Similarly, Jia et al. (2007) examined the proteome changes up to 24 h postmortem in bovine *longissimus thoracis* muscle. Results demonstrated that 47 protein spots changed during the first 24 h postmortem. Application of 2DE in the study identified approximately 1000 individual protein spots.

2.2 Tenderness

Picard et al. (2015) reported 21 potential biomarkers for tenderness in beef, which included heat shock, metabolic, structural, oxidative resistant, and proteolytic proteins. In addition, Picard et al. (2015) discussed underlying mechanisms, biomarker discovery, biomarker evaluation, and validation. Gagaoua, Terlouw, Boudjellal, & Picard (2015) determined correlation networks among these protein biomarkers and reported that PRdx6 were correlated with Hsp20 ($r=0.53$, $P<0.01$) and μ-Calpain ($r=0.49$, $P<0.01$). These authors went on to explain that antioxidant proteins and heat shock proteins appear to play key roles in the development of

86 tenderness. Franco et al. (2015) evaluated the effects of pre-slaughter stress on proteome changes
87 in bovine *longissimus thoracis*. Ten proteins were differentially expressed in dark firm and dry
88 (DFD) beef in comparison to normal beef. Seven of these proteins were structural-contractile and
89 3 proteins were metabolic in nature. These authors reported that highly phosphorylated fast
90 skeletal myosin light chain 2 isoforms were more abundant in control beef in comparison to DFD
91 meat and these were the most sensitive biomarkers that were detected for the incidence of DFD
92 meat.

93 2.3 Color

94 Muscle-specificity in beef color stability was investigated by Joseph et al. (2012) using
95 proteomic tools. These authors compared the sarcoplasmic protein profile of color-stable
96 *longissimus lumborum* (LL) and color-labile *psoas major* (PM), and correlated the differentially
97 abundant proteins with color traits. Metabolic enzymes (β-enolase and triose phosphate
98 isomerase), antioxidant proteins (thioredoxin, peroxiredoxin-2, dihydropteridine reductase,
99 aldose reductase, and peptide methionine sulfoxide reductase) and chaperones (heat shock
100 protein-27 kDa, heat shock protein-1 B-70 kDa, and stress-induced phosphoprotein-1) were more
101 abundant in color-stable LL compared with color-labile PM. Moreover, proteins having a
102 positive correlation with redness (aldose reductase, creatine kinase, and β-enolase; r = 0.64 -
103 0.72) and color stability (peroxiredoxin-2, peptide methionine sulfoxide reductase, and heat
104 shock protein-27 kDa; r = 0.87 - 0.92) were overabundant in LL, whereas the protein having
105 negative correlation with redness was overabundant in PM (mitochondrial aconitase 2, r = -
106 0.59). These findings indicated that the greater abundance of antioxidant and chaperone proteins
107 in LL compared to PM was responsible for the greater color stability of LL. Previous research
108 has indicated that antioxidants can improve meat color stability by limiting lipid oxidation and

myoglobin oxidation (Renerre, Dumont, and Gatellier, 1996). Further, Wu et al. (2016) examined the sarcoplasmic proteome profile of LL and PM in Chinese Luxi yellow cattle, and reported differential abundance of several glycolytic and antioxidant proteins between the muscles.

Proteomic approaches have been utilized to examine the molecular basis of intramuscular variation in beef color stability (Nair et al., 2016). For example, beef semimembranosus is a large muscle in beef hindquarters exhibiting intramuscular differences in color stability. Steaks from the outside region (OSM) of the *semimembranosus* is color-stable during retail display, whereas steaks from the inside region (ISM) of the *semimembranosus* is color-labile. Due to the location in the carcass, OSM and ISM demonstrate variation in postmortem temperature decline and pH drop during carcass chilling which in turn could be contributing to the color stability variations (Sammel, Hunt, Kropf, Hachmeister, & Johnson, 2002). Proteome analysis indicated that the ISM steaks had greater abundance of glycolytic enzymes (fructose-bisphosphate aldolase A, phosphoglycerate mutase 2 and β-enolase) than their OSM counterparts. The increased levels of glycolytic enzymes indicated the possibility of a rapid pH decline in postmortem ISM, which in combination with its high temperature during carcass chilling could have an adverse effect on myoglobin redox stability (Suman & Joseph, 2013; Faustman, Sun, Mancini, & Suman, 2010; Mancini & Hunt, 2005; Suman, Nair, Joseph, & Hunt, 2016), thereby compromising the meat color stability.

Another factor that can cause variation in beef color stability of beef is the animal effect (King et al., 2011). The biochemical basis of this animal-to-animal variation in color stability was examined using proteomic tools (Canto et al., 2015). These authors utilized beef LL from ten color-stable and ten color-labile carcasses for sarcoplasmic proteome analysis. Glycolytic

enzymes (phosphoglucomutase-1, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase M2) were more abundant in color-stable steaks and had a positive correlation with redness and color stability. These glycolytic enzymes could have a positive impact on NADH regeneration, which in turn helps to stabilize meat color.

The effect of breed (Romagnola × Podolian, Podolian, and Friesian) and aging time (1, 7, 14, and 21 days) on color and sarcoplasmic proteome profile of beef *longissimus* muscles was also examined using proteomic tools (Marino et al., 2014). Aging increased lightness (L^* values), whereas redness (a^* values) varied with breed. The response to aging was also breed-specific, with steaks from the Podolian and Romagnola × Podolian bulls having a greater redness with aging, whereas redness of from Friesian young bulls were not affected by aging. Mass spectrometric analysis indicated that the abundance of several proteins (β-enolase, creatine kinase M-type, fructose-bisphosphate aldolase B, glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, glutathione S-transferase P and protein DJ-1) decreased during aging, whereas those of others (phosphoglycerate kinase 1, β-enolase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase B, creatine kinase M-type, adenylate kinase isoenzyme 1, peroxiredoxin-6, peroxiredoxin-2, superoxide dismutase, histidine triad nucleotide-binding protein) was influenced by breed type. The authors suggested that the proteome analysis could provide the basis for the development of protein markers for meat quality. A recent proteomic study (Clerens et al., 2016) of four muscles (LL, PM, *semitendinosus*, and *infraspinatus*) from New Zealand-raised Angus steers identified twenty-four protein spots with intensity differences, whereas peptidomic analysis identified forty-four peptides, indicating clear distinction between the proteome profile of muscles.

3. Application of proteomics to pork quality

In pigs, proteomics has been applied to evaluate postmortem protein degradation/ modification, meat quality (PSE, WHC, pH), and meat color. Lametsch, Roepstorff, & Bendixen (2002) used proteomics (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) to identify protein degradation in *Longissimus dorsi* pig muscle. Nine different proteins including three structural proteins (actin, myosin heavy chain, and troponin T) and six metabolic proteins (glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyltransferase) increased in concentration during postmortem storage with the exception of 43 kDa fragment of troponin T, which decreased in concentration over storage time. Lametsch et al. (2003) used proteomics to explain the postmortem changes in porcine muscle and its relationship to meat tenderness by measuring Warner-Bratzler shear force. Results from the study indicated that actin fragments (2, 6, 7), myosin heavy chain, myosin light chain II (MLC II), and triose phosphate isomerase I fragment were overabundant after 72 h postmortem in comparison to 24 h postmortem and were correlated to shear force values (r = 0.49 - 0.59 for TPI, and -0.64 to -0.44 for others). Laville et al. (2009) evaluated three genotypes of F2 intercross pigs (NN, Nn, nn) with respect to Warner-Bratzler shear force and protein expression. The results indicated that nn muscles (homozygous mutation) had faster pH decline than NN (homozygous normal) or Nn (heterozygous) muscles, which may be partially explained by lower abundance of small heat shock proteins and myofibrillar proteins.

The proteomic approach has also been utilized to characterize the color of fresh pork. Earlier investigations by Laville et al. (2005) characterized the pale, soft, and exudative (PSE) zones in deep regions of ham (*semimembranosus* muscle) and reported that there was greater proteolysis in the PSE zone. Moreover, the authors reported that the variations in protein in PSE

zones resembled those induced by the acceleration of post-mortem glycogenolysis. Futher research by Hwang, Park, Kim, Cho, & Lee (2005) used proteomic methods to assess postmortem proteolysis in pork *longissimus* muscle to determine the relationship between *L** value and protein proteolysis. Results indicated that eighteen proteins were negatively correlated (r = -0.73 to -0.43) with *L** value. These proteins included actin α and α1, a-b crystalline, α1 actin precursor, myosin light chain 1, troponin T slow type isoform sTnT1 and sTnT2, and cofilin 2. All of these proteins were under expressed after 7 days postmortem. In addition, Sayd et al. (2006) characterized the sarcoplasmic proteome of pale and dark pork meat from the *semimembranosus* muscle using 2-dimensional electrophoresis and tandem mass spectrometry, and reported that twenty-two proteins were differentially expressed between the light and dark groups. Mitochondrial and respiratory enzymes (ATP-ase, succinate dehydrogenase, aldehyde dehydrogenase, glucose regulated protein-58kDa, and NADH dehydrogenase) were overabundant in normal (dark) muscle, indicating that metabolism in the dark muscle was predominantly oxidative. In contrast, pale (light) muscle exhibited an overabundance of glycolytic enzymes such as enolase-1, enolase-3, glycerol 3-phosphate dehydrogenase, creatine kinase, and glutathione transferase. These results indicated that the over-abundance of sarcoplasmic proteins in dark muscle are related to the inhibition of myoglobin denaturation and post-mortem pH decline.

4. Application of proteomics to poultry meat quality

Poultry proteomics has been applied to elucidate the role of diet on growth and meat quality (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012). Corzo, Kidd, Dozier, Shack, & Burgess (2006) reported the effect of dietary amino acid scarcity on the muscle proteome. In addition, researchers in the European Union (Doherty et al., 2004) have used

proteomic tools to explain changes in muscle proteomes during the growth of laying hens. Furthermore, Molette, Remignon, & Babile (2005) studied the proteomic basis of BUT9 turkeys and reported differences in fast and normal glycolyzing breast muscles and their relationship to meat quality, and suggested that the differences in proteomes were indicative of differences in meat quality. This study highlighted the potential of proteomic methods to elucidate the biochemical basis for color, water-holding capacity, and texture of broiler meat. Researchers used 2DE and isoelectric focusing (IEF) to determine that there were differences in three protein spots between normal and fast glycolyzing birds, and the proteins were identified as myosin heavy chain, actin fragments, and glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

Mekchay, Teltathum, Nakasathien, & Pongpaichan (2010) studied the proteomic analysis in Thai native and commercial broiler chicken muscles to determine the relationship between the protein composition and tenderness. Results indicated that glycolytic enzymes such as pyruvate kinase, phosphoglycerate mutase, and triosephosphate isomerase are related to meat quality.

In another study, a proteomic approach was used for the identification of chicken meat that was mechanically recovered and hand deboned. Results suggested that the amount of hemoglobin can be used as a marker to differentiate mechanically recovered chicken meat from deboned chicken meat (Surowiec, Koistinen, Fraser, & Bramley, 2011).

Proteomic characterization of the sarcoplasmic proteins in the *pectoralis major* and *supracoracoideus* breast muscles was conducted for two different chicken genotypes, which included Ross 708 commercial broilers and Leghorn chicks, Hyline W-36. Results suggested that glycogen phosphorylase, enolase, elongation factor 1, creatine kinase, fructose-bisphosphate aldolase, and glyceraldehyde 3-phosphate-dehydrogenase were different in the two strains during breast muscle growth (Zapata, Reddish, Miller, Lilburn, & Wick, 2012). Phongpa-Ngan et al.

(2011) studied the proteomic analysis and differential expression in proteins that were extracted from chicken with varying growth rates (slow vs. fast growing) and water-holding capacities (low vs. high). Proteins identified from chickens using two dimensional electrophoresis and mass spectrometry included metabolic enzymes (creatine kinase, pyruvate kinase, triosephosphate isomerase, ubiquitin), housekeeping proteins (heat shock protein), and contractile proteins (myosin heavy chain, actin). Serum albumin precursor, creatine kinase M type, and protein DJ-1 were overabundant in breast meat from slow growing broilers, and ubiquitin was less abundant in slow growing broilers in comparison to fast growing broilers. Heat shock protein, pyruvate kinase muscle isozyme, and two triosephosphate isomerase spots had greater abundance in high WHC and one triosephosphate isomerase spot had greater abundance in the low WHC group.

Current research includes characterizing biochemical pathways in live muscle, pre-rigor meat and post-rigor meat to explain the biochemical pathways and mechanisms associated with the development of PSE and woody breast through the evaluation of myofibrillar and sarcoplasmic proteomes in PSE and woody broiler breast meat; biochemical and proteomic differences are being evaluated for genetic strains that produce woody breast meat to characterize the genetics and biochemical pathways that are consistent with the production of woody breast meat. The proteome basis and biochemical pathways of woody breast meat and PSE meat are being elucidated to aid engineering strategies to prevent the incidence of PSE and woody breast meat and improve poultry meat quality, ultimately enhancing the competitiveness of the US poultry industry.

4.1 Woody breast meat

Since 2013, the broiler industry has been inundated with a breast meat defect that is characterized by hardened areas and pale ridge-like bulges at both the caudal and cranial regions

247 of the breast. “Woody breast” was classified as slight, moderate, and severe in the United States
248 (Owens, 2016; Tijare et al., 2016). Sihvo, Immonen, & Puolanne (2014) characterized this defect
249 as “wooden breast” in Europe. Woody breast samples have reduced yields due to reduced water
250 holding capacity and are pale, less red and more yellow than normal breast meat (Mudalal,
251 Lorenzi, Soglia, Cavani, & Petracci, 2015). In addition, woody breast meat was characterized by
252 a greater weight and a larger cross-sectional area (Dalle Zotte et al., 2014). Wooden breast
253 muscle typically had increased traits such as degenerative and atrophic fibers, variability in fiber
254 size, floccular/vacuolar degeneration and lysis of fibers, mononuclear cell infiltration, lipidosis,
255 and interstitial inflammation (de Brot et al., 2016; Soglia et al., 2016; Velleman & Clark, 2015).
256 Differences between the whole muscle proteomes of woody and normal breast meat samples
257 were evaluated from 3 poultry processing plants that harvest Ross 708 and Cobb 700 broilers.
258 From each processor, twelve samples characterized as moderately woody (Owens, 2016) and 12
259 normal breast pieces were sampled. Image analyses of the whole muscle proteome gels revealed
260 eight abundant protein spots between normal and woody breast (*pectoralis major*) meat. Myosin
261 regulatory light chain 2 and 14-3-3 protein gamma were overabundant ($P < 0.05$) in woody
262 breast meat. Myosin regulatory light chain 2 concentration was correlated with increased L*
263 value, increased cook loss, and less tender meat. 14-4-4 protein gamma contributes to the
264 regulation of myosin light chain kinase, muscle contraction, and rigor mortis, and is also
265 associated with less tender meat (Fu, Subramanian, & Masters, 2000; Lametsch et al., 2006).
266 Increased abundance of serum albumin indicates increased oxidative stress in muscle cells from
267 woody breast meat. Protein deglycase-DJ-1 was overabundant in woody breast meat.
268 Overabundance of this protein indicates both an increased need for deglycation of amino acids

and oxidative stress (Sayd et al., 2006; Wallimann et al., 1998). In humans, a deficiency in protein deglycase-DJ-1 is associated with Parkinson's disease (Sekito et al., 2006).

Phosphoglycerate mutase 1 was six times as abundant in woody breast meat in comparison to normal breast meat. This enzyme converts 3-phosphoglycerate into 2-phosphoglycerate within the glycolysis pathway. This may be an important indicator of glycolytic differences between normal and woody breast meat since it does not seem to be a protein that is needed in abundance in comparison to other enzymes involved in the glycolytic pathway. Carbonic anhydrase III was the second most abundant protein in woody breast meat at 2.5 times that of normal meat. Carbonic anhydrase III is an indicator of increased glycolysis and increased oxidative stress in skeletal muscle (Vasilaki et al., 2007). Creatine kinase M-type and triosephosphate isomerase were more abundant in normal breast meat. Creatine kinase phosphyorylates creatine during times of rest and provides a rapid-short term supply of ATP during intense muscle activity and the rigor process (Wallimann et al., 1998). Creatine kinase is also overabundant in color-stable beef (Nair et al., 2016), which indicates that it has relatively higher concentrations in beef and chicken that have normal color and quality.

Triosephosphate isomerase is an important enzyme in glycolysis since it converts dihydroxyacetone acetone phosphate to glyceraldehyde-3 phosphate, which increases the rate of glycolytic metabolism and promotes NADH and ATP production (Kim & Dang, 2005).

Since the proteomic differences between normal meat and woody breast meat have been demonstrated, further research is being conducted to elucidate the biochemical pathways and genetic expression differences that exist when woody breast meat is produced. Proteomic analyses will be conducted for different broiler breeds that do and do not produce woody breast meat and proteomic analysis will be evaluated in the live animal, right after slaughter, and after

rigor mortis to evaluate the impact on genetics and biochemical mechanisms on the production of woody breast meat. In addition, meat quality analysis and sensory characterization of the resulting meat will be evaluated and multivariate analyses will be utilized to relate meat quality to protein expression at various times in the meat production process.

4.2 Pale, soft, and exudative (PSE) poultry meat

In a previous study by Desai et al. (2016), results indicated that 15 different proteins were differentially expressed between PSE and normal breast meat, as presented in Table 1. In this study, male Hubbard × Cobb 500 broilers (n = 1,050) were raised on ad-libitum feed and water for 8 weeks at the Mississippi State University poultry farm and then harvested at the Mississippi State University Poultry Processing plant. Normal breast meat samples were characterized by a 24 h postmortem pH (pH_{24}) of 5.8 to 6.2 and a CIE L^* at 24 h postmortem (CIE L^*_{24}) of 45 to 55, and pale, soft, and exudative (PSE) breast meat samples were characterized by a pH_{24} of 5.4 to 5.7 and a CIE L^*_{24} of 55 to 65. Myofibrillar and sarcoplasmic proteomes were isolated and profiled using 2DE. Phosphoglycerate mutase-1, alpha-enolase, ATP-dependent 6-phosphofructokinase, and fructose 1, 6-bisphosphatase were overabundant in normal meat (Table 1). The other eleven protein spots were over-abundant in PSE meat samples and were identified as actin alpha, myosin heavy chain (in 2 different spots), phosphoglycerate kinase, creatine kinase M type (in 2 different spots), beta-enolase, carbonic anhydrase 2, proteasome subunit alpha, pyruvate kinase, and malate dehydrogenase (Table 1) (Desai et al., 2016).

4.2.1 Glycolytic enzymes

313 *Phosphoglycerate kinase (PGK) and beta-enolase.* Phosphoglycerate kinase (PGK) and beta-
314 enolase were over-abundant in PSE meat (Table 1). This is an indicator of increased glycolytic
315 activity and lower pH. In addition, PGK and beta-enolase were negatively correlated with pH_{24}
316 and were positively correlated with L^*_{24} and shear force (Table 1).

317 *Pyruvate kinase M type (PKM).* Pyruvate kinase M type (PKM) was over-abundant in PSE meat.
318 PKM is a key enzyme in the last step of the glycolytic cycle, in which phosphoenol pyruvate is
319 converted to pyruvate (Fontanesi et al., 2008). The overabundance of PKM was positively
320 correlated with L^*_{24} and negatively correlated with pH_{24} (Table 1).

321 *Phosphoglycerate mutase-1(PGM-1).* Phosphoglycerate mutase-1 (PGM-1), an enzyme that
322 converts 3-phosphoglycreate into 2-phosphoglycerate using 2, 3 biphosphoglycerate as a
323 cofactor in the glycolytic cycle, was over-abundant in normal meat (Qiu, Zhao, Xu, Yerle, &
324 Liu, 2008), which was negatively correlated with L^*_{24} and positively correlated with pH_{24} (Table
325 1).

326 *Alpha-enolase*. Alpha-enolase was over-abundant in normal meat. Di Luca, Elia, Hamill, &
327 Mullen (2013) examined the proteome profile of pork *longissimus* muscles with varying levels of
328 drip loss and reported the presence of alpha-enolase in drip. The proteome of drip loss was not
329 taken into consideration in this study. In the future, it will be interesting to determine if alpha-
330 enolase is greater in the drip loss from PSE breast meat when compared to normal meat.

331 *ATP dependent 6-phosphofructokinase (PFK).* In the glycolytic cycle, ATP dependent 6-
332 phosphofructokinase (PFK) is a rate limiting enzyme that converts fructose-6-phosphate to
333 fructose-1, 6-biphosphate (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Ohlendieck,

2010). PFK abundance was overabundant in normal meat, negatively correlated with L^*_{24} and b^*_{24}, and positively correlated with pH_{24} (Table 1).

Fructose 1, 6-bisphosphatase (FBP). Fructose 1, 6-bisphosphatase (FBP) and aldolase are catalysts in the hydrolysis of fructose 1, 6-bisphosphate to fructose 6-phosphate (Rakus, Pasek, Krotkiewski, & Dzugaj, 2004). Proteomic data indicates that glycolytic enzymes (phosphor-glycerate mutase-1, alpha-enolase, ATP-dependent 6-phosphofructokinase, and fructose 1, 6-bisphosphatase) are over-abundant in normal meat.

4.2.2 Myofibrillar Proteins

Actin alpha and myosin heavy chain were over-abundant in PSE broiler meat and positively correlated with shear force (Table 1). Similarly, Choi, Ryu, & Kim (2007) reported that fast-glycolyzing pigs yielded *porcine longissimus* that was overabundant in myosin heavy chain, and fast glycolyzing pigs are more likely to produce PSE meat than normal glycolyzing pigs. In addition, the myosin heavy chain (fast/slow) ratio was negatively correlated to early postmortem muscle pH ($r = -0.51$). Similarly, myosin heavy chain was over-abundant in PSE meat in our study and was negatively correlated with pH_{24} (Table 1). Lametsch et al. (2003) evaluated the relationship between tenderness and postmortem proteome changes in porcine muscles and reported that myosin and actin heavy chain spot intensities was negatively correlated ($r = -0.44$ to -0.55) to tenderness. In contrast, myosin light chain spot intensity was positively correlated ($r = 0.49$ to 0.59) with Warner-Bratzler shear force.

4.2.3 Malate dehydrogenase (MDH)

355 Malate dehydrogenase functions in the NAD/NADH coenzyme system to catalyze the
356 interconversion of oxaloacetate and malate and has an important role in the malate/aspartate
357 shuttle across the mitochondrial membrane (Minarik, Tomaskova, Kollarova, & Antalik, 2002).
358 Laville et al. (2007) reported that there were fourteen sarcoplasmic proteins that differed in
359 abundance between tough and tender *porcine longissimus* muscles and fourteen proteins were
360 differentially abundant between the two groups, and MDH was over-abundant in the tough
361 group. In addition, te Pas et al. (2013) identified MDH as a potential marker in pork *longissimus*
362 *dorsi* muscles with varying ultimate pH and drip loss. In our study, the over-abundance of MDH
363 in PSE meat was positively correlated with L^*_{24} and shear force and negatively correlated with
364 pH_{24} (Table 1).

365 *4.2.4 Creatine kinase M-type (CKM)*

366 Creatine kinase M-type was over-abundant in PSE meat. CKM converts creatine
367 phosphate into creatine and ATP (Phongpa-Ngan et al., 2011; van de Wiel & Zhang, 2007).
368 Laville et al. (2005) reported that 16 proteins differed in abundance between myofibrillar and
369 sarcoplasmic protein fractions of pig *semimembranosus* muscle from PSE and normal meat.
370 These authors also reported that myosin light chain, creatine kinase, and troponin T were
371 overabundant in PSE meat when compared with control meat. The over-abundance of CKM in
372 PSE meat in the current study was positively related to L^*_{24} and shear force and negatively
373 related to pH_{24} (Table 1).

374 *4.2.5 Carbonic anhydrase 2*

375 Carbonic anhydrase 2 was over-abundant ($P < 0.05$) in PSE meat. Carbonic anhydrase 2
376 catalyzes the reversible hydration of carbon dioxide (Hamelin et al., 2007). Damon et al. (2013)

examined the association between gene expression and meat quality in pork *longissimus* muscle whole muscle proteome extracts and reported that carbonic anhydrase gene expression was positively correlated with drip loss and L^* value and negatively correlated with ultimate pH. Similar results were observed in our study, in which carbonic anhydrase was positively correlated with L^*_{24} and shear force and negatively correlated with pH_{24} (Table 1).

4.2.6 Proteasome Subunit Alpha

Proteasome subunit alpha is involved in postmortem protein degradation (Sentandreu, Coulis, & Ouali, 2002). In the present study, the overabundance of proteasome subunit alpha in PSE meat was positively correlated with shear force values and negatively correlated with pH_{24} (Table 1). This indicates that the presence of proteasome subunit alpha could be a biomarker for tough meat.

Research demonstrated the overabundance of proteins that are involved in glycolytic, muscle contraction, proteolytic, ATP regeneration, energy metabolism and CO_2 hydration in PSE breast meat. This overabundance may be related to the meat quality differences between normal and PSE breast meat. Based on the protein markers identified in the present study, future studies will focus on identifying the biochemical pathways and genes responsible for differences in protein abundance and their relationship to poultry meat quality.

5. Quality defects in channel catfish

There are two known factors that contribute to the red-color defect in catfish fillets. These two items include pre-harvest stress and disease. The stress induced during capture, transport, and harvest of channel catfish can lead to red-color defect in its fillets. Bacterial contamination of fish with *Aeromonas* species can also occur when the fish are

399 immunosuppressed, which also contributes to the formation of red-catfish fillets. Proteomic tools
400 were employed to characterize the myofibrillar and sarcoplasmic proteomes in normal and
401 reddish catfish fillets and to examine the proteome basis of this red-color defect in channel
402 catfish (Desai et al., 2014). It was hypothesized that the red color defect may be similar to DFD
403 beef and pork since it has been associated with exposure to ling-term stress. Analysis of the
404 muscle proteome (sarcoplasmic and myofibrillar proteins) indicated that the only protein that
405 was differentially abundant between normal and reddish fillets was hemoglobin, which had a 22-
406 fold greater abundance in red-catfish fillets. These results lead to the conclusion that the red-
407 color defect in catfish fillets is primarily due to the over-abundance of hemoglobin. The
408 overabundance of hemoglobin could be due to rupturing of blood vessels due to stress and/or
409 contamination with a hemolytic pathogen. These results that the red color defect is not similar to
410 the production of DFD beef and/or pork.

411 In a previous study by Ciaramella, Nair, Suman, Allen, & Schilling (2016), the impact of
412 dissolved oxygen concentration, temperature, socking and transport stress were evaluated for
413 their effects on fillet quality. During a one week acclimation period, experimental temperatures
414 (25°C and 33°C, 1°C/day) and high (H) and low (L) dissolved oxygen (DO) levels (>5 mg/L and
415 ~2.5 mg/L, respectively altered at a rate of 10% saturation/day) were manipulated to achieve four
416 environmental treatments representing a control (25-H), oxygen stress (25-L), temperature stress
417 (33-H) and severe stress (33-L). Catfish were exposed to these conditions for 4 weeks and then
418 harvested. At this time, fillets were either slaughtered immediately, socked for 10 h and then
419 slaughtered, or socked and then transported for 4 h prior to slaughter.

420 A total of 147 spots were detected in fillets from fish reared under oxygen stress
421 combined with handling stress (25-L-ST), and 13 spots were differentially abundant from

422 controls (25-H). For the 33-H-ST treatment, 86 spots were detected with 9 differentially
423 abundant from controls (25-H). A total of 108 protein spots were isolated from the fillets of fish
424 subjected to severe environmental stress and handling (33-L-ST) with 6 differentially abundant
425 from controls. Thirty-five protein spots were differentially abundant among all treatments when
426 compared to the control fillets.

427 *5.1 Handling Stress*

428 Alpha actin sarcomeric/cardiac, F-actin capping protein subunit A, and Troponin T are
429 all structural proteins that were down regulated in comparison to fillets from the unstressed
430 control fish. The decreased incidence of structural proteins are indicative of increased
431 proteolytic activity and muscle degradation in the stressed fish, which has previously been
432 documented following crowding stress in fish (Silva et al., 2012; Veiseth-Kent, Grove,
433 Færgestad, & Fjæra, 2010). Annexin A4 was down-regulated in the stress handled fish in
434 comparison to the control and has been associated with the regulation of membrane proteins,
435 exocytosis, and ion channels (Piljić & Schultz, 2006; Yeh & Klesius, 2010).

436 The abundance of proteins involved in energy metabolism, including
437 phosphoglucomutase-1, L-lactate dehydrogenase A chain and creatine kinase were down
438 regulated in fillets due to handling stress. This is in contrast to the upregulation of metabolic
439 enzymes in fish fillets following acute stress (Morzel, Chambon, Lefevre, Paboeuf, & Laville,
440 2006; Silva et al., 2012; Veiseth-Kent et al., 2010). The decreased abundance of triose-phosphate
441 isomerase and glyceraldehyde-3-phosphate dehydrogenase may be due to long term adaptation
442 that redirected surplus glycerol to glucose instead of pyruvate (Alves et al., 2010). Decreased
443 abundance of pyruvate dehydrogenase and enolase following stress and the redirection of surplus
444 glycerol to glucose indicates a metabolic shift to a more ketogenic pathway (Alves et al., 2010).

445 Handling stress including socking and transport decreased the redness and color intensity
446 of catfish. Proteomic analysis revealed decreased expression of several proteins, including alpha
447 actin sarcomeric/cardiac, F-actin capping protein subunit A, and Troponin T (Ciaramella et al.,
448 2016). These results are similar to those reported in pork, in which structural proteins were less
449 abundant in PSE pork, which is lighter in color and associated with short term stress (Laville et
450 al., 2005; Laville et al., 2009).

451 *5.2 Effect of oxygen stress*

452 The pH value and hue angle increased, and redness, yellowness and chroma values
453 decreased when fish were reared under low oxygen conditions prior to socking and transport
454 stress. Exposure to low-dissolved oxygen led to a lesser abundance of structural proteins
455 including alpha-actinin-3 and tropomyosin alpha-1 chains. Alpha-actinins maintain the integrity
456 and maintenance of the sarcomere (Zhang et al., 2011) by linking titin and actin filaments
457 together (Atkinson et al., 2000). Tropomyosin alpha-1 chain is an essential component of
458 muscle contraction regulation (Galinska-Rakoczy et al., 2008). Hypoxic stress led to less
459 abundance of creatine kinase and L-lactate dehydrogenase, which are indicators that the fish
460 were conserving energy (Smith, Houlihan, Nilsson, & Brechin, 1996). In addition, down
461 regulation occurred in proteins involved in protein turnover and cellular lysis such as elongation
462 factor, proteasome subunit alpha, programmed cell death 6-interacting protein and fetuin B. The
463 reduced expression of these proteins is likely due to the down regulation of energy requiring
464 activity when oxygen is low.

465 Exposure of fish to oxygen stress caused increased expression of several proteins in the
466 fillets that are involved in metabolic processes, including glycerol-3-phosphate dehydrogenase,
467 AMP deaminase and glyceraldehyde-3-phosphate dehydrogenase. The up-regulation of these

glycolytic enzymes is consistent with the effect of stress that was reported by previous researchers (Morzel et al., 2006; Silva et al., 2012; Veiseth-Kent et al., 2010) and allows for increased anaerobic production of ATP in response to the hypoxic and handling stressors.

5.3 Effect of temperature stress

High temperature stress prior to socking and transport increased redness and fillet pH and decreased shear force and yellowness. The increase in redness indicates that temperatures of 33 ^{o}C or above likely contribute to the production of red fillets. The structural proteins actin alpha sarcomeric/cardiac, alpha actinin-3, and M-protein were down-regulated in fish fillets that were subjected to heat stress high temperature (33 °C) prior to handling when compared to 25-H. M-protein is necessary for the structure of striated muscle and as an anchor for titin. Phosphoglucomutase-1 and L-lactate dehydrogenase function in the glycolytic pathway (Nelson & Cox, 2008a) and adenylate kinase isoenzyme-1 regulates cellular ADP concentrations and the replenishment of ATP (Nelson & Cox, 2008b). These enzymes were down regulated, likely to conserve energy. AMP deaminase was up regulated in thermally stressed fish, which is indicative of stress induced energy expenditures (Silva et al., 2012; Veiseth-Kent et al., 2010).

Exposure to a combination of low dissolved oxygen and heat stress resulted in increased pH and hue angle and decreased redness, yellowness and chroma values. However, there was no difference in lightness and texture between fillets from the 25-H and the 33-L treatments. When low oxygen and high temperature rearing were combined, actin alpha sarcomeric/cardiac, alpha-actinin-3 and M-protein striated muscle were all down regulated, similar to what was observed when fish were exposed to high temperature or low oxygen alone. When fish were exposed to high temperature and low oxygen, fructose bisphosphate aldolase A, mitochondrial creatine kinase and a partial sequence of a proteasome subunit were down-regulated and annexin A6 was

491 upregulated. Increased annexin concentrations are linked to disease in channel catfish (Yeh &
492 Klesius, 2010) and thus could also be an indicator of hypoxia stress as they were upregulated in
493 all fish experiencing hypoxia in the present study.

494 Increased proteolytic activity as a result of heat and low dissolved oxygen stressors was
495 likely the cause of decreased fillet color intensity (redness, yellowness, and chroma). Fish that
496 were reared under low oxygen conditions yielded fillets with a pronounced increase in the
497 abundance of metabolic proteins. Contrary to what was expected, exposure to low oxygen and
498 high temperature produced the fewest changes in the muscle proteome after exposure to socking
499 and transportation stress. This suggests that exposure to long term environmental heat and low
500 oxygen stress prior to handling improves the fish's ability to respond to subsequent stress events
501 such as socking and transport. Inability to reproduce the red fillet condition under controlled
502 conditions indicates that the production of reddish fillets in ponds is likely a combination of heat
503 stress, low oxygen stress, and disease. Further research needs to be conducted to determine how
504 the combination of stress and the subsequent disease that may result from immunosuppression
505 contributes to the production of red catfish fillets.

506 **4. Conclusions**

507 Proteomic methods are useful in exploring potential biomarkers that can be linked to
508 meat quality defects. In order to work towards minimizing these defects, biomarkers need to be
509 linked to genetics, nutrition, and environment to explain the factors that lead to the quality
510 defect. This is true for beef discoloration and toughness, pale, soft, and exudative pork and
511 chicken and woody broiler breast meat. In addition, for these defects, instrumental meat quality,
512 and sensory characterization is also necessary to fully elucidate the cause and the effect on the
513 product. With respect to the red color defect in catfish, proteomic analysis links over abundance

514 in hemoglobin as the cause of the red color defect. However, since it has proven difficult to
515 reproduce the red condition in the laboratory, epidemiological and pathophysiologic studies need
516 to be conducted along with stress in order to determine the inherent causes of this quality defect.
517 Proteomic methods can be used to provide pertinent information with respect to meat quality.
518 Future research needs to be linked to other omic methodologies, quality determinations, and
519 sensory data in order to work towards eliminating quality defects from meat production.

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Table 1 Functional roles of differentially abundant sarcoplasmic and myofibrillar proteins in normal and PSE broiler breast (*pectoralis major)* meat identified by liquid chromatography-electrospray ionization-tandem Mass Spectrometry (LC-ESI-MS/MS) and their relationships to different meat attributes. This data is adapted from original research published by Desai et al., (2016).

Protein	Functional category	Over-abundant category	Correlation coefficient		
			pH_{24}	L^*_{24}	Shear force
Actin, alpha skeletal muscle	Muscle contraction	PSE	-0.65*	0.56	0.73*
Alpha-enolase	Glycolytic enzyme	Normal	0.73*	-0.65*	-0.66*
ATP-dependent 6-phosphofructokinase	Glycolytic enzyme	Normal	0.78*	-0.90*	-0.34
Beta-enolase	Glycolytic enzyme	PSE	-0.94*	0.93*	0.68*
Carbonic anhydrase 2	Hydration of CO_2	PSE	-0.83*	0.90*	0.70*
Creatine kinase M-type	ATP regeneration	PSE	-0.81*	0.80*	0.88*
Fructose 1,6-bisphosphatase (Fragment)	Glycolytic enzyme	Normal	0.67*	-0.55	-0.42
Malate dehydrogenase	Energy metabolism	PSE	-0.88*	0.81*	0.53*
Myosin heavy chain, skeletal muscle	Muscle contraction	PSE	-0.78*	0.67*	0.66*
Phosphoglycerate mutase 1	Glycolytic enzyme	Normal	0.61*	-0.57*	-0.41
Phosphoglycerate kinase	Glycolytic enzyme	PSE	-0.90*	0.87*	0.58
Proteasome subunit alpha type	Proteolytic enzyme	PSE	-0.66*	0.62	0.70*
Pyruvate kinase PKM	Glycolytic enzyme	PSE	-0.65*	0.66*	0.31

*$P < 0.05$