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MEAT QUALITY FOR FURTHER PROCESSING

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- Histology, composition, and quality traits of chicken Pectoralis major muscle affected by wooden breast abnormality. 2016. **Soglia F.**, Mudalal S., Babini E., Di Nunzio M., Mazzoni M., Sirri F., Cavani C. and Petracci M. *Poultry Science, 95: 651-659.*
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ABSTRACT

In the past few decades, the meat industry of the Western countries has experienced an overall increase in the demand of poultry meat. Therefore, genetic selection programs were carried out for several years in order to improve the production traits in broiler chickens and obtain high-growth rate and breast yield hybrids. However, the profound changes in muscle structure and its metabolic status led to a remarkable increased incidence of several muscular myopathies and abnormalities mainly affecting the *Pectoralis major* muscles. Among them, according to their incidence, the white-striping (WS), the wooden breast (WB) and the poor cohesion (PC) or spaghetti meat defects are of relevant importance.

Thus, eight studies were carried out during this PhD project aimed at deepening the current knowledge concerning histological features, quality traits and technological properties of broiler *Pectoralis major* muscles affected by emerging muscular abnormalities, clarifying their respective peculiarities and similarities as well as investigating the underlying mechanisms involved in their occurrence. In detail, four studies were performed in order to investigate the implications of WS and WB abnormalities, occurring alone or combined within the same *Pectoralis major* muscle, on i) quality traits ii) histological features and composition iii) technological properties and chemical composition of meat and iv) gene expression. In addition, considering the incomplete knowledge concerning the mechanisms responsible for harder consistency associated with the occurrence of WB, three studies were carried out in order to i) measure sarcomere length and investigate the effect of storage time on ii) muscle structure, textural properties and iii) evolution of the main proteolytic indicators in *Pectoralis major* muscles affected by WB abnormality. At last, since no information was available on PC defect (frequently associated with WS), a study was carried out in order to evaluate the implications of its occurrence on muscle histology and meat quality traits.

With regard to histological features, the findings of the present PhD project evidenced that, irrespective of the type of muscle abnormality, similar histological alterations, including profound degenerative myopathic changes and atrophic fibers, were found in association with occasional regenerative processes. In addition, variability in fibers cross-sectional area coupled with proliferation of loose connective tissue and fat deposition were observed within the endomysial and perimysial spaces of the *Pectoralis major* muscles affected by muscular abnormalities in which also interstitial edema and inflammatory cells infiltrates were identified. These profound changes in muscle architecture might contribute in explaining the altered textural properties of the WB. Indeed, the longer sarcomeres measured in WB were speculated to result from a lower tension originating from fibres detachment.

As for quality traits, in spite of their higher ultimate pH value that should result in improved water holding capacity of meat, the pectoral muscles affected by muscular abnormalities exhibited severely impaired technological properties with the alterations being more pronounced within the superficial layer of the cranial portion of the *Pectoralis major* muscles when more than one abnormality coexist. Accordingly, an increased proportion ($P < 0.05$) and mobility of the extra-myofibrillar water fraction was observed. With regard to chemical and mineral composition, an overall higher ($P < 0.05$) moisture, fat and collagen content to the detriment of protein and total heme pigments levels were found in abnormal muscles. Furthermore, increased ions contents and alterations in sodium and calcium homeostasis were found, whereas only negligible modifications in fatty acids profile and an overall reduced ($P < 0.001$) Δ5 and Δ6 desaturase activity were observed. In addition, if compared to normal, 204 differentially expressed genes related to muscle development, polysaccharide metabolic processes, glucose metabolism, proteoglycans synthesis, inflammation and calcium signalling pathways were found in WS/WB.

The results concerning the effect of a 7-days storage on textural properties, particle size distribution and evolution of the main proteolytic indicators evidenced that the superficial layer of WB exhibited higher ($P < 0.05$) 40 and 80% compression values measured at the earliest sampling time. In addition, the 80% compression values suggested a tendency of progressive softening process taking place within the superficial layer of the WB samples from 10 to 72 h *post-mortem* ($P < 0.001$). As for particle size distribution, the increased deposition of extracellular matrix and fibrosis in WB might contribute in explaining the different fragmentation patterns observed between the superficial and the deep layer in the WB samples, with the superficial part exhibiting a higher amount of larger particles and an increase in particles with larger size during storage, in comparison with normal. The results for Western Blot against desmin revealed that at 10 h *post-mortem*, the WB cases exhibited significantly larger ($P < 0.05$) amounts of desmin. Then, a sharp decrease of the intact desmin band coupled with a progressive accumulation of its 39-kDa degradation fragment were observed without any significant difference among the groups. Thus, the findings of the present study suggested that the hardened consistency of the WB cases is mainly structural and it is only partially counteracted by endogenous proteolytic processes taking place during the *post-mortem* period. Thus, according to the outcomes of the present research it is reasonable to hypothesise the existence of a complex network of biological changes that, acting simultaneously, are responsible for the phenotypic features and the consistent impairment of muscular metabolism of the abnormal muscles.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Muscle and meat

Meat is defined as the flesh of animals used as foods (Laurie and Ledward, 2006). After slaughtering, the muscle tissue is converted from an extensible and metabolically active system to an inextensible and inactive one with a speed and an extent that significantly affect both technological properties and quality traits of meat (Greaser and Guo, 2012). A diagram displaying the structural organization of a skeletal muscle is shown in **Figure 1.1**. Muscle is a highly organized tissue. The whole skeletal muscles are normally enclosed by a thick, collagenous and nearly inextensible connective tissue layer, the epimysium and connected to bone by tendons. Moving forward, a thinner layer of connective tissue, the perimysium, separates the muscle into bundles (also known as fasciculi), which are composed of multinucleated fibers encased in a thin layer of connective tissue referred as endomysium (Huff-Lonergan and Lonergan, 2005; Pearce *et al.*, 2011; Greaser and Guo, 2012).

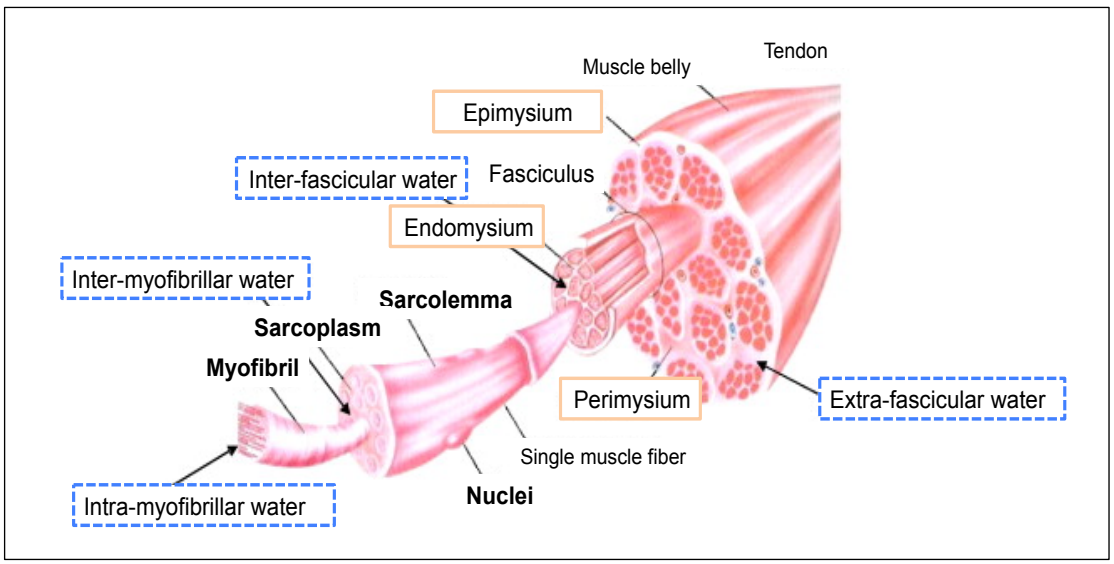


Figure 1.1 Structural organization of a skeletal muscle (adapted from Pearce *et al.*, 2011).

The latter, being adherent to the sarcolemma, consists of a basement membrane connected to a reticular layer surrounded by matrix-embedded fine collagen fibrils (Bailey and Light, 1989). Each muscle fiber consists of numerous single strands, the myofibrils, which in turn, are comprised of thick (predominantly based on myosin) and thin filaments (with actin, troponin and tropomyosin being their major components) (Greaser and Guo, 2012; Rawn, 1989).

Myofibrils are string-like structures composed of repeating units, the sarcomeres. A sarcomere is defined as an assembly of thick and thin filaments between adjacent Z-lines and composing the fundamental contractile unit of the muscle tissue (Davies, 2004). According to the literature, the mean sarcomere length measured in broiler breast fillets may vary between 1.61 and 1.80 μm in *pre-rigor* muscles (before chilling) (Papinaho *et al.,* 1996; Yu *et al.,* 2005; An *et al.,* 2010) and within the range between 1.8 and 2.0 μm in *post-rigor* muscles (after chilling) (Yu *et al.,* 2005; Wattanachant *et al.,* 2005).

As previously demonstrated by Swartz *et al*. (2009), the microscopic appearance of a muscle fiber is remarkably influenced by the organization of its intracellular components. Indeed, an inner myosin-containing A-band (about 1.6 μm in length) enclosed by a thin-filament-containing I-band (about 1.0 μm in length) were observed (Swartz *et al.,* 2009). Although not visible by electron microscopy, other two cytoskeletal filamentous proteins are present within the sarcomere: titin and nebulin. Extending from the middle of the sarcomere to the Z-line, titin is a giant elastic protein protecting the muscle tissue from over-stretch. On the other hand, being attached to the Z-line, nebulin contributes in regulating the length of the thin filaments (Greaser and Guo, 2012). As showed in **Figure 1.2**, a myomesins and titin containing M-line is included within the middle of each A-band and exerts a relevant role in composing and maintaining the sarcomeric structure.

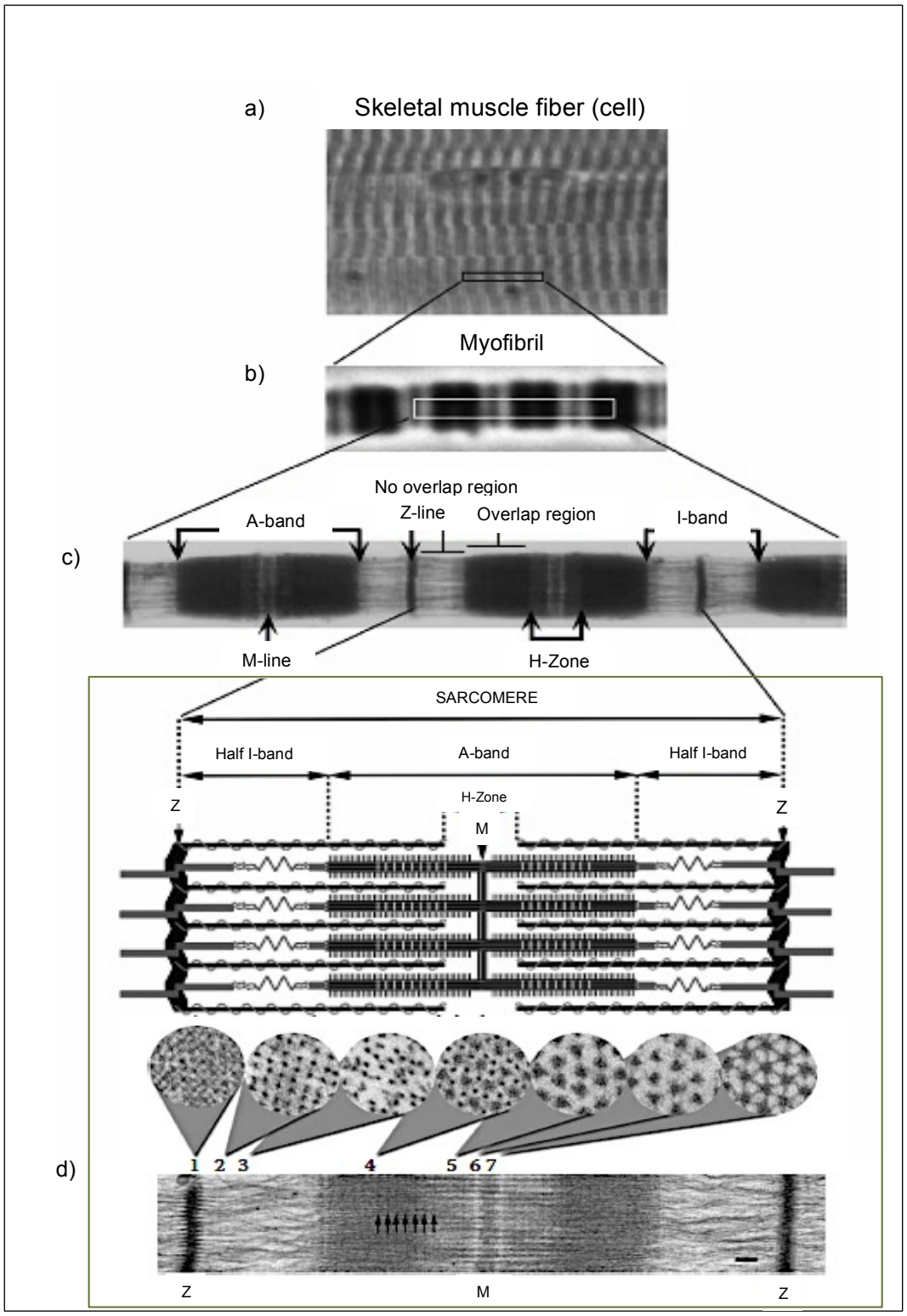


Figure 1.2 Longitudinal section of skeletal muscle displaying structural organization of muscle fibers (a,b). A schematic representation of a sarcomere, the contractile unit composing the muscle tissue, is reported in c) and whereas the sarcomeric organization of rabbit *Psoas* muscle is showed in d). The different regions highlighted in both cross-and longitudinal-sections (d) are ascribed to: 1. Z-lines; 2. I-band close to the Z-line; 3. I-band; 4. Overlap region of the A-band; 5. Non-overlap region of the A-band; 6. Bare H-zone of the A-band; 7. M-band; Bar = 100 nm. (adapted from Swartz *et al.,* 2009).

Each I-band comprises an inner dense Z-line and a flanking, low-density no-overlap region of the thin filaments resulting from the contiguous half-sarcomere. In addition, a lower density H-zone might be observed within myofibrils exhibiting long sarcomeres (Swartz *et al.,* 2009; Greaser and Guo, 2012). Other transverse intermediate filaments (such as desmin, talin, filamin and vinculin) can be observed in the I-band region, laterally linked the myofibrils and connected them to the sarcolemma. In addition, connecting the Z-line proteins of continuous myofibrils, desmin exerts a relevant role in maintaining sarcomere structure (Small *et al.,* 1992; Taylor *et al.,* 1995). Within this context, the mass of a given muscle depends on both number and size of fibers being its major component.

1.2 Fundamental basis of avian muscular tissue development

The growth of biological tissues is directly or indirectly regulated by various hormones controlling their function (Villee, 1960). In detail, muscle growth involves:

a) the synthesis of tissue-specific protein complexes;

b) their precise alignment into the fibers;

c) the subsequent differentiation and development of the fibers in order to accomplish both muscle type and function (Lawrie and Ledward, 2006).

1.2.1 Pre-hatch growth

Skeletal muscle development initiates from the germinal mesodermal layer of the developing embryo. Becoming more prominent, the mesodermal cells begin to organize into cuboidal clusters known as somities (Weaver, 2012). Muscle cells initially consist of a mass of fusiform-shaped, undifferentiated and closely spaced cells (Lawrie and Ledward, 2006). As the development proceeds, two types of cells could be observed: i) the former exhibiting the morphology of the primitive connective tissue and the latter ii) entering the myogenic pathway, proliferating and assuming a bipolar spindle-shaped ascribable to primitive muscle cells (myoblasts). The latter after differentiating and beginning to express specific proteins, fuse to form multinucleated myotubes and muscle fibers (Rehfeldt *et al.,* 2000; Lawrie and Ledward, 2006). Secondary myotubes are then formed as a consequence of

the organization, alignment and fusion of other myoblasts promoted by the primary ones (**Figure 1.3**).

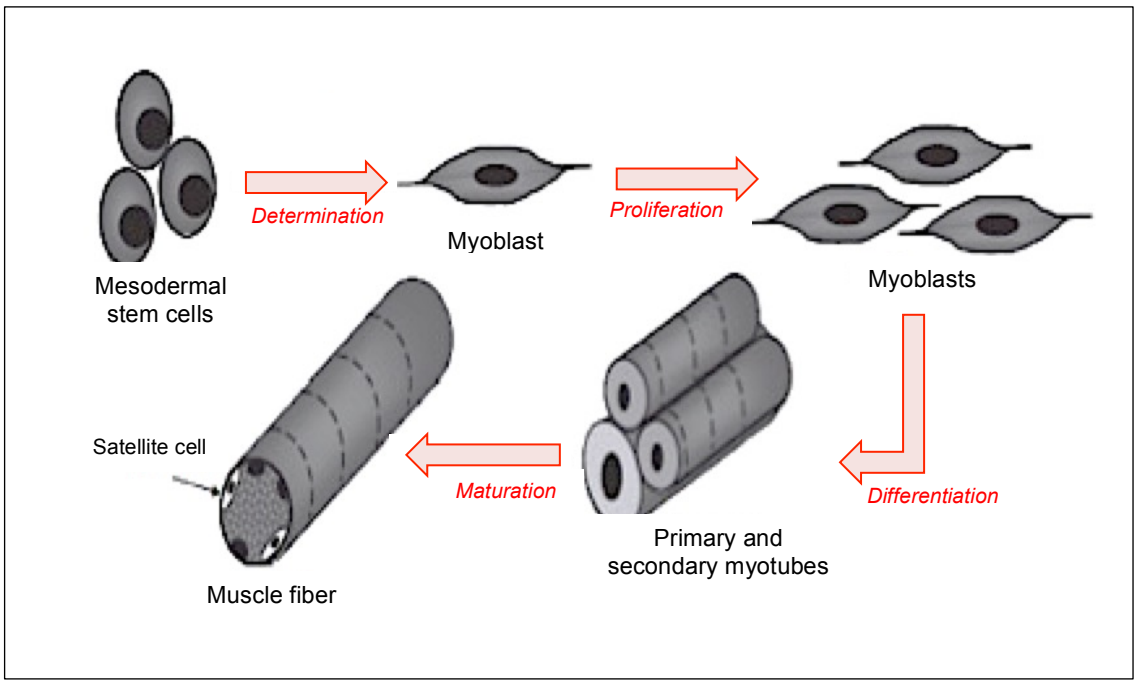


Figure 1.3 Diagram displaying the main events leading to myogenesis (adapted from Weaver, 2012).

During embryogenesis, another population of myoblasts, not involved in fibers formation and surrounding the myofibres, might be observed. These are termed as satellite cells and being able to further divide are considered as a source of new myonuclei during *post*-natal development (Moss and Leblond, 1971; Schultz, 1974). During the embryonic development, the number of myofibrils within each muscle fiber increases by longitudinal fission of the original fibril (Maurer, 1894). The process of radial growth of muscle fibers proceeds through a longitudinal splitting of the myofibrils and is commonly termed as myofibrillogenesis. According to Stromer *et al*. (1974) the two newly formed myofibrils, in turn, enlarge and after synthesizing additional contractile, regulatory and structural proteins become functional. On the other hand, the passive tension exerted by the growing bones stimulates the serial addition of sarcomeres, which are generally added at the distal end of the existing myofibres thus resulting in a longitudinal growth of muscle fiber (William and Goldspink, 1971).

1.2.2 *Post*-hatch development of muscle tissue

At *post*-hatch, the increase in skeletal muscle mass is primary based on the enlargement of muscle fibers, which have been developed during the embryonic period (hypertrophy). Contextually, a proliferation of satellite cells allows incorporating new myo-nuclei into the muscle fibers. Indeed, several authors demonstrate that, since the formation of muscle fibers takes place prenatally, muscle fiber number remains unchanged in both mammals and birds after birth (Rehfeldt *et al.,* 2000). This hypertrophic growth is a function of protein synthesis and degradation. Indeed, in order to generate a net accumulation of protein and a consequent increase in muscle mass, protein synthesis must exceed the degradation rate. On the other hand, muscle atrophy occurs if proteins degradation proceeds faster than synthesis (Weaver, 2012). Within this context, and interesting phenomenon was observed: at the end of the growth period, muscle fiber number inversely correlates with thickness. As a result, higher fiber numbers are associated with reduced *post*-natal growth rate of the individual muscle fibers. In addition, both fiber number and thickness positively correlate with muscular cross-sectional area (Rehfeldt *et al.,* 2000).

In a previous study performed by Aguilar *et al*. (1983), a sigmoid curve was adopted in order to describe the progressive development and growth of broiler chickens (**Figure 1.4**). This phenomenon typically exhibits an initial exponential development phase, an intermediate or transitory phase and a final development step in which a gradual reduction in growth rate following an asymptotic increase in body weight is observed. Hence, different selection criteria may exert a relevant role in determining the chronology of the events occurring during growth and thus resulting in distinctive growth curve per each broiler strains (Scheuermann *et al.,* 2003).

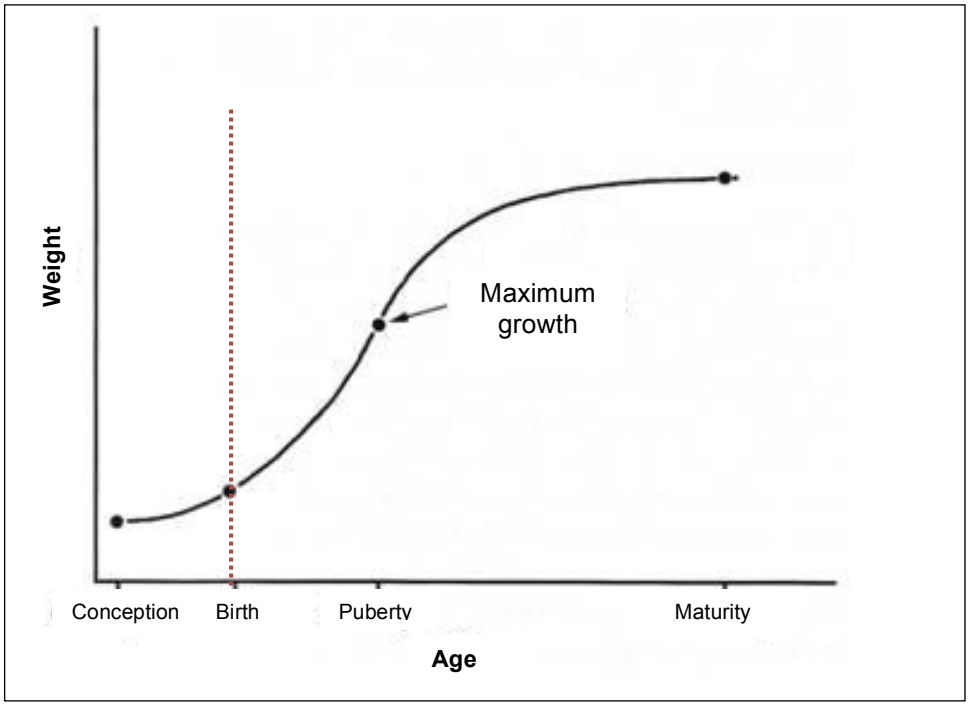


Figure 1.4 Progressive development of body weight of broiler chickens (adapted from Aberle *et al.,* 2012).

Although a reduction in skeletal development might be observed at the inflection point of the growth curve, muscle growth continues leading to a constant increase in meat yield (Moran, 1999). Within this step, since bone growth is not completed, muscle growth proceeds through enlargement and elongation of the myofibres (Ono *et al.,* 1993). According to the literature, after a rapid increase up to 15 days of age, breast yield exhibits a continuous even if less pronounced growth (Iwamoto *et al.,* 1993; Ono *et al.,* 1993). In addition, since they are not required to function immediately breast muscles development is not completed before hatching. Therefore, in order to compensate for the delayed development during the embryonic period, pectoral muscles exhibited a remarkable growth rate after hatching (Ono *et al.,* 1993).

1.3 Selection for growth rate and muscle hypertrophy in broilers

In the past few decades, the meat industry of the Western countries has experienced an overall increase in the demand of chicken meat. This phenomenon might be explained considering the nutritional profile (high-

protein and low-fat content, low-cholesterol and a balanced n-6 to n-3 polyunsaturated fatty acids ratio together with functional components) and the suitability for further processing, resulting from neutral flavour, consistent texture and light colour, that typically distinguish the poultry meat. In addition, both the cheap price and the absence of cultural and religious issues have exerted a relevant role in increasing the demand of chicken meat (Petracci *et al.,* 2013, 2015). Within this context, a remarkable improvement in both the efficiency and the production traits have been observed as a result of the progressive development of industrialization and the consequent specialisation of broiler meat production chain that took place starting from the end of the Second World War (**Table 1.1**) (Petracci *et al.,* 2017).

		Broiler production performances				
Year	Production *(.000 tons)*	*market age (d)*	*market live weight (kg)*	*average daily gain (g/d)*	*feed to live weight ratio (g/g)*	*mortality (%)*
1945	-	84	1.37	16.36	4	10
1955	-	70	1.39	19.89	3	7
1965	9,365	63	1.58	25.06	2.4	6
1975	16,326	56	1.71	30.46	2.1	5
1985	27,293	49	1.90	38.79	2	5
1995	46,352	47	2.12	45.07	1.95	5
2005	70,259	48	2.44	50.75	1.95	4
2015	96,338	48	2.83	58.97	1.89	4.8

Table 1.1 Trend in world broiler meat production (FAO, 2016) and development of broiler performances in the US (adapted from Petracci *et al.,* 2016).

Indeed, in order to fulfil the worldwide increasing demand of poultry meat, a genetic improvement of animals was required. Therefore, a genetic selection mainly focused on improving the production traits of broiler chickens was carried out for several years. As a result, the genetic selection for high-growth rate and breast yield hybrids led to an overall increased production profitability achieved through a 50% reduction (to 5 weeks) of the number of

rearing days necessary to obtain market body weight broilers (Dransfield and Sosnicki, 1999; Havenstein *et al.,* 2003; Hafez and Hauck, 2005) (**Figure 1.5**). In addition, the findings of previous studies performed by Havenstein *et al*. (2003a,b) evidenced that Ross 308, representing the most popular commercial broiler hybrid, were five-times larger than the 1957 Randombred ones.

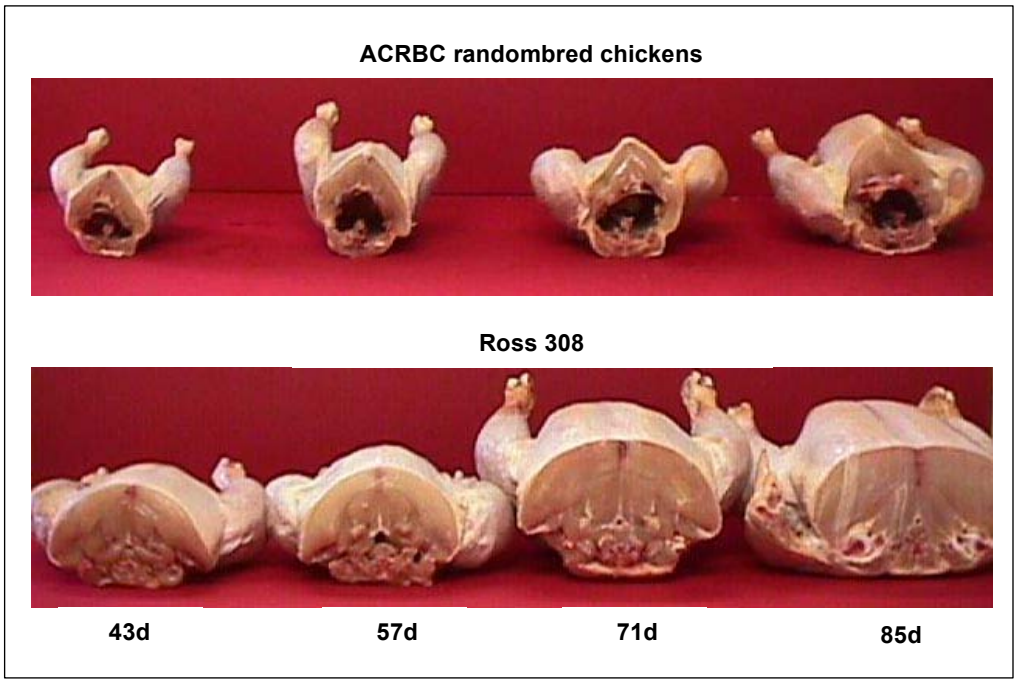


Figure 1.5 Carcass development of Ross 308 broiler hybrid (selected for improved breast yield and growth rate) in comparison with Randombred chickens (ACRBC) according to Havenstein *et al*. (2003a,b).

Within this context, as a consequence of the remarkable changes in broilers growth rate, feed conversion and carcass yield progressively increased over time (Havenstein *et al.,* 2003a). Selection practices performed breeding companies exerted a relevant role in improving production traits and efficiency for broiler chickens (Sherwood, 1977; Havenstein *et al.,* 1994). Indeed, not only metabolism but also poultry development rates were significantly changed (Buzala and Janicki, 2016).

In detail, being the most valuable portion composing the broiler chicken carcass, the quantitative selection practices carried out within the past 50 years has mainly focused on pectoral muscles (Scheuermann *et al.,* 2003).

1.3.1 Effects on muscle characteristics and meat quality traits

Currently, the commercial broiler strains are the result of successful selection programs carried out in order to obtain fast-growing hybrids exhibiting improved production traits such as slaughter weight, feed conversion efficiency and breast yield (*See Table 1.1*). Within this context, the increased body weight achieved in modern broiler lines has been achieved through an overall increase in muscle weight and yield, which, in turn, might be related to an enlargement of muscle fibers diameter (**Figure 1.6**).

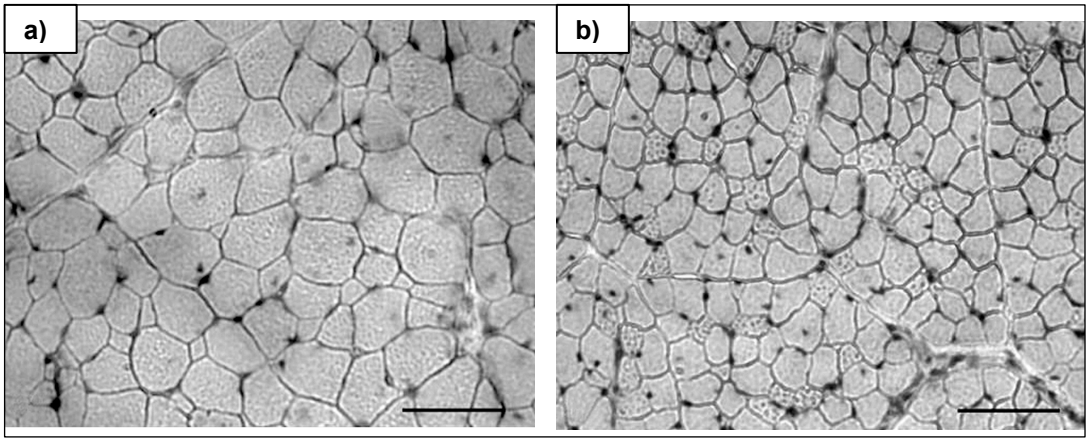


Figure 1.6 Cross-sectional slides of *Pectoralis major* muscles from broilers (a) and Leghorn-type chickens (b). Bars = 50 µm (adapted from Scheuermann *et al*. 2004).

Conversely to what previously presumed, Rehfeldt *et al*. (2000) demonstrated that, although muscle fibers formation occurs prenatally, their number is not merely genetically determined but might also be influenced by those environmental factors, which can affect prenatal myogenesis (Rehfeldt *et al.,* 2000). Several studies demonstrated that selection for increased growth rate and breast yield broilers led to a relevant increase in muscle fiber diameter through hypertrophy of the existing fibers without excluding the possibility of an increase in muscle fiber length (Rémignon *et al.,* 1995; Burke and Henry, 1997; Scheuermann *et al.,* 2004; Berri *et al.,* 2001). Accordingly, myofibers number was found to significantly correlate with breast yield thus demonstrating that an increase in myofibres number might be related to an improved breast yield (Scheuermann *et al.,* 2004).

The remarkable increase in muscle fibers cross-sectional area (CSA) observed within the pectoral muscles of high-growth rate and breast yield

broilers led to several changes affecting both the muscle tissue itself and the quality traits of the resulting meat. A negative genetic correlation was found between glycogen content (*in vivo*) and breast muscle development (Berri *et al.,* 2005). Indeed, an overall decrease in both rate and extent of *post-mortem* pH decline was observed in high growth rate and breast yield hybrids (Dransfield and Sosnicki, 1999; Berri *et al.,* 2001; Duclos *et al.,* 2007). This phenomenon led to a delayed acidification and grater ultimate pH values (Berri *et al.,* 2001, 2007). According to previous observations, muscular glycogen content was found to be primarily determined by the overall muscle growth rather than to the diameter of its constituting fibers (Henckel, 1996; Berri *et al.,* 2007). Indeed, as a consequence of the exceptionally shortened growth period achieved for broiler chickens, pectoral muscles might be more prone to develop hypoxic conditions (Henckel, 1996). This resulting in a shift of the energetic metabolism towards glycolytic pathways (even in case of light movements) might lead to glycogen depletion and affect the *peri-mortem* processes determining meat quality (Henckel, 1996; Hoving-Bolink *et al.,* 2000). Besides, the overall reduction in capillary density and capillary-to-fibers ratio, resulting in impaired oxygen supply and metabolic waste product displacement, might exacerbate this condition (Dransfield and Sosnicki, 1999; Hoving-Bolink *et al.,* 2000). On the other hand, in a recent study performed by Berri *et al*. (2005) on chicken breast muscles, a negative correlation was found between glycolytic potential and carcass leanness, thus demonstrating that selection for increased growth rate and carcass leanness would result in higher ultimate pH values.

As for meat quality traits, selection for growth and muscle development was associated with a significant decrease in chicken breast meat colour intensity (Le Bihan-Duval *et al.,* 1999, 2001; Berri *et al.,* 2001, 2007) in agreement with previous observations performed on turkeys (Santé *et al.,* 1991) and ducks (Baéza *et al.,* 1997). These differences could be partly attributed to a decreased heme pigment content within the *Pectoralis major* muscles of selected birds (Le Bihan-Duval *et al.,* 1999, 2001; Berri *et al.,* 2001*,* 2007). Indeed, redness positively correlates with the amount of myoglobin (and thus heme-iron) (Boulianne and King, 1995) that is remarkably lower within the type IIB-white-glycolytic fibers composing the

pectoral muscles rather than within the oxidative-red-ones (Lieber, 1992). On the other hand, contrasting results were obtained in previous studies investigating the effect of selection for increased growth rate and muscle size on meat tenderness. Indeed, although according to Verdiglione and Cassandro (2013) fiber size seemed not to exert any relevant effect on meat tenderness, several studies evidenced a positive correlation between fiber size and meat toughness. Thus, as the muscle fibers cross-section increases, a significant decrease in meat tenderness was observed (Lonergan *et al.,* 2003; Chen *et al.,* 2007; Branciari *et al.,* 2009; Zhao *et al.,* 2011). On the contrary, Duclos *et al*. (2007) and Berri *et al*. (2007) observed higher tenderness in breast muscles exhibiting larger fibers cross-sectional areas. In addition, muscle fibers cross-sectional area was found to significantly affect water-holding/-binding capacity of meat. In detail, the increased fibers diameter resulting from selection for growth performances and body weight indirectly led to reduced drip, thaw and cooking losses. Hence, the *Pectoralis major* muscles exhibiting fibers with larger diameters would be potentially better adapted for further processing than the muscles displaying small fibers cross-section (Duclos *et al.,* 2007).

Phenotypic and genetic correlation coefficients evidenced that an increase in plasma creatine kinase activity (considered as an indicator of stress associated to musclular tissue dysfunctions) was associated with enlargement of muscle fibers diameter (Remignon *et al.,* 1996; Berri *et al.,* 2007; Sandercock *et al.,* 2006, 2009). Such an increase was also suggested to reflect the protein turnover closely related to faster muscle growth rate (Berri *et al.,* 2007). Additionally, as a consequence of adaptive responses to increased tissue metabolic demand, intracellular cation homeostasis was remarkably altered in broiler chickens. Indeed, if compared to their unselected counterpart, the increased sodium concentration found within the muscle tissue of fast-growing broilers might contribute to alter muscular cells functions (Sandercock *et al.,* 2009). Furthermore, as a consequence of being involved in injuries and disease states, raised sodium content within the skeletal muscle tissue may initiate the degenerative processes leading to reduction of meat quality (Sandercock and Mitchell, 2004).

1.4 Meat abnormalities

The selection for increased growth performances and breast-yield carried out over the past 30 years largely affected both muscle structure and its metabolic status leading to an overall increased occurrence of several muscular abnormalities mainly affecting the pectoral muscles. Indeed, as a consequence of being predominantly composed by type IIB, white-glycolytic fibers displaying an anaerobic metabolism, breast muscles are more prone to develop metabolic related defects or myopathies affecting the nutritional value, sensorial and technological quality of resulting breast meat.

1.4.1. Deep pectoral myopathy

The deep pectoral myopathy (DPM), also known as Oregon disease or green muscle disease, being firstly observed in 1968 and subsequently studied at the Oregon State University, was described as a “degenerative myopathy” affecting the deep pectoral muscle. In detail, DPM was described as an ischemic necrosis affecting the *Supracoracoideus* or *Pectoralis minor* muscle (**Figure 1.7a**) as a consequence of its anatomical compartmentalisation (Jordan and Pattinson, 1998). Indeed, an increased internal pressure within the pectoral muscle (in response to muscle exercise) results in the occlusion of cranial and pectoral arteries responsible for blood supply within the muscle tissue inducing necrosis (Petracci and Cavani, 2012). Although this condition was firstly described in spent turkeys and chickens, it was also observed in fast-growing birds about 30 years ago (Siller, 1985).

Nowadays, although there are no reported cases in slow- and medium-growing chickens, the occurrence of this myopathy still represents a relevant quality issue for the poultry plants processing fast-growing broilers.

1.4.2 PSE-like condition

It is widely recognized that both rate and extent of *post-mortem* pH decline as well as the *post-mortem* metabolism contribute to the variation in fresh meat quality and its processing suitability. Within this context, meat exhibiting low ultimate pH values (<5.7), referred to PSE (Pale, Soft and Exudative)-like meat (**Figure 1.7b**), typically displayed impaired water-

holding/binding capacity (Barbut *et al.,* 2009). Several studies have been performed in order to investigate the underlying mechanisms involved in the occurrence of PSE-like syndrome in broilers. PSE-like meat were found to originate from protein denaturation and a consequent reduction in protein functionality resulting from a fast muscle acidification occurring in early *post-mortem* muscles (30 minutes after the death of animal) when the temperature of the muscle tissue is still high.

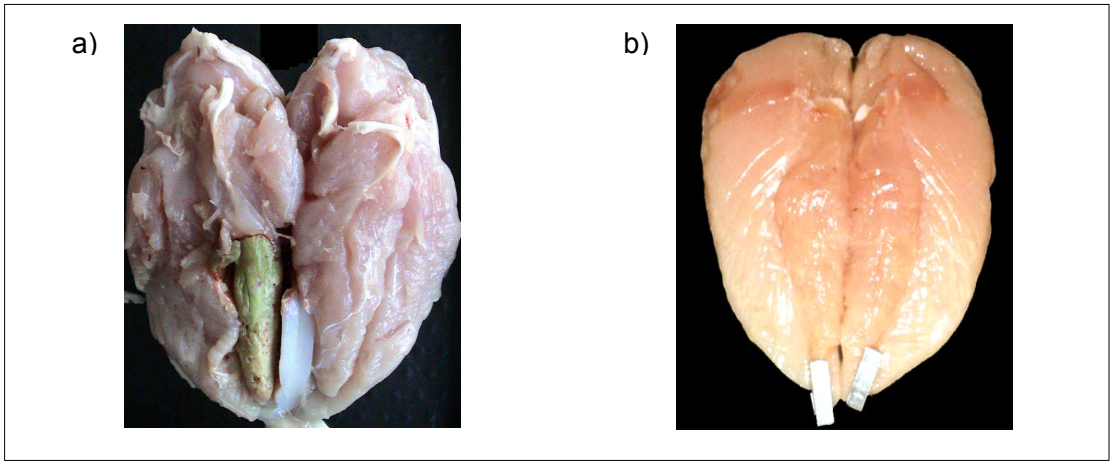
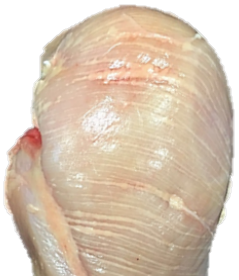


Figure 1.7 Images representing pectoral muscles affected by DPM (a) and PSE-like syndrome (b) (adapted from Petracci and Cavani, 2012).

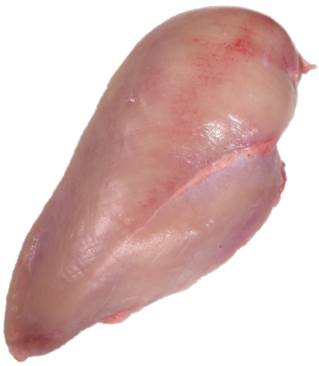
1.4.3 Growth-related muscle abnormalities

In the recent years, a new group of muscular abnormalities mainly affecting high-breast yield and fast-growing broilers have appeared. They include: i) the white striping defect exhibiting white striations parallel to the muscle fibres on the ventral surface of breast fillets (Kuttappan *et al.,* 2009); ii) the wooden breast condition (often associated with the white striping defect) in which the affected muscle are visually hard, out bulging and exhibit pale colour (Sihvo *et al.,* 2014), and iii) the poor cohesion or "spaghetti meat" abnormality described as the tendency toward separation of the muscle fiber bundles composing the *Pectoralis major* muscle (Sirri *et al.,* 2016) (**Figure 1.8**).



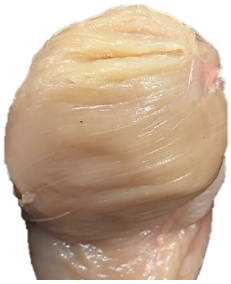
White Striping

White striations of variable thickness parallel to the muscle fibres direction on the ventral surface of breast fillets (Kuttappan *et al*., 2009)



Wooden Breast

Macroscopically pale, out-bulging and hardened *Pectoralis major* muscles (Sihvo *et al*., 2014).



Poor Cohesion or "Spaghetti Meat"

Impaired integrity and tendency towards separation of the muscle fiber bundles within the cranial part of the *Pectoralis major* muscle (Sirri *et al*., 2016).

Figure 1.8 Emerging muscle abnormalities (adapted from Petracci *et al*., 2017).

The aetiological causes leading to the occurrence of muscular abnormalities are currently unknown.

1.4.3.1 Histological features and possible causative mechanisms

According to previous studies, irrespective of the type of abnormality, the histological features of the skeletal muscles affected by muscular myopathies were found to overlap. Indeed, similar histological alterations including profound degenerative myopathic changes (focal and diffuse necrosis) resulting in atrophic fibers which typically loose their cross-striations were found in association with occasional regenerative processes,

as proved by the presence of thin fibers exhibiting faint cross-striations and nuclear rowing. In addition, variability in fibers cross-sectional area, proliferation of loose connective tissue and fat deposition (fibrosis and lipidosis) were observed within the endomysial and perimysial spaces in which also interstitial edema and inflammatory cells infiltrates were identified (Kuttappan *et al.,* 2013a; Sihvo *et al.,* 2014, 2017; Trocino *et al.,* 2015; Mazzoni *et al*., 2015; Soglia *et al.,* 2016a; de Brot *et al.,* 2016). Similar lesions were previously found to affect other muscles composing legs and backs of the carcass (Zimerman *et al.,* 2012; Kuttappan *et al.,* 2013b).

Recent studies demonstrated that giant, hyaline (hyper-contracted) and necrotic fibers might be observed also within the *Pectoralis major* muscles showing no macroscopic lesions ascribable neither to the white striping nor to the wooden breast defects (Mazzoni *et al.,* 2015; Soglia *et al.,* 2016a). The presence of necrotic fibers has previously been reported in fast-growing broilers (Soike and Bergmann, 1998; MacRae *et al.,* 2006) and turkeys (Sosnicki *et al.*, 1991) since 90s. Similarly, although typically observed within the *Pectoralis major* muscles affected by muscle myopathies, giant fibers are commonly found in the histological cross-sections of fast-growing hybrids (Sihvo *et al.*, 2014, 2017; Mazzoni *et al.*, 2015). Indeed, previous studies demonstrated that selection for increased growth rate and breast yield led to a relevant increase in muscle fiber diameter by inducing hypertrophy and hyperplasia of the existing fibers (*See Par. 1.3.1*) (Rémignon *et al.*, 1995; Burke and Henry, 1997; Scheuermann *et al.*, 2004; Berri *et al.*, 2007) which, in turn, is frequently associated to a higher incidence of giant fibers (Dransfield and Sosnicki, 1999; Le Bihan-Duval, 2003; Miraglia *et al.*, 2006). Thus, the presence of myofibres of different diameter and giant fibers alone should not be considered as a criterion to distinguish between the unaffected cases and muscles affected by muscular abnormalities. Mild-to-severe structural abnormalities were found within the *Pectoralis major* muscles of fast-growing broiler hybrids (Mazzoni *et al.*, 2015). Thus, not only the fillets noticeably affected by muscle abnormalities but also the macroscopically unaffected cases exhibited structural modifications such as mild myodegeneration in association with the presence

of abnormal fibers (Soike and Bergmann, 1998; Mahon, 1999; Mazzoni *et al.*, 2015; Sihvo *et al.*, 2017).

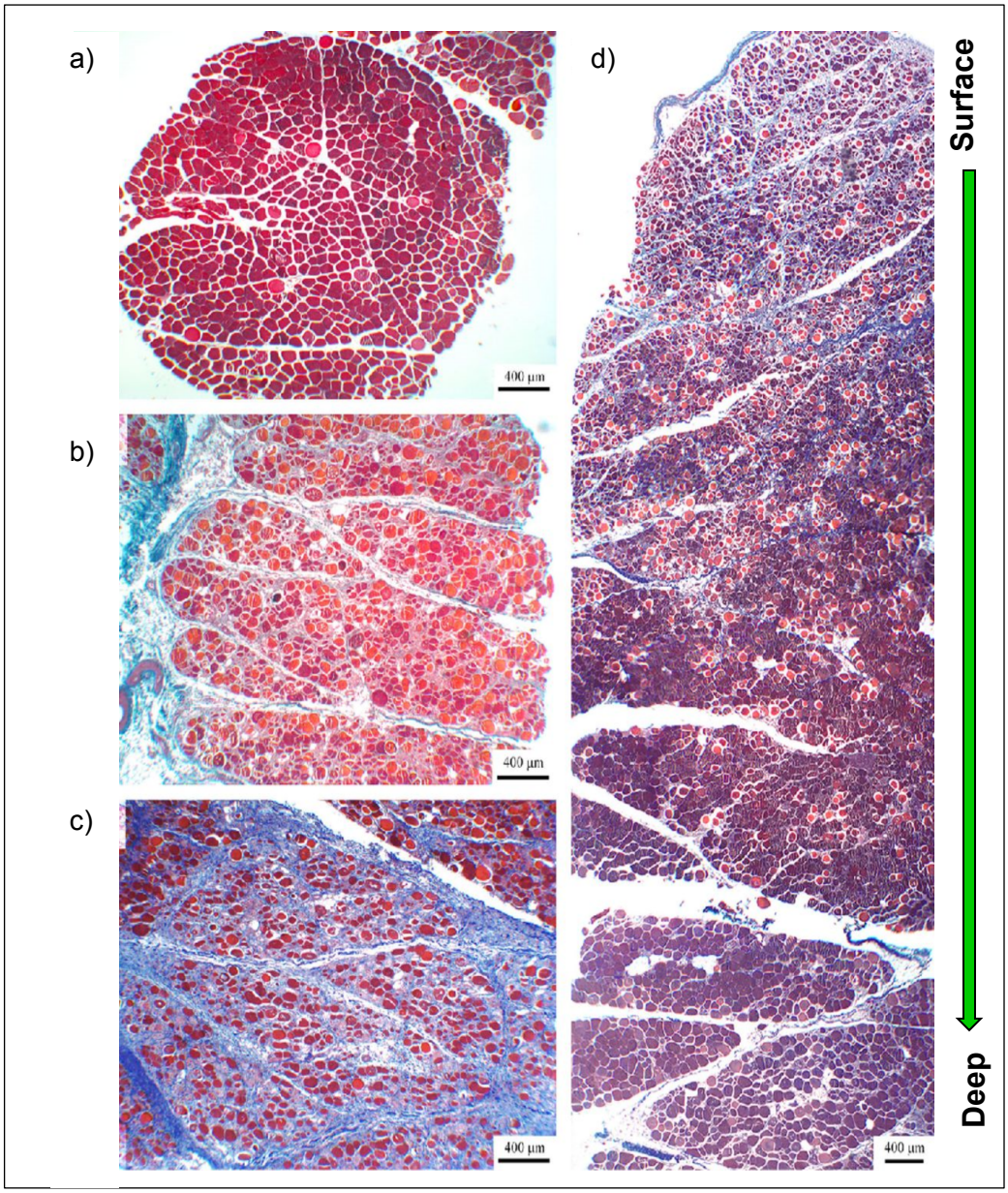


Figure 1.9 Cross-sections displaying samples mildly (a), moderately (b) and severely (c) affected by muscle abnormalities (Masson's Trichrome). Muscle fibers exhibiting polygonal shape, well package and similar size can be observed within the mildly affected cases (a). Fibers with different diameter and thickening of the perimysial connective tissue (b). An overall reduction in muscle fibers number can be observed in severely affected cases. In addition, muscle fibers devoicing from their polygonal shape (rounded), exhibiting variable size and separated or replaced by a loose or more organized connective tissue. A gradual progression of the histopathological lesions moving from the superficial towards the deep portion of the *Pectoralis major* muscle (adapted from Soglia *et al*., 2016a).

It is thus clear that a complex aetiology is associated with the occurrence of these recent muscular myopathies and abnormalities affecting the fast-growing genotypes.

Although several histological investigations have been performed to describe the main microscopic processes taking place within the muscles affected by abnormalities, the histological traits are not specific enough to distinguish the different muscle myopathies from each other. In previous studies performed by Kuttappan *et al*. (2013a) and Ferreira *et al*. (2013), profound degenerative myopathic lesions leading to myofibres degeneration and occasional regeneration, floccular/vacuolar degeneration, mild mineralization and inflammation of the interstitial spaces with edema as well as lymphocytes and macrophages infiltrations were associated with the occurrence of white-striping abnormality. In addition, the white-striped muscles simultaneously exhibited polyphasic changes involving muscle fibers fragmentation and phagocytosis, even if proliferation of connective tissue was not evident in broilers slaughtered at earlier ages. The hematologic and serologic profiles assessed on white-striped meat suggested that the occurrence of this muscular abnormality could not be associated neither to infectious or inflammatory mechanisms nor to stress condition. However, the increased levels of creatine kinase in association with serologic enzymes profile observed in white-striped meat are similar to those observed for other degenerative conditions such as stress and muscular dystrophies resulting in muscle damage (Kuttappan *et al.*, 2013b).

Several studies investigated the histological traits of the wooden breast cases (Sihvo *et al.*, 2014, 2017; Mazzoni *et al.*, 2015; Soglia *et al.*, 2016a). It could not be possible to identify specific histological patterns for distinguishing between the white-striping and the wooden breast cases. Indeed, polyphasic myodegeneration, rounded fibers and nuclear internalization often associated with lymphocytic infiltrations and occasional regenerative processes were observed within the wooden *Pectoralis major* muscles. In addition, proliferation and diffuse thickening of the endomysial and perimysial connective tissue associated with granulation tissue and increased deposition of loose connective tissue (fibrosis) and fat deposition

were found to affect the wooden breast fillets (Sihvo *et al.*, 2014; Mazzoni *et al.*, 2015; Soglia *et al.*, 2016). In addition, in agreement with the Kuttappan *et al*. (2013a), Sihvo *et al*. (2014) observed vasculitis and irregular perivascular infiltrations of lymphocytes (sometimes disrupting the vascular wall) affecting also the endomysial and perymisial connective tissue of the wooden breast cases (Mazzoni *et al.*, 2015). Since the occurrence of the white-striping and the wooden breast abnormality is frequently combined within the same *Pectoralis major* muscle, Soglia *et al*. (2016a) investigated whether the simultaneous occurrence of white-striping and wooden breast defects influeces the histological traits of skeletal muscle. A complete reorganization of the skeletal muscle structure involving the replacement of damaged and necrotic muscle fibers (accompanied by interstitial inflammatory infiltrates) with proliferation of connective tissue was found. In addition, as a result of the degenerative and contextual regenerative processes taking place, muscle fibers exhibited rounded profile, variable cross-sectional area and myofibres number (Soglia *et al.*, 2016a). However, both morphological characteristics and histopathological lesions observed within the affected cases were not uniform through the *Pectoralis major* muscle and gradually decrease moving from the external surface towards the inner section (about 1-cm-deep) of the *Pectoralis major* muscles leading to remarkable modification of the muscular architecture (Soglia *et al.*, 2016a; Clark and Velleman, 2016).

Within this context, since these muscle myopathies and abnormalities led to similar histological features, a common underlying mechanism responsible for their occurrence might be hypothesised. Thus, different studies have been performed in order to identify the underlying mechanisms at the basis of these alterations. According to the schematic diagram reported in **Figure 1.10**, it can be assumed that in case of white-striping abnormality, the increased growth rate (especially of breast muscles) achieved through the selection of fast-growing hybrids might have result in altered muscle metabolism, overstretching and/or ischemia of the muscular tissue leading to muscle damage and inducing reparative responses. Additionally, the increased free radical production and accumulation of intracellular calcium may alter the integrity of muscle fibre membrane and promote protein degradation through activation of proteases and lipases.

This process might lead to degenerative and necrotic processes that overtake the regenerative capacity of muscle thus leading to lipidosis and fibrosis (Kuttappan *et al.*, 2009).

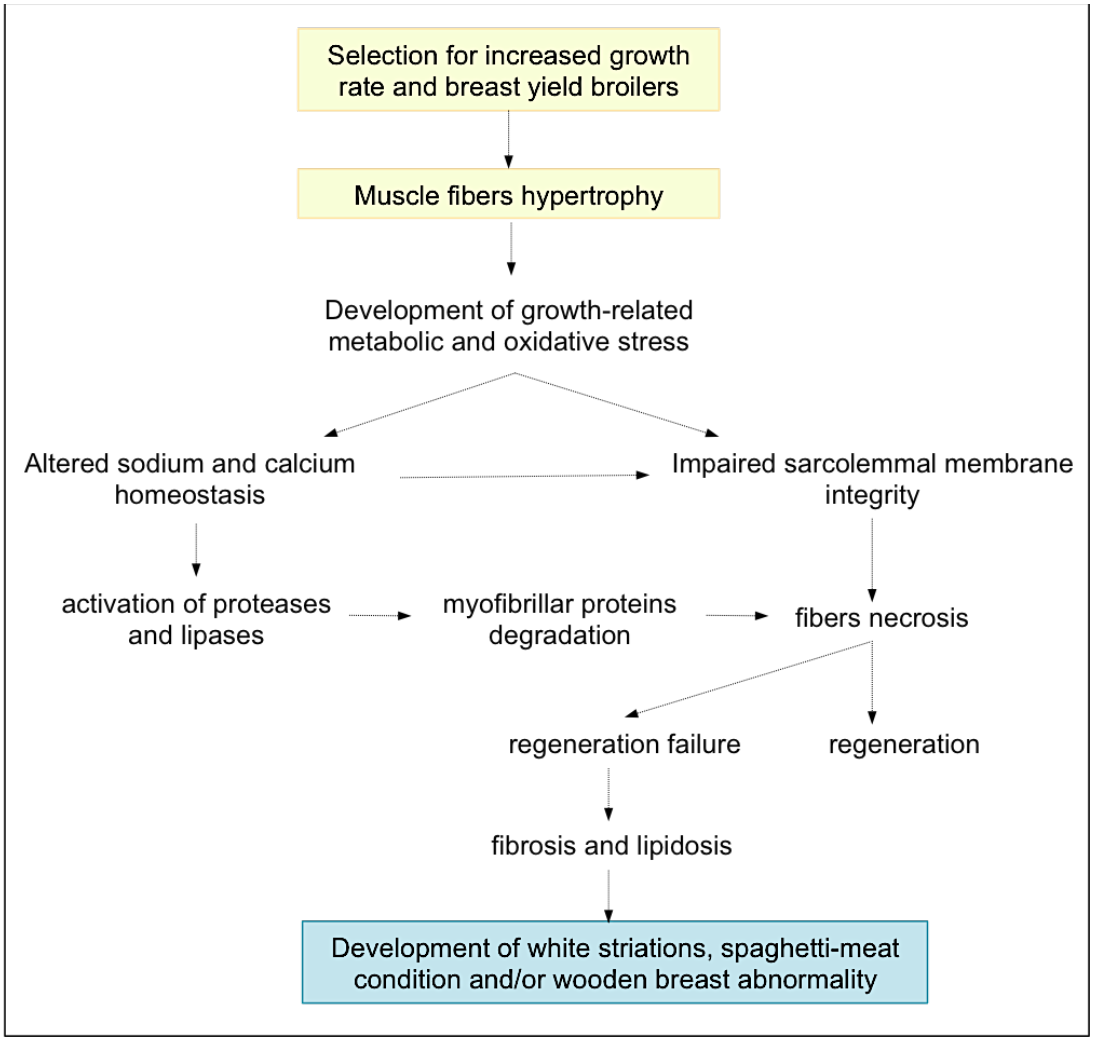


Figure 1.10 Schematic diagram displaying the mechanism hypothesised to underlie the occurrence of muscle abnormalities (adapted from Petracci *et al.*, 2017).

A similar underlying mechanism was hypothesised to be the basis for the wooden breast abnormality. In detail, a higher amount of glycolytic fibers with increased cross-sectional area and reduced capillary-to-fiber ratio was observed within the *Pectoralis major* muscles of fast-growing broilers (Hoving-Bolink *et al.*, 2000). As a result, both muscular oxygenation (hypoxic condition) and metabolic waste products displacement might be impaired leading to an excessive accumulation of reactive oxygen species responsible

for oxidative stress and involved in initiating inflammatory processes within the muscle tissue (Mutryn *et al.*, 2015; Zambonelli *et al.*, 2016). Then, complex biological reactions and regenerative processes aimed at contrasting muscle inflammation and limiting necrosis and apoptosis take place. Once more, altered calcium homeostasis and glucose metabolism originate when the degenerative processes resulting from inflammation overtake the regenerative capacity of the muscle tissue. Although to date it is reasonably difficult to define which features of the disease appear primarily and secondarily, a differential expression of several genes is associated with the occurrence of muscular abnormalities. In detail, several genes not only exerting a relevant role in inflammatory processes, extracellular-matrix synthesis (with particular reference to proteoglycans) and muscle development but also involved in polysaccharides metabolic pathways, glucose metabolism and calcium signalling pathway were up- or down-regulated within the abnormal Pectoralis major muscles (Mutryn *et al.*, 2015; Zambonelli *et al.*, 2016). Intriguingly, the increased expression of hypoxia-related genes (Mutryn *et al.*, 2015; Zambonelli *et al.*, 2016) in association with the presence of tubular structures resulting from neovascularization accompanying the myodegenerative processes in affected muscles corroborate the central role exerted by hypoxia in promoting muscle myopathies and abnormalities.

1.4.3.2 Implications on meat quality features

As expected, the occurrence of muscle myopathies and abnormalities severely affected quality traits and technological properties of meat (Kuttappan *et al.*, 2012, Petracci *et al.*, 2014; Mudalal *et al.*, 2015; Soglia *et al.*, 2016a,b; Zambonelli *et al.*, 2016) with the alterations being more pronounced when more than one abnormality coexists within the same muscle (Soglia *et al.*, 2016a,b; Zambonelli *et al.*, 2016). The main implications of muscular abnormalities on meat quality features are summarised in **Table 1.2b**.

Within this context, while only a minimal effect is exerted by the PSE-like condition (Qiao *et al.*, 2002), the occurrence of muscle myopathies and abnormalities significantly altered the proximate composition of meat thus

affecting its nutritional value. Indeed, if compared to their unaffected counterpart, abnormal muscles exhibited an overall higher amount of moisture, fat and collagen to the detriment of protein content (Kuttappan *et al.*, 2012; Soglia *et al.*, 2016a,b; Zambonelli *et al.*, 2016). Besides, not only a 3-fold increase in energy deriving from fat but also elevated collagen-to-total protein ratio led to a significantly lowered nutritional value of severe white-striped meat (as a consequence of the low digestibility of collagen and of some amino acids deficiencies) (Petracci *et al.*, 2014; Mudalal *et al.*, 2014). Overall, these differences are likely ascribed to the progressive myodegeneration and regenerative processes, resulting in fibrosis and lipidosis, typically observed within the abnormal muscles. Indeed, while an increased fat (lipidosis) and connective tissue (fibrosis) deposition might respectively account of the higher fat and collagen content, the remarkably elevated moisture level might be attributed to the moderate-to-severe edema resulting from the inflammatory processes (Petracci *et al.*, 2014; Sihvo *et al.*, 2014; Soglia *et al.*, 2016a). Hence, both myodegeneration and the presence of histological lesions may have lead to the extremely reduced protein content observed within the abnormal muscles (Petracci *et al.*, 2014; Soglia *et al.*, 2016a). With regard to minerals content, consistent with the mechanism leading to Duchenne muscular dystrophy in mammal, increased ion levels and alterations in sodium and calcium homeostasis were observed and associated with the development of muscle damage thus promoting the occurrence of muscular abnormalities (Sandercock and Mitchell, 2004; Wallace and McNally, 2009; Soglia *et al.*, 2016a). With regard to fatty acids profile, the occurrence of muscle abnormalities led to significantly higher amount of linoleic acid (Soglia *et al.*, 2016b) previously found in *mdx* mice and positively correlated with sarcolemmal damage and oxidative stress as well (Tuazon and Henderson, 2012). However, although as a whole the total poly- and mono-unsaturated fatty acids contents were found to be similar in pectoral muscles from normal and dystrophic chickens, the last exhibited an overall reduction in Δ5 and Δ6 desaturase activity leading to lower amount of EPA, DPA and other intermediate products from elongation and desaturation (Jordan *et al.*, 1964; Soglia *et al.*, 2016b). Interestingly, in spite of their significantly higher fat content, a lower amount of SFA was associated with

the occurrence of muscle abnormalities (Kuttappan *et al.*, 2012; Soglia *et al.,* 2016b). Considering these findings and the relatively low amount of heme pigments observed within the abnormal *Pectoralis major* muscles, the potential pro-oxidant activity of heme-iron released from the globin molecule of a damaged porphirin ring and the contextual exposure of phospholipids resulting from the structural changes associated to the severe myopathic lesions was hypothesised to affect oxidative stability (lipid oxidation and protein carbonylation level) of meat (Soglia *et al.*, 2016a). As a consequence of the physical and chemical changes (including protein aggregation, polymerization and intra- and inter-molecular cross-linking) induced by oxidation (Davies *et al*., 1987; Fagan *et al*., 1999; Lund *et al*., 2007; Kim *et al*., 2010) an overall decrease in protein solubility and functionality was observed within the abnormal muscles (Mudalal *et al.*, 2014; Bowker and Zhuang, 2016).

With regard to meat quality traits, altered colour and ultimate pH values were observed within the *Pectoralis major* muscles affected by abnormalities. Indeed, as a direct consequence of the strong fibrotic response and the lower amount of heme pigments, increased yellowness and pale colour might be observed in abnormal muscles. If compared to their unaffected counterpart, the affected cases revealed a remarkably higher ultimate pH values which, although associated with a lower glycogen content (Mutryn *et al.*, 2015), were hypothesised to arise from a change in glucose utilization rather that in its availability (Zambonelli *et al.*, 2016; Abasht *et al.*, 2016). Indeed, although several factors suggest the occurrence of hypoxic conditions, there was not an expected increased conversion of pyruvate into lactate (Zambonelli *et al.*, 2016). Even if high ultimate pH values might significantly increase water holding and processing attitudes of meat, since microbial growth strongly depends on pH, they may compromise the microbiological stability of meat (Barbut *et al.*, 2008). Within this context, it seems reasonable to hypothesise that microbial shelf-life of meat affected by muscle abnormalities could be remarkably reduced as a consequence of their higher ultimate pH values. Moreover, in spite of the higher ultimate pH that should result in improved water holding capacity of meat, the pectoral muscles affected by muscular abnormalities exhibited severely impaired

technological properties (marinate uptake, cooking loss and yield), as showed in **Table 1.2c** (Mudalal *et al.*, 2014; Petracci *et al.*, 2014; Mudalal *et al.*, 2015; Tijare *et al.*, 2016; Soglia *et al.*, 2016a,b; Tasoniero *et al.*, 2016). Indeed, reduced water holding and water binding capacities are associated with the occurrence of muscle abnormalities and likely linked to an overall reduction in protein functionality, with more pronounced effect being exerted by the wooden breast rather that the white striping defect (Mudalal *et al.*, 2014, 2015; Bowker and Zhuang, 2016). This phenomenon might be partly due to protein aggregation and cross-linking following oxidation (Soglia *et al.*, 2016b) and to the overall substantial reduction and altered profile of muscular contractile and sarcoplasmic proteins typically observed within the abnormal muscle tissues (Mudalal *et al.*, 2014; Soglia *et al.*, 2016a; Bowker and Zhuang, 2016). Dealing with that, the overall impairment of the water holding capacity of meat was corroborated by NMR relaxation properties examining both the relative intensity and the T_2 transverse relaxation time for the three proton populations (bound, intra- and extra-myofibrillar) identified within the muscle tissue. According to our study, the remarkable increased proportion and mobility of the extra-myofibrillar water fraction (the potential drip of the meat) may account for the lower water holding ability of the abnormal cases during processing and storage (Soglia *et al.*, 2016b).

The occurrence of muscle abnormalities not only alters the visual appearance of meat impairing consumer acceptance (Kuttappan *et al.*, 2012) but also significantly affects its textural properties. Overall, regardless of freshness, cooking and the degree of abnormality, the textural properties of meat, are severely affected by the occurrence of muscle myopathies and abnormalities (Petracci *et al.*, 2013; Mudalal *et al.*, 2015; Soglia *et al.*, 2016a; Chatterjee *et al.*, 2016). However, since extensive poor cohesion (fiber bundles separation) frequently affected the white-striped areas, textural differences were more pronounced with the occurrence of wooden breast rather than white striping abnormality (Petracci *et al.*, 2013; Mudalal *et al.*, 2015). If compared to their unaffected counterpart, abnormal muscles exhibited higher compression and MORSE forces as well as increased hardness and chewiness in case of raw and cooked meat, respectively (Petracci *et al.*, 2013; Mudalal *et al.*, 2015; Soglia *et al.*, 2016a; Chatterjee *et*

al., 2016). These changes in textural properties of meat might be explained by the profound alterations affecting the muscle fiber itself as well as the reduced water holding capacity of meat leading to muscle shrinkage and increased packing density of fibers following cooking (Wattanachant *et al.*, 2004; Huff-Lonergan and Lonergan, 2005). On the other hand, the thermally labile cross-links composing the newly deposed connective tissue might contribute to explain the absence of significant differences in the shearing properties of cooked unaffected and affected muscles (assessed by Allo-Kramer shear test) (Mudalal *et al.*, 2015).

The main histological features, quality traits and technological properties of *Pectoralis major* muscles affected by muscular abnormalities are summarized in **Table 1.2**.

EMERGING MUSCLE ABNORMALITIES	
a) HISTOLOGICAL FEATURES	
• profound degenerative myopathic changes • presence of abnormal fibers exhibiting rounded profile • occasional regenerative processes • variability in fibers cross-sectional area • proliferation of loose connective tissue (fibrosis) • fat deposition (lipidosis) • interstitial edema • inflammatory cells infiltrates • vasculitis	Kuttappan *et al.*(2009, 2013); Sihvo *et al.* (2014, 2017); Mazzoni *et al.*, 2015; Soglia *et al.* (2016a); Clark and Velleman (2016); Kawasaki *et al.* (2016); Radaelli *et al.* (2017)
b) QUALITY TRAITS	
• impaired nutritional value • higher amount of moisture, fat and collagen	Petracci *et al.*, 2014; Mudalal *et al.*, 2014 Kuttappan *et al.*, 2012; Soglia *et al.*, 2016a; Soglia *et al.*, 2016b; Zambonelli *et al.*, 2016
• reduced protein content and decreased protein solubility and functionality	Petracci *et al.*, 2014; Mudalal *et al.*, 2014; Mudalal *et al.*, 2015; Bowker and Zhuang, 2016
• increased ion levels and altered sodium and calcium homeostasis	Sandercock and Mitchell, 2004; Wallace and McNally, 2009; Soglia *et al.*, 2016a; Zambonelli *et al.*, 2016;
• higher amount of linoleic acid	Soglia *et al.*, 2016b;
• reduced Δ5 and Δ6 desaturase activity	Jordan *et al*., 1964; Soglia *et al.*, 2016b
• lower content of anserine, carnosine and creatine	Sundekilde *et al.*, 2017
• increased yellowness and pale colour	Kuttappan *et al.*, 2009; Soglia *et al.*, 2016a;
• increased ultimate pH	Petracci *et al.*, 2013b; Mudalal *et al.*, 2015; Trocino *et al.*, 2015; Mutryn *et al.*, 2016; Abasht *et al.*, 2016; Soglia *et al.*, 2016a; Zambonelli *et al.*, 2016, Tasoniero *et al.*, 2016.
• reduced and altered profile of muscular contractile and sarcoplasmic proteins	Mudalal *et al.*, 2014; Soglia *et al.*, 2016a; Bowker and Zhuang, 2016
• reduced oxidative stability	Soglia *et al.*, 2016b
c) TECHNOLOGICAL PROPERTIES	
• reduced water holding and water binding capacities: impaired marinate uptake, cooking loss and yield	Mudalal *et al.*, 2014; Petracci *et al.*, 2014; Mudalal *et al.*, 2015; Trocino *et al.*, 2015; Tijare *et al.*, 2016; Soglia *et al.*, 2016a; Soglia *et al.*, 2016b; Tasoniero *et al.*, 2016.
• increased proportion and mobility of extra-myofibrillar water fraction	Soglia *et al.*, 2016b
• elevated compression and MORSE forces (raw meat) • increased TPA hardness and chewiness (cooked meat)	Petracci *et al.*, 2013b; Mudalal *et al.*, 2015; Soglia *et al.*, 2016a; Chatterjee *et al.*, 2016; Tasoniero *et al.*, 2016

Table 1.2 Histological features, quality traits and technological properties of meat affected by emerging muscle abnormalities.

1.4.3.3 Rearing factors affecting the occurrence of muscle abnormalities

In the past few years, several studies have been performed in order to investigate the main factors involved in the occurrence of white striping defect (**Table 1.3**). Since muscle abnormalities predominantly occur in fast-growing genotypes selected for improved breast yield and body composition (Petracci *et al.*, 2013; Trocino *et al.*, 2016), both growth rate and muscle hypertrophy were hypothesised to exert a relevant role in promoting their manifestation. Within this context, not only the overall incidence of muscle abnormalities is higher in flocks belonging to high-growth rate and breast yield hybrids but also increases at increasing weight at slaughter (Kuttappan *et al.*, 2012; Lorenzi *et al.*, 2014; Kindlein *et al.*, 2015; Ferreira *et al.*, 2015). On the other hand, according to Trocino *et al.* (2015) male broilers seemed to be more prone to develop the wooden breast abnormality. However, the eventual interactions existing between gender and weight at slaughter are not fully understood. Indeed, since male broilers normally achieved higher weight at slaughter than females, body weight might exert a more significant effect in comparison with gender. On the other hand, an overall reduction in the occurrence of muscle abnormalities was observed as an indirect consequence of reduced growth rate and/or slaughter weight.

In addition, according to their very limited impact on the overall incidence of muscle abnormalities, both dietary and prophylactic treatments seem not to be directly related with the occurrence of muscular abnormalities (Kuttappan *et al*., 2012a,b; Ferreira *et al*., 2014; Cemin *et al*., 2015; Christensen *et al*., 2015; Trocino *et al*., 2015; Livingstone *et al*., 2016). Thus, although a strong non-genetic component was recently found for all breast myopathies and abnormalities (Bailey *et al*., 2016), no effective management and dietary solutions are available to mitigate the occurrence of these abnormalities without negatively affect live production performances (Bailey *et al*., 2015).

Variable	Incidence of muscular abnormalities	References
Genotype	High > Standard breast-yield	Petracci *et al*. (2013), Trocino *et al*. (2016)
Body weight at slaughter	High > Low	Lorenzi *et al*. (2014), Kindlein *et al*. (2015), Ferreira *et al*. (2016)
Growth rate	Fast > Slow	Kuttapan *et al*. (2012a, 2013)
Gender	Males > Females Males = Females	Lorenzi *et al*. (2014) Trocino *et al*. (2015)
Dietary supplementation		
Crude protein level	High > Low[1]	Kuttapan *et al*. (2012a)
Vitamin E	No effect	Kuttapan *et al*. (2012b)
Selenium	No effect	Ferreira *et al*. (2016)
Lysine	High > Low	Cruz *et al*. (2017)
L-arginine	No effect	Christensen *et al*. (2015)
Feeding plan		
Early dietary restriction	No effect	Trocino *et al*. (2015)
Full dietary restriction	*Ad libitum* > Feed restricted[1]	Livingston *et al*. (2016)
Coccidiosis control	Anticoccidial ≈ Vaccination Anticoccidial > Vaccination	Unpublished data, Dalle Zotte *et al*. (2015)

Table 1.3 Live production factors involved in the occurrence of emerging muscle abnormalities.

CHAPTER 2
EVALUATION OF MEAT QUALITY TRAITS

2.1 Morphometric measurements

Morphometric measurements (expressed in mm) were assessed through an electronic calliper on each *Pectoralis major* muscle according to the procedure described by Mehaffey *et al*. (2006) with slight modifications. In detail, as showed in **Figure 2.1**, fillet length (L), width (W) and height (assessed in three different positions, H1, H2 and H3) were determined and defined as follows:

Length	**L**	The longest dimension of the fillet
Width	**W**	The longest side-to-side distance within the middle part of the fillet
Height 1	**H1**	Measured at the thickest point of the cranial part
Height 2	**H2**	Measured at half distance of fillet length
Height 3	**H3**	Measured at the end of the caudal part, 1-cm toward dorsal direction

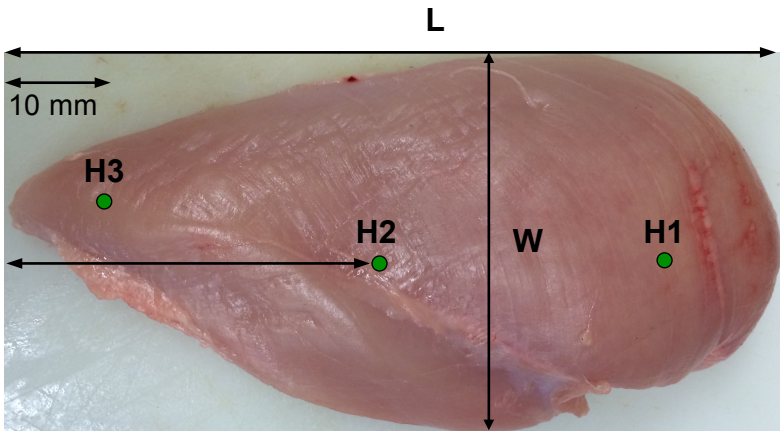


Figure 2.1 Protocol applied to assess morphometric measurement on *Pectoralis major* muscle.

2.2 pH

Ultimate pH value significantly affects both quality traits and technological properties of meat such as colour, water-holding/binding capacity and microbial shelf-life.

Being the negative logarithm of the hydrogen ion activity, ultimate pH designates the hydrogen ion activity of a given solution assuming that the activities of H^+ and OH^- equalize their concentrations (Barbut, 2015).

$$\mathrm{pH} = -\log[\mathrm{H}^+] = \log \frac{1}{[\mathrm{H}^+]}$$

With regard to meat, hydrogen ions result from lactic acid dissociating (Honikel, 2004; Barbut 2015) as follows:

$$CH_3\text{-}CHOH\text{-}COOH \longleftrightarrow CH_3\text{-}CHOH\text{-}COO^- + H^+$$

In living tissues, lactic acid is produced through an anaerobic metabolic pathway and reconverted into glucose (liver) or broken down into water and CO_2 through a specialized enzyme system (heart) (Aberle *et al.*, 2012). Once the circulation stops, lactic acid accumulation takes place until glycogen reserves are depleted/inaccessible or glycolytic enzymes are inactivated by the lowered pH values (Lyon and Buhr, 1999). A remarkable pH decline occurs during the conversion of muscle to meat. In detail, as a consequence of lactic acid accumulation, muscle pH falls from values exceeding 7.0 (in living tissues) to a metabolic ultimate value of 5.4 below which the glycolytic enzymes are inactivated (**Figure 2.2**) (de Fremery and Lineweaver, 1962; Stewart *et al.*, 1984; McGinnis *et al.*, 1989; Barbut, 2015). As a consequence of being predominantly constituted of type IIB-white-glycolytic fibers, the ultimate pH values of broilers *Pectoralis major* muscles (5.8 achieved 2-3 h *post-mortem*) significantly differ from the ones measured in red leg muscles (5.6 at 8 h *post-mortem*) (Stewart *et al.*, 1984). Indeed, the rate and extent of *post-mortem* pH decline plays a relevant role in determining meat quality traits and colour development.

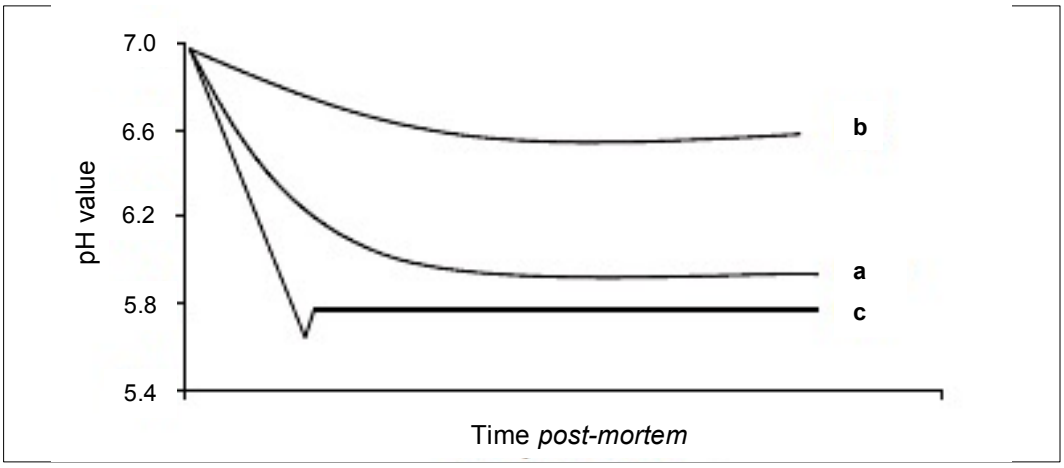


Figure 2.2 Rate and extent of *post-mortem* pH decline in *Pectoralis major* muscles under physiologic conditions (a) and within the muscles displaying the DFD (b) and PSE-like (c) syndrome (adapted from Barbut, 2015).

In some cases, as a consequence of glycogen stores depletion before slaughtering, lactic acid does not accumulate within the muscle tissue resulting in high ultimate pH values (around 6.0) achieved in very short time. The resulting meat, referred to as DFD (Dark, Firm and Dry), exhibit dark colour, firm texture and dry appearance. The last condition is linked to the high ultimate pH value that, in its turn, being far from the isoelectric point of muscle proteins leads to an increased water-holding capacity (Barbut *et al.*, 2015; Shen *et al.*, 2009). On the opposite, a rapid pH decline occurring at the beginning of the *post-mortem* process (within the first hour after slaughtering) when the muscle temperature is still high (> 35°C) leads to protein denaturation (myosin) resulting in the development of the PSE (Pale, Soft and Exudative)-like condition (Barbut *et al.*, 2015). In detail, the impaired functionality of the partially denatured myosin molecules leads to an overall reduced water-holding capacity of meat displaying increased fluid losses (exudative meat). In addition, affecting the water binding properties of muscle proteins, the reduced ultimate pH value observed in PSE-like meat alters the physical structure of meat and, accordingly, its light reflecting properties. Therefore, the increased proportion of light diffracted by the loose muscle structure resulted in paler muscles (Briskey, 1964; Barbut, 2015).

Ultimate pH value was assessed by using the iodoacetate method proposed by Jeacocke (1977). In detail, as showed in **Figure 2.3**, a thin

cross-sectional slice was excised from the cranial part of each *Pectoralis major* muscle. Subsequently, after being manually minced, 2.5 grams of meat were homogenized by Ultra-Turrax T25 basic (IKA-Werke, Staufen, Germany) (30 s at 13,500 rpm) in 25 mL of 5 mM sodium iodoacetate and 150 mM potassium chloride solution (pH 7.0). The pH was then determined on the homogenate by using a Jenway 3510 pH-meter previously calibrated at pH 4.0 and 7.0.

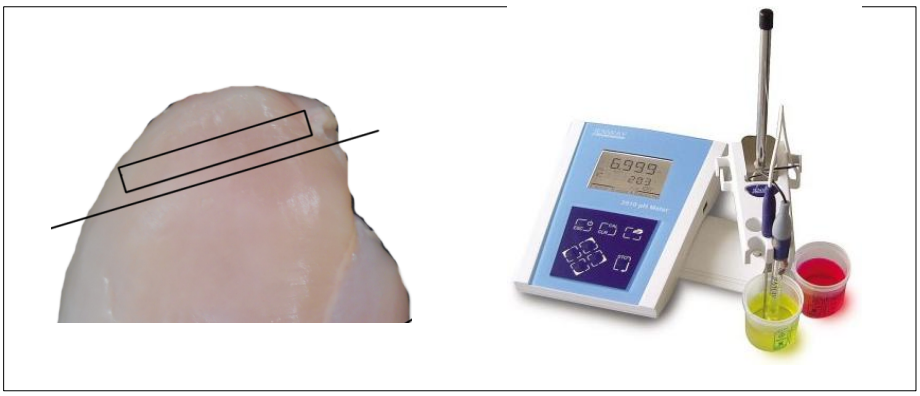


Figure 2.3 Thin cross-sectional slice excised from the cranial part of the *Pectoralis major* muscle for evaluating ultimate pH by using a Jenway 3510 pH-meter.

2.3 Colour

The pigment primarily responsible for meat colour is myoglobin, a globular single-chain protein located in the sarcoplasm. However, small amounts of haemoglobin as well as cytochrome C and ribonucleases may play a minor role in determining meat colour (Castiliego *et al.*, 2012; Mancini, 2013).

Myoglobin is a monomeric globular protein consisting of a peptide colourless portion (globin) and an oxygen-binding prosthetic group (heme) containing a flat porphyrin ring and an iron atom (Fe^{2+}) in its inner core, placed within a hydrophobic pocket created by the apoprotein (**Figure 2.4**). As a consequence, since the vinyl groups are oriented towards the hydrophobic core created by the apoprotein, the globin portion not only confers water solubility but also protects the prosthetic heme group from oxidation.

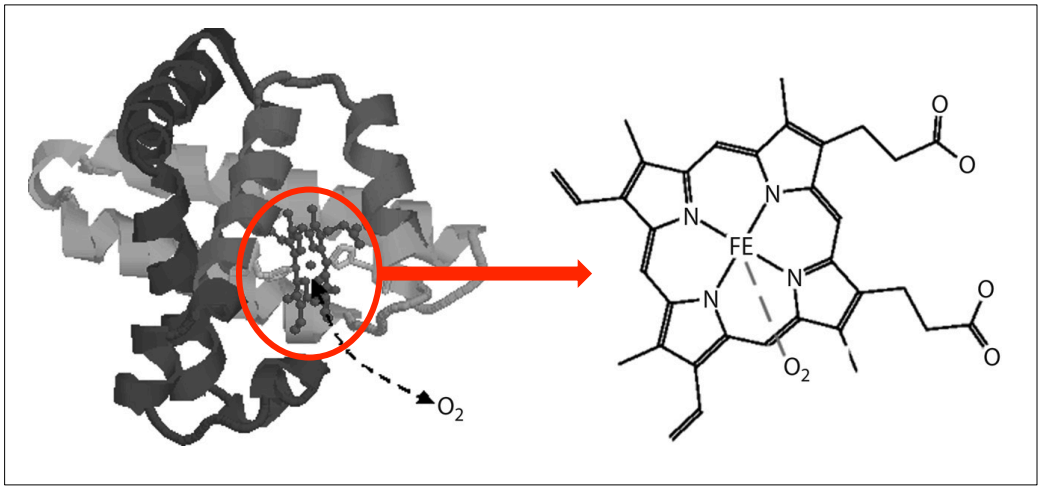


Figure 2.4 Schematic representation of the myoglobin molecule containing a flat porphyrin ring and an iron atom within the hydrophobic pocket created by the apoprotein (adapted from Castiliego *et al.*, 2012).

The iron atom is coordinated to the pyrrole nitrogen of heme by four non-covalent bonds and attached to the proximal histidine H93 of the protein. Indeed, the attachment of the prosthetic heme group to the apoprotein composing the myoglobin molecule is ascribed to the interactions established between iron and the proximal histidine residue H93. In addition, since the distal histidine may interact with small molecules (such as oxygen and carbon monoxide) capable of penetrating the hydrophobic pocket and directly interact with the iron atom itself, a sixth coordination bond may be potentially formed. Furthermore, the spatial relations between heme and the bound small molecules are controlled by a distal histidine H64 located within the hydrophobic pocket of the apoprotein and influencing the chemical dynamics related to colour. Indeed, the redox state of iron and its reversible transition from a reduced ferrous (Fe^{2+}) to an oxidized ferric state (Fe^{3+}) but also its ability to bind different ligands, exert a relevant role in determining meat colour. In detail, the iron atom located within the center of heme's ring can reversibly binds different ligands such as diatomic oxygen (O_2), carbon monoxide (CO) or nitric oxide (NO).

As a consequence, four water-soluble chemical forms of myoglobin, each conferring a different colour, might be observed (Stewart *et al.*, 1965; Suman and Joseph, 2013):

1) the purple-red deoxymyoglobin (Mb^{2+}),

2) the bright red oxymyoglobin (OMb^{2+}) (typical of fresh meat),
3) the brown metmyoglobin ($MetMb^{3+}$)
3) Carboxymyoglobin (CO Mb^{2+}).

		Oxidation	Sixth ligand	Colour	Globin
Myoglobin	Mb^{2+}	Fe^{2+}	H_2O	Purple-red	Native
Oxymyoglobin	OMb^{2+}	Fe^{2+}	O_2	Bright red	Native
Metmyoglobin	$MetMb^{3+}$	Fe^{3+}	H_2O	Brown	Native
Denatured globin		Fe^{3+}	H_2O	Grey	Denatured

Table 2.1 Redox states, ligands and iron valence conferring different colours to the myoglobin molecule.

Since a lack of electron donors might be observed under very low oxygen partial pressures, ferrous iron (Fe^{2+}) tends to ionically interact with water conferring a dark-red purple colour to the meat (native myoglobin). On the other hand, after exposing meat cut to air, myoglobin oxygenation (referred to also as “blooming”) might occur leading to the formation of oxymyoglobin. Indeed, at higher oxygen tensions, O_2 tends to occupy the sixth coordination site and bind to ferrous iron that maintains the lower oxidation state:

$$Mb^{2+} + O_2 \Leftrightarrow OMb^{2+}$$

Although this reaction is influenced by several factors such as pH, temperature, O_2 partial pressure and possible competitors, in meat, OMb^{2+} might be rapidly converted to Mb^{2+} and *vice versa*. However, the conversion from OMb^{2+} to Mb^{2+} might require a two-step reaction:

$$OMb^{2+} \rightarrow MMb^{3+} \rightarrow Mb^{2+}$$

Once the ferrous iron (Fe^{2+}) is further oxidized to its ferric (Fe^{3+}) counterpart, although its capability to bind molecular O_2 is lost, complexes with other molecules (such as in case of $MetMb^{3+}$) might be formed. Indeed, the interaction between iron and a water molecule results in the formation of a covalent bond involving a hydroxyl group and leading to a shift of meat colour toward brown of an extent directly related to the proportion of $MetMb^{3+}$ and to the amount of meat surface containing a high percentage of $MetMb^{3+}$. Since the formation of $MetMb^{3+}$ is often associated with the occurrence of

autoxidation, superoxide anion (O_2^-) might be formed and, in its turn, converted into hydrogen peroxide (H_2O_2) through spontaneous dismutation (Tajima and Shikama, 1987).

$$OMb^{2+} + H_2O + H^+ \Leftrightarrow MetMb^{3+}\ HO_2 \xrightarrow[dismutation]{} H_2O_2$$

This process was demonstrated to be markedly influenced by oxygen partial pressure (with a maximum within the range from 1 to 1.4 mmHg) (George and Stratmann, 1952; Gill and McGinnis, 1995), pH (favoured in the acidic conditions typically exhibited by meat) (Shikama and Sugawara, 1978) and temperature (Brown and Mebine, 1969). In addition, autoxidation of OMb^{2+} to $MetMb^{3+}$ in *post-mortem* muscles might also be promoted by an increase in the amount of free radicals (resulting from microbial growth and processing) as well as by accumulation of ions and microbial metabolites (Renerre, 1990; Giroux *et al.*, 2001; Osborn *et al.*, 2003). Indeed, as a consequence of the overall reduction in $MetMb^{3+}$ reductase activity and increased protein denaturation, pigment oxidation is enhanced at high salt concentrations (Stewart *et al*.,1965b).

The Gage Canadian Dictionary defines colour as “the sensation produced by the different effects of waves of light striking the retina of the eye”. According to this definition “different colours are produced by rays of light having different wavelengths”.

A typical reflectance spectrum for chicken thigh meat is showed in **Figure 2.5**. Since almost all/most of the blue and green lights are absorbed and only small amounts of yellow, moderate amounts of orange and larger amounts of red lights are reflected, the overall colour appears to be red. In detail, this is a direct consequence of the resonant nature of the conjugated double bonds of the heme group (Castiliego *et al.*, 2012).

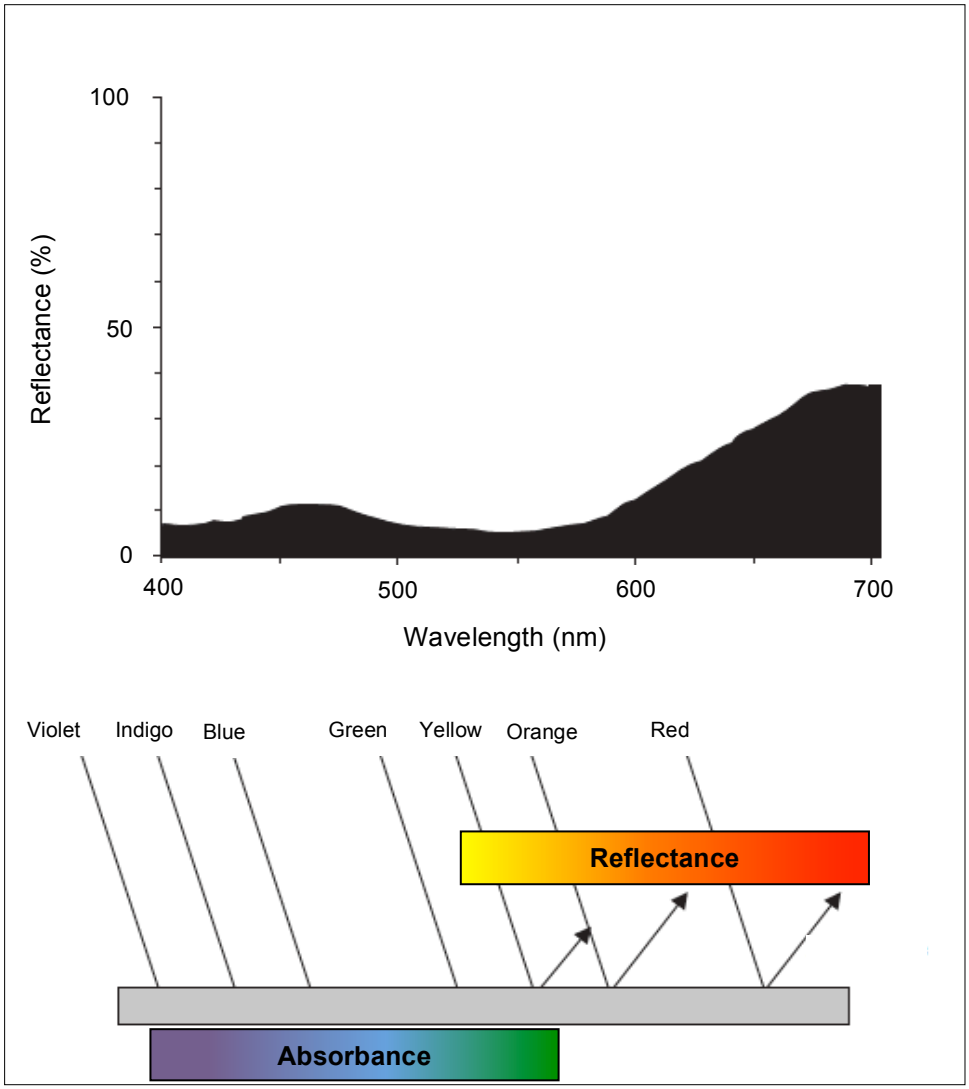


Figure 2.5 Spectral reflectance (%) and absorbance typical of chicken thigh meat (adapted from Barbut, 2002b).

In the past years, several methods have been used by the poultry industry to measure and express colour. Among them visual, chemical-spectral photometric methods (including direct pigment quantification) and reflectance colorimetry were the most widely used with the last being the most common one at the present days.

A visual description of meat colour was introduced in the early 1900s and developed to score colour of poultry skin and of egg yolk. According to this method, after developing appropriate standards and assigning numbers, colour was expressed on a linear scale. However, although these visual

standards have been widely used by the red meat industry (pork colour scale used in various abattoirs to evaluate pork meat quality), such system is not commonly applied for evaluating poultry meat.

The chemical-spectral photometric procedures are based on the chemical extraction of the different meat pigments and their subsequent quantification (*See Par. 2.7.2.5*).

Reflectance colorimetry is the most widely used method to assess poultry meat colour with its major advantages being its accuracy, objectivity and reproducibility.

Three main terms are conventionally used to describe the human's perception of colour: i) Lightness (L*) describing the lightness of the colour itself (dark or light), ii) Hue (H*) defining a primary colour such as red, green or blue and iii) Chroma (C*) or saturation indicating how vivid or dull the colour is (Wyszecki and Stiles, 1967). The relationship existing between these three terms might be observed in **Figure 2.6**.

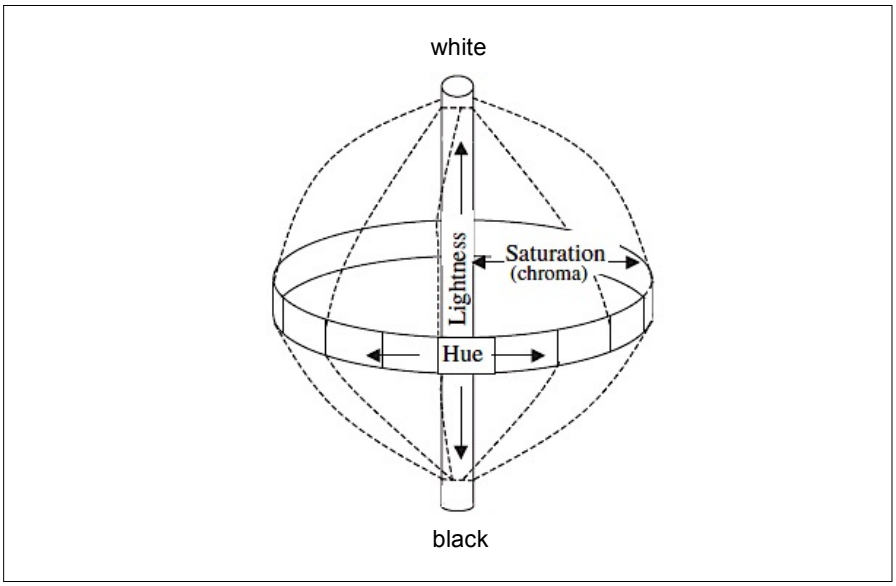


Figure 2.6 Three-dimensional colour space displaying the relationship between Hue (H*), Lightness (L*) and Chroma (C*) (CIE, 1976).

Over the years, different systems have been progressively developed. In 1931 the CIE (Commission Internationale de l'Eclairage) expressed colour by the additive mixture of three primary colours, the so-called tristimulus values X (red), Y (green) and Z (blue) as viewed by a "standard observer" (human)

under defined illumination and viewing conditions. This system is based on the theory that receptors for the three primary colours might be identified in human's eye and that colours are seen as a mixture of these three components. Although this system provides a useful tool to define colour, it is not easy to graphically visualize the results since they don't correlate with the human's perception. As a result, CIE defined other colour spaces in order to clearly define colour.

Apart from the Hunter Lab solid scale, the most popular colour space is the CIE LAB one (illustrated in **Figure 2.7**) defining colour as a combination of three parameters: Lightness (L*), Redness (a*) and Yellowness (b*). The CIE LAB colour space was defined in 1976 in order to minimize the problem observed within the chromaticity diagram in which equal distances did not correspond to equal perceived colour differences. The L* value is an expression of the lightness ranging from 0 (black) to 100 (white). Positive a* values indicate red colour with the higher values denoting more red items. On the other hand, negative a* values indicate green colour. The positive and negative b* values denote yellow and blue items, respectively.

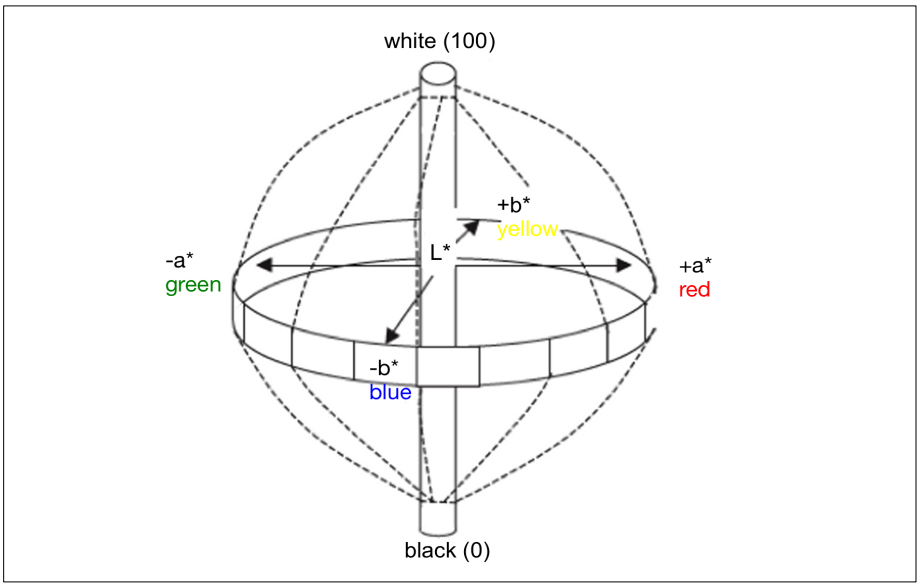


Figure 2.7 Three-dimensional spherical LAB colour space system used to define colour as a function of three parameters: L*, a* and b* (CIE, 1976). The vertical axis shows lightness (100 = white) to darkness (0 = black), a* represents Redness (+a*) to Greenness (-a*) and b* Yellowness (+b*) to Blueness (-b*), respectively.

Hue and Chroma can be calculated as a function of a* and b* as follows:

$$\text{Chroma} = C^* = \sqrt[2]{a^2 + b^2}$$

$$\text{Hue} = H^* = tan^{-1}\ \frac{b^*}{a^*}$$

In the experiments performed within this thesis, the colour profile of the *Pectoralis major* muscles was assessed through a reflectance colorimeter equipped with an illuminant source C and previously calibrated with a reference colour standard white ceramic tile (Y = 93.9, x = 0.3130, and y = 0.3190).

Colour measurement was performed (in triplicate) at 24 h *post-mortem* on the bone-side (ventral) surface of each *Pectoralis major* muscle by using a Chroma Meter CR-400 (Minolta Corp., Milan, Italy) and expressed according to the CIE LAB system as lightness (L*), redness (a*) and yellowness (b*).

2.4 Water Holding Capacity

Corresponding to approximately 75% of the total muscle mass, water represents the main component of the lean muscular tissue (Offer and Knight, 1988). Water-holding capacity (WHC) is defined as the aptitude of meat to hold all or part of its own water (constitutive water or added during processing) when mechanical or physical forces are applied to the system (Honikel *et al.*, 2009).

Approximately 85% of water is located within the intra-myofibrillar compartment and immobilized within the protein-dense myofibrillar protein network (in the space between thick and thin filaments) (Huff-Lonergan and Lonergan, 2005) by charged or hydrophilic side chains of amino acids and capillary forces (Honikel, 2009) On the other hand, the remaining 15% is located outside the myofibrillar compartment within the extra-myofibrillar spaces (Hamm, 1975; Lawrie, 1998).

Three water fractions ascribed to the extra-myofibrillar compartment might be identified: i) the inter-myofibrillar water (located in the spaces between the myofibrils within the sarcoplasm), ii) the inter-fascicular water (located between the muscle fibers and the inter-fascicular space) (Hamm,

1975; Offer and Knight, 1988; Offer *et al.*, 1989) and iii) the extra-fascicular water (in the extra-fascicular space in between the muscle fasciculi) (Schaefer *et al.*, 2000). Thus, the water composing the muscular tissue can be classified into three fractions:

1) Protein-associated water (or bound water). Having a highly reduced mobility as a consequence of being tightly bound by the charged hydrophilic groups on muscle proteins, bound water does not move towards other compartments even when severe mechanical or physical forces are applied (Offer and Trinick, 1983; Huff-Lonergan and Lonergan, 2005).
2) Immobilised water (or entrapped water). Accounting for about 85% of the myowater it is located within the thick filaments and in the spaces between the thick and the thin filaments composing the myofibril (Honikel *et al.*, 1986). Immobilised water typically exhibits a less ordered molecular orientation towards the charged groups and is bound by both steric effect (resulting from the attraction between the filaments) and hydrogen bonds with muscle proteins (Huff-Lonergan and Lonergan, 2005).
3) Free water. Held only by the intermolecular forces established between the liquid and the surrounding matrix (capillary forces), typically exhibits a molecular orientation that is not related to the position of the charged groups. Since mobility of this water fraction is unimpeded, free water can be easily mobilised through the application of minor physical and mechanical forces (Honikel *et al.*, 2004).

More in detail, Kinsella *et al*. (1989) proposed the existence of six different types of water in food protein systems including the a) structural water (tightly bound to protein molecules and essentially unavailable for chemical reactions), b) hydrophobic hydration water (located around the apolar amino acid residues), c) monolayer water (absorbed by the protein groups and available for some reactions), d) unfreezable water, e) capillary water (held by surface tension forces) and f) hydrodynamic hydration water (loosely surrounding proteins) whose amount and degree of binding is deeply affected by pH. Within this context, not only the specific molecular structure of a protein but also its conformation might affect water-holding/binding capacity

of meat (Kinsella *et al.*, 1989). Indeed, the side chains of the amino acids, protruding from the main polypeptide chain, might be neutral as well as positively or negatively charged in relation to the type of amino acid residue and environmental pH value. Since these reactive charged groups (responsible for water binding) are extensively reduced after slaughter as a result of pH drop Hedrick *et al*. (1994), the resulting overall reduction in WHC *post-mortem* might be ascribed to three main factors:

a) The net charge effect refers to the total amount of amino acidic charged groups on the protein molecules available for water binding. As showed in **Figure 2.8**, as a consequence of lactic acid accumulation in *post-mortem* meat, pH tends to equalize the isoelectric point (pH value at which the number of positively and negatively charged groups is equal) for muscle proteins resulting in fewer charged groups available for water binding. On the other hand, at pH values far from the isoelectric point, the net charge is greater and consequently a higher number of charged group is available for binding water molecule leading to increased WHC (Barbut, 2002c; Huff-Lonergan and Lonergan, 2005).

b) The steric effect refers to the repulsive phenomenon observable in charged side chains exhibiting a similar charge and thus repelling each other. This would lead to the establishment of bigger spaces between the fibers filaments in which the water molecules might be bound. Being related to a high proportion of negatively or positively charged groups resulting in repulsive phenomenon, the steric effect might be observed at both side of the isoelectric point (Huff-Lonergan and Lonergan, 2005).

c) The ion exchange takes place when the enzymatic degradation of the cellular structure (during aging) leads to a re-distribution of ions implying the replacement of some divalent cations, such as calcium, with monovalent ions, such as sodium. Since divalent cations are capable of neutralizing two negatively charged side groups, replacing them with monovalent ions result in a greater number of free charged groups available for binding water molecules and thus increasing the WHC of meat.

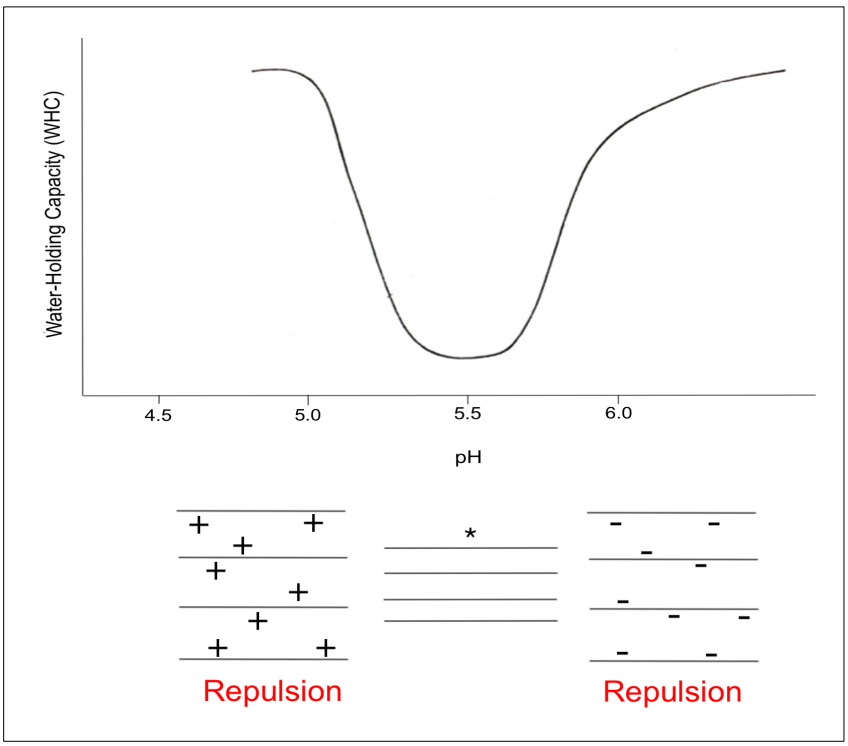


Figure 2.8 Effect of muscle pH on water-holding capacity of meat. In detail, influencing the distribution of charged groups on the myofilaments and the dimension of intra-myofilamental spaces, pH exerts a relevant role in determining WHC (adapted from Barbut *et al*., 2002a).

Besides pH, the eventual addition of sodium chloride to a meat system is known to significantly affect its WHC. Indeed, as a result of both the solubilization of the myofibrillar protein fraction (salt-soluble) and the addition of negatively charged chloride ions to the system, the WHC curve is remarkably shifted towards the left (**Figure 2.9**). However, adding above 5% of sodium chloride has a dramatic consequence and resulted in the "salting-out" effect: proteins will aggregate, fold and their charged side groups would not be available anymore for binding water.

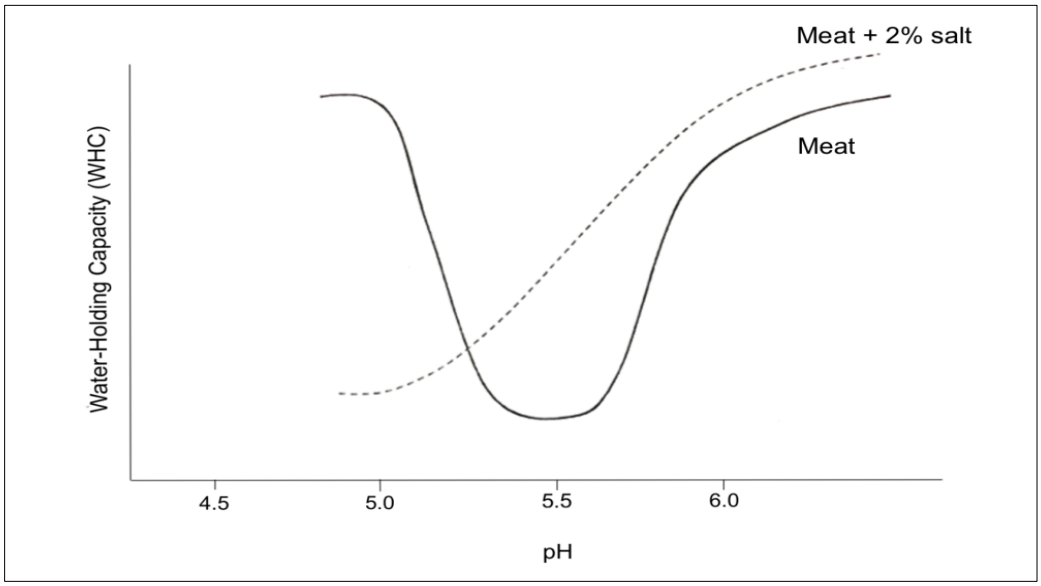


Figure 2.9 Effect exerted by salt-addition on water-holding capacity of meat (adapted from Barbut *et al*., 2002a).

The water-holding capacity of fresh and marinated meat was assessed by determining several parameters.

2.4.1 Drip loss

"Drip loss" is defined as the percentage of fluid released by raw meat via passive exudation during its refrigerated storage (Petracci and Baeza, 2011). Drip formation and the consequently water loss originate from changes in the myofibrils volume induced by both pH drop and attachment of myosin heads to actin filaments at *rigor* (Honikel *et al.*, 2004). In detail, water moves firstly from the intra-myofibrillar towards the inter-myofibrillar spaces and then into one of two other extra-myofibrillar compartments, the inter- and extra-fascicular spaces (Honikel *et al.*, 1986; Penny, 1975). In addition, as a consequence of a 1.6 fold increase in the volume of the extra-myofibrillar spaces hypothesised to occur at 24 h *post-mortem* (Currie and Wolfe, 1983; Heffron and Hegarty, 1974; Huff-Lonergan and Lonergan, 2005), the development of longitudinal channels and gaps between the muscle fibers has been observed and considered as the primary place from where drip and purge loss originate (Offer *et al.*, 1989; Purslow *et al.*, 2001).

According to the sampling protocol displayed in **Figure 2.10**, a 8 × 4 × 2 cm sub-sample was excised from each *Pectoralis major* muscle, individually weighted and, after being introduced in plastic boxes over sieved plastic rack, stored at 4 ± 1°C for 48 h. Then, after being cleaned from any superficial liquid accumulation, each sample was re-weighted and drip loss calculated as a percentage of the initial sample weight according to the formula:

$$\text{Drip loss (\%)} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100$$

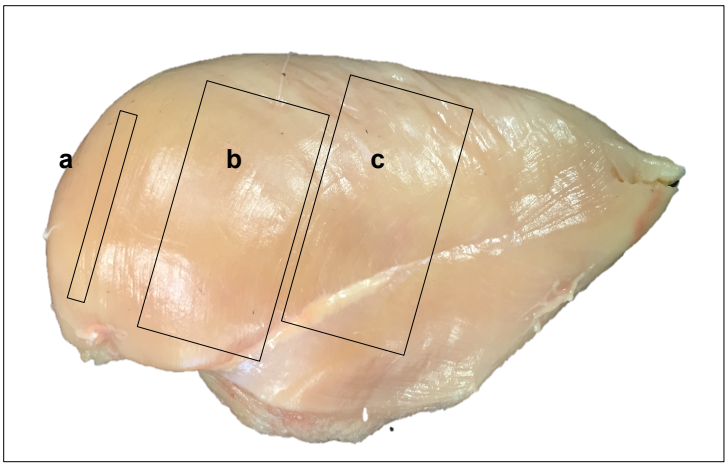


Figure 2.10 Schematic diagram displaying the sampling positions considered for excising the sub-samples used to assess ultimate pH value (a) and quality traits and technological properties of raw (b) and marinated (c) *Pectoralis major* muscles.

2.4.2 Marinade uptake

The amount of marinade solution retained by raw meat during marination is termed as "marinade uptake" and calculated as the percentage of weight gain by each individual sample in relation with its initial weight according to the formula:

$$\text{Marinade uptake (\%)} = \frac{(\text{Marinated wt} - \text{Raw meat wt})}{\text{Marinated wt}} \times 100$$

Marination performances were assessed on a 8 × 4 × 2 cm sub-samples (weighting approximately 60 grams) (**Figure 2.10**) that, after being individually labelled were marinated through the addition of:

a) 15% (in weight) of marinade solution containing 7.6% sodium chloride and 2.3% sodium tripolyphosphate, or

b) 20% (in weight) of marinade solution containing 6% sodium chloride and 1.8% sodium tripolyphosphate.

The marination process was performed by using a small-scale vacuum tumbler (model MGH-20; Vakona Qualitat, Lienen, Germany) set in order to achieve a total tumbling time of 46 min (three working cycles of 13 min/cycle and two pause cycles of 3 min/cycle) under vacuum (-0.95 bar). After tumbling, samples were weighted and weight difference (before and after tumbling) used to calculate marinade uptake.

2.4.3 Purge loss

Being the exact counterpart of drip loss, purge loss describes the percentage of fluid (basically marinade solution) released by marinated meat via passive exudation during its refrigerated storage. In order to assess purge loss, after tumbling, the samples were placed on sieved plastic racks in covered plastic boxes and stored at 4 ± 1°C for 48 h. Then, after removing any eventual superficial liquid accumulation, each sample was re-weighted and purge loss calculated as a percentage of the initial marinated sample weight according to the formula:

$$\text{Purge loss (\%)} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100$$

2.4.4 Cooking loss and yield

Considering the structural basis of water-holding (*See Par. 2.4*), the swelling as well as the transverse and longitudinal shrinkage of the myofibrils composing the muscle tissue consequent to the application of heat treatments exert a relevant role in determining the WHC of meat (Offer and Knight, 1988). In detail, after heating, structural changes such as the destruction of cell membranes (Rowe, 1989), the transversal and longitudinal shrinkage of muscle fibres themselves, the aggregation (and gel formation) of the sarcoplasmic proteins fraction and both shrinkage and solubilisation of the connective tissue are observed (Cheng and Parrish, 1976; Jones *et al.*, 1977; Bendall and Restall, 1983). The specific temperature at which these conformational changes take place is known as denaturation temperature and commonly investigated through differential scanning calorimetry (DSC).

A typical curve displaying the three main thermal transition zones of a muscle tissue is showed in **Figure 2.11**. In detail, exhibiting its maximum between 54 and 58°C, the first transition zone was attributed to myosin denaturation resulting in the formation of large globular aggregates (Martens and Vold, 1976; Wright *et al.*,1977; Tornberg, 2005).

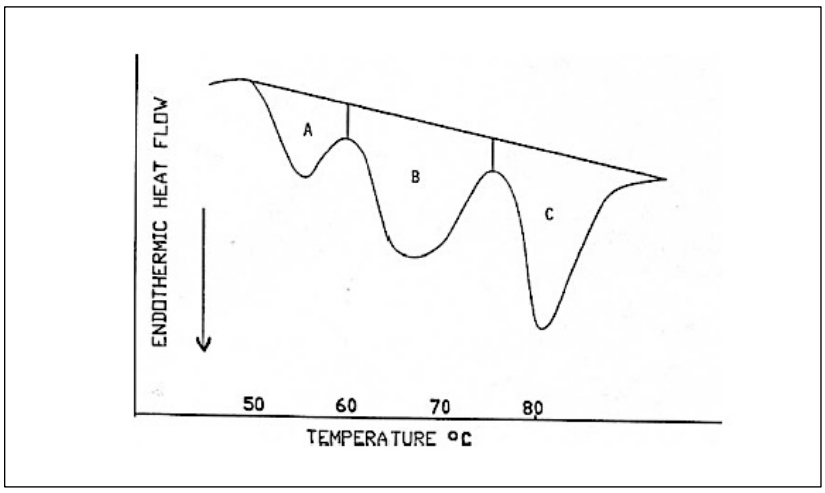


Figure 2.11 Typical thermal curve of a muscle tissue in which three main transitions zones ascribed to: A) the myosin subunits, B) the sarcoplasmic proteins and collagen and C) actin (Findlay *et al.*, 1989).

The second transition, occurring in the range between 65 and 67°C was attributed to sarcoplasmic proteins aggregation (Wright *et al.*, 1977) and collagen denaturation (Martens and Vold, 1976; Stabursvik *and* Martens, 1980), the last involving first a structural impairment (resulting from the breakage of the hydrogen bonds) and a subsequent shrinkage of collagen fibers up to one-quarter of their resting length. At last, having a transition temperature comprise within the range between 80 and 83°C, the third transition zone was assigned to actin (Findlay *et al.*, 1989). Thus it might be stated that heating results in conformational changes that, affecting the muscular proteins and the muscle structure itself, lead to an overall reduction in WHC of meat.

In order to assess cooking loss (%), defined as the amount of fluid released by both raw and marinated meat after a heat treatment has been applied, both raw and marinated samples were individually packaged under vacuum (99%) and cooked at 80°C in a water bath (Combes *et al.*, 2003). In

detail, to avoid the development of any thermal gradient moving from the external surface toward the inner core of the samples, cooking time was defined as the time necessary to attain a temperature of 80°C within the inner core of the samples. After cooking, the samples were allowed to equilibrate to room temperature, reweighed and cooking loss calculated according to the following equation:

$$\text{Cooking loss (\%)} = \frac{(\text{Fresh weight} - \text{Cooked weight})}{\text{Fresh weight}} \times 100$$

As for marinated samples, total yield percentage, reflecting the overall processing yield, was calculated as the ratio between cooked (after marination) and raw meat weights according to the following equation:

$$\text{Total yield (\%)} = \frac{Cooked\ weight}{Fresh\ weight} \times 100$$

2.4.5 NMR-relaxation properties

Proton transverse relaxation (T_2) decay curves in breast meat samples were recorded at the operating frequency of 20MHz with a Bruker (Milan, Italy) Minispec PC/20 spectrometer using the Carr-Purcell-Meiboon-Gill (CPMG) pulse sequence described in our previous study (Petracci *et al.*, 2012). All the measurements were performed at a constant temperature of 24 °C on a meat sample having a weight of about 600 mg and a height not exceeding the active region of the radio frequency coil. The CPMG decays were normalized by the sample weight and transformed into relaxograms (i.e. continuous distributions of relaxation times) through the program UPEN (Borgia *et al.*, 1998). As showed in **Figure 2.12**, each relaxogram was then interpreted in terms of bound, intra-myofibrillar and extra-myofibrillar water proton pools in agreement with our previous study (Petracci *et al.*, 2012) and consolidated literature (Bianchi *et al.*, 2004).

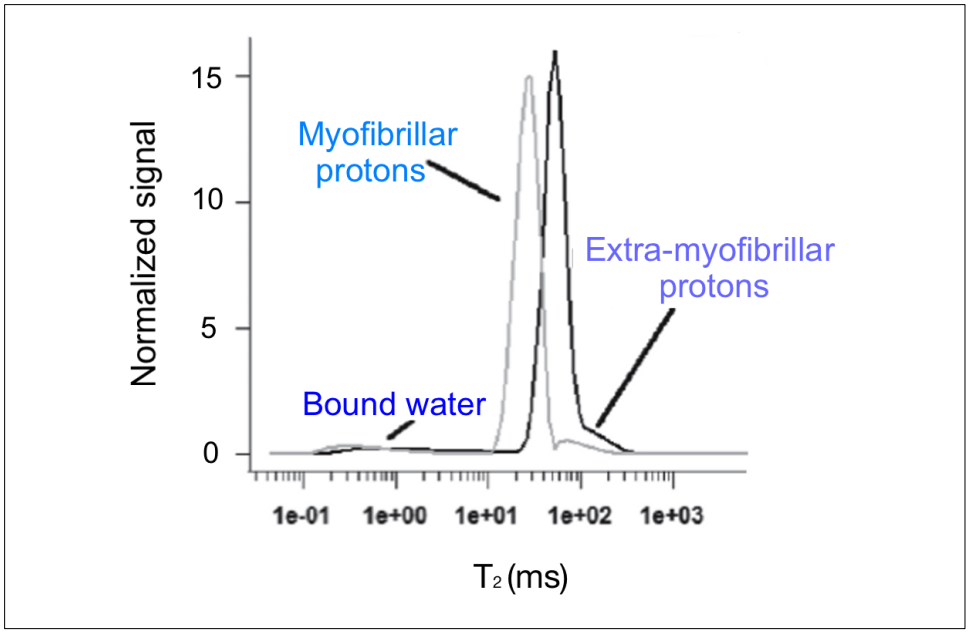


Figure 2.12 Transverse relaxation time spectra (T2) obtained for raw (black line) and cooked (grey line) meat samples (adapted from Petracci *et al.*, 2012).

To separately observe such protons populations, the relaxograms were fit to the sum of four exponential curves and the two with intermediate T_2, describing the behaviour of intra-myofibrillar protons, merged as explained in detail by Brown (1989).

2.5 Tenderness

Tenderness is one of the most important meat quality traits affecting consumers' acceptance. Factors, such as deboning time, age at slaughter, genetic strain as well as cooking (*See Par. 2.4.4*), significantly affect the myofibrillar and the connective tissue components responsible for meat tenderness (Lawrie, 1998; Northcutt *et al.*, 2001; Cavitt *et al.*, 2004; Owens and Meuellenet, 2010). In addition, since *post-mortem* metabolism significantly affects the myofibrillar components, understanding the development of *rigor mortis* is of great importance. Indeed, stiffening, loss of muscle extensibility and elasticity as well as muscle shortening result from the formation of permanent acto-myosin bonds occurring *post-mortem* (Hedrick *et al.*, 1989). Within this context, meat tenderness is primarily influenced by the state of sarcomeres (the basic contractile units within the

muscle tissue) when the permanent acto-myosin cross-bridges are formed (Fennema, 1996). Indeed, sarcomere shortening naturally occurs as a consequence of an increased number of acto-myosin bonds formed within the *post-mortem* period. However, sarcomere shortening and meat toughening were more pronounced when induced by early deboning practises (Goodwin, 1984; Cavitt *et al.*, 2004). On the contrary, the connective tissue component does not play a major role in determining broiler meat tenderness. Indeed, although the fibrous collagen structure typically exhibits thermally stable intermolecular cross-links resulting in a high tensile strength, mature cross-links form as animal gets old. Thus, since in broilers mature cross-links have not been formed yet, collagen is not thermally stable and melt during cooking (Owens and Meuellenet, 2010).

Texture was defined as “the sensory manifestation of the structure of the food and the manner in which this structure reacts to the applied forces” (Szczesniak, 1990). It is thus clear that the instrumental methods applied to measure meat texture should reflect its sensory perception.

Many instrumental methods might be used to mechanically evaluate meat texture. Before 1963, since texture was not considered as a multiparameter trait, its assessment was primarily based on finding a single measurement whereby texture could be evaluated. This led to the development of devices, such as the Warner-Bratzler shear blade and the Allo-Kramer shear cell commonly used to assess poultry meat texture. Then, as the multiparameter nature of texture became apparent, multiple measurements and imitative instrumental tests, such as the texture profile analysis (TPA) method, were developed (Owens and Meuellenet, 2010).

The textural properties of the *Pectoralis major* muscles considered within this thesis were assessed by using different methods and devices.

2.5.1 Allo-Kramer shear test

The Allo-Kramer shear instrument was developed in the early 1950s and consists of a hydraulic press, a moving test cell and a stationary cell component. In its turn, the moving test cell consists of 3 mm thick and 3 mm

apart multiple blades (10 in case of poultry meat testing) moving into the stationery cell at the bottom of which the sample is located. Having this conformation, the Allo-Kramer device first compresses and subsequently shears the meat sample leading to a typical force-deformation diagram displayed in **Figure 2.13**.

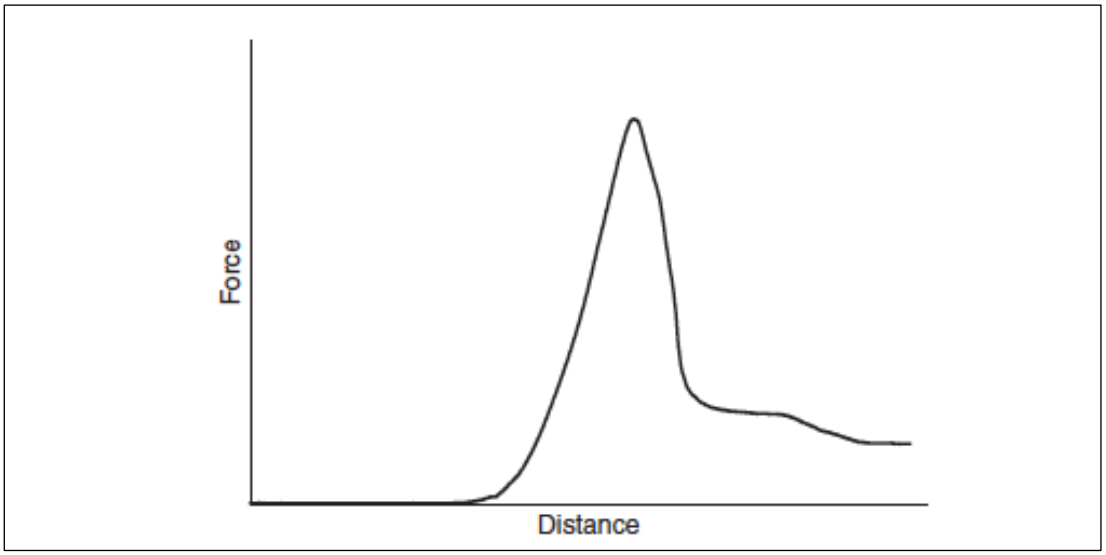


Figure 2.13 Typical force-deformation diagram obtained by shearing the meat samples through an Allo-Kramer shear cell (Barbut, 2009).

Allo-Kramer shear test was performed according to the following procedures:

a) on sub-samples of cooked fillets by using a TA-HDi Heavy Duty texture analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK) using a 25 kg loading cell equipped with a 10 blade Allo-Kramer shear cell, according to the procedure described by Sams *et al.* (1990). After being individually packaged and cooked under vacuum at 80°C in a water bath, a sub-sample (4 × 2 × 1 cm) was excised from each *Pectoralis major* muscle, weighted and sheared with a test speed of 500 mm/min. The shear values were then expressed as kilograms of force per gram of sample.

b) on both the superficial and the deep layer of cooked fillets by using an Instron Model 6625 (Instron Co., Canton, MA) equipped with an Allo-Kramer shear cell and a 5 kN load cell. After being individually packaged and cooked under vacuum at 75°C in a water bath. A total of six (20 × 20 × 6 mm) sub-

samples (3 from the superficial and 3 from the deep layer) were excised from each *Pectoralis major* muscle, weighted and sheared with a test speed of 40 mm/min. The results were expressed as Newtons of force per gram of sample.

However, since not only the instrumental conditions, but also sample orientation was found to significantly affect the shearing forces (Smith *et al.*, 1988), sample orientation was standardize and each sample sheared perpendicularly to its fibers direction.

2.5.2 Warner-Bratzler shear test

Warner-Bratzler shear test was performed on 4 × 1 × 1 cm sub-samples excised parallel to the muscle fibers direction and sheared perpendicularly to them by using a TA-HDi Heavy-Duty Texture Analyser (Stable Micro Systems Ltd), equipped with a with a 5 kg loading cell and a Warner-Bratzler shear blade (Sams *et al*., 1990). The actual cross-sectional area at the shearing point was measured with an electronic calliper and shear values expressed in kg.

2.5.3 Compression test

Compression test was performed in order to objectively describe the typical hardened consistency associated with the occurrence of the Wooden Breast abnormality. Thus, compression force was assessed on both raw and cooked *Pectoralis major* muscles according to the following procedures:

a) A raw cylindrical meat cut (2.5 cm diameter and 1 cm high, weighting about 5 grams) was sampled from the caudal part of each fillet. Then, the sample was compressed to 40% of its initial height by using a TA-HDi heavy-duty texture analyser (Stable Micro Systems Ltd, Godalming, Surrey, UK) equipped with a 25 kg loading cell connected to a 50 mm DIA cylinder aluminium probe. The test speed was 1 mm/s, whereas the pre- and post-test speeds were both 3 mm/s. The compression value was recorded as the maximum force required to compress 40% of the initial height of the sample and expressed in kg.

b) The compression test was performed in triplicates on both the superficial

and the deep layer of raw and vacuum-packaged and cooked (core temperature 75°C) samples. The test was performed on a 2 × 1 × 1 cm sample by using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd, Godalming, Surrey, UK) set to compress the sample in a longitudinal conformation applying the force perpendicular to the muscle fibers (Lepetit and Culioli, 1994) using a modified compression device (Campo *et al.,* 2000). The sample was compressed to 95% of its initial height with a speed of 50 mm/min. Then, the forces recorded after compressing the sample at 40 and 80% of its initial height were extracted in order to estimate the contribution given by the myofibrils and the connective tissue, respectively (Lepetit and Culioli, 1994; Campo *et al.*, 2000).

2.5.4 Texture Profile Analysis (TPA)

Establishing parameters correlated with the sensory perception, TPA was developed in the early 1960s. In detail, a cylindrical sample is compressed through a two-cycle compression, to a certain predetermined deformation with an intermediate pressure release.

Texture profile analysis was performed on a cylindrical meat sample (3 cm diameter × 0.8 cm height) excised from the 8 × 4 × 3 cm sample designed for cooking loss determination (**Figure 2.10**) and double compressed up to 50% of its initial height. The test was run by using a TA-HDi Heavy Duty texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK) equipped with a 50 kg loading cell and a 5 cm diameter cylindrical aluminium probe. Then, for each sample, the TPA parameters of Hardness, Cohesiveness, Springiness, Gumminess and Chewiness (defined in **Table 2.2**) were obtained by elaborating the double compression diagram (force/deformation) displayed in **Figure 2.14** (De Campos *et al.*, 2008; Lyon *et al.*, 2010).

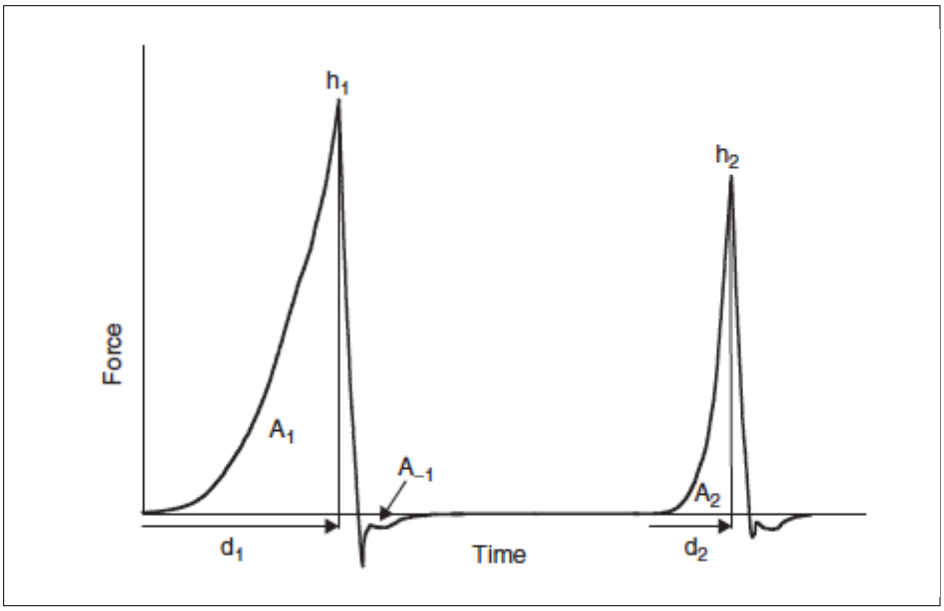


Figure 2.14 Typical force-deformation diagram obtained by double-compressing the meat samples by TPA.

Parameter	Definition	Sensory Perception
Hardness (kg or N)	Peak force during the first compression cycle, **h1**	Force required to compress the food between the teeth (molar)
Cohesiveness	Ratio of the energy during the second compression (to peak force) to the one during the first compression cycle, **A2/A1**	Deformation degree to which a food is compressed between the teeth (molar) from the second bite, prior rupture
Adhesiveness (kg or N)	Negative force area representing the energy required to pull the compression plate away from the sample, **A-1**	
Springiness	Height recovered by the sample during the time elapsing between the end of the first compression cycle and the beginning of the second one, **d2/d1**	Ability of the sample, once deformed, to recover its initial non-deformed condition
Gumminess (kg or N)	Product of Hardness × Cohesiveness	
Chewiness (kg or N)	Product of Gumminess × Springiness	Force required to disintegrate a semisolid food until it is ready to be swallowed

Table 2.2 TPA parameters and their corresponding sensory perception.

As previously stated, considering the minor contribution given by the connective tissue, the myofibrillar component of the muscle is the main factor exerting a relevant role in determining poultry meat tenderness. Thus, not only sarcomere length but also the extent of the proteolytic processes taking place within the *post-mortem* period were assessed.

2.5.5 Sarcomere length

Sarcomere length, considered as a marker of the contractile state of the muscle tissue, was found to be highly correlated with meat tenderness assessed by MORSE and Allo-Kramer shear tests (Cavitt *et al.*, 2004) (*See Par. 2.5*). As shown in **Figure 2.15**, sarcomere length can vary within the range from 1.6 to 3.6 µm, being the first the width of the thick filaments with the Z-lines touching the end of the myosin and the second a fully extended sarcomere with little or no overlap between the thick and thin filaments (Kerth, 2013).

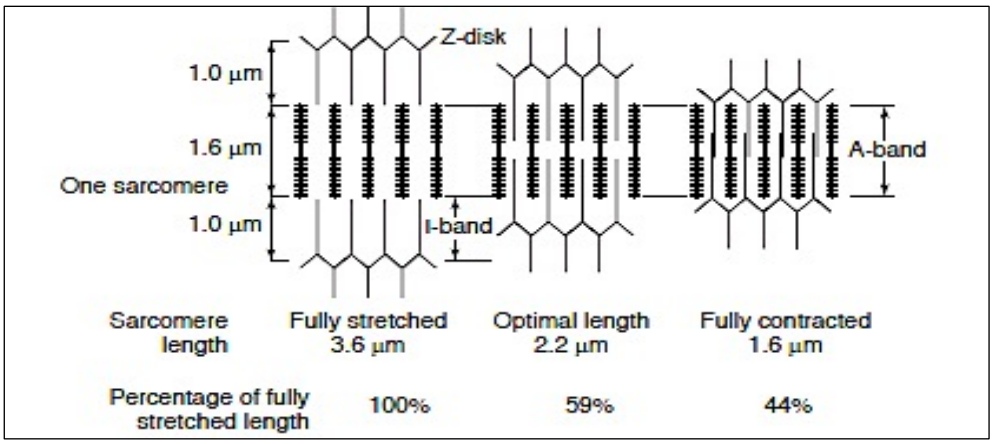


Figure 2.15 Sarcomere length and contractile state (Kerth, 2013).

Sarcomere length has been previously measured by different methods on muscle types belonging to various animal species such as lamb *Longissimus thoracis et lumborum* (Hopkins and Thompson, 2001), pork *Longissimus* (Pomponio and Ertbjerg, 2012), beef *Semimembranosus* (Farouk *et al.*, 2012) and chicken breast and leg (Yu *et al.*, 2005) muscles. Many methods were developed for measuring sarcomere length. Among them, the

microscopic examination of a histological-embedded section and the laser diffraction method are the most widely used. The last, assuming a cylindrical shape for both myofibrils and muscle fibers, is based on measuring the projected diffraction bands generated by a collimated monochromatic light passing through the sample. In detail, the arrangement of the actin and myosin filaments within the sarcomere structure results in a diffraction pattern used as a coefficient to calculate sarcomere length (Yeh *et al.*, 1980).

Sample preparation was carried out following the procedure described by Liu *et al.* (2014) with slight modifications. Briefly, 10 ml of formalin solution (35 g/L formaldehyde in 85 mM phosphate buffer) were added to 0.6 grams of *Pectoralis major* muscle and homogenized for 30 seconds with a Potter-Elvehjem-Type Tissue Grinder with PTFE Pestle (Tomas Scientific, Swedesboro, NJ). After that, sarcomere length was determined measuring the laser diffraction pattern generated by a drop of the homogenate placed under a Novette 1570-0 Helium-neon gas laser (Uniphase, Manteca, CA) according to the equation proposed by Cross *et al.* (1980):

$$\text{Sarcomere length } (\mu\text{m}) = \frac{\left(632.8 \times 10^{-3} \times \text{D} \times \sqrt{\left(\frac{T}{D}\right)^2 + 1}\right)}{\text{T}}$$

where D being the distance (in mm) from the specimen-holding device to the screen and T being the separation (in mm) between the zero and the first maximum band.

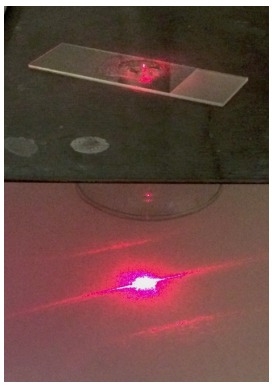


Figure 2.16 Diffraction pattern generated by a collimated monochromatic light passing through the homogenised sample.

According to this equation, the shorter the diffraction pattern projected onto the screen is, the longer the sarcomeres are. On the other hand, short sarcomeres will result in wider distance between the zero line and the maximum band.

Sarcomere length and *post-mortem* proteolysis of the main myofibrillar proteins account for the main variation observed in meat tenderness (Koomaraie *et al.*, 2002). Indeed, several evidences suggested that the proteolytic degradation of key myofibrillar proteins responsible for maintaining the structural integrity of the myofibril and involved in: i) inter-myofibrillar bonds (desmin and vinculin), ii) intra-myofibrillar bonds (titin, nebulin and troponin T), iii) connecting myofibrils to the sarcolemma (vinculin and dystrophin), and iv) attaching the muscle cells to the basal lamina (laminin and fibronectin) would result in myofibrils weakening and, thus, meat tenderization (Price, 1991; Koomaraie *et al.*, 2002).

2.6 *Post-mortem* protein degradation

Meat tenderizes after slaughter due to the presence of endogenous proteolytic enzymes. The process has been suggested to be multi-catalytic in nature (Ouali *et al.*, 2013; Kemp and Parr, 2012) including the action of calpains, cathepsins, caspases and the proteasome. Strong evidences suggest the involvement of the calpain system in *post-mortem* cleavage of myofibrillar and myofibrillar-associated proteins (Koohmaraie and Geesink, 2006; Huff-Lonergan *et al.*, 2010).

Calpains are a large family of calcium-dependent proteases divided into two groups according to their ubiquity or tissue-specific distribution (Sorimachi and Suzuki, 2001). In mammalian muscles two ubiquitous isoforms have been identified and termed as μ- and m-calpain referring to the micro- and millimolar calcium concentration required for their activation (Lee *et al.*, 2008). Calpains are heterodimers consisting of a 30 kDa subunit and a large catalytic site having molecular mass of approximately 80 kDa. At low ionic strength and in the presence of calcium, calpains are activated through partial autolysis that initially reduces the mass of the 80 kDa subunit to 76 kDa, through a 78 kDa intermediate, and the mass of the 30-kDa subunit to

18-kDa (Suzuki *et al.*, 1995; Lee *et al.*, 2007). This partial autolysis reduces the calcium requirement for proteolytic activity but does not affect its specificity. On the other hand, further autolysis leads to more extensive degradation of the large sub-unit and results in a loss of its proteolytic activity (Inomata *et al.*, 1986; Edmunds *et al.*, 1991; Koohmaraie, 1992a; Veiseth *et al.*, 2001).

In broiler pectoral muscles, although the isoform homologous to m-calpain is not translated, a calpain isoform exhibiting calcium sensitivity intermediate between those of mammalian μ-calpain and m-calpain and, thus termed as μ/m-calpain, was observed (Sorimachi *et al.*, 1995). In addition, according to previous studies performed by Lee *et al*. (2007; 2008), a calpain band exhibiting a slightly greater mobility than that of μ/m-calpain was detected through casein zymography and, thus, ascribed to a partially autolyzed μ/m-calpain form (Zhao *et al.*, 2016).

Considering that the *post-mortem* proteolytic degradation of the myofibrillar proteins might significantly affect meat quality, protein degradation was extensively studied.

2.6.1 Myofibril Fragmentation Index

Myofibril Fragmentation Index (MFI) defines the degree of myofibril fragmentation to shorter fragments during meat aging. Involving the measurement of the turbidity of samples adjusted to a common protein concentration (Olson *et al.*, 1976), this index reflects the degradation of key structural proteins composing the I-band of the sarcomere (Taylor *et al.*, 1995) and highly correlates with tenderness (Culler *et al.*, 1978). MFI was determined according to the procedure described by Culler *et al.* (1978) with slight modifications (Lametsch *et al.*, 2007).

In detail, 1.5 grams of meat were placed in ice-cold vessels and homogenized in 15 mL of ice-cold buffer (pH 7.0 at 4-5°C) composed as follows: 0.1 mM KCl, 1 mM EDTA, 1 mM NaN_3, 25 mM potassium phosphate (7 mM KH_2PO_4 and 18 mM K_2HPO_4). Samples were homogenized (13,500 rpm for 30 s), in two burst, using an Ultra-Turrax T25 (IKA Labortechnik,

Germany) with a 30 s break in ice. The homogenates were centrifuged at 1,000 × g for 15 min at 4°C, the supernatant discarded and the resulting myofibrils re-suspended in 10 mL of ice-cold buffer. The procedure was repeated twice and after removing connective tissue and debris by filtering the resulting myofibrils through a 1 mm mesh, protein concentration was assessed by Bradford assay (Bradford, 1976) (*See Par. 2.7.2.4*). Aliquots (in triplicate) of the suspensions were diluted to a final protein concentration of 0.5 mg/mL and the absorbance immediately measured at 540 nm with a 6715 Split Beam UV/Vis Scanning Spectrophotometer (Jenway, UK). Then, MFI was calculated as follows:

$$MFI = Abs\ 540\ nm \times 150$$

2.6.2 Casein zymography

Casein zymography was used to evaluate calpain activity and determine the effect of *post-mortem* storage on the proteolytic activity of μ- and m-calpains in lamb and porcine *Longissimus dorsi* muscles (Veiseth *et al.*, 2001; Pomponio *et al.*, 2008; Pomponio and Ertbjerg, 2012). Proteins were separated under denaturing but non-reducing conditions and then, after incubation with a calcium-containing buffer in order to activate the calpain, allowed to re-nature and consume the substrate (casein immobilized within the gel).

Within this thesis, calpain activity was determined according to the procedure described by Pomponio and Ertbjerg (2012). Each sample was run in duplicate by using 12.5% casein precast gels (Bio-Rad Laboratories, Hercules, CA).

Briefly, one volume (15 μl) of Sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT; pH 6.8) was mixed with 3 volumes (45 μl) of sample. After loading 15 μl of sample, the separation was carried out at 80 V for 3 hours at 4°C in 25 mM Tris, 192 mM glycine, 1 mM EDTA (pH 8.3) running buffer. The gels were subsequently incubated in 100 mL of incubation buffer (50 mM Tris, 10 mM monothioglycerol, 4 mM $CaCl_2$; pH 7.5) and shaken at room temperature for 1 hour (3 changes of buffer). Gels were

then washed overnight (16 h) in 20 mM Tris and 10 mM EDTA (pH 7.0) in order to stop the calpain activity. After staining with Coomassie Brilliant Blue G-250 and destaining with water (overnight) the calpain activity was visualized as clear bands against a blue background. The result for each band was expressed as relative intensity (%) considering the band assigned to native μ-m calpain in NB samples at 10 h *post-mortem* as 100%.

2.6.3 Western-Blot against desmin

Western Blot is a widely used analytical procedure to detect and quantify specific proteins in a muscle sample. First introduced by Burnette (1981), Western Blot against myosin and titin was successfully applied to study protein degradation occurring in beef muscles (Bandman and Zdanis, 1988). Within this thesis, Western-Blot against desmin was considered. In the past years, a number of *post-mortem* changes in muscle proteins have been identified. In detail, the myofibrillar proteins such as desmin, troponin T, titin, nebulin, and vinculin all will become partially or completely degraded during the first week *post-mortem* (Huff-Lonergan *et al.*, 2010).

Having a molecular mass of 53 kDa and attaching the myofibrils to the Z-disk, desmin is responsible for maintaining the lateral structural integrity of the muscle fiber and is considered one of the main target proteins for calpain (Lazarides and Hubbard, 1976; Li *et al.*, 1997). In addition, intact desmin was found to be negatively correlated with the 76-kDa-product resulting from calpain autolysis. In addition, previous studies performed on porcine, bovine and poultry muscles demonstrated that intact desmin positively correlates with drip loss (Verrez-Bagnis *et al.*, 1999; Kristensen and Purslow, 2001; Melody *et al.*, 2004; Huff-Lonergan *et al.*, 2005; Zhang *et al.*, 2006; Wojtysiak and Połtowicz, 2006; Wojtysiak *et al.*, 2008).

2.6.3.1 Protein extraction procedure

Myofibrils were extracted following the procedure described by Joo *et al.* (1999) with slight modifications.

One gram of frozen (-80°C) *Pectoralis major* sample was homogenized using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) (13,500 rpm for 30 s) in 10 mL of cold Rigor buffer (75

mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA) (pH 7.0). The homogenate was centrifuged at 10,000 × g (4°C for 20 min) and the supernatant containing mainly sarcoplasmic protein discarded. The procedure was repeated twice and the final pellet re-suspended in 10 mL of cold Rigor buffer. The protein concentration was determined in triplicates by using RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) and a standard curve prepared with Bovine Serum Albumin (BSA) as a reference.

2.6.3.2 SDS-PAGE separation

In order to avoid any variability in the amount of proteins loaded within each lane, protein concentration was adjusted to 1.54 µg/µl by adding different amount of *rigor* buffer. Then, the samples for SDS-PAGE analysis were prepared according to the procedure described by Liu *et al*. (2014). In detail, 12.5 µl of NuPAGE® LDS (Lithium Dodecyl Sulfate) Sample Buffer (4×) and 5 µl of NuPAGE® Sample Reducing Agent (10×) supplied by Invitrogen (Carlsbad, CA) were added to 32.5 µl of myofibrillar protein samples and the mixture heated at 100°C for 3 min in a dry bath heater. Eight µl of sample (corresponding to 8 µg of protein) and 4 µl of Novex® Sharp Pre-Stained Protein Standard were loaded in a NuPAGE® Novex® 7% (35 kDa-180 kDa) Tris-Acetate gel (Invitrogen, Carlsbad, CA). Gels were assembled in a XCell SureLock® Mini-Cell electrophoresis chamber and run for 80 min at 150 V by using Tris-Acetate Buffer (Invitrogen, Carlsbad, CA). After staining with Coomassie Brilliant blue R-250 and acquiring images by a digital camera, myofibrillar protein bands were identified using a Protein Simple AlphaImager® HP System, CA.

2.6.3.3 Blotting

After being separated through the SDS-PAGE gel (*see par. 2.6.3.2*), the myofibrillar proteins were electrically transferred to an ImmobilonTM-FL Transfer Membrane (Millipore, Bedford, MA) by a semi-dry transfer system. The blotting process was run at 30 V for 1 h at 4°C. After blocking with in 20 mL TBS solution (50 mM Tris, 150 mM NaCl; pH 7.5) and 1 g (50 g/L) of skimmed milk powder at room temperature for 1 h, the membrane was washed with TBS-T (50 mM Tris, 150 mM NaCl, 0.5 g/L Tween-20; pH 7.5)

for 10 min. Then, in order to detect desmin, membranes were subsequently incubated at room temperature for 1 h with 4 µl of a primary antibody, a mouse antidesmin monoclonal antibody (DE-R-11; Santa Cruz, CA, USA) (Christensen *et al.*, 2004), diluted at 1:5,000 in TBS-T and 0.4 gram (20 g/L) skimmed milk powder. After that, membranes were subsequently incubated in dark with 1 µl of a secondary antibody, a Donkey anti-mouse IgG (H+L) antibody (IRDye® 800 CW; Santa Cruz, CA, USA) diluted in 15 mL TBS-T, 0.3 g (20 g/L) of skimmed milk powder and 15 µl (0.01 g/L) SDS for 1 h. After blocking the reaction, the Western Blot membranes were scanned with a LI-COR® Odyssey Infrared Imaging System-CLx (LI-COR Cop, Lincoln, NE) at 800 nm and the relative intensities of each band calculated considering as 100% the intensity of the desmin band detected in NB at 10 h *post-mortem*.

2.6.4 Particle size analysis

Particle size analysis is based on the theory that, when homogenized, the muscle tissue will give a specific fragmentation pattern reflecting its original structure. Protein degradation occurring in *post-mortem* muscles was previously measured by assessing MFI (Olson, *et al.*, 1976) that reflects the degradation of key structural proteins in the I-band of the sarcomere (Taylor *et al.*, 1995). However, the main disadvantage of this method is the time-consuming steps necessary to prepare the sample. In previous studies performed by Lametsch *et al*. (2007) and Karumendu *et al*. (2009), the multi-angle light scattering generated by particles of different sizes was used, instead of the traditional turbidity method, to estimate myofibril fragmentation in porcine and ovine *Longissimus thoracis* and *lumborum* muscles with the major advantage being the simple sample preparation required to perform the analysis.

Laser diffraction is a widely used technique to describe the particle size distribution for materials the particles of which are ranging in size from nanometres to millimetres. According to the Mie theory of light scattering, a laser beam passing through a homogenized-dispersed sample is scattered with an angular variation that is dependent on the particle size: small and high scattering angles result from large and small particles, respectively (Malvern, 2015). Moreover, since particle size can be measured on a meat

homogenate without any further sample preparation, the analysing time can be reduced up to four times (Lametsch *et al.*, 2007).

The particle size distribution was analysed following the procedure proposed by Lametsch *et al*. (2007) with slight modifications.

Each sample (2.5 grams) was homogenized in 20 mL of cold phosphate buffer (100 mM KCl, 20 mM Potassium Phosphate (pH 7.0), 1 mM EDTA, 1 mM MgCl2) at 13,500 rpm using an Ultra-Turrax T25 basic (IKA-Werke, Staufen, Germany). Particle size was measured using a Malvern Mastersizer 3000 (Malvern Instruments Ldt, Worchester, UK). The instrument measures the particle size distribution based on the angular variation in intensity of light scattered as a laser beam passes through a wet dispersed particulate sample. According to the concept of the equivalent sphere, particle size is defined by the diameter of an equivalent sphere having the same volume as the actual particle. The instrument was connected to a wet dispersion unit containing water and the sample was added drop-wise to the unit while stirring before measuring. The refractive index was set to 1.46, absorption coefficient to 0.01, and the particles were considered as non-spherical. After obtaining an obscuration level of 12, ten measurements were collected during ten seconds while stirring. The parameters reported were D10, D50, D90, which are related to the volume-based particle size distribution for which 10, 50, and 90%, respectively, of the particles are smaller than this size. In addition, the volume moment mean diameter D [4,3] in which the proportion of particles for each size is weighted according to their volume when calculating the mean size were reported.

2.7 Chemical composition

2.7.1 Moisture

The moisture content of raw meat was assessed according to AOAC official methods (AOAC, 1990). Briefly, about 5 grams of finely minced meat were accurately weighted in aluminium pans (heated for 1 h at 105°C and allowed to equilibrate to room temperature in desiccator in order to achieve stable weight) and dried in conventional oven for 16 h at 105°C. After allowing to equilibrate to room temperature in desiccator, the samples were

weighted and moisture content calculated according to the following equation:

$$\text{Moisture (\%)} = \frac{(Initial\ weight - Dry\ weight)}{Initial\ weight} \times 100$$

2.7.2 Proteins

Proteins are major organic compounds of the muscle tissue and are responsible for the structural organization and the biological functions of muscle in living animals.

According to their solubility in solutions exhibiting different ionic strengths, muscular proteins can be classified into to three main categories: myofibrillar proteins, sarcoplasmic proteins and the connective tissue or stromal proteins (Toldrà and Reig, 2006; Lee *et al.*, 2010; Sathe, 2012; Kauffman, 2012). Representing approximately 60% of the total protein composing the muscle tissue, myofibrillar proteins are soluble in saline solutions of high-molarity (about 0.6 M). On the contrary, constituting approximately 30% of the muscular proteins and consisting mainly of metabolic enzymes and endogenous proteinases, sarcoplasmic proteins are soluble in water or saline solutions of low ionic strength (< 50 mM). At last, being made up of a very heterogeneous group of constituents responsible for the structural integrity of the muscle and its attachment to the other anatomical elements, connective tissue proteins are mostly insoluble in the above-mentioned solvents (Schreurs, 2000). A summary of the main proteins, their localization and role within the muscle tissue is provided in **Table 2.3**.

Protein	Localization and main role in muscle
Myofibillar proteins	
Myosin	The main constituent of thick filaments
Actin	The main constituent of thin filaments
Titin	Throughout entire sarcomere; responsible for the longitudinal integrity of the sarcomere
Nebulin	Along thin filaments
Tropomyosin	Coiled around thin filaments in association with actin and troponin; regulatory protein
Troponin T, C, I	Coiled around thin filaments in association with actin and tropomyosin; regulatory protein
Vinculin	Attachment of myofibrils to sarcolemma
Filamin, synemin, Z nin, C,H,X,F I proteins	In the Z line; contributes to its high density
Desmin	In Z line; links adjacent myobibrils at the level the Z line
α, β, γ and eu-actinin	Proteins regulating the physical state of actin
Sarcoplasmic proteins	
Mitochondrial enzymes	Enzymes involved in the respiratory chain
Lysosomal enzymes	Digestive hydrolases (catepsines, lipase, phospholipase, etc.)
Myoglobin	Natural pigment of meat
Connective tissue proteins	
Collagen	Protein giving support, strength, and shape to fibers
Elastin	Protein that gives elasticity to such tissues as capillaries, nerves, tendons

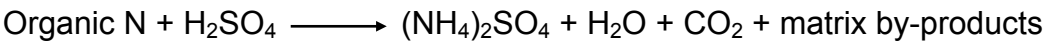
Table 2.3 Localization and function of the main protein composing the muscle tissue.

2.7.2.1 Total crude protein content

Protein content was determined according to the Kjeldahl method (AOAC, 1990). Based on quantifying the total nitrogen content deriving from proteins, amine as well as ammonia and urea nitrogen fractions, the Kjeldahl method is based on a three steps procedure involving an acid digestion of the sample, distillation and titration of the resulting ammonia complex.

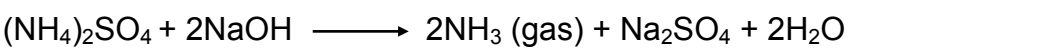
About 0.5 grams of finely minced meat were accurately weighted and placed within a Kjeldahl tube in which half “Kjeltabs” (containing 3.5 grams of K_2SO_4 and 3.5 grams of selenium) and 12 mL of a 96% sulphuric acid and 4% ortho-phosphoric acid solution were added. The addition of an inorganic salt (K_2SO_4) to the digest elevates the boiling point of the H_2SO_4 significantly

increasing the rate of organic decomposition in the digestion mixture and shortening the time required for digestion. The samples were digested gradually increasing the temperature from 80 to at 420°C (50°C/15 min) for 3 h and ammonium sulphate was generated according to the following equation:

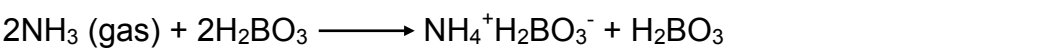


$$\text{Organic N} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + H_2O + CO_2 + \text{matrix by-products}$$

Distillation and titration were performed by using a Vapodest® 50s distil-titrator unit. After adding 50 mL of distilled water the excess acid solution is neutralized through the addition of 30% NaOH solution. Ammonia gas starts to liberate and after being condensed in the distillation unit is captured by boric acid forming an ammonium-borate complex:

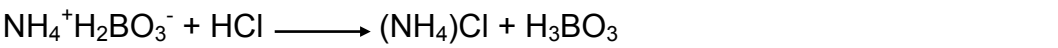


$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3\,(gas) + Na_2SO_4 + 2H_2O$$



$$2NH_3\,(gas) + 2H_2BO_3 \longrightarrow NH_4^+H_2BO_3^- + H_2BO_3$$

Then, after titrating the distilled ammonia by using 0.2 N HCl, protein content was calculated by multiplying the amount of organic nitrogen by a conversion factor of 6.25 (typical for meat and meat products) and expressed as a percentage:



$$NH_4^+H_2BO_3^- + HCl \longrightarrow (NH_4)Cl + H_3BO_3$$

$$\text{Nitrogen content (\%)} = \frac{(S-B)\times N \times 1.4007}{Sample\ weight\ (g)}$$

With, S being the mL of HCl required to titrate the sample, B the mL of HCl required to titrate the blank and N the normality of the acid solution.

Protein content (%) = Nitrogen content (%) × 6.25

2.7.2.2 SDS-PAGE analysis of the sarcoplasmic and myofibrillar fractions

Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a widely used technique applied in order to separate proteins according to their molecular weight. When proteins are separated in the presence of SDS and denaturing agents, they become fully denatured (assume a long, rod-like shape instead of a complex tertiary conformation) and dissociate from each other. In addition, since SDS non-covalently binds to proteins imparting an overall negative charge and masking their intrinsic charge, the rate at which SDS-bound protein migrates within the gel depends primarily on its size, thus enabling molecular weight estimation.

Myofibrillar and sarcoplasmic protein extracts were prepared following the procedure described by Liu *et al*. (2014) by homogenizing (30 s at 13,500 rpm, in ice) two grams of frozen *Pectoralis major* muscle in 40 mL of cold Rigor Buffer (75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2mM EGTA; pH 7.0). Each sample was run in duplicates. The homogenate was centrifuged for 10 min at 10,000 × g at 4°C and the supernatant collected as the sarcoplasmic protein fraction. The procedure was repeated twice and the resultant pellet, composed by myofibrillar proteins, was re-suspended by homogenization in 20 mL of cold *rigor* buffer. Protein concentration of each extract was adjusted to 1.0 mg/mL and each sample was mixed 1:1 (v/v) with sample buffer (50 mM Tris-HCl, 8M Urea, 2 M Thiourea, 75 mM DTT, 3% (v/v) SDS) (Fritz *et al.*, 1989). SDS-PAGE analysis was run

a) in duplicates, on 15 µg of proteins according to the procedure described by Laemmli (1970) by using 7.5% polyacrylamide hand-cast gels. A molecular weight marker (Precision Plus Standard Proteins, All Blue Prestained, Bio-Rad) was loaded into each gel to assess the molecular weight of the protein bands. Gels were run on a Bio-Rad Mini Protean II electrophoresis apparatus at 110 V constant voltage for about 1 hour by using a Tris-Gly running buffer containing 50 mM Tris, 0.384 M glycine, and 0.1% (wt/v) SDS (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250 (1g/L) containing 40% (v/v) methanol and 10% (v/v) acetic acid in distilled water and destained in distilled water. Gel images were acquired by using a GS-800™ Calibrated Densitometer (Bio-Rad) to quantify

the relative abundance of each protein band.

b) in order to determine the relative abundance and MW of the sarcoplasmic protein fraction by using a Mini-Protean TGX any kDa Stain-Free Gel (BioRad) (4 µg proteins/well). At the same time, in order to accurately quantify the bands ascribed to myofibrillar proteins typically exhibiting both low and high relative abundance, the myofibrillar protein samples were run twice at different protein loads on gels with different polyacrylamide concentrations. Thus, 6 and 22 µg of the extracted samples were respectively loaded in 7.5% (to quantify the bands of high abundance) and 12% (to quantify the bands of low abundance) Mini-Protean TGX Stain-Free Gel (BioRad), selected according to their migration charts. Gels were settled in a Mini-PROTEAN Tetra Cell (BioRad) and run for one h at 100 V with a Tris-Gly running buffer containing 50 mM Tris, 0.384 M glycine, and 0.1% (wt/v) SDS (Laemmli, 1970). After acquiring the images by using a ChemiDocTM MP System (BioRad), proteins molecular weight were determined by comparing their relative mobility against those of the reference proteins (included in the molecular weight marker) and protein bands identified according to the literature by comparing their MW with that obtained by mass spectrometry (Lan *et al.*, 1995; Huang *et al.*, 2011; Zapata *et al.*, 2012). Band fluorescence was analysed by Image Lab Rev 4.0 software. A calibration curve (R^2 value ranging from 0.98 to 0.99) obtained by loading increasing protein concentrations (from 0.125 to 2.0 mg/mL) was prepared on each gel and protein bands exhibiting a fluorescence intensity within the range of the calibration curve were quantified as relative abundance.

2.7.2.3 Protein solubility

Protein solubility is a “thermodynamic parameter defining the concentration of protein in a saturated solution that is in equilibrium with a solid phase, either crystalline or amorphous, under a given set of conditions” (Cohn and Edsall, 1943; Arakawa and Timasheff, 1985). According to its fundamental role in determining several functional properties of meat, such as gelation, emulsification, adhesion and water binding, protein solubility plays a major role in meat processing. Indeed, being fundamental in order to obtain valuable processed meat products (such as comminute and

restructured products), protein solubility is primarily dependent on the interactions between the myofibrillar proteins and the other meat components. In literature, the terms “protein solubility” and “protein extractability” are often considered as synonyms. Indeed, as previously observed by Xiong (2004), once solubilised proteins can also be easily extracted.

Protein solubility might be affected by a number of extrinsic (pH, ionic strength, temperature, the presence of additives) and intrinsic (primarily the amino acids on the protein surface) factors (Riès-kautt and Ducruix, 1997). As a direct consequence of their small dimension, the distribution of charged and non-charged polar groups on their amino acid side-chains and their globular structure, the sarcoplasmic proteins exhibit the highest solubility and naturally dissolves in water or aqueous solutions of low ionic strength (Sathe, 2012). On the other hand, the complex structural arrangement and the interactions existing among the polypeptides composing the myofibrillar proteins confers them a substantial insolubility at low ionic strength. Similarly, the thermally stable cross-links typically observed within collagen (Xiong, 2004) require protracted heat treatments and/or acid/alkaline hydrolysis to partially solubilise it. As previously stated, protein solubility might be influenced by several factors such as pH and ionic strength. Protein solubility is remarkably influenced by the balance of hydrophobic and hydrophilic amino acids exposed on protein surface itself, with the presence of hydrophilic groups conferring good water solubility (*See Figure 2.8*). In addition, the presence of charged amino acidic residues exerts a relevant role in supporting protein solubility. Indeed, lacking of a net charge, proteins are least soluble at pH values equalising the isoelectric point (pI) for muscle proteins (pH = pI). On the other hand, as pH increases (pH > pI) or decreases (pH < pI) far from the isoelectric point for muscle proteins, protein solubility increases.

Because a protein contains multiple charged groups, at low ion concentration its solubility slightly increases as salt (commonly NaCl) is added. This salting-in phenomenon might be explained considering that the additional ions shield protein's multiple ionic charges weakening the

attractive forces between the individual protein molecules, which are responsible for protein aggregation and precipitation. However, a salting-out effect can be observed as higher salt concentrations are achieved. Indeed protein surface charges are screened and surrounded by small ionic elements (such as sodium and calcium), this effect primarily results from the added ions (salt) competing with the protein molecules for solvent (water). Thus, at very high salt concentrations significantly less solvent is available to dissolve other substances, including proteins. However, decreasing the isoelectric point of myosin, high salt concentrations increased the net charge associated to meat proteins (Totosaus *et al.*, 2002; Shen and Swartz, 2010). Overall, the myofibrillar proteins composing the type IIB-white fibers exhibit a higher solubility level in comparison with their type IA-red counterpart. This difference might be ascribed to their different structural arrangement. Indeed, different isoforms of α-actinin (the most important structural protein composing the Z-disks) and other minor structural proteins (such as M-protein, C-protein, H-protein, etc.) as well as slightly less Z-disks were observed within the myofibrils composing the type IIB-white fibers. In addition, white and red fibers are constituted of different fiber type-specific myosin isoforms displaying different morphological features and solubility (Xiong, 2004).

Protein solubility was determined following the procedure described by Warner *et al*. (1997) with slight modifications (Bowker and Zhuang, 2013) and based on extracting the different protein fractions by using solutions of different ionic strengths. In detail, to measure sarcoplasmic protein solubility 1 gram of *Pectoralis major* sample (in triplicates) was homogenized (13,500 rpm × 30 s) by Ultra-Turrax T25 basic (IKA-Werke, Staufen, Germany) in 10 mL of cold 25 mM potassium phosphate buffer (pH 7.2). Samples were then incubated at 4°C for 20 h and, after centrifuging (2,600 × g for 30 min at 4°C) and decanting the supernatant, protein concentration was measured by using the Bradford assay (*see par. 2.7.2.4*) with bovine serum albumin as a standard. Similarly, total protein solubility was determined by homogenising the meat samples in a 1.1 M KI and 0.1 M potassium phosphate buffer (pH 7.2). Then, myofibrillar protein solubility was calculated as the difference in

solubility of total proteins and sarcoplasmic proteins fraction.

Standard curves were created (**Figure 2.18**) by using bovine serum albumin for both total and sarcoplasmic proteins by plotting the absorbance measured at 595 nm of five samples (y-axis) against their known-concentration (mg/mL) (x-axis). In detail, in order to obtain a good linearity, five concentration-points within the range between 0.125 and 1.0 mg/mL (0.125, 0.250, 0.500, 0.750 and 1.0 mg/mL) were considered.

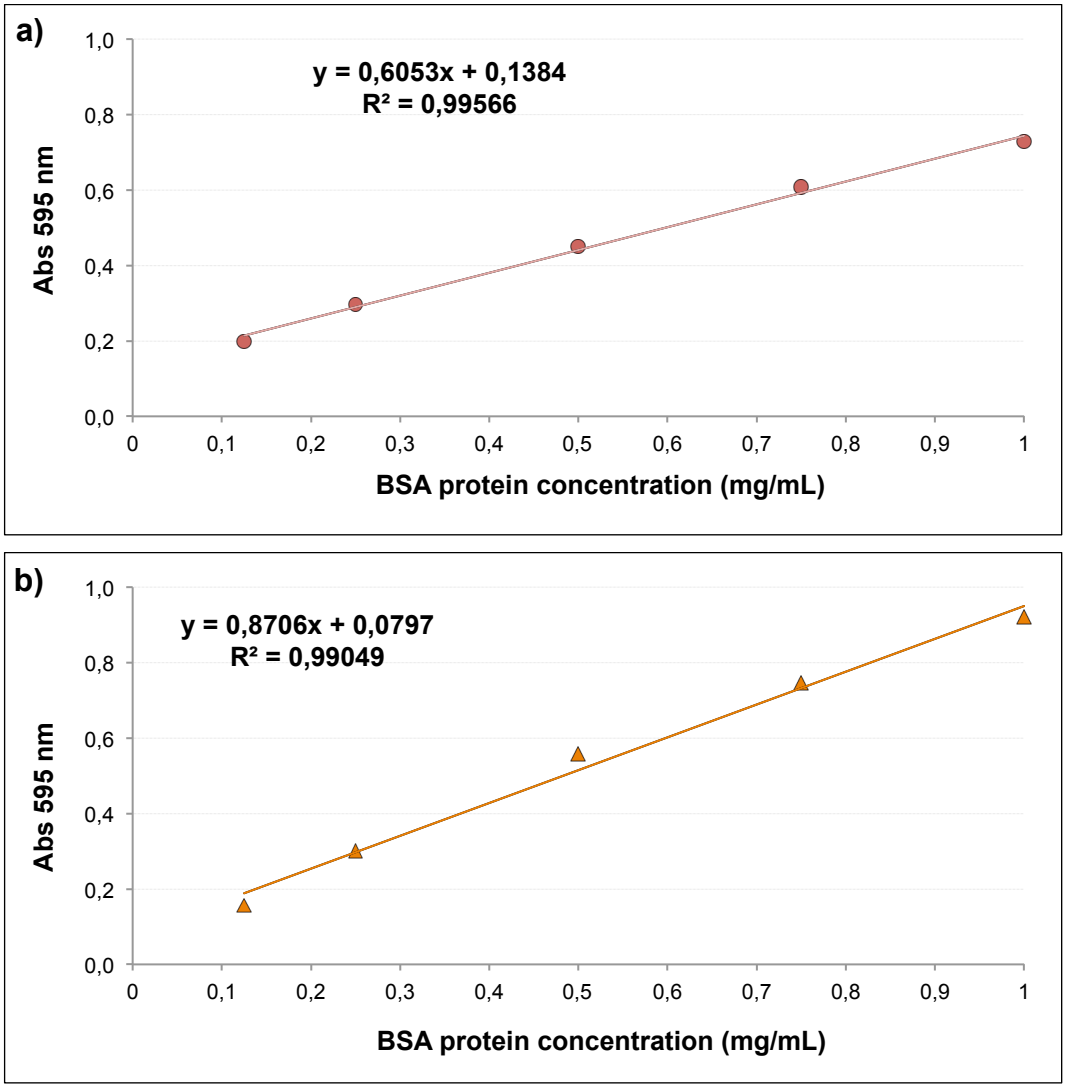


Figure 2.18 Standard curves created by using bovine serum albumin for both total (a) and sarcoplasmic proteins (b).

2.7.2.4 Bradford assay

The Bradford assay is a protein determination procedure based on the binding of Coomassie Brilliant Blue G-250 dye (**Figure 2.19**) to proteins (Bradford, 1976). The dye exists in three forms: cationic (red), neutral (green) and anionic (blue) (Compton and Jones 1985).

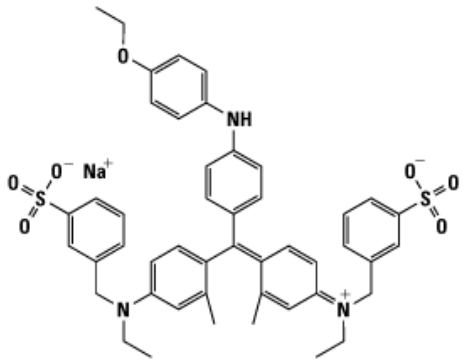


Figure 2.19 Structure of the Comassie Brilliant Blue G-250 Dye.

Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Abs max = 470 nm). However, when the dye binds to the protein, it is converted to a stable un-protonated blue form (Abs max = 595 nm) (Reisner *et al.*, 1975; Fazekes de St. Groth *et al.*, 1963; Sedmack and Grossberg, 1977) that is detected at 595 nm by using a spectrophotometer or a microplate reader (**Figure 2.21**). The blue colour can be measured at any wavelength between 575 and 615 nm. However, at the two extremes (575 and 615nm) there is a loss of about 10% in the measured amount of colour (absorbance) compared to that obtained at 595nm (**Figure 2.20**).

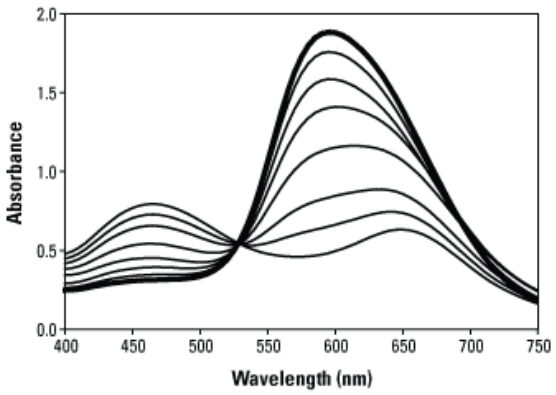


Figure 2.20 Absorbance spectrum of the Coomassie Brilliant Blue G-250 dye binding proteins and exhibiting a maximum absorbance at 595 nm.

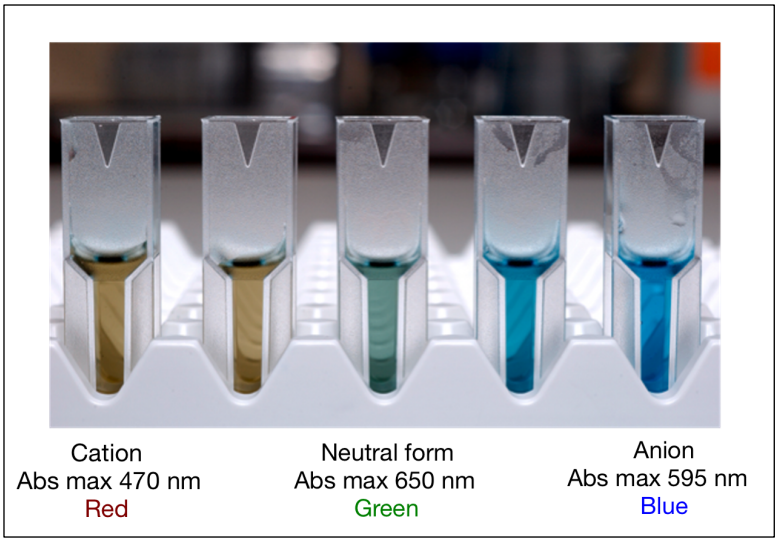


Figure 2.21 Different forms of Coomassie Brilliant Blue G-250 dye.

Coomassie Brilliant Blue G-250 dye primarily binds to basic and aromatic amino acid residues within the protein (primarily arginine, lysine and histidine) (Compton and Jones, 1985). Since Spector (1978) demonstrated that the extinction coefficient of a dye-albumin-complex solution was constant over a 10-fold concentration range, Beer's law may be applied for accurately quantify protein concentration once defined an appropriate dye-to-sample volume ratio.

In this study, protein concentration was determined by using the Quick Start Bradford Protein Assay (Bio-Rad) with Bovine Serum Albumin (BSA) as standard. In any protein assay, the ideal protein to use as a standard is a purified preparation of the protein being assayed. However, in the absence of such an absolute reference protein, another protein must be selected as a relative standard. Since, the best relative standard to use is one that gives a colour yield similar to that of the protein being assayed, we selected BSA exhibiting a linear range within 125-1,000 µg/ml.

The standard protocol can be performed in three different formats: 5 mL and 1 mL cuvette assay and a 250 µl microplate assay. The 1x dye reagent was removed from 4°C storage and, allowed to equilibrate to ambient temperature, was inverted a few times before use. Concentration of protein

standards and unknown sample solutions was measured in triplicates by adding 20 µl of sample/standard to 1 mL of 1x dye reagent. After incubating the tubes at room temperature for at least 5 min (but less than 1 h), the absorbance against a blank sample was read at 595 nm by using a 6715 Split Beam UV/Vis Scanning Spectrophotometer (Jenway, UK).

A standard curve (**Figure 2.22**) was created by plotting the absorbance measured at 595 nm of five BSA-samples (y-axis) against their known-concentration (mg/mL) (x-axis). In detail, in order to obtain a good linearity, five concentration-points within the range between 0.125 and 1.0 mg/mL (0.125, 0.250, 0.500, 0.750 and 1.0 mg/mL) were considered.

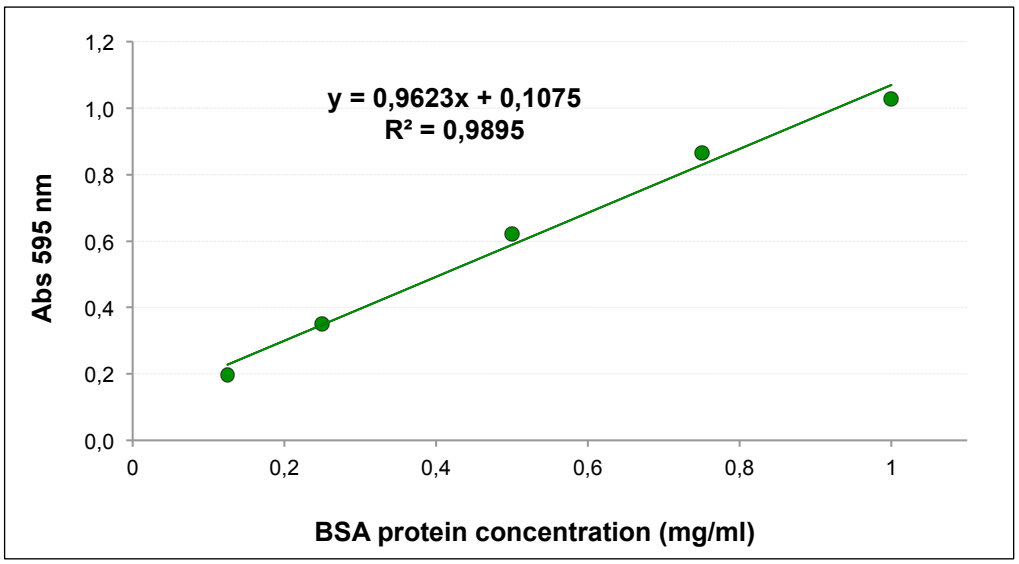


Figure 2.22 Standard curve created in order to quantify protein concentration by using the Bradford assay.

2.7.2.5 Total heme pigments

Total heme pigments were analysed following the procedure described by Hornesey (1956) and expressed as µg hematin/g of meat. This analysis is based on reading the absorbance of an ematin chlorohydrate extract obtained after extracting the heme-group from pigments by using aceton and hydrochloric acid. In detail, 5 grams of meat were homogenized (30 sec at 13,000 rpm) in 20 mL of an aceton:water solution (20:1) and after the addition of 0.5 mL of 37% HCl samples were incubated for 1 h (in a dark

place). Then, samples were filtrated through a Whatman #1 filter paper and the absorbance read at 640 nm (against blank). Total heme pigments were calculated as follows:

$$\text{Ematin (ppm)} = \frac{679.2 \times \text{Abs } 640 \text{ nm} \times 5}{\text{weight (g)}}$$

2.7.2.6 Total collagen content

Collagen fibrils are the major fibrous component of the intramuscular connective tissues. Although several collagen types have been identified, types I and III are the main types involved in fiber formation (observed within the endomysial, perimysial and epimysial layer) whereas type IV represents the main component of the basement membrane linking the fibrous layer of the endomysium to the muscle cell membrane (sarcolemma).

Collagen types contain globular domains and share a common structure of left-handed triple helical segments composed of three α-polypeptide chains, ranging in size from 10 to 150 kDa. As showed in **Figure 2.23**, each α-chain exhibits a repeating tripeptide sequence of Gly – x – y in which x and y are often occupied by proline and hydroxyproline (Bateman *et al.*, 1996).

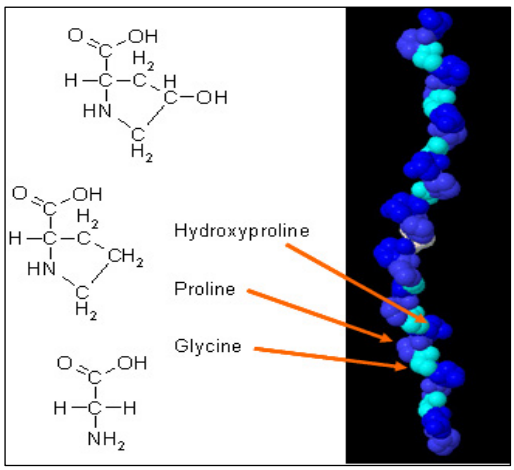


Figure 2.23 Collagen structure.

Within this context, since the amount of hydroxyproline composing the amino acidic sequence of collagen is fixed, collagen content can be derived quantifying hydroxyproline in muscle tissue (Jackson and Cleary, 1967; Gallop and Paz, 1975).

Collagen content was determined by using a spectrophotometric-based hydroxyproline assay firstly described in 1950 (Bateman *et al.*, 1996) and subsequently modified by Kolar (1990). The samples were hydrolysed with sulphuric acid, oxidized by chloramine-T and after reacting with 4-dimethylaminobenzaldehyde, leading to the formation of a purple-red compound, the amount of hydroxyproline was quantified reading the absorbance of the resulting solution at 558 nm.

Four grams of finely minced meat were carefully weighted in a 250 mL round bottom flask and added with 30 mL of a 25% sulphuric acid solution. Then, after covering the round bottom flask with an evaporating glass dish, the samples were heated in oven at 105°C for 16 h in order to digest collagen. The hydrolysed samples (still hot) were diluted with distilled water into a 500 mL volumetric flask and shook to ensure homogeneity. Then, after filtering about 50-70 mL of sample by Whatman 1 filter papers, 5 mL of filtrate were diluted with distilled water into a 100 mL volumetric flask. Two- mL of the final diluted sample were transferred to a test tube in which 1 mL of oxidizing agent (sol. B) were added. The samples were incubated in the dark for 20 min and after adding 1 mL of colorimetric solution (sol. C), the tubes were left to complete the colour development in a water bath (60°C) for 15 min. At last, the absorbance was read at 558 nm by using a 6715 Split Beam UV/Vis Scanning Spectrophotometer (Jenway, UK) and the hydroxyproline content (HYP %) of each sample calculated as follows:

$$\text{HYP (\%)} = \frac{(h \times 2.5)}{(m \times V)}$$

where:

- h = hydroxyproline content (expressed as mg/mL) of the filtrate, derived from calibration curve,
- m = sample weight (in grams)
- V = mL of filtrate diluted to 100 mL

The amount of collagen was then calculated by multiplying the percentage of hydroxyproline by a conversion factor of 8.

The oxidizing agent (sol. B) and the colorimetric solution (sol. C) were prepared as follows:

- Buffer solution (pH 6.0) (A) = 30 g of citric acid monohydrate, 15 g of sodium hydroxide and 90 g of sodium acetate trihydrate were accurately weighted and dissolved in 500 mL of distilled water. Then, 290 mL of 1-propanol were added and after adjusting the final pH to 6.0, the volume was completed with distilled water. If stored at 4°C this solution is stable for about two months.
- Oxidizing solution (B) = 1,4 g of chloramine-T were accurately weighted and dissolved into a 100 mL volumetric flask with the Buffer solution (A). If stored at 4°C this solution is stable for one week.
- Colorimetric reagent (C) = 10 g of 4-(Dimethylamino)benzaldehyde were accurately weighted and transferred into a beaker. Then, 35 mL of perchloric acid 60% and 65 mL of 2-propanol were slowly added while stirring until the content is completely dissolved. One-day stability If stored at 4°C.

2.7.3 Lipids

In muscle foods lipids are a mixture of non-polar components (mainly acylglycerides and cholesterol), free fatty acids and polar lipids (phospholipids and sphingolipids) (Ruiz *et al.*, 2004).

Consisting of a glycerol-based structure linked to three long-chain fatty acids by an ester bond, triglycerides in meat can be classified as simple (when three identical fatty acids are bound to the glycerol molecule) or mixed (in case of different fatty acids). Palmitic (16:0), stearic (18:0) and oleic acid (18:1n-9), representing 20-25%, 10-30% and 30-55% of the total lipids in meat, respectively, are the main fatty acids composing animal triglycerides. In addition, minor amount of myristic (14:0), palmitoleic (16:1n-7), linoleic (18:2n-6), and α-linoleic acid (18:3n-3) can be observed. The fatty acids composition of meat is directly related to animal species and to diet (Stephen and Tume, 2008). If compared to both beef and lamb meat, a higher content of polyunsaturated fatty acids was found within the triglycerides of poultry

muscles (Calkins and Hodgen, 2007). These, leading to unsaturated volatile aldehydes, would contribute to the specific aroma of poultry meat (Noleau and Toulemonde, 1987; Mottram, 1991).

Representing 0.5 to 1% of the total muscle lipids, phospholipids are located within the cell membranes in which they carry out both structural and functional activities. However, this lipid fraction is more prone to oxidize than its neutral counterpart (triglycerides). From a structural point of view, phospholipids exhibit the same structure as triglycerides with a phosphoric acid group interpolated between the glycerol ester and the third fatty acid.

As previously stated, lipids in muscle foods are a mixture of non-polar components (mainly triglycerides and cholesterol), free fatty acids and polar lipids (phospholipids and sphingolipids) (Ruiz *et al.*, 2004). Thus, all the chemical and physical treatments applied for lipids extraction are based on removing them from their binding sites within cell membranes, lipoproteins and glycolipids. In the past years, several methods have been developed for total lipid extraction with the most widely used in meat and meat products being the Soxhlet method, the official AOAC-recommended method (AOAC, 1990) and that based on lipid extraction perfomed through the use of chloroform and methanol solution described by Folch *et al*. (1957). Whatever the method applied is, the solvents used for extracting fat should have a high solubility for all lipid compounds and be sufficiently polar (Smedes *and* Askland, 1999).

2.7.3.1 Soxhlet method

Total crude fat content was assessed on 1.5 grams of finely minced meat, accurately weighted and placed in the bottom on an extraction thimble, covered with cotton wool. A schematic diagram displaying the lipid extraction procedure according to Soxhlet method is showed in **Figure 2.24**. After adding 50 mL of diethyl ether into previously weighted aluminium cups (dried in oven at 105°C for 1 h and let lo equilibrate at room temperature in desiccator) the thimble was placed in a Soxhlet liquid/solid extractor and lowered inside the cup containing the extraction solution.

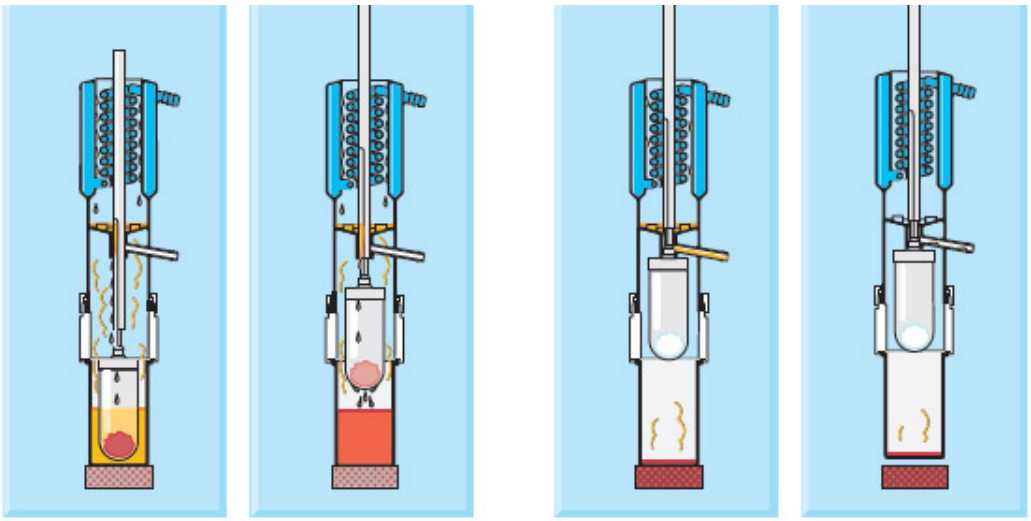


Figure 2.24 Schematic diagram displaying the lipid extraction procedure according to Soxhlet method.

The extraction was performed by recirculating the distilled extraction solvent after condensation for 45 min at 95°C (Boiling: phase 1). After raising up the thimble, a rinsing phase aimed at efficiently remove the remaining soluble matter by dripping the distilled extraction solution over the sample was carried out for 45 min (Rinsing: phase 2). Then, recirculation of the distilled solvent was stop and the cups (containing lipids dissolved in diethyl ether) dried (15 min) over a heating block in order to remove most of the extraction solvent from fat (Recovery: phase 3). The remaining extraction solvent was subsequently collected from the condenser and re-used). The aluminium cups containing lipids were subsequently dried at 85°C for 1 h and, after allowing them to equilibrate to room temperature in desiccator, the lipid content was calculated as follows:

$$\text{Crude fat content (\%)} = \frac{Weight_{C+F} - Weight_{C}}{Sample\ weight} \times 100$$

where $Weight_{C+F}$ and $Weight_{C}$ refer to the weight of the cup containing the extracted lipids (after drying) and the weight of the empty cup, respectively.

2.7.3.2 Folch method

Lipid extraction following the Folch *et al*. (1957) method was performed in duplicates by using an extraction ratio of 20 parts chloroform:methanol (1:1, v/v) to 1 part of meat sample. Briefly, after adding

50 µL of a BHT-methanol solution (12 mg/ml) 10 g of finely minced and frozen sample were mixed with 100 ml of chloroform:methanol (1:1, v/v) solution in a 500 mL glass bottle with screw-cap. and homogenised by Ultra-Turrax at 21,500 rpm for 2 min. The bottle was then placed in oven at 60°C for 20 min before adding 50 mL chloroform and homogenising the sample as before (21,500 rpm for 20 min). The homogenate was then filtered (under vacuum) in order to remove the solid residues mainly composed of proteins. After adding 50 mL of a 0.88% KCl solution, the samples were transferred into a separating funnel and left overnight in order to separate the upper aqueous phase from the lower chloroformic one. The lower chloroform-lipid phase was then filtered through anhydrous sodium sulphate and collected into a flask. After incubating for about 5 h with sodium sulphate anhydrous, the samples were filtered by filter paper and dried by using a rotary evaporator under vacuum.

Lipid content was gravimetrically determined as the ratio between the amount of lipid extracted (into the flask) and the weight of the sample from which they have been extracted, as follows:

$$\text{Lipid content (\%)} = \frac{Weight_F - Weight_I}{Sample\ weight} \times 100$$

2.7.3.3 Fatty acids profile

Transesterification of lipid to fatty acids methyl esters was performed with KOH/methanol solution according to the method described by Christopherson and Glass (1969). Then, fatty acids methyl esters (FAME) were separated by using a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer Instruments, LLC, Shelton, USA) equipped with a Varian CP-SIL88 capillary column (50 m length; 0.25 mm i.d.; 0.20 mm film thickness) (Varian Inc., Walnut Creek, CA, USA) and a flame ionization detector (FID). The operating conditions of the instruments were set as follows: the oven temperature was kept at 140°C for 1 min, increased up to 150°C at a rate of 5°C/min and kept for 10 min, then increased up to 180°C at a rate of 2°C/min and kept for 20 min. The temperatures of both injector and detector units were set at 200°C. Helium, used as a carrier gas, was set at a constant flow of 1.7 ml/min. To identify FAMEs, their retention times were compared to

those of a PUFA-2 FAME standard from animal source (Supelco, Bellefonte, PA, USA).

2.7.4 Ash and minerals

Ashes represent the inorganic residues remaining after that both water and all organic matters are removed by heating. Thus, the ash content directly reflects the total amount of minerals within the muscle tissue. Among the analytical procedures developed to determine the ash content, the most widely used is based on the principle of dry ashing. According to the dry ashing procedure, water and all other volatile components of meat are vaporized, whereas organic substances are burned in presence of oxygen and converted to oxides, sulphates, phosphates, chlorides and silicates. Although most minerals exhibit a fairly low volatility, some (such as iron, lead and mercury) are volatile and might be partly lost during incineration.

Before starting the analysis, ashing sample crucible were heated in a muffle furnace at 525°C for 1 h and, after equilibrating at room temperature in desiccator, accurately weighted. Five-grams of finely minced meat were placed into the ashing crucible and dried in air oven at 105°C for 2 h. Then, the samples were moved into a muffle furnace at 200°C for 1 h and then incinerate at 525°C for 4 h. After cooling to 200°C, the samples were allowed to equilibrate to room temperature in desiccator and ash content expressed on a wet basis, as follows:

$$\text{Ash (\%)} = \frac{M\ ash}{M\ wet} \times 100$$

With M_{ash} being the mass of the ashed samples and M_{wet} the original mass of the wet sample.

Elemental analysis (Ca, Na, P, K, and Mg) was performed using the Inductively Coupled Plasma Optical Emission Spectrometry method (ICP-OES) after microwave digestion of 3.0 g of meat. Samples were digested by microwave using a Milestone ETHOS ONE oven (Milestone, Sorisole, Italy) using 4 mL nitric acid and 1 mL hydrogen peroxide. All reagents were from Merck (Darmstadt, Germany); acids were of Suprapur grade. Elements were

quantified by ICP-OES using a Perkin Elmer Optima 2100 DV instrument, coupled with a CETAC U5000AT+ ultrasound nebulizer for mercury. Two blanks were run during each set of analysis to check for chemical purity, and accuracy of the method was verified with reference materials (CRM GBW 09101, human hair control, Shanghai Institute of Nuclear Research Academia Sinica; CRM 201505 and 201605 Trace Element Whole Blood, Seronorm, Billingsad, Norway). All values of the reference materials were within certified limits. Instrumental detection limits were expressed as wet weight (w.w.). Concentrations of elements in tissues were expressed as mg kg^{-1} breast muscle wet weight.

2.7.5 Free Calcium

Free calcium concentration was determined on both the superficial and deep layer of NORM and WB samples following the procedure described by Pomponio and Ertbjerg (2012) with slight modifications.

Briefly, 25 g of chopped breast meat were centrifuged at 18,000 × g for 30 min at 4°C and the supernatant collected. After that, 60 µl of 4 M KCl were added to an aliquot (3 ml) of the supernatant in order to provide a background ionic strength and the free calcium concentration was measured using a calcium Ion Selective Electrode (ISE) equipped with a reference electrode (perfectIONTM Combination Calcium Electrode, Mettler Toledo AG, Switzerland) having a measurement range from 0.5 µM to 1.0 M. All measurements were run in duplicates at a constant temperature (20 ± 1°C) and a calibration curve was prepared before performing each run in order to convert the output in mV to a calcium concentration in mM.

2.8 Oxidation

The *post-mortem* changes occurring within the muscle tissue include an increase in protein and lipid oxidation levels. Affecting several meat quality traits such as odour, flavour, colour and texture, oxidation is one of the main processes responsible for quality deterioration of muscle foods. Although lipid oxidation in meat has been extensively studied for many years, the influence and the mechanisms of protein oxidation has been largely unexplored (Lund *et al.*, 2011; Zhang *et al.*, 2013).

Oxygen-mediated oxidation in muscle foods proceeds through free-radical reactions comprising an initiation, propagation and termination step (Allen *et al*., 1996; Lund *et al*., 2011). Within this context, since both lipids and proteins are recognized as target molecules for reactive oxygen species (ROS), both lipid oxidation and protein carbonylation level were assessed in order to evaluate the oxidative stability of chicken meat.

2.8.1 Lipid oxidation

Hydroperoxides are the first intermediary products originating from lipid oxidation proceeding through the free radical mechanism (Guillen-Sans and Guzman-Chozas, 1998), which include a primary and a secondary oxidation phase. The primary oxidation phase includes an initiation, propagation and termination steps. In detail, a free radical (L•) is formed from a triglyceride or a free fatty acid molecule by its inter-reaction with O_2 in the presence of an initiator (heat, light and high-energy radiation) (*Initiation step*).

$$LH + O_2 \rightarrow L\bullet + HOO^-$$

$$LH \rightarrow L\bullet + H\bullet$$

The free radical, L•, can subsequently react with O_2 to generate a peroxide radical that, in its turn, after reacting with a triglyceride or a free fatty acid produces hydroperoxyde molecules. The newly formed free radical resulting from this reaction can reinitiate the process leading to propagation (*propagation step*).

$$L\bullet + O_2 \rightarrow LOO^-$$

$$LOO^- + LH \rightarrow LOOH + L\bullet$$

This reaction chain, propagating itself, terminates (*termination step*) when an inactive substance is generated by the inter-reaction of two radicals, as follows:

$$L\bullet + L\bullet \rightarrow L{-}L$$

$$LOO\bullet + L\bullet \rightarrow LOOL$$

Although at the beginning lipid oxidation takes place slowly and uniformly (primary phase), an increased reaction rate is observed within the secondary phase in which carbonyl compounds and a large number of aldehydes, mainly responsible for off-flavours, are formed. As the hydroperoxydes resulting from lipid oxidation are degraded into secondary reaction products

(such as alcohols, aldehydes, ketones and hydrocarbons) malondialdehyde (MDA), a three-carbon dialdehyde is formed. The level of MDA generated in meat can be determined according to the thiobarbituric acid-reactive substances (TBARS) assay (Jo and Ahn, 1998).

The TBA test was firstly described by Kohn and Liversedge (1944) who observed that, when heated in acidic conditions with 2-thiobarbituric acid, aerobically oxidized brain tissue developed a brilliant red colour exhibiting an absorption maximum at 532 nm. On the other hand, in 1954 Turner *et al*. applied for the first time the TBA assay on pork meat.

Within this thesis, TBARS were measured according to the method of Salih *et al*. (1987), with some modifications by Utrera *et al*. (2014).

A 5 grams sample (in triplicates) excised from each *Pectoralis major* muscle was homogenized in 15 mL of trichloroacetic acid (5%, w/v) and 0.5 mL butylated hydroxytoluene (4.2% in ethanol, w/v) in ice bath. The homogenization was performed by an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) at 13,500 rpm for 30 s. After filtering the slurry through a filter paper (Whatman 40, GE Healthcare), an aliquot of 2 mL filtrate (in duplicate) was mixed with 2 mL thiobarbituric acid (0.02 M) in a test tube and boiled in a water bath (100°C) for 40 min. After cooling, absorbance was read at 532 nm by using a 6715 Split Beam UV/Vis Scanning Spectrophotometer (Jenway, UK). A standard curve was prepared (**Figure 2.25**) by using increasing amount of 1,1,3,3-tetraethoxypropane and used to calculate the amount of MDA within each sample. Then, TBARS content was expressed in ppm as mg of malondialdehyde/kg meat.

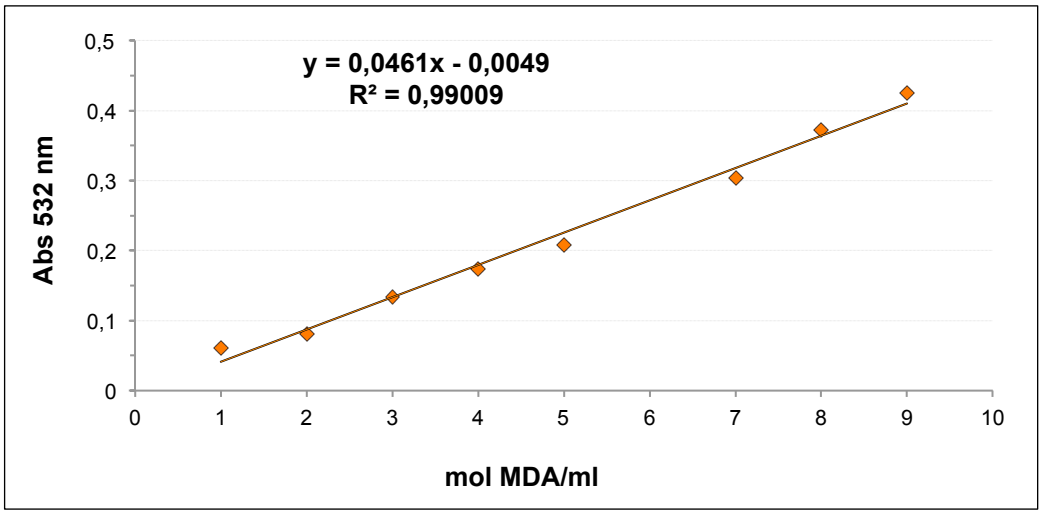


Figure 2.25 standard curve prepared by using increasing amount of 1,1,3,3-tetraethoxypropane and used to calculate the amount of MDA within each sample.

2.8.2 Protein oxidation

Protein oxidation is considered to be an ongoing deteriorative process during storage of fresh and processed meat and it is associated with increased meat toughness and reduced water-holding capacity. Protein oxidation proceeds *via* a free radical chain reaction similar to that of lipid oxidation (Lund *et al.*, 2011) in which a protein carbon-centered radical (P•) is generated from the abstraction of a hydrogen atom by reactive oxygen species (ROS). In the presence of oxygen, this radical is further converted into a peroxyl radical (POO•) and into an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another molecule. Subsequently, alcoxyl radical (PO•) and its hydroxyl derivative (POH) are generated by further reactions with HO_2•.

Overall, the reaction of radicals with proteins and peptides in the presence of oxygen lead to alterations in both proteins backbone and amino acid side chains. In detail, as showed in **Figure 2.26**, protein oxidation results in protein cross-linking and in the formation of various oxidation products such as aromatic hydroxylation, thiol oxidation and formation of carbonyl groups in the amino acid side chains, the latter being widely employed as a general marker for protein oxidation in different muscle foods.

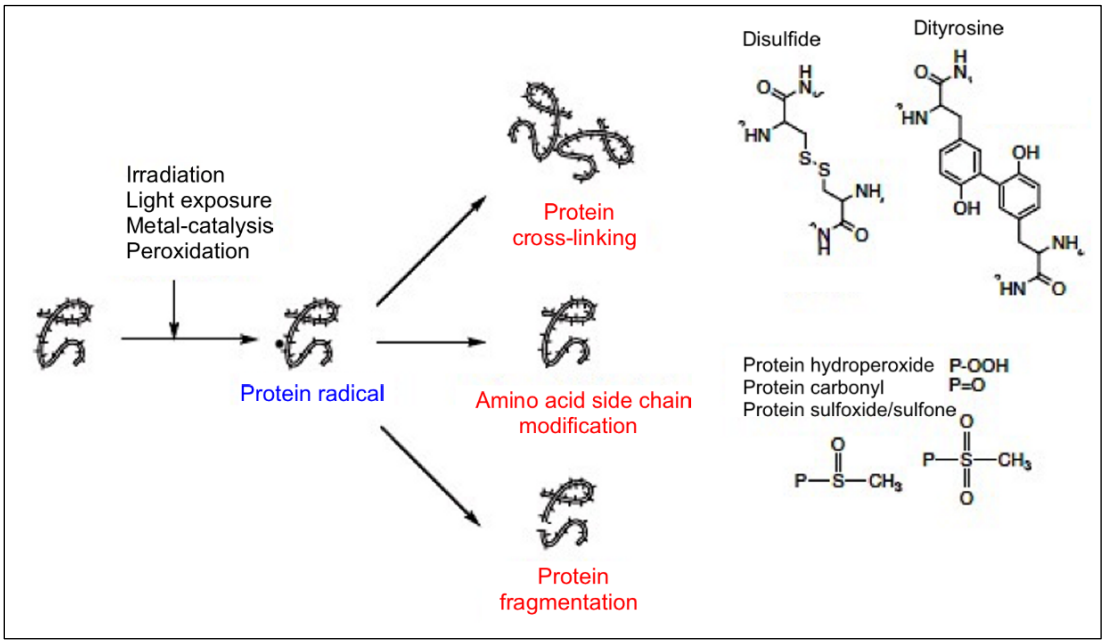


Figure 2.26 Oxidation products resulting from protein oxidation (adapted from Lund *et al*., 2011).

Advanced techniques exist to quantify protein oxidation based either on a spectrofluorometry assay or the measurement of the specific oxidation markers in the form of the carbonyl products a-aminoadipic and c-glutamic semialdehydes (Armenteros *et al*., 2009; Requena *et al*., 2001). The carbonyl species can also be detected after reacting with 2,4-dinitrophenylhydrazine (DNPH) to form protein-bound 2,4-dinitrophenylhydrazones. Oxidation of muscle-derived proteins is also studied immunochemically following derivatization with DNPH, by using anti-dinitrophenyl antibodies (Clausen *et al*., 2009; Rowe *et al*., 2004). In the reaction with DNPH, the formed hydrazones can be quantified spectrophotometrically by measuring the optical absorbance of DNPH at 370 nm using an absorption coefficient (e) of 22,000 M^{-1} cm^{-1}. The sensitivity of the direct measurement is lower than the immunoblot-based procedures, but the colorimetric assay also has advantages such as low cost, no need of advanced equipment and being less time-consuming. Measurements based on the DNPH detection by spectrophotometry enable detection of protein-bound carbonyls in beef (Lindahl *et al*., 2010; Zakrys-Waliwander *et al*., 2012), pork (Morzel *et al*., 2006), chicken (Soyer *et al*., 2010) and fish (Eymard *et al*., 2009). The method is also used to follow protein oxidation in meat after freezing

(Leygonie *et al*., 2012; Soyer *et al.*, 2010; Xia *et al*., 2009) and cooking (Gatellier *et al*., 2010) and in some processed meat products (Ganhao *et al*., 2010; Rodriguez-Carpena *et al*., 2011). The method is robust, but nevertheless attention to the methodology is needed to ensure a precise reflection of the oxidation level of the proteins and to avoid artefacts influencing the results. The detection of protein-bound carbonyls in muscle and meat is challenging due to the presence of proteins with low solubility in myofibrils and connective tissue. In addition, oxidation leads to protein cross-linking that results in aggregates with low solubility.

Muscle structural proteins are especially prone to oxidation reactions resulting in formation of carbonyls (Pazos *et al*., 2011). Therefore, carbonylated structural proteins may account for a considerable proportion of the total oxidized proteins in meat. Carbonyls buried in large protein aggregates and insoluble protein in suspension may, however, not react adequately with DNPH. In the standard method, proteins are precipitated with trichloroacetic acid (TCA) before derivatization with DNPH. We investigated the effect of increasing the solubility of the TCA precipitated protein before derivatization. The treatment included the addition of 5% sodium dodecyl sulfate (SDS), heat treatment at 100°C for 10 min and ultrasonication for 30 min at 40°C (Soglia *et al.*, 2016c).

The novel DNPH-based method developed (Soglia *et al.*, 2016c) was based on the traditional spectrophotometric DNPH assay described by Levine *et al.* (1990) with the major difference being the addition of three treatments to the pellet obtained after TCA precipitation: (a) the addition of 400 µL 5% SDS, followed by (b) heating at 100°C for 10 min and (c) ultrasonication at 40°C for 30 min. The effect of the sequential addition of each step was individually tested in order to understand the contribution to the overall carbonyl labelling attained with the modifications, including the combination of all the additional treatments that will be referred to as the novel method. It should be noted that these treatments were applied to the precipitated proteins from the muscle extract in order to increase the solubility. Moreover, these added steps resulting in protein unfolding lead to the exposure of carbonyl groups from the inner core of the proteins that can

subsequently react with DNPH. To validate the ability of the novel method to reflect an increase in carbonyl content, both methods were applied on minced beef with increasing level of protein oxidation induced by the addition of 100 mM NaClO.

The novel method was as follows: 1 gram (in triplicates) of muscle sample was homogenized in 10 ml of ice-cold 0.15 M KCl solution. Homogenization was performed on ice using an Ultra-Turrax T25 basic (IKA® – WERKE, Labortechnik, Staufen, Germany) homogenizer at 9,500 rpm for 30 s. Five aliquots (100 µL /each) of the homogenate were mixed with 1 ml of 10% TCA and centrifuged at 5,000 × g for 5 min (Eppendorf Centrifuge 5424). After removing the supernatant, 400 µL of 5% SDS were added to the pellet that was subsequently heated at 100°C for 10 min and ultrasonicated (ultrasonic cleaner, VWR US600TH, Leuven, Belgium) at 40°C for 30 min. The samples (3 replicates) were then treated with 0.8 ml of 0.3% (w/v) DNPH in 3 M HCl while 0.8 ml of 3 M HCl were added to the blank (2 replicates). After 30 min incubation, 400 µL of 40% TCA were added to precipitate the proteins and the supernatant was separated by centrifugation at 5000 × g for 5 min. After removing the supernatant, the pellet was washed three times with 1 ml of ethanol-ethyl acetate (1:1, v:v) solution by centrifugation at 10,000 × g for 5 min. These washing steps aimed to remove any free DNPH that could interfere with the spectrophotometric measurement giving an overestimation of the carbonyl content. After the final wash, the resulting pellets were dried with nitrogen and subsequently dissolved in 1.5 ml of 6 M guanidine hydrochloride in 20 mM NaH_2PO_4 (pH 6.5). After incubation overnight at 4°C, the absorbance at 280 and 370 nm was measured with a UV–VIS 1800 spectrophotometer (Shimadzu Corporation, Japan) at 25°C in order to quantify protein concentration and carbonyl content, respectively. Carbonyl content, expressed ad nmol/mg of protein, was calculated according to the following equation (Levine *et al*., 1994) with slight modifications aimed at considering the potential interference given by the hydrazone at 280 nm: $\text{Carbonyls} = \frac{\text{Abs } 370 - \text{Abs } 370_b}{22{,}000 \times [\text{Abs } 280 - (\text{Abs } 370 - \text{Abs } 370_b) \times 0.43]} \times 10^6$

CHAPTER 3
IMPLICATION OF WHITE-STRIPING AND WOODEN BREAST ABNORMALITIES ON QUALITY TRAITS OF BROILER *PECTORALIS MAJOR* MUSCLES

In the past few decades, a remarkable increase in the incidence of abnormalities mainly affecting the *Pectoralis major* muscle was observed. Among them, white-striping (WS) and wooden-breast (WB), respectively defined as the appearance white striations parallel to muscle fibres (Kuttappan *et al.*, 2012) and visually hard, out-bulging and pale areas (Sihvo *et al.*, 2014) on the ventral surface of *Pectoralis major* muscles, are of relevant importance and normally result in meat downgrading.

Within this context, three studies were conducted in order to investigate

- The implications on quality traits of raw and marinated chicken meat (*Animal, 9: 728-734, 2015*) (par. 3.1).
- The histological features, composition and quality traits of the *Pectoralis major* affected by muscle abnormalities (*Poultry Science, 95: 651-659, 2016*) (par. 3.2).
- The oxidative stability, composition and fatty acids profile as well as the NMR-relaxation properties of the abnormal muscles (*Food Research International, 89:1071-1076, 2016*) (par. 3.3).

In addition a fourth study was carried out in order to evaluate:

- Quality traits and gene expression in *Pectoralis major* muscles contextually affected by WS and WB abnormalities and compare them with their unaffected counterpart (*Poultry Science, 95: 2771-2785, 2016)* (par. 3.4).

3.1 Implications of white striping and wooden breast abnormalities on quality traits of raw and marinated chicken meat

Previous studies demonstrated that the white striping and the wooden breast abnormalities shear similar histological features (Sihvo *et al*., 2014). However, although the main quality traits of the white-striped breast fillets have been previously investigated (Kuttappan *et al*., 2012a; Petracci *et al*., 2013a; Petracci *et al*., 2014), no information were available on the effect exerted by the occurrence of wooden breast on quality traits of meat. Therefore, this study aimed at evaluating the effect exerted by white-striping and wooden breast abnormalities, occurring alone or combine within the same *Pectoralis major* muscle, on quality traits and technological properties of raw and marinated meat.

3.1.1 Materials and Methods

Two individual trials were conducted using a total of 192 boneless, skinless, *Pectoralis major* muscles from 52-day-old male Ross 708 broilers (3.7 kg live weight) selected from the deboning area of a commercial processing plant at 3 h *post-mortem*. According to the presence of white-striping and wooden breast abnormalities, the fillets were grouped into four classes based on the criteria proposed by Kuttappan *et al*. (2012b) and Sihvo *et al*. (2014), as follows:

- Normal (N): fillets with neither hardened areas nor white striations on the surface;
- White-striping (WS): fillets that superficially exhibited medium-to-thick white striations on the ventral surface;
- Wooden breast (WB): fillets with diffuse hardened areas and pale ridge-like bulges at the caudal end;
- White-striping and wooden breast (WS/WB): fillets affected by both WS and WB abnormalities.

In each trial, 24 samples / group were selected and transported under refrigerated conditions to the laboratory. Upon receipt at the laboratory, superficial fat, cartilage and connective tissues were trimmed off and the fillets were subsequently stored at 2 to 4°C throughout handling and

measurements. Then, at 24 h *post-mortem* the main quality traits and technological properties of both raw and marinated meat were assessed according to the sampling protocol displayed in **Figure 3.1**, as follows:

Meat quality traits	
Colour	Par. 2.3
Morphometric measurements	Par. 2.1
pH	Par. 2.2
Compression test (kg)	Par. 2.5.2 (a)
Technological properties (Raw meat)	
Drip loss	Par. 2.4.1
Cook loss	Par. 2.4.4
Colour (after cooking)	Par. 2.3
Warner-Bratzler shear test (kg)	Par. 2.5.2
Technological properties (Marinated meat)	
Marinade uptake	Par. 2.4.2
Purge loss	Par. 2.4.3
Cook loss	Par. 2.4.4
Colour (after cooking)	Par. 2.3
Warner-Bratzler shear test	Par. 2.5.2

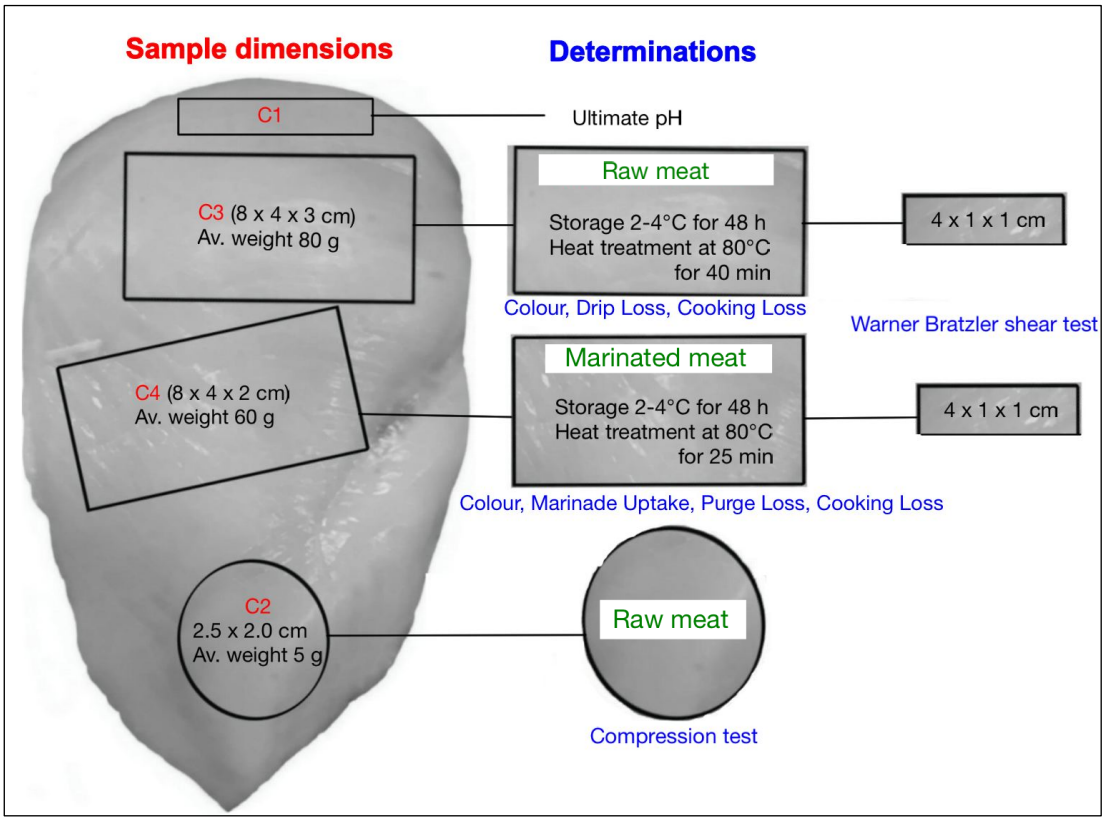


Figure 3.1 Sampling protocol adopted to assess quality traits and technological properties of both raw and marinated meat. In detail: C1 was used to assess ultimate pH; C2 was used for compression test; C3 and C4 were used to assess the technological properties of raw and marinated meat, respectively.

3.1.2 Statistical analysis

Data were analysed by using the one-way ANOVA option of the GLM procedure present in SAS software (SAS Institute Inc, 1988) and by testing the main effects for type of meat quality abnormality (NORM, WS, WB and WS/WB) and replication, as well as the interaction term on meat quality traits. Means were separated using Tukey's honestly test of the GLM procedure (SAS Institute Inc, 1988).

3.1.3 Results

3.1.3.1 Weight, dimension and texture of raw fillets

The results for weight, morphometric measurements and compression of breast fillets are shown in **Table 3.1**.

Table 3.1 Effect of WS and WB abnormalities on weight, dimension and compression force of raw *Pectoralis major* muscles.

Parameter	NORM	WS	WB	WS/WB	sem	*P-value*
Weight (g)	244.7^{b}	305.5^{a}	298.7^{a}	318.3^{a}	3.4	< *0.001*
Length (L)	38.1^{b}	45.7^{a}	43.9^{a}	45.7^{a}	0.4	< *0.001*
Width (W)	24.7^{c}	31.2^{b}	30.5^{b}	33.8^{a}	0.4	< *0.001*
Top Height (H1)	8.2^{b}	8.7^{b}	11.0^{a}	11.6^{a}	0.2	< *0.001*
Middle Height (H2)	195.0	196.5	196.8	196.5	0.9	NS
Bottom Height (H3)	78.7	81.5	80.3	79.9	0.6	NS
Compression force (kg)	2.02^{b}	2.28^{b}	4.02^{a}	3.33^{a}	0.15	< *0.001*

N = 48 samples / group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups (P < 0.05). NS = not significant. sem = standard error of mean.

Fillets affected by WS, WB and WS/WB abnormalities had significantly higher (P < 0.001) weights in comparison with the NORM ones. Although none of the abnormalities had any significant effect on either fillets length or width WB and WS/WB samples exhibited the highest H1 (P < 0.001). As for raw meat texture, if compared to NORM and WS (that did not differed), WS/WB and WB showed significantly higher compression values (2.02 and 2.28 *vs*. 3.33 and 4.02 kg; P < 0.001).

3.1.3.2 Quality traits of raw breast meat

The quality traits of non-marinated breast meat samples are shown in **Table 3.2**.

WS/WB had the highest (P < 0.001) ultimate pH values (6.04). In addition, although the ultimate pH values of WB did not differ from their NORM counterpart, the latter exhibited significantly lower (P < 0.05) values than WS. Considering colour of raw meat, if compared to both WS and WS/WB, the WB group showed higher lightness values (L*) (54.9 and 55.2 *vs*. 57.0, respectively; P < 0.001), whereas NORM exhibited an intermediate value. Moreover, although Redness was not affected by the occurrence of muscle abnormalities, WB fillets had the highest (P < 0.05) Yellowness values, whereas there were no significant differences among the other groups.

Table 3.2 Effect of WS and WB abnormalities on quality traits of raw and cooked *Pectoralis major* muscles.

Parameter	NORM	WS	WB	WS/WB	sem	*P-value*
Raw meat						
pHu	5.80^{c}	5.90^{b}	5.87^{bc}	6.04^{a}	0.01	*< 0.001*
Lightness (L*)	56.0^{ab}	54.9^{b}	57.0^{a}	55.2^{b}	0.2	*< 0.001*
Redness (a*)	1.76	1.72	1.67	1.70	0.05	NS
Yellowness (b*)	2.72^{b}	2.70^{b}	3.27^{a}	2.64^{b}	0.08	*0.017*
Drip Loss (%)	0.93^{b}	0.72^{c}	1.19^{a}	1.03^{b}	0.03	*< 0.001*
Cooked meat						
Cooking Loss (%)	21.6^{c}	24.7^{b}	28.0^{a}	29.5^{a}	0.4	*< 0.001*
Shear force (kg)	2.37	2.35	2.19	2.21	0.04	NS
Lightness (L*)	84.0	83.7	84.0	83.5	0.1	NS
Redness (a*)	1.64	1.81	1.79	1.85	0.03	NS
Yellowness (b*)	8.01^{b}	8.30^{ab}	8.43^{a}	8.47^{a}	0.05	*0.001*

N = 48 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups (P < 0.05). NS = not significant. sem = standard error of mean.

Compared with the NORM group, WS/WB fillets had similar drip losses, whereas WB and WS fillets exhibited significantly higher (P < 0.05) and lower (P < 0.05) values, respectively. In general, all types of abnormalities showed higher (P < 0.001) cooking losses compared with NORM fillets. In particular, WB or WS/WB fillets had the highest (P < 0.001) cooking losses. However, the presence of abnormalities had no effect on the shear force or colour of cooked meat with the only exception of yellowness, which was higher (P < 0.05) in WB and WS/WB than in NORM fillets.

3.1.3.3 Quality traits of marinated breast meat

The results of marinade uptake and quality traits of breast meat following tumbling are presented in **Table 3.3**.

All parameters were significantly (P < 0.001) affected by the occurrence of WS and WB, alone or combined within the same *Pectoralis major* muscle, with the only exception being the colour parameters of cooked meat. Indeed, if compared to NORM, all the abnormal groups showed lower (P < 0.001) marinade uptake values. In detail, WB and WS/WB showed lower marinade uptakes than the WS group (6.94 and 6.24 *vs*. 9.33%, respectively;

P < 0.001). In addition, together with the impaired ability to absorb marinade solution, WB and WS/WB fillets also exhibited greater cooking losses if compared with both WS and NORM (17.4 and 18.7 *vs.* 15.3 and 15.0%, respectively; P < 0.001). On the other hand, fillets affected by WS/WB showed significantly lower (P < 0.001) purge loss in comparison with NORM and WB fillets, whereas WS showed a somewhat lower value than NORM and WB, but not statistically significant.

Considering the shear force of cooked meat, the samples exhibiting both WS and WB defects showed the highest shear force values in comparison with both NORM and WS groups (1.63 *vs*. 1.25 and 1.38 kg, respectively; P < 0.001), whereas WB exhibited an intermediate value (1.45 kg).

Table 3.3 Effect of WS and WB abnormalities on quality traits and technological properties of raw and cooked marinated *Pectoralis major* muscles.

Parameter	NORM	WS	WB	WS/WB	sem	*P-value*
Raw meat						
Marinade Uptake (%)	13.15^{a}	9.33^{b}	6.94^{c}	6.24^{c}	0.26	*<0.001*
Purge Loss (%)	1.30^{a}	1.20^{ab}	1.30^{a}	1.10^{b}	0.02	0.007
Cooked meat						
Cooking Loss (%)	15.3^{b}	15.0^{b}	17.4^{a}	18.7^{a}	0.2	*<0.001*
Total Yield (%)	94.5^{a}	92.0^{b}	87.3^{c}	85.6^{c}	0.4	*<0.001*
Shear force (kg)	1.25^{b}	1.38^{b}	1.45^{ab}	1.63^{a}	0.03	*<0.001*
Lightness (L*)	84.6	84.8	84.8	84.2	0.1	NS
Redness (a*)	2.14	1.88	1.84	1.91	0.04	NS
Yellowness (b*)	10.9	10.9	10.9	11.2	0.1	NS

N = 48 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups (P < 0.05). NS = not significant. sem = standard error of mean.

3.2 Histology and composition of chicken *Pectoralis major* muscle affected by white-striping and wooden breast abnormality

The white-striping abnormality has been the subject of several studies investigating its impact on consumer acceptability (Kuttappan *et al.*, 2012a), histological traits (Kuttappan *et al.*, 2013a), chemical composition (Kuttappan *et al.*, 2012b; Petracci *et al.*, 2014), protein profile (Mudalal *et al.*, 2014) and

processing abilities (Petracci *et al.*, 2013a). On the other hand, only few studies have evaluated the technological traits (Mudalal *et al.*, 2015) and suitability for further processing of breast muscles affected by WB and there is a lack of information concerning the mineral composition of muscle exhibiting WS and WB abnormalities. Thus, this study was performed in order to evaluate the impact of WB abnormality, occurring alone or combined with WS within the same *Pectoralis major* muscle, on histology and chemical composition of meat with special emphasis on mineral levels and protein profile, which may contribute in explaining the altered texture and water-holding properties of the affected meat.

3.2.1 Materials and Methods

According to the presence or absence of WS, WB and wooden and white-striped (WB/WS) muscle abnormalities, 96 boneless, skinless, *Pectoralis major* muscles were selected, in 2 replicates, from 52-day-old male Ross 708 broilers (3.7 kg live weight) in the deboning area of a commercial processing plant at 3 h *post-mortem*. Consistent with the criteria proposed by Kuttappan *et al.* (2012a) and Sihvo *et al.* (2014) and according to the criteria adopted in the previous study (*see par. 3.1*) the fillets were graded by visual appearance and manual palpation into 4 classes: Normal, WS, WB and WS/WB (in which only severe cases were considered).

3.2.1.1 Muscle histology

Histological evaluations were performed on 3 samples / group for each replication for a total of 24 samples. Approximately one cm^3 from the cranial part of each *Pectoralis major* muscle (from the skin surface) was immediately removed and fixed in 10% formalin buffer for 24 h at room temperature. Specimens were oriented for transverse fiber sectioning, dehydrated in a graded series of ethanol, and embedded in paraffin. From each sample, serial transverse sections (6 µm thick) were obtained, mounted on polylysine-coated slides, and stained with Masson's trichrome. For each section of muscle, the presence of abnormal fibers (giant fibers, fibers with hyaline degeneration, and damaged fibers with round profile) in 10 primary myofibers fascicles (PMF) were assessed, and the levels of myodegeneration were

graded according the same criteria adopted in our previous study (Mazzoni *et al.*, 2015) and showed in **Figure 3.2**:

• Score F1 – mild: abnormal fibers ranging from 2 to 4 for each PMF;

• Score F2 – moderate: abnormal fibers ranging from 5 to 10 for each PMF;

• Score F3 – severe: abnormal fibers represent the majority of the fibers for each PMF.

3.2.1.2 Quality traits

Subsequently, all breast muscles (16 samples / group / replication) were packaged and transported under refrigerated conditions to the laboratory. At 24 h *post-mortem*, the *Pectoralis major* muscles were trimmed from superficial fat and connective tissues and used to assess the main quality traits, technological properties of both raw and marinated meat, as follows:

Meat quality traits	
Colour	Par. 2.3
Compression test (kg)	Par. 2.5.3 (a)
Composition	
Moisture	Par. 2.7.1
Protein	Par. 2.7.2
Fat	Par. 2.7.3.1
Collagen	Par. 2.7.2.6
Ash Minerals (Na, Ca, Mg, K, P)	Par 2.7.4
SDS-PAGE analysis	Par. 2.7.2.2 (b)
Technological properties (Raw meat)	
Drip loss	Par. 2.4.1
Cook loss	Par. 2.4.4
Colour (after cooking)	Par. 2.3
TPA	Par. 2.5.4
Technological properties (Marinated meat)	
Marinade uptake	Par. 2.4.2
Cook loss	Par. 2.4.4

3.2.2 Statistical analysis

The results were statistically evaluated with the ANOVA option of the

GLM procedure present in SAS software (1988). The main effects of meat abnormality (NORM, WS, WB, and WB/WS) and replication, as well as the interaction term on meat quality traits, were evaluated. Means were separated using Tukey's HSD test (multiple range test) of the GLM procedure (SAS Institute, 1988) and, according to the not significant P-values found, the interaction terms were not considered.

3.2.3 Results

3.2.3.1 Histological Analysis

Overall, the histological observations of the pectoral muscles, showed in **Figure 3.2**) correlate with the macroscopic lesions adopted as classification criteria during muscle sampling. Indeed, all NORM breasts not showing either hardened area or white striations on the surface had myofibers with a normal polygonal profile and endo- and peri-mysial connective tissues without remarkable alterations: PMF showed few abnormal fibers (F1). On the other hand, abnormal fibers devoicing from their typical polygonal profile (rounded fibers) were found in correspondence to diffuse hardened areas in WB and with-striations in WS samples. These fibers exhibited variable cross-sectional areas (large fibers and small ones were concomitantly detected) and nuclear internalization (F2). In addition, proliferation and thickening of the perimysial network (fibrosis) as well as an increase in intramuscular fat deposition (lipidosis) were observed. In some cases, multifocal degenerative aspects of some fibers were identified together with inflammatory cell infiltration (F3). At last, both WS and WB/WS samples exhibited profound degenerative myopathic lesions together with replacement of the chronically damaged muscle with adipocytes and fibrosis. In the present study, the microscopic observations showed a complete reorganization of the skeletal muscle structure characterized by replacement of muscle fibers with boundless proliferation of peri- and endo-mysial connective tissue (as evidenced by staining with Masson's trichrome). As a result of the severe fibrosis, connective tissue was the most abundant tissue in PMF. Rounded-profile degenerative or necrotic fibers appearing decreased in both number and cross-sectional area, showed a rounded profile and were

by an interstitial inflammatory infiltrate. Interestingly, a clear and gradual decrease of the histopathological lesions and a modified muscle architecture were observed moving from the surface toward the inner section of the F3 samples (**Figure 3.2D**).

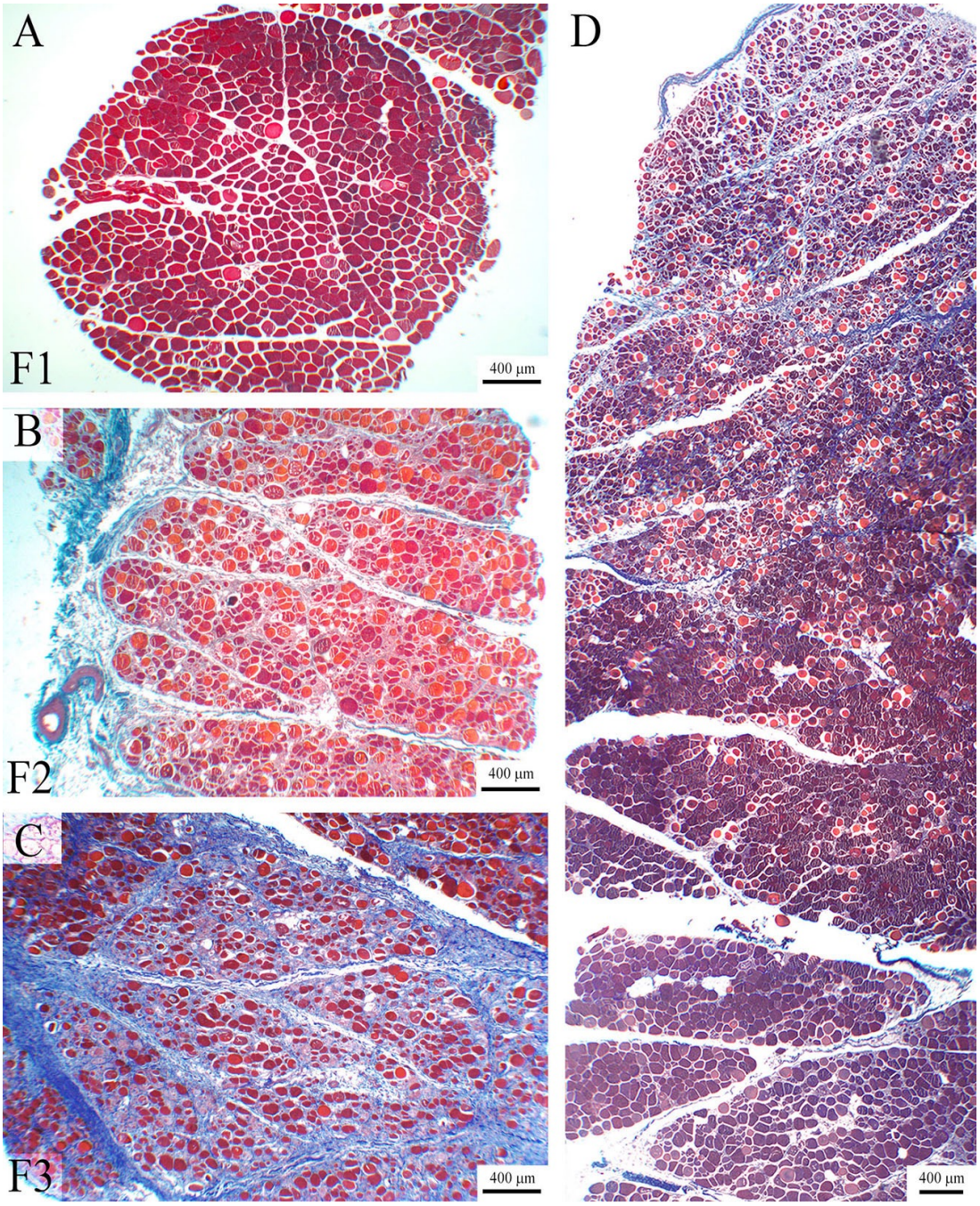


Figure 3.2 Images of mild (A), moderate (B), and severe (C) samples in the histological scale (ranging F1 to F3) used to score the levels of myodegeneration (Masson's Trichrome). A = The polygonal muscle fibers in an F1 score are well packaged and relatively of the same size. B = The fibers show different diameter and the perimysial connective tissue is thickened. C = The number of muscle fibers is reduced; variably sized muscle fibers are rounded and separated or replaced by a loose or more organized connective tissue. In (D) a sample is represented (score F3) with gradual progression of the histopathological lesions. From the surface (upper part image) up within the muscle (lower part of the image) the histological lesions are gradually disappearing (Masson's Trichrome). Score F1 – mild: abnormal fibers ranging from 2 to 4 for each PMF (A); Score F2 – moderate: abnormal fibers ranging from 5 to 10 for each PMF (B); Score F3 – severe: abnormal fibers represent the majority of the fibers for each PMF (C).

3.2.3.2 Chemical Composition (Proximate, Mineral, and Collagen)

The occurrence of WS and WB abnormalities significantly affect the proximate and mineral compositions of breast meat (**Table 3.4**) with the changes being more pronounced when the WS and WB abnormalities occur together within the same *Pectoralis major* muscle.

The findings for chemical composition evidenced that the fillets affected by muscle abnormalities exhibited higher fat and collagen content coupled with an overall reduction in proteins. In detail, if compared to NORM, WB and WS/WB samples exhibited significantly higher ($P < 0.001$) moisture content. Moreover, a significant ($P < 0.001$) decrease in protein and ash was found moving from NORM to WS/WB samples, whereas fat and collagen levels revealed an opposite trend.

Table 3.4 Effect of WS and WB abnormalities on chemical and mineral composition of *Pectoralis major* muscles.

Parameter	NORM	WS	WB	WS/WB	sem	*P-value*
Moisture (g kg^{-1})	741^{b}	742^{b}	753^{a}	751^{a}	1.7	*<0.001*
Protein (g kg^{-1})	228^{a}	219^{b}	214^{b}	204^{c}	1.9	*<0.001*
Fat (g kg^{-1})	8.7^{c}	13.6^{b}	12.5^{b}	19.8^{a}	0.78	0.007
Ash (g kg^{-1})	13.7^{a}	13.1^{ab}	12.6^{bc}	12.1^{c}	0.16	*<0.001*
Collagen (g kg^{-1})	10.9^{c}	11.4^{bc}	11.8^{b}	12.6^{a}	2.8	*<0.001*
Na (mg kg^{-1})	393^{b}	475^{b}	637^{a}	741^{a}	37.9	*<0.001*
Ca (mg kg^{-1})	84^{b}	103^{ab}	201^{a}	131^{ab}	21.9	*<0.05*
Mg (mg kg^{-1})	364^{ab}	406^{a}	350^{ab}	320^{b}	9.0	*<0.05*
K (mg kg^{-1})	3,754^{ab}	4,117^{a}	3,794^{ab}	3,611^{b}	70.0	*<0.05*
P (mg kg^{-1})	2,202^{a}	2,238^{a}	2,059^{ab}	2,079^{b}	29.1	*<0.05*

N = 32 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups ($P < 0.05$). sem = standard error of mean.

With regard to minerals, the content of sodium (Na), calcium (Ca), magnesium (Mg), potassium (K) and phosphorous (P) of the *Pectoralis major* muscles considered within this study were remarkably affected by the occurrence of muscular abnormalities. In detail, a significantly higher amount of Na was measured in WB and WS/WB samples in comparison with the NORM and WS ones (637 and 741 *vs.* 393 and 475 mg kg^{-1}; $P < 0.001$). In addition, if compared to NORM, WB samples revealed an increased amount

of Ca whereas the WS and WS/WB ones exhibited intermediate values. At last, although it was not possible to identify any clear trend, WS/WB fillets exhibited the lowest Mg, K and P content.

3.2.3.3 Electrophoretic Analysis

The results of SDS-PAGE quantification of sarcoplasmic and myofibrillar proteins obtained from NORM, WS, WB, and WB/WS fillets were expressed as relative abundance to avoid small differences in protein loading (**Tables 3.5 and 3.6**). Moreover, in order to minimize the inter-gel variability, calibration curves were created by loading increasing amounts of standard BSA within each gel. All the curves had a good linearity ($R^2 > 0.98$) and any band above or below the linear range of the densitometer was not considered for quantification.

Overall, the occurrence of WS and WB abnormalities exerted a relevant effect on the sarcoplasmic and myofibrillar proteins profile of meat. With regard to myofibrillar proteins, it was possible to estimate the relative abundance of 9 bands with MWs ranging from 16 to 220 kDa (**Table 3.5**).

Table 3.5 Effect of WS and WB abnormalities on the relative abundance (%) of the main myofibrillar proteins identified within the *Pectoralis major* muscle.

Protein	MW (kDa)	NORM	WS	WB	WS/WB	sem	*P-value*
Myosin Light Chain-3	16	14.1	14.6	14.1	15.8	0.69	NS
Myosin Light Chain-2	19	1.2	3.0	1.1	1.7	0.22	NS
Myosin Light Chain-1	27.5	12.5^{a}	10.1^{ab}	9.3^{ab}	8.4^{b}	0.48	*<0.05*
Troponin T (fragment)	29	4.1	4.5	4.9	5.0	0.16	NS
Tropomyosin	34	4.7	4.3	5.2	4.6	0.14	NS
Actin	42	34.1	32.2	34.4	34.6	0.91	NS
Desmin	53	5.2	6.1	6.6	6.3	0.25	NS
Troponin T	70	4.9	6.7	6.7	6.5	0.33	NS
Myosin Heavy Chain	220	15.9	17.8	15.6	15.4	0.91	NS

N = 32 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups ($P < 0.05$). sem = standard error of mean.

It is interesting to note that the myofibrillar proteins profile did not differed

among the groups with the only exception being the LC1 slow-twitch light chain myosin (27.5 kDa). In detail, if compared to NORM, the electrophoretic band ascribed to the LC1 fragment revealed a remarkably lower intensity in WS/WB samples.

With regard to sarcoplasmic proteins, the relative abundance of 11 bands with MWs raging from 25 to 114 kDa and 10 bands ranging in size from 25 to 90 kDa was estimated in affected and NORM samples, respectively (**Table 3.6**).

Table 3.6 Effect of WS and WB abnormalities on the relative abundance (%) of the main sarcoplasmic proteins identified within the *Pectoralis major* muscle.

Protein	MW (kDa)	NORM	WS	WB	WS/WB	sem	*P-value*
PGAM	25	7.2^{a}	7.2^{a}	6.3^{ab}	6.2^{b}	0.14	*<0.05*
TPI 1	26.4	7.3	7.3	7.3	6.8	0.14	NS
CA	31.8	9.6	9.0	9.9	9.0	0.28	NS
LDH	34	18.6^{b}	18.6^{b}	21.2^{ab}	22.3^{a}	0.44	*<0.05*
GAP	36	11.2^{b}	13.1^{a}	13.3^{a}	12.8^{a}	0.30	*<0.05*
ALD	39	7.1^{b}	8.4^{ab}	8.6^{ab}	9.8^{a}	0.31	*<0.05*
CK	43	10.9^{a}	10.1^{ab}	9.5^{b}	9.6^{b}	0.14	*<0.001*
PGI	58	7.7^{a}	7.8^{a}	7.3^{a}	5.4^{b}	0.33	*<0.05*
PK	68	5.6	4.9	4.5	4.7	0.18	NS
GP	90	13.6^{ab}	11.7^{b}	11.7^{b}	14.4^{a}	0.35	*<0.05*
Ca^{2+}- ATPase	114	n.d.^{b}	3.2^{a}	3.3^{a}	2.7^{a}	0.30	*<0.001*

N = 32 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups ($P < 0.05$). sem = standard error of mean. n.d. = not detected.

Overall, the findings revealed that the sarcoplasmic protein profile was remarkably affected by the contextual appearance of both WS and WB abnormalities. In detail, if compared to NORM, WS/WB samples exhibited an increased relative abundance of the electrophoretic bands ascribed to phosphoglycerate mutase (PGAM; 7.2 *vs*. 6.2%; $P < 0.05$), creatine kinase (CK; 10.9 *vs*. 9.6%; $P < 0.001$) and glycogen phosphorilase (GP; 7.7 *vs*. 5.4%; $P < 0.05$) coupled with a lower amount of lactate dehydrogenase (LDH; 18.6 *vs*. 22.3%; $P < 0.05$), glyceraldehyde dehydrogenase (GAP; 11.2 *vs*. 13.3%; $P < 0.05$) and aldolase (ALD; 7.2 *vs*. 9.6%; $P < 0.05$).

3.2.3.4 Texture Profile Analysis

The findings for TPA on cooked meat revealed that significantly higher ($P < 0.05$) hardness, gumminess, springiness and chewiness values were measured in WS/WB and WB, the latter exhibiting the highest springiness and chewiness values (**Table 3.7**).

Table 3.7 Effect of WS and WB abnormalities on the textural traits of cooked *Pectoralis major* muscle.

	NORM	WS	WB	WS/WB	sem	*P-value*
Hardness (kg cm^{-2})	19.1^{b}	17.4^{b}	22.1^{a}	21.6^{a}	0.53	*<0.05*
Cohesiveness	2.80	2.81	2.87	2.93	0.07	NS
Gumminess (kg cm^{-2})	52.5^{b}	51.5^{b}	63.3^{a}	61.3^{a}	2.0	*<0.05*
Springiness (mm)	1.66^{a}	1.63^{a}	1.66^{a}	1.58^{b}	0.02	*<0.05*
Chewiness (kg mm)	89.3^{c}	84.3^{c}	107.3^{a}	97.8^{b}	3.7	*<0.05*

N = 32 samples/group. $^{a-c}$ = Mean values followed by different superscript letters differ significantly among the groups ($P < 0.05$). sem = standard error of mean.

3.3 Functional property issues in broiler breast meat related to emerging muscle abnormalities

In order to understand whether any difference between NORM and affected cases could be observed, this study aimed at evaluating the effect of WS and WB muscle abnormalities, occurring alone or combined within the same *Pectoralis major* muscle, on proximate composition, fatty acid profile, lipid and protein oxidative stability as well as the interactions between water molecules and muscle structure, studied through the T_2 relaxation times signals obtained by time domain nuclear magnetic resonance (TD-NMR).

3.3.1 Materials and Methods

Twenty boneless and skinless *Pectoralis major* muscles were selected from the same flock of high breast-yield broilers (males) in the deboning area of a commercial processing plant. Consistent with the criteria proposed by Kuttappan *et al.* (2012a) and Sihvo *et al.* (2014) and according to the criteria adopted in the previous study (see par. 3.1) the fillets were graded by visual appearance and manual palpation into four classes including 5 NORM, 5 WS, 5 WB and 5 WS/WB samples.

At 24 h *post-mortem*, each fillet was trimmed to remove any superficial fat and connective tissue and sampled in order to determine the NMR T_2 relaxation time. Subsequently, the residual part of each fillet was finely minced for 15 s using a grinder in order to obtain a homogeneous meat sample which was stored at −20 °C and used to assess:

Moisture	Par. 2.7.1
Protein	Par. 2.7.2.1
Fat	Par. 2.7.3.2
Collagen	Par. 2.7.2.6
Fatty acids profile	Par. 2.7.3.3
NMR-relaxation properties	Par. 2.4.5
Hematin	Par. 2.7.2.5
Protein oxidation	Par. 2.8.2
Lipid oxidation	Par. 2.8.1

3.3.2 Statistical analysis

The results from proximate composition, fatty acids profile and oxidation of proteins and lipids were statistically evaluated with the ANOVA option of the GLM procedure present in SAS software (1988). The main effect of meat abnormality (NORM, WS, WB, and WB/WS) was evaluated and means separated using the Tukey's HSD test (multiple range test) of the GLM procedure (SAS Institute, 1988). On the other hand, Wilcox test was preferred for interpreting T_2 data, to take advantage of a priori information about relative intensities of intra- and extra-myofibrillar proton pools, which in abnormal samples were expected to be respectively lower and higher than in the corresponding normal ones.

3.3.3 Results

The occurrence of WS and WB muscle abnormalities, either alone or combined within the same sample, exerted a relevant effect on proximate composition of meat. As shown in **Table 3.8**, if compared to NORM fillets, abnormal samples exhibited an overall higher moisture, fat and collagen content to detriment of protein.

Table 3.8 Effect of WS and WB abnormalities on proximate composition of chicken *Pectoralis major* muscles.

	NORM	WS	WB	WS/WB	sem	*P-value*
Moisture (%)	73.78^{c}	74.78^{bc}	77.26^{a}	74.99^{b}	0.34	*<0.001*
Protein (%)	24.65^{a}	22.98^{b}	21.60^{b}	22.06^{b}	0.36	*<0.01*
Fat (%)	0.85^{c}	1.27^{b}	1.07^{b}	1.66^{a}	0.08	*<0.001*
Collagen (%)	1.15^{b}	1.20^{b}	1.42^{a}	1.34^{ab}	0.04	*<0.05*

N = 5 samples / group. $^{a-c}$Mean values followed by different superscript letters significantly differ among the groups (P < 0.05). sem = standard error of mean.

In details, higher moisture and collagen levels were found in WB and WS/WB samples. As for fat, the difference was more pronounced when the WS and WB abnormalities were combined within the same fillet. In fact, even if WS and WB cases did not differ between each other, a substantial increase in fat content was observed going from NORM to WS/WB fillets (0.85 *vs.* 1.66%; P < 0.001). On the other hand, protein content was remarkably lower (P < 0.01) in all abnormal samples which did not differ from each other.

Similarly, the occurrence of WS and WB muscle abnormalities considerably affected the fatty acids profile of meat (**Table 3.9**). Overall, no significant differences were found in total monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) contents. However, the total amount of saturated fatty acid (SFA) was found to be higher in NORM samples than in WS ones (33.03 *vs*. 29.45%; P < 0.05), whereas WB and WS/WB cases did not differ between each other. Referring to the total amount of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), although no significant differences were found between WS and WB cases, a relevant decrease was observed going from NORM to WS/WB samples (1.43 *vs*. 0.89%; P < 0.05). Dealing with that, significant differences (P < 0.01) were found concerning the Δ5 andΔ6 desaturase activity among the four groups. In detail, the Δ5 and Δ6 desaturase activity was reduced when the WS and WB abnormalities were combined within the same fillet, while NORM and WB cases revealed the highest values. Nevertheless, concerning the amount of C18:2 n-6 and C18:3 n-3 fatty acids, involved as precursors for the synthesis of n-6 and n-3 long-chain PUFA, an increasing trend (P < 0.05) was found going from NORM to WS/WB samples (31.30 *vs*. 35.42 and 2.36 *vs*. 3.05%, respectively).

Table 3.9 Effect of WS and WB abnormalities on fatty acids profile of the *Pectoralis major* muscles.

	NORM	WS	WB	WS/WB	sem	*P-value*
C14:0	0.47	0.43	0.46	0.49	0.013	NS
C14:1	0.06	0.07	0.07	0.06	0.005	NS
C15:0	0.10	0.09	0.10	0.10	0.004	NS
C16:0	21.91	20.05	20.40	20.07	0.327	NS
C16:1 cis	0.18	0.15	0.18	0.17	0.015	NS
C16:1 trans	1.62	2.08	2.08	1.64	0.145	NS
C17:0	0.38	0.40	0.40	0.48	0.016	NS
C17:1	0.09	0.08	0.08	0.08	0.003	NS
C18:0	8.29	7.02	8.46	7.35	0.283	NS
C18:1 n-9 cis	22.06	24.88	23.11	23.50	0.521	NS
C18:1 n-9 trans	0.64	0.59	0.58	0.45	0.030	NS
C18:2 n-6 cis	31.30^{b}	33.37^{ab}	31.41^{b}	35.42^{a}	0.613	*<0.05*
C18:2 cis9-trans11	0.02	0.01	0.02	0.02	0.003	NS
C18:3 n-6	0.01	0.01	0.01	0.01	0.001	NS
C20:0	0.20	0.22	0.20	0.21	0.007	NS
C18:3 n-3	2.36^{b}	2.74^{ab}	2.61^{ab}	3.05^{a}	0.077	*<0.05*
C20:1 cis	0.20	0.22	0.21	0.18	0.007	NS
C20:2	0.82	0.59	0.74	0.56	0.043	NS
C20:4 n-6	4.25^{a}	3.26^{ab}	3.98^{ab}	2.73^{b}	0.210	*<0.05*
EPA	0.17^{a}	0.12^{ab}	0.16^{ab}	0.11^{b}	0.008	*<0.05*
C20:3 n-6	0.07^{a}	0.05^{ab}	0.06^{ab}	0.04^{b}	0.005	*<0.05*
C24:0	1.07^{ab}	0.83^{b}	1.24^{a}	0.79^{b}	0.060	*<0.05*
C24:1	0.23^{a}	0.14^{b}	0.20^{ab}	0.14^{b}	0.013	*<0.05*
DPA	0.81^{a}	0.65^{ab}	0.85^{a}	0.54^{b}	0.045	*<0.05*
DHA	0.44	0.31	0.37	0.24	0.030	NS
Altri	1.64	1.23	1.52	1.18	0.088	NS
Σ(EPA+DPA+DHA)	1.43^{a}	1.08^{ab}	1.37^{ab}	0.89^{b}	0.077	*<0.05*
Δ5 e Δ6 desaturase	16.18^{a}	12.01^{ab}	15.24^{a}	9.82^{b}	0.816	*<0.01*
Σ SFA	33.03^{a}	29.45^{b}	31.78^{ab}	29.89^{ab}	0.524	*<0.05*
Σ MUFA	25.06	28.21	26.50	26.21	0.617	NS
Σ PUFA	40.26	41.10	40.20	42.72	0.553	NS
Σ PUFA n-3	3.78	3.82	3.98	3.94	0.060	NS
Σ PUFA n-6	36.46	37.27	36.19	38.76	0.518	NS
n-6/n-3	9.65	9.78	9.10	9.86	0.136	NS

N = 5 samples / group. $^{a-c}$Mean values followed by different superscript letters significantly differ among the groups ($P < 0.05$). sem = standard error of mean. NS = not significant.

The results for total heme pigments, lipid oxidation and protein carbonylation level are shown in **Table 3.10**.

Table 3.10 Effect of WS and WB abnormalities, occurring alone or combined within the same *Pectoralis major* muscle, on lipid and protein oxidation (TBARS and carbonyls) and total heme pigments (hematin) of meat.

Parameter	NORM	WS	WB	WS/WB	sem	*P-value*
Ematin (mg/kg of meat)	41.51^{a}	25.71^{c}	31.52^{b}	26.42^{c}	1.554	*<0.001*
TBARS (mg MDA/kg of meat)	0.22^{b}	0.35^{ab}	0.41^{a}	0.29^{ab}	0.027	*<0.05*
Carbonyls (nmol/mg protein)	1.11^{b}	1.12^{b}	1.35^{a}	1.28^{a}	0.012	*<0.001*
Protein concentration (mg/ml)	0.85^{a}	0.81^{ab}	0.76^{b}	0.67^{c}	0.019	*<0.001*

N = 5 samples / group. $^{a-c}$Mean values followed by different superscript letters significantly differ among the groups (P<0.05). sem = standard error of mean.

In comparison with NORM, an overall decrease ($P < 0.001$) was found in WS, WB and WS/WB samples concerning the total amount of heme pigments expressed as ematin (mg/kg of meat). Moreover, in the present study, even if no significant differences were found in WS and WS/WB samples, the WB group exhibited higher ($P < 0.05$) TBARS value in comparison with NORM (0.41 *vs*. 0.22 mg MDA/kg of meat). At last, a similar trend was observed for protein carbonylation level. In detail, higher carbonyls were measured in WB and WS/WB samples than in NORM and WS ones (1.11 and 1.12 *vs*. 1.35 and 1.28 nmol/mg of protein; $P < 0.001$).

The relative intensity and T_2 transverse relaxation time for proton populations identified by TD-NMR in the samples are in **Table 3.11**. Although no significant difference was found concerning the relative intensity of bound water among samples, both WS and WB samples exhibited higher extra/intra myofibrillar water ratio, with the highest values observed when WS and WB abnormalities were combined within the same *Pectoralis major* muscle. Such increased ratio led to a relative intensity of extra-myofibrillar proton pools, which in abnormal breasts was 18.75% to 124.6% higher than in the normal counterpart. Similarly, if compared to NORM samples, in abnormal breasts, each of the three proton pools was also characterized by significantly higher T_2 relaxation time, with the highest values observed in WS/WB fillets.

Table 3.11 Effect of WS and WB abnormalities on relative intensity (R.I.) and T_2 relaxation time of the protons populations identified through TD-NMR.

	Bound water		Intra-myofibrillar water		Extra-myofibrillar water	
	R.I.	T_2	R.I.	T_2	R.I.	T_2
NORM	3.90 ± 0.33	1.49 ± 0.40	93.69 ± 0.96	46.50 ± 3.78	2.40 ± 1.19	286.28 ± 12.89
WS	3.81 ± 0.32	1.84 ± 0.50	92.12 ± 2.02	48.55 ± 5.76	4.08 ± 2.08*	296.79 ± 23.97
WB	3.93 ± 0.10	2.34 ± 1.03*	92.21 ± 0.81**	50.98 ± 10.43	3.85 ± 0.90*	325.73 ± 28.47**
WS/WB	3.71 ± 0.43	4.70 ± 2.44**	90.90 ± 2.47**	63.50 ± 14.45**	5.39 ± 2.36**	363.85 ± 36.76**

N = 5 samples / group. * = P < 0.05; ** = P < 0.01;

Discussion and conclusions (par. 3.1, 3.2, 3.3)

In these studies, a global investigation of the main effects exerted by the WS and WB abnormalities, occurring alone or combined within the same *Pectoralis major* muscle in broiler chickens, was developed.

In agreement with a previous research (Mitchell, 1999) the findings of these studies evidenced that growth rate might represents one of the most important factors involved in the occurrence of these abnormalities. The relationship existing between muscle growth and the occurrence of WS and WB abnormalities was investigated by assessing weight and morphometric measurements of each *Pectoralis major* muscle. Overall, if compared to their NORM counterpart, all samples affected by muscle abnormalities exhibited significantly higher weights and greater thickness. Consistent with previous findings obtained by Kuttappan *et al.* (2013a), the results of the present study revealed that, similarly to WS, the occurrence of WB was associated with heavier and thicker *Pectoralis major* muscles. As a consequence, this evidence suggested that, within the same flock, the broilers displaying pectoral muscles with higher weights are more prone to the occurrence of WS and WB muscle abnormalities. This can further support the hypothesis that selection for growth rate and breast yield exerts a major role in the occurrence of these emerging aberrations (Petracci and Cavani, 2012). However, although the increased weight did affect neither length nor width, it remarkably influenced the thickness of the pectoral muscles. Overall, this is consistent with previous studies (Lubritz, 1997; Brewer *et al.*, 2012) in which the impact of fillet weight on muscle thickness was greater than that on

length and width. In detail, if compared to both NORM and WS, the ridge-like bulge at the caudal end of the WB samples, previously described by Sihvo *et al.* (2014), led to remarkable increase in the bottom height (H3) that could thus be proposed as a parameter to identify the WB cases. In addition, the hardened consistency that is typically associated with the occurrence of WB and clearly perceived by manual palpation of the meat can be instrumentally quantified through compression test. Indeed, regardless of the presence of WS, WB exhibited higher instrumental hardness than their NORM and WS counterparts that did not differ between each other.

With regard to meat colour, alterations were observed within the *Pectoralis major* affected by muscular abnormalities. WB showed lighter colour (L*) if compared to both WS and WS/WB fillets, whereas NORM exhibited intermediate values. These differences might be partly explained considering the lower amount of heme pigments as well as both the different ultimate pH values and muscle tissue modifications following the strong fibrotic response and the histological degeneration observed within the abnormal muscles. Indeed, higher ultimate pH values were measured in WS without any relevant colour changes detected in comparison with NORM (Petracci *et al*., 2013a). On the other hand, although in agreement with Sihvo *et al.* (2014), WB exhibited higher Lightness and Yellowness, no relevant changes in Lightness were found in WS/WB. This may be explained by the high ultimate pH values observed in *Pectoralis major* muscles affected by both abnormalities, which should be directly associated with an increased meat darkness (Swatland, 2008) and that is partially counteracted by the effects of the muscle abnormalities. Indeed, it seems reasonable to hypothesise that the complete reorganization of the skeletal muscle structure (histologically observed) in *Pectoralis major* affected by abnormalities, might alter the light diffraction responsible for colour perception.

SDS-PAGE analysis revealed a general modification of the glycolytic enzymes in WS/WB *Pectoralis major* muscles. Indeed, an increased expression of the glycolytic enzymes lactate dehydrogenase (LDH), glyceraldehyde dehydrogenase (G3P), aldolase (ALD) and glycogen phosphorilase (PYGL) was observed in WS/WB samples. However, among the glycolytic enzymes, the magnesium-dependent enzymes

phosphoglycerate mutase (PGAM), phosphoglucose isomerase (GPI) and pyruvate kinase (KPYM) were less expressed in WS/WB samples. Furthermore, WB and WB/WS samples exhibited significantly lower pyruvate kinase (68 kDa) and creatine kinase (43 kDa) levels, the latter being used as an indicator of muscle damage. In detail, according to Mitchell (1999), the lower creatine kinase levels can be considered a direct consequence of impaired contractile and metabolic muscular functions resulting from elevated calcium concentrations that inducing cellular breakdown lead to a subsequent loss of its intracellular components. Furthermore, in agreement with Sandercock and Mitchell (2004), the altered Na homeostasis may play a key role in the development of both WS and WB abnormalities. Indeed, the increased Na^{+} and Ca^{2+} levels observed in WB and WB/WS samples might lead to the development of muscle damage resulting in the leakage and loss of various enzymes such as creatine kinase. In addition, if compared to NORM, WS, WB and WB/WS samples exhibited a significantly higher relative abundance of the calcium ATPase (SERCA, 114 kDa), which catalyses the hydrolysis of ATP coupled with the transport of calcium ions across the membrane (Periasamy and Kalyanasundaram, 2007). This outcome is in agreement with Mutryn *et al.* (2015) who recently found that ATPase, Ca transporting, cardiac muscle, slow twitch 2 (ATP2A2) gene is up regulated in birds affected by WB abnormality. Indeed, this gene encodes for SERCA isoform 2a and 2b. Higher concentration of SERCA in WB samples may likely occur in response to increased amounts of intracellular calcium. Mutryn *et al.* (2015) found that parvalbumin is also over expressed in WB birds. This protein acts as a calcium-binder and exerts an essential role in regulating the calcium concentrations within muscle cells. Thus, it is possible that its up-regulation occurs as a compensatory effect to avoid an excessive calcium concentration that has been found to occur in other muscle disorders such as Duchenne dystrophy.

In spite of the increased synthesis of LDH enzyme in WS/WB samples, higher ultimate pH values were found in the *Pectoralis major* affected by muscle abnormalities. These findings, in agreement with previous studies performed by Kuttappan *et al.* (2009) and Petracci *et al.* (2013a) in which higher ultimate pH values were observed in *Pectoralis major* muscles

affected by WS and/or WB abnormality, might be partly explained by considering the negative genetic correlation found between glycogen content (*in vivo*) and breast muscle development (Berri *et al.*, 2005). Indeed, the overall decrease in both rate and extent of *post-mortem* pH decline observed in high growth rate and breast yield hybrids (Dransfield and Sosnicki, 1999; Berri *et al.*, 2001; Duclos *et al.*, 2007) led to delayed acidification and grater ultimate pH values (Berri *et al.*, 2001, 2007). In addition, although associated with a lower glycogen content (Mutryn *et al.*, 2015), the increased ultimate pH value was hypothesised to arise from a change in glucose utilization rather that in its availability (Abasht *et al.*, 2016) suggesting that there was not an increase in the transformation of pyruvate into lactate, as normally expected in hypoxic conditions.

With regard to drip losses, it could be hypothesised that the higher ultimate pH values observed in WS, WB and WS/WB samples should be responsible for an increased ability of meat to retain liquid during storage in comparison with NORM. However, although no modifications were found in either moderate or severe white-striped samples (Petracci *et al*., 2013a), WB had higher drip losses than NORM without any effect ascribable to their ultimate pH values. It could thus be speculated that, regardless of a slightly higher ultimate pH value, an extensive loss of membrane integrity and the contextual presence of a thin layer of fluid viscous material observed in WB (Sihvo *et al.*, 2014) might be responsible for the increased liquid loss during refrigerated storage of meat. However, the relevant increase in ultimate pH observed in WS/WB samples might partially counteract the impaired WHC of meat resulting from the occurrence of muscle abnormalities and result in drip losses similar to those of NORM. However, although the negative impact of breast abnormalities on raw meat quality properties seems to be mitigated by the concomitant rise in ultimate pH, the ability to bind marinade solutions and retain liquid during cooking in both non-marinated and marinated meat were also severely impaired. Within this context, previous studies demonstrated that, as a consequence of a dramatically impaired functionality of the myofibrillar protein fraction, the occurrence of WS was associated with higher cooking losses and lower marinade yields (Petracci *et al.*, 2013a; 2013b; 2014; Mudalal *et al.*, 2014). If compared to WS, the WB samples exhibited

more severely impaired water-holding and water-binding capacities (lower marinade uptake and higher cooking losses). Thus, it seemed reasonable to hypothesise that, if compared to WS, the WB abnormality results in more severe adverse effects on meat quality attributes with the contextual appearance of both abnormalities exerting the most detrimental effect.

The instrumental tests performed in order to investigate the effect exerted by the occurrence of muscle abnormalities on meat texture evidenced that, as shown by the dramatic increase in compression force values, the raw WB samples exhibited a very noticeable hardness. Therefore, compression test can be helpful to objectively establish the presence of the WB abnormality in raw *Pectoralis major* muscles. Intriguingly, the textural traits of WB and WS/WB meat were remarkably altered after applying a heat treatment and evidenced an increased hardness, gumminess, and chewiness values ascribable to the higher cooking losses resulting from an impaired WHC of meat. Indeed, since as a consequence of protein denaturation, heating is normally associated with shrinkage of the muscle structure leading to an increase in packing density of the fibers (Huff-Lonergan and Lonergan, 2005), higher cooking losses are associated with increased muscular shrinkage and meat toughness. At last, the shear force values of both non-marinated and marinated *Pectoralis major* muscles were modestly affected by the presence of WS and/or WB abnormalities. In particular, although higher compression force values were observed in raw WB, only negligible differences were observed for cooked meat. With regard to marinated meat, the differences among the experimental groups were more likely associated with the lower marinade uptake rather than to a direct effect of the WS and WB abnormality.

The histological observations performed on WS and WB *Pectoralis major* muscles evidenced a complete reorganization of the skeletal muscle structure involving the replacement of the damaged and necrotic muscle fibers with proliferation of connective tissue. Indeed, muscle fibers with an abnormal polygonal profile (rounded fibers) were found in correspondence to the white-striped and diffuse hardened areas in WS and WB samples. In addition, the polyphasic myodegeneration was often associated with lymphocytic infiltrations and occasional regenerative processes involving

proliferation and thickening of the endomysial and perimysial connective tissue associated with granulation tissue and increased deposition of loose connective tissue (fibrosis) and fat (Sihvo *et al.*, 2014; Mazzoni *et al.*, 2015). Similar histological features were previously observed in a study performed by Lopes-Ferreira *et al.* (2001) in which fibrosis and lipidosis developed as a result of hypoxia. Furthermore, in agreement with Kuttappan *et al.* (2013a) and Sihvo *et al.* (2014) vasculitis and irregular perivascular infiltrations of lymphocytes (sometimes disrupting the vascular wall) affecting also the endomysial and perymisial connective tissue were observed (Mazzoni *et al.*, 2015).

With regard to meat composition, overall, the occurrence of WS and WB abnormalities led to increased moisture, fat and collagen contents coupled with reduced protein level. Likely, these differences are linked to the alteration in muscle structure observed by examining the histological sections from WS and/or WB affected meats (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014; Velleman and Clark, 2015). In particular, the diffuse thickening of interstitial connective tissue (fibrosis) (Sihvo *et al.*, 2014; Velleman and Clark, 2015) and the increased deposition of intramuscular fat (lipidosis) (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014) corroborate the higher amount of collagen and fat found in WS, WB and WS/WB cases. In addition, the higher moisture content measured in WB samples might be explained considering both the possible occurrence of moderate to severe edema as a consequence of an inflammatory process (Sihvo *et al.*, 2014) and the increased deposition of extracellular matrix glycosaminoglycans found in wooden breast samples by Velleman and Clark (2015). The last, as a consequence of the very high negative charge resulting from being covalently bound to proteoglycan core proteins, ionically interact with water leading to relevant changes in water holding capacity of meat (Velleman and Clark, 2015). Moreover, in agreement with our findings, the profound myodegeneration and reorganization of skeletal muscle structure resulting in replacement of muscle fibers with adipose fat and connective tissue can support the lower protein content.

With regard to fatty acid profile, higher linoleic and α-linolenic acid levels were found in WS/WB samples if compared to NORM. Previous

authors have suggested that main degenerative features undergoing in WS and WB muscles are similar to those observed in muscular dystrophies (Petracci *et al.*, 2015; Velleman and Clark, 2015). Therefore it is interesting to note that significantly higher linoleic acid was also previously found in mdx mice and positively correlated with sarcolemmal damage and oxidative stress as well (Tuazon and Henderson, 2012). However, in the present study both total PUFA and MUFA content was found to be similar within the four groups. This might be due to the accumulation of synthesized PUFA (DHA) in muscle tissues (Ajuyah *et al.*, 1993). Besides, similar results were found in a previous study performed by Jordan *et al.* (1964) where the fatty acid composition of triglycerides was found to be very similar in pectoral muscles from normal and dystrophic chicken. Moreover, in spite of the higher fat content, WS, WB and WS/WB samples exhibited significantly lower amount of total SFA. Interestingly, similar results were found in a previous study performed by Kuttappan *et al.* (2012a) where lower amount of SFA were found in WS samples and no significant differences were observed in the total amount of PUFA between NORM and WS samples. Dealing with that, aside from the amount of precursors, the significant differences found in EPA, DPA and other intermediate products from elongation and desaturation might be related to an overall reduction in Δ5 and Δ6 desaturase activity for WS/WB samples. This was recently found comparing fast- and slow-growing broilers demonstrating a different expression of genes encoding for desaturase enzymes (Sirri *et al.*, 2011; Boschetti *et al.*, 2015). According to our results, since a different expression of the genes involved in main metabolic pathways and calcium homeostasis was found in WS/WB samples (Zambonelli *et al*., 2016), similarly, an overall reduction in the expression of genes encoding for Δ5 and Δ6 desaturase enzymes might be hypothesised.

The variations observed in the amount of total heme pigments, which are associated with catalase activity, might be responsible for the differences found in oxidative stability (Rhee *et al.*, 1996). In detail, a pro-oxidative activity of heme pigments was demonstrated at pH values of relevance for meat and meat products (Kendrick and Watts, 1969; Baron and Andersen, 2002). According to our results, the possibility of heme iron to act as a pro-oxidant rather than the concentration of myoglobin itself in chicken *Pectoralis*

major muscle seems to be responsible for the oxidative stability of meat. In particular, the disruption of muscle cell structure as a consequence of the degenerative myopathic lesions observed in samples affected by muscle abnormalities (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014) might result in damaged porphyrin ring with the consequent breakdown of heme molecule and the release of iron from globin (Miller *et al.*, 1994; Min and Ahn, 2005). In addition, the changes found in muscle structure of WS and/or WB affected meat might result in exposure of phospholipids that, therefore, accelerates the development of lipid oxidation processes (Ladikos and Lougovois, 1990). At last, the widely documented interaction between lipid and protein oxidation (Mercier *et al.*, 1998; Estévez *et al.*, 2008) might explain the increased carbonylation level found in WB and WS/WB cases. In detail, because the onset of lipid oxidation in meat and meat products takes place faster than the oxidative degradation of proteins (Lund *et al.*, 2011), the increased carbonylation level was likely promoted by lipid oxidative processes. Moreover, the reduced oxidative stability found in WB and WS/WB samples and the consequent altered functional properties of myofibrillar proteins (Xiao *et al.*, 2011) might contribute to the overall impaired water-holding and water-binding capacity as previously observed in WB and WS/WB fillets (Mudalal *et al.*, 2015).

Low resolution NMR has been successfully used for many years to investigate water-holding capacity and deepen the knowledge about the behaviour of water in case of meat abnormalities such as pale-soft-and-exudative condition (Mitchell *et al.*, 2014). An overall impairment in the water holding capacity of WS and WB fillets was found examining both the relative intensity and T_2 transverse relaxation time for the three proton populations (bound, intra- and extra-myofibrillar) identified within this study. Indeed, the higher intra-myofibrillar T_2 relaxation time found in WS/WB samples demonstrated a greater mobility of water within meat structure, which is likely caused by fibre degeneration and subsequent reduction of myofibrillar proteins in muscles affected by these abnormalities (Mudalal, *et al.*, 2014; Bowker and Zhuang, 2016). In addition, all abnormal samples had a higher proportion of extra-myofibrillar water, which was overall less tightly bound, as supported by the higher T_2 times found in WB and WS/WB groups. At last,

although the relative intensities were not modified, also the protons ascribed to bound water exhibited a higher T_2 in WB and WS/WB breasts. In agreement with our previous studies where higher cooking losses were associated with WS and/or WB, these findings demonstrated that a strong reduction in water holding capacity was found in meat affected especially by the WB abnormality (Mudalal *et al.*, 2015). Indeed, liquid losses during cooking are due to shrinkage of fibres giving rise to large gaps between the cooked muscle fibres (extra-myobrillar spaces), as well as at the level of the individual myofibrils (intra-myobrillar spaces) and concomitant to water expulsion from the myofibrillar matrix (Pearce *et al.*, 2011). Therefore, the higher proportion of extra-myofibrillar water and the greater mobility of intra-myofibrillar water likely led to increased losses of juice during cooking in abnormal breast meat.

3.4 Detection of differentially expressed genes in broiler *Pectoralis major* muscles affected by WS and WB abnormalities

The previous researches investigated the effect of WS and WB abnormality, occurring alone or combined within the same *Pectoralis major* muscle, on histological features, quality traits and technological properties of meat. However, considering the incomplete knowledge concerning the progressive development of muscular abnormalities, this study was carried out in order to investigate the main underlying mechanisms involved in the occurrence of WS and WB. In detail, this study aimed at quantifying the expression levels of genes involved in the main cellular metabolic pathways and subsequently compare the genomic transcription profile in WS/WB and NORM *Pectoralis major* muscles.

3.4.1 Materials and Methods

Twenty boneless and skinless *Pectoralis major* muscles were selected from the same flock of Ross 708 broilers (males, weighing around 3.7 kg) in the deboning area of a commercial processing plant within 2 h *post-mortem*. Birds belonging to this flock were farmed and slaughtered under commercial conditions according to Italian and European law for broiler chicken production. At slaughterhouse, the birds were electrically stunned in

agreement with the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing. All slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. Fillets were selected, evaluated for the presence/absence of muscle abnormalities, and classified as NORM and WS/WB according to the criteria proposed by Kuttappan *et al.* (2009) and Sihvo *et al.* (2014). In detail, 10 NORM fillets without any hardened area and white striations and 10 WS/WB samples exhibiting diffused, hardened areas and pale-bulging caudal end coupled with superficial white striations in the cranial part were packaged and transported to the laboratory under refrigerated conditions (0 to 2◦C). At the slaughterhouse, for the gene expression analysis, pieces of the 12 fillets showing the most extreme WS/WB phenotype among the 20 samples were chosen (6 NORM and 6 WS/WB), immediately frozen in liquid nitrogen, then stored at -80°C until RNA extraction. RNA was extracted using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA), as described in Davoli *et al.* (2011).

At 24 h *post-mortem* the samples were analysed as follows:

Raw Meat	
Morphometric measurements	Par. 2.1
pH	Par. 2.2
Colour	Par. 2.3
Drip Loss	Par. 2.4.1
Cooking Loss	Par. 2.4.4
Allo-Kramer shear force	Par. 2.5.1
Moisture	Par. 2.7.1
Protein	Par. 2.7.2
Fat	Par. 2.7.3.1
Collagen	Par. 2.7.2.6
Ash Minerals	Par. 2.7.4
SDS-PAGE	Par. 2.7.2.2 (b)
Marinated meat	
Uptake	Par. 2.4.2
Cooking Loss	Par. 2.4.4
Allo-Kramer shear force	Par. 2.5.1

3.4.2 Statistical analysis

Differences on meat quality and technological traits, composition as well as myofibrillar and sarcoplasmic protein profiles for WS/WB and NORM samples, were tested by 2-tailed Student's t-test. Statistical analyses were performed with SAS version 9.4 (SAS 9.4, Cary, NC. SAS Institute Inc.) and the nominal P-value ≤ 0.05 was considered as significance threshold.

3.4.3 Microarray Expression Profiling

Each extracted RNA was checked for integrity and quality using an Agilent BioAnalyzer 2100, retrotranscribed, amplified, labeled and applied to Affymetrix GeneChip Chicken Gene 1.1 ST v1 expression array by an outsource company (Cogentech Microarray Unit, Milan, Italy). All analytic procedures performed on microarray data were carried out using Partek Genomics Suite software, version 6.6 Copyright 2014 (Partek Inc., St. Louis, MO). Gene expression profiles from the 6 WS/WB biological replicates were compared to the 6 NORM biological ones in order to identify differentially expressed genes (DEGs) indicated with the genes fold change (FC) values between WS/WB an NORM broiler. FC filtering criteria combined with statistical t-test with FDR applied for multiple testing corrections were used to identify DEGs between the 2 conditions. The expression data obtained have been submitted to U.S. National Center for Biotechnology Information GEO database with the accession number GSE79276.

3.4.4 Validation by Quantitative Real Time-PCR

The results of the array expression analysis were validated by quantitative real-time PCR (qPCR). After DNAse treatment (TURBO DNA-freeTM, Ambion, Applied Biosystems), 1 µg of total RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturers' instructions. QPCR was performed on Rotor GeneTM 6000 (Corbett Life Science, Concorde, New South Wales, Australia) using 5 µL of SYBRR Premix Ex TaqTM (TAKARA Bio INC, Olsu, Shiga, Japan), 5 pmol of each primer, 2 µL of cDNA template diluted 1:10 and then was made up to the total volume of 10 µL with water. Rotor GeneTM 6000 protocol was optimized using specific annealing temperatures for each primer couple. The

samples were first used to assess the expression level of 3 candidate normalizing genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L32 (RPL32), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ).

The expression levels of these 3 genes were evaluated using NormFinder and GAPDH and YWHAZ, the 2 most stably expressed normalizing genes, used as reference genes. For each gene selected for validating the results of the expression array, an external primer pair to obtain the amplicon for the standard curve and an internal primer pair for the qPCR were designed. For the validation of the microarray results, 5 genes were chosen: crystalline alpha B (CRYAB), myoglobin (MB), glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE), utrophin (UTRN), and prostaglandin F receptor (PTGFR).

Threshold cycles obtained for the samples were converted by Rotor Gene 6000 to mRNA molecules/μL using for each gene the relative standard curve (Pfaffl, 2004; Zambonelli *et al.*, 2016). Moreover, the average mRNA molecules/μL for each sample was normalized dividing the gene mRNA molecules/μL by the geometric average of GAPDH and YWHAZ mRNA molecules/μL in the given sample, as described in Zambonelli *et al.* (2016). Differences on the expression level calculated for WS/WB and NORM samples were tested by 2-tailed Student's t test. Statistical analyses were performed with SAS version 9.4 (SAS 9.4, Cary, NC. SAS Institute Inc.) and the nominal P-value ≤ 0.05 was considered as significance threshold. Furthermore, in addition to the genes found differentially expressed, also ATPase sarcoplasmic/ endoplasmic reticulum Ca2+ transporting 2 (ATP2A2) gene expression was tested through qPCR, and its absolute expression normalized using GAPDH and YWHAZ as normalizing genes.

3.4.5 Functional Characterization

Functional annotation, classification and annotation clustering of selected gene sets were carried out by DAVID Tools 6.7 (Huang *et al.*, 2009a,b) using Biological Processes, Molecular Function gene ontology categories and KEGG pathways. A threshold for significance of $P < 0.05$ was considered to choose the significant functional categories. The precise

identification of the regulated snoRNAs was obtained by BLAST analysis (https://blast.ncbi. nlm.nih.gov/Blast.cgi?PAGE TYPE = BlastSearch) using blastn algorithm and standard parameters. In order to identify the codified name and the putative target genes of the differentially expressed micro-RNAs (miRNAs) the miRBase website (http://www.mirbase.org/) was consulted in order to check the predicted chicken target genes resulting by the inspection of the correspondences obtained using miRDB (http://mirdb.org/miRDB/index.html) and TargetScan (http://www.targetscan.org/) databases available in miRBase.

3.4.6 Results

3.4.6.1 Quality and Technological Traits

The effects of muscle abnormalities on fillet weight and dimensions are displayed in **Table 3.12**. Overall, WS/WB samples exhibited higher weight ($P < 0.001$) coupled with increased length, width, and middle height. As for raw meat quality (**Table 3.13**), WS/WB fillets were paler and revealed higher redness and ultimate pH values. Additionally, both raw and marinated WS/WB samples exhibited higher cooking losses ($P < 0.001$) and lower marinade uptake ($P < 0.001$). Besides, if compared with NORM group, a sharp increase in shear force value was measured in raw and marinated WS/WB fillets ($P < 0.001$).

Table 3.12 Effect of WS and WB abnormalities on weight, dimension and texture of chicken *Pectoralis major* muscles.

Parameter	NORM	WS/WB	sem	*P-value*
Weight (g)	218.27	301.51	11.262	≤ *0.001*
Top height (H1)[1] (mm)	37.82	44.31	0.959	NS
Middle height (H2)[2] (mm)	23.43	34.52	1.478	≤ *0.001*
Bottom height (H3)[3] (mm)	7.60	12.70	0.808	NS
Length (mm)	185.03	194.10	2.815	≤ *0.001*
Length (mm/g)	85.42	64.76	2.738	≤ *0.001*
Width (mm)	73.95	77.01	1.173	≤ *0.001*
Width (mm/g)	34.37	25.64	1.279	≤ *0.001*
Weight (g)	218.27	301.51	11.262	≤ *0.001*
Top height (H1)[1] (mm)	37.82	44.31	0.959	NS
Middle height (H2)[2] (mm)	23.43	34.52	1.478	≤ *0.001*

N = 10 samples / group. [1]H1 measured at the thickest point in the cranial part. [2]H2 measured at half distance of the breast length. [3]H3 measured as the vertical distance far from the end of the caudal part by 1 cm in a dorsal direction. sem = standard error of mean. NS = not significant.

Table 3.13 Effect of WS and WB abnormalities on quality traits of chicken *Pectoralis major* muscles.

Parameter	NORM	WS/WB	sem	*P-value*
Raw meat				
pHu	5.87	6.06	0.034	≤ *0.05*
Lightness (L*)	54.50	52.52	0.508	≤ *0.05*
Redness (a*)	0.82	1.41	0.157	≤ *0.05*
Yellowness (b*)	3.92	3.62	0.207	NS
Drip loss (%)	1.07	1.06	0.064	NS
Cooking loss (%)	21.45	34.04	1.733	≤ *0.001*
Allo-Kramer shear force (kg/g)	4.26	7.54	0.677	≤ *0.05*
Marinated meat				
Uptake (%)	18.33	7.44	1.344	≤ *0.001*
Cooking loss (%)	14.53	21.94	1.121	≤ *0.001*
Yield (%)	101.12	83.83	2.121	≤ *0.001*
Allo-Kramer shear force (kg/g)	2.13	4.08	0.277	≤ *0.001*

N = 10 samples / group. sem = standard error of mean. NS = not significant.

3.4.6.2 Chemical and Mineral Composition

The occurrence of WS/WB abnormalities exerted a relevant impact on proximate and minerals compositions of the affected muscles (**Table 3.14**). In particular, WS/WB fillets exhibited higher ($P < 0.001$) moisture, fat and collagen contents coupled with reduced ash and proteins ($P < 0.001$) levels.

As for mineral composition, no differences were found in potassium content whereas WS/WB samples had lower magnesium and phosphorus levels. Additionally, an increased amount of sodium ($P < 0.001$) and calcium was observed in WS/WB fillets.

Table 3.14 Effect of WS and WB abnormalities on proximate and mineral compositions of chickens *Pectoralis major* muscles.

Parameter	NORM	WS/WB	sem	*P-value*
Moisture (%)	74.64	76.82	0.39	*≤ 0.001*
Fat (%)	0.79	1.79	0.17	*≤ 0.001*
Ash (%)	1.46	1.19	0.05	*≤ 0.05*
Collagen (%)	1.16	1.35	0.04	*≤ 0.05*
Protein (%)	23.37	18.45	0.69	*≤ 0.001*
Mg (mg/100g)	35.99	32.59	0.79	*≤ 0.05*
K (mg/100g)	359.31	362.96	6.59	NS
P (mg/100g)	222.63	207.30	3.69	*≤ 0.05*
Na (mg/100g)	37.82	75.06	5.76	*≤ 0.001*
Ca (mg/100g)	7.81	11.32	0.69	*≤ 0.05*

N = 10 samples / group. sem = standard error of mean. NS = Not Significant.

3.4.6.3 Identification of Differentially Expressed Proteins

With regard to myofibrillar proteins pattern (**Table 3.15**), 9 bands having molecular weight from 16 to 220 kDa were identified. In detail, lower relative abundance of slow-twitch light chain myosin (LC1, 27.5 kDa) coupled with higher amount of 70 kDa myosin heavy chain (MHC) fragment were found in WS/WB samples. As for sarcoplasmic protein pattern (**Table 3.16**), eleven bands, having molecular weight ranging from 25 to 114 kDa, were detected and almost all the enzymes involved in glucose metabolism differed between WS/WB and NORM groups. In particular, compared with NORM, WS/WB fillets exhibited lower amount of phosphoglycerate mutase (PGAM, 25 kDa), creatine kinase (KCRM, 43 kDa), phosphoglucose isomerase (GPI, 58 kDa) and pyruvate kinase (KPYM, 68 kDa) together with higher relative abundance of lactate dehydrogenase (LDH, 34 kDa), glyceraldehyde dehydrogenase (G3P, 36 kDa), aldolase (ALDO, 39 kDa) and glycogen phosphorylase (PYGL, 90 kDa). In addition, WS/WB samples exhibited higher calcium-transporting ATPase sarcoplasmic reticulum type slow twitch skeletal muscle isoform (ATP2A2, 114 ATP2A2 gene).

Table 3.15 Effect of breast abnormalities on myofibrillar proteins composition of chicken meat.

Protein	MW (kDa)	NORM	WS/WB	sem	*P-value*
Myosin Light Chain-3	16	12.41	15.68	1.066	NS
Myosin Light Chain-2	19	3.58	3.50	0.398	NS
Myosin Light Chain-1	27.5	13.71	8.01	1.095	*≤ 0.05*
Troponin T (fragment)	29	4.00	4.96	0.292	NS
Tropomyosin	34	4.81	4.67	0.301	NS
Actin	42	33.81	36.88	1.774	NS
Desmin	53	5.25	6.29	0.398	NS
MHC-fragment	70	4.77	6.91	0.467	*≤ 0.05*
Myosin Heavy Chain	220	16.19	13.12	1.774	NS

N = 10 samples / group. sem = standard error of mean. NS = not significant.

Table 3.16 Effect of breast abnormalities on sarcoplasmic proteins composition of chicken meat.

Protein	MW (kDa)	NORM	WS/WB	sem	*P-value*
PGAM	25	7.44	5.54	0.33	*≤ 0.001*
TPIS	26.4	7.16	6.50	0.24	NS
CA	31.8	9.42	8.27	0.59	NS
LDH	34	18.76	22.85	0.85	*≤ 0.05*
G3P	36	10.33	13.26	0.62	*≤ 0.05*
ALDO	39	7.06	10.42	0.62	*≤ 0.05*
KCRM	43	11.23	9.89	0.32	*≤ 0.05*
GPI	58	8.31	4.86	0.67	*≤ 0.05*
KPYM	68	6.06	4.17	0.36	*≤ 0.05*
PYGL	90	13.28	15.79	0.59	*≤ 0.05*
AT2A2	114	0.00	1.86	0.35	*≤ 0.05*

N = 10 samples / group. sem = standard error of mean. NS = not significant.

3.4.6.4 Profiling of Differentially Expressed Genes

Comparing the gene expression profiles obtained for WS/WB vs. NORM samples, 207 differentially expressed genes (DEG) were found: 103 up-regulated and 104 down-regulated in WS/WB chickens. In order to confirm the results obtained with microarray, 5 genes (CRYAB, MB, GNE, UTRN, PTGFR) were selected and analyzed by qPCR. Then, the data were compared with the expression profiles obtained using microarrays (**Figure 3.3**). As showed in Figure 3.3, the expression levels assessed by microarray

and qPCR were in agreement for all genes and the findings were correlated correlation (R^2 = 0.91). Moreover, since a differential expression of the protein encoded by ATP_2A_2 was observed by SDS-PAGE analysis, the expression level of ATP_2A_2 mRNA was tested, although not included among the probes constituting the microarray.

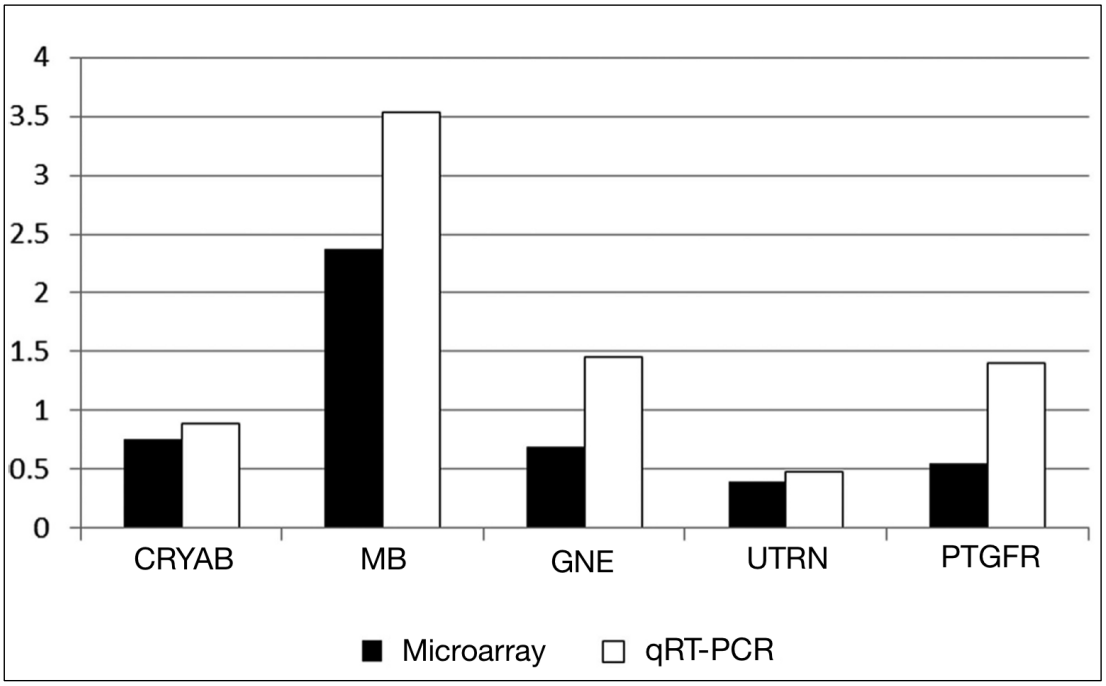


Figure 3.3 Validation by qPCR of 5 differentially expressed genes obtained by microarray analysis. Fold changes values obtained from microarrays, (black bars) and from qPCR data (white bars), for the 5 tested genes.

3.4.7 Discussion

The gene expression and the meat quality traits of affected chickens' *Pectoralis major* muscles were investigated, in order to evaluate the effects exerted by these pathologic conditions on the gene transcription levels and the fillets' biochemical composition.

On the whole, the results outlined the presence of a severely damaged *Pectoralis major* muscle, with WS/WB samples showing consistent changes in the expression level of genes related to muscle development, reactive oxygen species metabolism, oxidative stress and signal transduction, blood vessel morphogenesis, and polysaccharide metabolism. Considering the data reported in the present study, together with the results identified in literature (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014, Mazzoni *et al.*, 2015;

Mutryn *et al.*, 2015), a consistent impairment of normal muscle metabolism is evident for WS/WB defects and these abnormalities appear to be linked to a complex pathogenesis.

To date, the complex pathological framework characterizing WS/WB defects has made it extremely difficult to identify the underlying causes at the basis of the alterations. Despite agreement in the literature about WS and WB defects regarding the large number of histological, biochemical and metabolic alterations accompanying the occurrence of these breast muscle abnormalities, there is no consensus on the causes leading to the insurgence of this complex pathological framework.

The results obtained from the present research have been evaluated and discussed in the light of the knowledge reported to date in literature on WS and WB topics, aiming to outline an overall view of the pathological changes affecting *Pectoralis major* muscle showing in addition the gene networks and the biological evidences related to the occurrence of the WS/WB tissue alterations. Furthermore, the overall analysis of the variations obtained to date at the genetic, biochemical, biological, and histological levels allows defining some possible hypotheses on the mechanisms determining the onset of these myopathies.

3.4.7.1 Oxidative Stress

In the present research, the microarray showed an overall increase in the expression level of genes involved in the response against the accumulation of hydrogen peroxide and reactive oxygen species in WS/WB fillets, in agreement with the presence of an oxidative stress possibly linked to a muscle hypoxic condition. In particular, WS/WB samples presented increased expression levels of crystallin alpha B (CRYAB), adenosine deaminase (ADA), MB genes and a decreased activity of reactive oxygen species modulator 1 (ROMO1) gene, that are all involved in the response to reactive oxygen species. These results are in agreement with Mutryn *et al.* (2015) that identified a set of DE genes involved in myofibers reaction to oxidative stress in muscle samples of high breast yield chickens affected by WS/WB. The cause of this oxidative stress is not clear, although past studies can help defining some possible causes. One of the possible hypotheses

reported in literature indicates an inadequate breast muscle vascularisation as a possible key factor in the occurrence of WS/WB (Mutryn *et al.*, 2015). It is worth to note that in 1999, Dransfield and Sosnicki reported an increased proportion of glycolytic fibers with enlarged diameter in chicken lines selected for high growth rate and breast yield. Moreover, Hoving-Bolink *et al.* (2000) showed an intense reduction in both vascularization and capillary-to-fiber ratio in chicken hybrids selected for high growth rate and breast yield compared to unselected chicken lines. Based on these results, genetic selection in these chicken lines determined in muscles an inadequate blood vessel growth with the consequent impairment in oxygen supply and in the metabolic waste products displacement from breast myofibers. In agreement with Mutryn *et al.* (2015), the present results suggested that an excessive accumulation of reactive oxygen species (ROS) within the muscle tissue of WS/WB samples might be involved in initiating the inflammatory mechanism typically associated with WS and/or WB muscle abnormalities.

3.4.7.2 Inflammation and Myofiber Degeneration

The profound alterations and the inflammatory status observed in previous researches were confirmed in the present study where an increased transcription of genes coding for proteins involved in biological processes related to tissue alteration was detected. More precisely, immunoglobulin superfamily, member 10 gene (IGSF10), heat shock 105 kDa/110 kDa protein 1 (HSPH1) and heat shock 60 kDa protein 1 (Chaperonin) (HSPD1) were overexpressed in WS/WB samples, supporting the presence of the tissue inflammation. Moreover, the overexpression in WS/WBs of ADAM family metallopeptidase with thrombospondin type 1 motif 12 (ADAMTS12) and ADAM metallopeptidase with thrombospondin type 1 motif 19 (ADAMTS19) genes suggested the existence of a muscle tissue inflammation, as ADAMTS12 in particular is already known to be involved in the activation of inflammatory responses (Moncada-Pazos *et al.*, 2012). Similarly, the higher transcription levels of nuclear factor, interleukin 3 regulated (NFIL3) and snail family zinc finger 2 (SNAI2), as genes encoding for hindering-cell death molecules (Keniry *et al.*, 2014), could reveal the attempt to limit apoptotic processes and necrosis of the cells. Thus, the onset

of a complex biological reaction aimed at contrasting the inflammatory process with the activation of anti-inflammatory responses was observed in WS/WB samples. These inflammatory and necrotic processes were previously found in WS and WB breast muscles (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014; Mutryn *et al.*, 2015) and degenerative processes of muscular nerve growth were hypothesized. Indeed, the microarray analysis evidenced in WS/WB broilers reduced transcription levels of the genes deafness autosomal recessive 31 (DFNB31), syntaxin 3 (STX3), neurogenin 1 (NEUROG1), SLIT and NTRK-like family member 6 (SLITRK6), wingless-type MMTV integration site family member 7A (WNT7A), and EPH receptor A2 (EPHA2), involved in neuron genesis and differentiation as indicated by DAVID analysis. Another differentially expressed gene found in the present study is interleukin 1 beta (IL1B), which encodes for a member of the interleukin 1 cytokine family. This protein exerts a central role as mediator of the inflammatory response and is involved in a variety of cellular activities (including cell proliferation, differentiation and apoptosis). Although the findings of the present study outlined an inflammatory process affecting WS/WB samples, a down-regulated transcription of IL1B gene was found. Despite the majority of the literature reports in muscles affected by inflammation an increased expression level of the IL1B gene (Dinarello, 1998; Li *et al.*, 2008), in some cases this gene was found downregulated during some chronic pathological situations (Karli *et al.*, 2014). Moreover it is possible to hypothesize that the reduced transcription level of IL1B identified within the WS/WB samples could be linked to the low level of vascularization of the WS/WB samples as IL1B was also found to play a relevant role in the angiogenic processes promoting the emergence of new capillaries from pre-existing blood vessels (Dinarello, 1996; Voronov *et al.*, 2003). Furthermore, as interleukin 1 has a pyrogenic role and its involvement in the pain sensation during inflammation has been evidenced, the down-regulation of IL1B transcription might be responsible for the lack of symptoms in chickens affected by WB/WS abnormalities during the broiler farming period. Some genes identified as differentially expressed have been reported in literature to be associated with the development of myopathies. The over-expression of PLN observed in WS/WB samples was related in mice muscles to an altered

phenotype similar to the centronuclear myopathy identified in human muscles (Fajardo *et al.*, 2015). Centronuclear myopathy is a congenital myopathy characterized by centrally located nuclei, a peculiarity that was already described in WS/WB affected muscles (Sihvo *et al.*, 2014). Moreover, an impaired activity of AT_2A_2 protein might be involved in the WS/WB phenotype with a loss of adhesion among myocytes and the presence of abundant connective tissue replacing the muscle cells. This phenomenon is for some instances similar to a human pathology, Darier-White disease (Savignac *et al.*, 2011), which is characterized by a loss of adhesion between epidermal cells and keratinization leading to apoptosis of the same cells. The causative mutation of the human disease was found in one of the transcripts of ATP_2A_2 gene.

3.4.7.3 Myofibers Regeneration

In the present study, several genes involved in muscle development and cell differentiation were found differentially expressed within the WS/WB cases. In particular, the over-expression of CSRP3 and PTGFR as well as the down-regulation of P2RY1 gene could be associated respectively to muscle fibers synthesis (Kong *et al.*, 1997; Jansen and Pavlath, 2008) and myogenesis (Krasowska *et al.*, 2014). The cascade pathway of PTGFR, PLN, GNAQ, PLCB2, and PLCD1 has been also related to mechanisms aimed at regenerating damaged muscle. The combined activity of these 5 DEG triggers several downstream metabolic pathways that increase muscle cells volume (Horsley and Pavlath, 2003, 2004; Hindi *et al.*, 2013) and contribute to the regeneration of myofibers upon injury, suggesting a possible role of these genes in trying to repair the effect of severe *Pectoralis major* myopathies such as WB and WS. Additionally, WS/WB fillets showed an increased mRNA level of the gene FAM64A. The expression of this gene was reported to be associated with rapidly proliferating tissues during mouse embryogenesis (Archangelo *et al.*, 2008). Based on this previous finding, FAM64A overexpression may be associated to the regeneration processes taking place in the damaged breasts, and its upregulation could be determined by gga-miR-196-5p that identifies FAM64A as a specific target gene. Similarly, also increased mRNA levels of PLXNA1 and PRRX2 genes,

up-regulated in WS/WB samples, have been related to proliferating fetal fibroblasts and developing tissue (White *et al.*, 2003; Hota and Buck, 2012). As suggested for FAM64A, we hypothesize that PLXNA1 and PRRX2 expression changes may be respectively linked to the regulation exerted by gga-miR-205a and gga-miR-1600. Anyway, these hypotheses will need further studies to be proven.

On the whole, the results obtained from the microarray, with DE genes from pathways linked to muscle differentiation and development, can be interpreted as the evidences of an activation of muscle cells regenerative processes in response to the degenerative status. These tissue regenerative processes (nuclear rowing and cell multi-nucleation) have been evidenced within the WS/WB muscles in previous histological observations (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014). Furthermore, a remarkable increase in the amount of ATP_2A_2 (**Table 3.16**) coupled with an overexpression of ATP_2A_2 gene and MB gene, was detected in WS/WB samples similar to what was observed by Mutryn *et al.* (2015) in muscles affected by WB abnormality. The higher expression of ATP_2A_2 and MB might be the result of a shift from type IIb towards slow twitch type I fibers in abnormal breast muscles. This reorganization of the tissue might be explained considering that similar regenerative mechanisms, exhibiting an overall increase in slow-twitch fibers and the apoptosis of the fast-twitch ones, were previously observed in mice dystrophic muscles (Massa *et al.*, 1997). In addition, the up-regulation in the transcription of acetyl-CoA acyltransferase 2 (ACAA2) gene, encoding for a protein exerting a relevant role in the metabolic pathway leading to mitochondrial beta oxidation of fatty acids, might support the hypothesized shift of WS/WB muscles towards oxidative metabolism.

3.4.7.4 Impaired Muscle Ion Homeostasis

WS/WB samples showed relevant changes in their chemical composition, with an overall modification in mineral content. In particular, the increased sodium content may be related to the more elevated transcription of solute carrier family 9, subfamily A (NHE7, cation proton antiporter 7) member 7 (SLC9A7) gene, as it encodes a sodium and potassium/ proton antiporter (Kagami *et al.*, 2008). Additionally, also increased levels of calcium

were identified in WS/WB samples, supporting the existence of the intracellular calcium build-up already hypothesized by Mutryn *et al.* (2015) on the basis of the altered transcription levels identified for genes involved in calcium homeostasis. In agreement with this hypothesis, several genes linked to intracellular ion homeostasis were found differentially expressed: the up-regulation of genes linked to the activation of G protein-coupled purinergic receptors was observed in cascade in WS/WB samples. The over-expression of PTGFR coupled with the increase in guanine nucleotide binding protein (G protein) alpha 11 (GNAQ) and G protein-coupled receptor 1 (GPR1) might be related to the up-regulation of phospholipase C beta 2 (PLCB2) and phospholipase C delta 1 (PLCD1) genes (Figure 3), involved in the processes leading to the increase of Ca^{2+} in the cells. According to Bucheimer and Linden (2004), the G protein-coupled receptors activate β phospholipase enzymes, resulting in an increased intracellular Ca^{2+} concentration and altered ions homeostasis. Within this framework, the reduced expression of calcium homeostasis modulator 3 gene (CALHM3), the higher synthesis of ATP_2A_2 protein and the increased transcription of its relative gene ATP_2A_2 and PLN gene can produce in WS/WB samples an overall alteration in calcium signalling pathway, contributing to the inflammatory processes. An over-expression of ATP_2A_2 was also noticed by Mutryn *et al.* (2015) in breast muscles affected by WB abnormality. Among the differentially expressed miRNAs, ggamiR- 1600 may be the putative regulator of PLN gene expression, suggesting that for calcium signalling pathway a higher level of regulation might be responsible of relevant changes in the molecular mechanisms involved in the origin of the WB/WS myopathy.

Anyway, on the basis of the whole literature produced to date, it is not possible to formulate a certain assumption about the causes leading to this overall impairment in muscle ion homeostasis. The differential expression of genes related to the purinergic receptors pathways that we found in WS/WB samples may be one of the primary causes of the inflammation or, most likely, one of the effects of muscle tissue structural changes related to WS/WB abnormalities and the results of the activation of the purinergic receptors from the ATP released in the extracellular matrix spaces from damaged fibers (Bucheimer and Linden, 2004; Eltzschig *et al.*, 2012).

3.4.7.5 Altered Glucose Metabolism, Lipidosis, Fibrosis, and Proteoglycan Synthesis

The SDS-PAGE results revealed an intensified glycolytic activity in WS/WB samples, with higher amount of glycolytic enzymes such as lactate dehydrogenase (LDH), glyceraldehyde dehydrogenase (G3P), aldolase (ALDO) and glycogen phosphorylase (PYGL). Among the glycolytic enzymes, the magnesium-dependent enzymes phosphoglycerate mutase (PGAM), phosphoglucose isomerase (GPI) and pyruvate kinase (KPYM) were less expressed in WS/WB samples. The inadequate level of disposable magnesium in affected muscles could be linked to the lower translation of these magnesium-dependent enzymes in affected samples. A general modification of the glycolytic enzymes expression was evident in WS/WB *Pectoralis major* muscles. Despite the increased synthesis of LDH enzyme in WS/WB samples, a higher ultimate pH in the affected breast muscles was observed, suggesting that there was not an increase in the transformation of pyruvate into lactate, as normally expected in hypoxic conditions. In addition to the modifications in glycolytic enzymes synthesis, we found by microarray analysis that in WS/WB samples the genes GNE, glycogen branching enzyme (GBE1), UDPglucose 6-dehydrogenase (UGDH), and protein phosphatase 1, catalytic subunit, beta isozyme (PPP1CB), involved in polysaccharide metabolic processes were over-expressed. GNE enzyme plays an essential role in hexosamine pathway, in particular for the biosynthesis of N-acetylneuraminic acid, a precursor of sialic acids. This evidence might suggest an alternative utilization of fructose 6-phosphate, produced by PGI enzyme during glycolysis. Indeed fructose 6-phosphate can undertake the glycolysis pathway or can be used as the initial substrate of the hexosamine and hexuronic acid pathways, resulting in collagen, proteoglycans and glycosaminoglycans synthesis. This shift towards hexosamine pathway was also described by Du *et al.* (2000) as a consequence of the accumulation of ROS species, which otherwise exerted an inhibitory effect on glycolysis. On the other hand, UGDH converts UDP-glucose to UDP-glucuronate and thereby participates in the biosynthesis of glycosaminoglycans such as hyaluronan (a common component of the

extracellular matrix).

The over-expressions of UGDH, GNE, GBE1, PPP1CB and interphotoreceptor matrix proteoglycan 2 (IMPG2) genes identified in the present research, together with the observed fibrosis described on the same samples, could explain the increased presence of collagen and proteoglycans in the areas affected by WS/WB lesions. These findings agree with the evidences reported in literature for WS and/or WB abnormalities (Kuttappan *et al.*, 2013a; Sihvo *et al.*, 2014; Mutryn *et al.*, 2015; Velleman and Clark, 2015). Additionally, the increased collagen and fat contents observed in WS/WB samples were in agreement with previous findings (Kuttappan *et al.*, 2013a; Mudalal *et al.*, 2014). A similar situation was described by Lopes-Ferreira *et al.* (2001), who noticed in hypoxic conditions the development of both fibrosis and lipidosis within the skeletal muscle, with a replacement of the lost fibers with the collagen synthesis and lipid deposition.

3.4.8 Conclusions

On the whole, the findings of the present study revealed that, at the gene level, a complex aetiology is associated with the occurrence of WS and WB muscle abnormalities.

In WS/WB breast muscles, there is evidence of differentially expressed genes related to several functional categories: muscle development, polysaccharide metabolic processes, glucose metabolism, proteoglycans synthesis, inflammation, and calcium signalling pathway. By combining the functional roles for the differentially expressed genes, we hypothesized a network of biological changes that are acting simultaneously and are responsible for the phenotypic evidences of these myopathies. The present results, combined with the existing knowledge, allowed to draw a scheme (**Figure 3.4**) describing a possible progression of the biological processes cascade hypothesized to be involved in the development of WS/WB myopathies. Although the cause of these myopathies is still unclear, the majority of the results reported in literature suggest that selection criteria more and more addressed towards fast growing and high breast yield broilers could be involved in the occurrence of the breast oxidative stress that triggers

the cascade of WS/WB related muscle alterations. The data obtained in the present research can be useful for the clarification of the WS/WB pathogenesis and further studies have to be planned to disentangle the complex aetiology behind these myopathies.

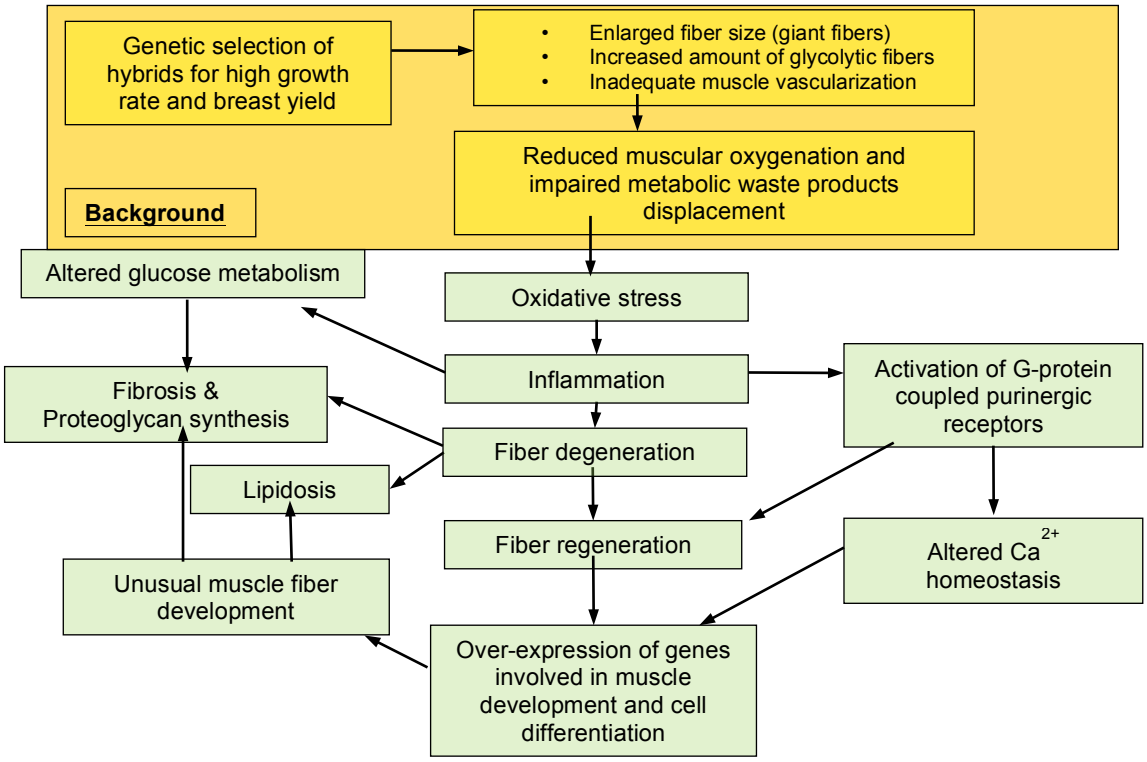


Figure 3.4 Schematic representation of one of the possible etiologies responsible for the occurrence of WS and WB abnormalities.

CHAPTER 4

IMPLICATIONS OF WOODEN BREAST ABNORMALITY ON BROILER PECTORALIS MAJOR MUSCLES DURING STORAGE

This research topic was developed during my 4 months-research period in Finland at the Department of Food and Environmental Sciences of the University of Helsinki. In detail, considering the incomplete knowledge concerning the underlying mechanisms responsible for the hardened consistency that is typically associated with the occurrence of wooden breast abnormality, this study aimed at investigating

- Sarcomere length in *Pectoralis major* muscles affected by Wooden Breast in order to evaluate whether the contractile state of the muscle tissue exerts a role in the development of the hard and out-bulging areas associated with the occurrence of the Wooden Breast condition (*Submitted for publication to Poultry Science*) (par 4.1).

The effect of a 7-days refrigerated storage on

- Muscle structure, textural properties, particle size distribution and (par. 4.2.1) (*Submitted for publication to Poultry Science*)
- Evolution of the main proteolytic indicators in broiler *Pectoralis major* muscles affected by Wooden Breast abnormality (Manuscript under preparation) (par. 4.2.2).

4.1 Sarcomere length in Wooden Breast broiler chickens

The abnormally hard muscle consistency – before and after the onset of *rigor* – is a distinctive feature for WB (Sihvo *et al.*, 2014, 2016) but, in general, is not common in other degenerative myopathies (van Vleet and Valentine, 2007; Klasing *et al*., 2008). In normal conditions, the hardened consistency exhibited by the skeletal muscles after the onset of *rigor mortis* is generated by strong actomyosin bonds in association with the eventual

contraction of sarcomeres (Marsh and Leet, 1966; Marsh and Carse, 1974; Wheeler and Koohmaraie, 1994). On the other hand, the underlying mechanism responsible for the typical hardness in WB is currently unknown.

Muscle shortening (and thus sarcomere length) has been widely applied as index for meat tenderness and indirectly for other quality indices, such as water-holding capacity. Marsh and Carse (1974) demonstrated that the ability of muscle to resist against stretching would linearly increase in the range between 0 and 40% of sarcomeres shortening. Thus, shorter sarcomeres would result in harder raw meat. Within this context, it seemed reasonable to hypothesize that shorten sarcomeres might be partly responsible for the hardened consistency typically exhibited by the Wooden Breast muscles. Thus, this study aimed to compare the sarcomere lengths in *Pectoralis major* muscles in order to verify whether any differences might be observed between the unaffected cases and WB broiler chickens.

4.1.1 Materials and Methods

At a slaughterhouse, 39-day old, broiler chickens of a high-yield hybrid (Ross 508) were slaughtered, chilled to 2°C and the breast fillets cut off according to the commercial procedure. At 3 hours *post-mortem*, a total 20 *Pectoralis major* muscles were selected by visual appearance and manual palpation: 10 WB cases, exhibiting diffusely hardened consistency and pale colour, and 10 unaffected fillets (NORM) of normal consistency and without any macroscopic changes.

The muscles were kept at 0-2°C until sampled in the middle area of each fillet at 10 hours *post-mortem*. All samples, 1 × 1 × 1 cm in size, were excised from the ventral surface of the fillet (facing the skin), excluding the most superficial 5 mm. Then, sarcomere length was determined according to the procedure described by Liu *et al.* (2014) (*See par. 2.5.5*).

4.1.2 Statistical analysis

The data were analysed by the Shapiro-Wilk's test for normality. The difference in sarcomere length between the unaffected and WB cases was tested with the Student's t-test. All the statistical tests were performed using SAS Software (SAS Institute Inc., Cary, NY).

4.1.3 Results and Discussion

This study compared the sarcomere lengths in *Pectoralis major* muscles of NORM and WB broiler chickens as measured by laser diffraction method. The WB cases exhibited 13% longer sarcomeres than the NORM ones (1.91 *vs*. 1.69 µm; $P < 0.001$). These findings are in agreement with previous results obtained on WB fillets by Tijare *et al.* (2016). The longer sarcomeres observed in the hardened WB cases indicate that the mechanism responsible for the hardening in WB differs from the traditional *rigor* state of the muscle, in which the hardness is induced by sarcomeres contraction (Marsh and Leet, 1966; Marsh and Carse, 1974; Wheeler and Koohmaraie, 1994).

Our result on WB muscles is in accordance with the electron microscopic study of Ashmore *et al.* (1988) on *Patagialis* muscle dystrophy in chickens in which the affected muscles exhibit 22 and 25% longer sarcomeres at 2 and 8 weeks of age, respectively, than the controls. Ashmore *et al.* (1988) suggested that in dystrophic *Patagialis* muscles, the alteration of sarcomere length results from an increased tension towards the muscle fibres during the growth period. The amount of tension is an important factor in normal muscle physiology, since the addition of new sarcomeres at the end of an existing myofibre is stimulated by the tension exerted by the longitudinal growth of bones (Goldspink, 1971). Within this context we suggest another hypothesis: the myofibres degeneration and detachment from each other observed in chronic WB cases (Sihvo *et al.*, 2014) might induce an amount of tension that is lower than the one that normally induce an increase in sarcomere number. As a result, the remaining functional fibres observable within the WB affected areas exhibited longer sarcomeres.

4.1.4 Conclusions

In conclusion, wooden breast muscles exhibited longer sarcomeres than their unaffected counterparts. Since it is known that the longitudinal tension stimulates the serial addition of sarcomeres, we speculate that a lower longitudinal tension might result from fibres detachment thus leading to

longer sarcomeres in WB muscles.

4.2 Effect of a 7-days refrigerated storage on broiler *Pectoralis major* muscles affected by Wooden Breast abnormality

Meat tenderizes after slaughter due to the presence of endogenous proteolytic enzymes. The process has been suggested to be multi-catalytic in nature (Ouali *et al.*, 2013; Kemp and Parr, 2012) including the action of calpains, cathepsins, caspases and the proteasome. There is strong evidence for the involvement of the calpain system in *post-mortem* cleavage of myofibrillar and myofibrillar-associated proteins (Koohmaraie and Geesink, 2006; Huff-Lonergan *et al.*, 2010) and the best-characterized isoforms in mammalian muscle are μ-calpain, m-calpain and the muscle-specific calpain-3. A special variant, termed μ/m-calpain, might be observed in birds skeletal muscles and exhibiting a calcium sensitivity intermediate between those of mammalian μ-calpain and m-calpain (Sorimachi *et al.*, 1995). In a previous study performed on chicken, Lee et al. (2007, 2008) described a calpain band in casein zymograms with slightly greater motility than that of μ/m-calpain and identified as partial autolyzed μ/m-calpain (Zhao *et al.*, 2016). The calpain system in broilers has been previously studied in relation to factors such as animal growth rate (Piórkowska *et al.*, 2015) and *early post-mortem* pH decline rate (Huang *et al.*, 2016).

Within this context, although different studies were performed in order to evaluate the effect exerted by WB abnormality on meat quality, there is no evidence concerning its impact on the proteolytic processes taking place during *post-mortem* period. Thus, considering the lack of information concerning the activity of proteolytic enzymes in WB samples, this study aimed at investigating the effect of a 7-days storage of broiler *Pectoralis major* muscles on free calcium concentration, calpain activity and proteolysis. Both the superficial and the deep layers of each *Pectoralis major* muscles were studied in order to understand whether any peculiar traits might be found according to the intra-filet sampling location.

4.2.1 Effect of a 7-days refrigerated storage on muscle structure, textural properties and particle size distribution in broiler *Pectoralis major* muscles affected by Wooden Breast abnormality

4.2.1.1 Materials and Methods

A total of 180 *Pectoralis major* muscles were randomly selected at 3 hours *post-mortem*, from 37 to 38-day-old male broilers Ross 508, in the deboning area of a commercial processing plant. The samples were classified by two experienced people by manual palpation as Normal (NORM) and Wooden Breast (WB) according to the criteria of Sihvo *et al*. (2014). After trimming the muscles from visible fat and connective tissue they were packed into loose polyethylene bags and stored at 4 ± 1°C until 10, 24, 72, 120, 168 h *post-mortem*. At each sampling time, 18 NORM and 18 WB fillets were cut in order to separate the superficial from the deep layer and used to assess texture (on both raw and cooked meat), measure the shear force of cooked meat and perform particle size analysis.

The histological evaluation was performed on a total of 18 *Pectoralis major* muscles (9 NORM and 9 WB cases) selected at 3 hours *post-mortem*, from 35-day-old broilers (Ross 308, males, having an average weight of 2.5 kg), in the deboning area of a commercial processing plant. After being stored for 10, 72 and 168 h *post-mortem*, each fillet was sampled and cut in order to separate the superficial from the deep layer according to the sampling protocol summarized in **Figure 4.1**. In detail, the “superficial layer” was defined from about 0.2 cm to 1.2 cm below the breast muscle surface. Besides, the “deep layer” was defined from about 1.5 cm to .5 cm below the breast muscle surface, few millimetres away from the superficial one. Then, both the superficial and the deep layer of each fillet was immediately fixed in a 10% buffered formalin solution for 24 h at room temperature. Specimens were oriented for transverse fiber sectioning, dehydrated in a graded series of ethanol, and embedded in paraffin. From each sample, eight transverse sections (6 μm thick) were obtained, mounted on polylysine-coated slides and stained with Masson’s trichrome. The presence of abnormal fibers (fibers

exhibiting hyaline degeneration and damaged fibers with round profile) was assessed in 10 primary myofiber fascicles (PMF) randomly selected from the eight transverse sections and the levels of myodegeneration graded (score F1, F2 and F3, respectively) according to the same criteria adopted in previous studies (Mazzoni *et al*., 2015; Soglia *et al*., 2016a; Sihvo *et al*., 2017).

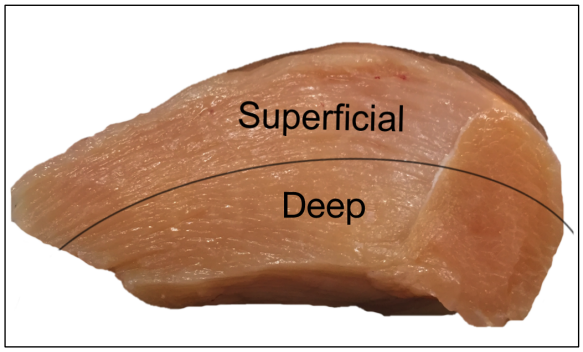


Figure 4.1 Sampling procedure adopted for each *Pectoralis major* muscle in order to separate the superficial layer from the deep one.

Then, after excising the sample for muscle histology, sub-samples were excised from the superficial and deep layer of each sample and used to assess

Compression test	Par. 2.5.3 b
Allo-Kramer shear force	Par. 2.5.1 b
Particle size analysis	Par. 2.6.4

Being the WB samples diffusely affected cases, our sampling protocol led us to analyse both the lesion (surface) and the non-lesion (deep layer) areas of the WB samples and compare them with their corresponding portion within the NB.

4.2.1.2 Statistical analysis

The findings were statistically evaluated with the Two-Ways ANOVA option of the GLM procedure present in SAS software (1988). The main effects of meat abnormality coupled with the sampling position (NB superficial, NB deep, WB superficial, WB deep), the storage time and their interactions were evaluated and means separated using the Tukey's HSD

test (multiple range test) of the GLM procedure (SAS Institute, 1988).

4.2.1.3 Results and Discussion

4.2.1.3.1 Macroscopic observation and histology

In the present study, the differences in the histological features observed between the superficial and the deep part of the WB fillets, previously reported (*See Par. 3.2.3.1*), were further investigated.

The WB muscles sampled after 10, 72 and 168 h *post-mortem* were macroscopically hard and exhibited a transparent exudate and/or hemorrhages (*petechiae* or small hemorrhages) on the surface in agreement with Sihvo *et al.* (2014) and Trocino *et al.* (2015). On the contrary, the NORM samples did not show any superficial lesions.

As to the microscopic observations, according to the PMF myodegeneration (**Figure 4.2**), the NORM breasts were graded as F1 and F2 (two samples) with the F1 samples exhibiting some abnormal fibers and intermingled normal muscle fibers and the F2 ones revealing an increased number of rounded fibers with characteristic hyalinization and nuclear internalization. On the other hand, the WB samples exhibited severe myodegeneration (F3) and fibrosis both in the superficial and in the deep part. In addition, in agreement with previous studies, many histopathological features such as hyaline and vacuolar degeneration, necrosis and lysis and plentiful inflammatory infiltrate were observed (Sihvo *et al.*, 2014; Trocino *et al.*, 2015; de Brot *et al.*, 2016). No changes in the histological features were observed with storage time at 10, 72 and 168 h *post-mortem* either in NORM or WB samples.

The main causes and/or consequences associated with the increase of interstitial connective tissue (resulting in fibrosis) might be attributed to a reduced microcirculation and subsequent impaired muscle fiber metabolism and oxygen supply leading to ischemia (Sosnicki and Wilson, 1991; Kuttappan *et al.*, 2013a; Petracci *et al.*, 2015; Trocino *et al.*, 2015) and hypoxia (Hoving-Bolink *et al*., 2000; Joiner *et al*., 2014). In this regard, the WB cases exhibited a differential expression of the genes associated with hypoxia as assessed by Mutryn *et al.* (2015) through a RNA-sequencing

analysis.

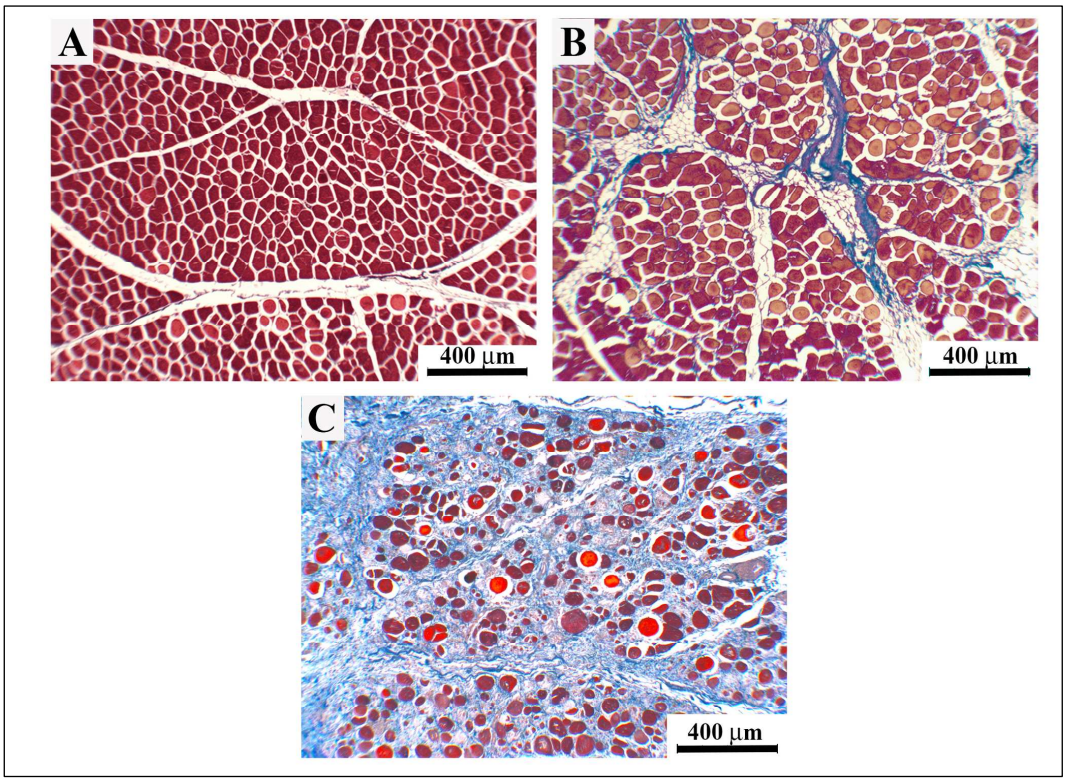


Figure 4.2 Histological representation of levels of myodegeneration.
Score F1 – mild histogical changes: abnormal fibers ranging from 2 to 4 for each primary myofiber fascicle (PMF) (A); Score F2 – moderate: abnormal fibers ranging from 5 to 10 for each PMF, and connective and fatty tissue infiltrates the perimysial space (B); Score F3 – severe: abnormal fibers represent the majority of the fibers for each PMF, and the muscle fibers exhibit high variability in size. Muscle fibers are immersed in an abundant connective tissue (C).

In addition, although micro-ischemia was previously linked to the reduced endomysial and perimysial spaces and capillary density (and capillary to fiber ratio) (Sosnicki and Wilson, 1991; Dransfield and Sosnicki, 1999; Velleman *et al.*, 2003), the findings of the present study revealed an accumulation of endomysial and perimysial fibrotic tissue and a subsequent increase in the spaces between the muscle fibers. Similar histological features exhibiting endomysial fibrosis and an increase in capillary-to-myofiber distance were previously observed in *mdx* mice (Desguerre *et al.*, 2009; Latroche *et al.*, 2015). In addition, an increase in the inter-capillary distance and an impaired angiogenesis were respectively found in dystrophic 7-10 month of age golden retriever and *mdx* mice (Nguyen *et al.*, 2005; Matskas *et al.*, 2013). Thus, since the increase of connective tissue might be

associated with a lower capillary density and a longer inter-capillary distance, fibrosis seems to be one of the main factors linked to the impairing of the microvascular architecture in dystrophic skeletal muscles. Thus, the physical barrier and/or distance between the capillary/microvessel contour and the myofiber, might contribute in explaining the different degree of myodegeneration observed in the superficial and the deep part of the *Pectoralis major* muscles affected by WB abnormality: being more distant from the blood vessels responsible for oxygen translocation, the superficial part is more severely affected.

4.2.1.3.2 Texture

The peak forces recorded when compressing both the raw and the cooked meat samples by 40 and 80% of their initial height are shown in **Table 4.1**.

With regard to raw meat, a similar trend was found in the values measured either at 40 or 80% of compression. In detail, considering the compression values measured 10 h *post-mortem*, no significant differences were found between the deep layer of the WB cases and either the superficial or the deep layer of the NORM. Notwithstanding, the superficial layer of the WB cases exhibited pronounced higher 40 and 80% compression values measured at the earliest sampling time of 10 h. Although the 40% compression measurements did not reveal any evolution in raw meat tenderness during storage, compression to 80% of the initial height showed that there is a tendency for a progressive softening process taking place within the superficial layer of the WB samples from 10 to 72 h *post-mortem*. Increased compression values have been previously reported in the caudal portion of the *Pectoralis major* muscles affected by Wooden Breast abnormality (Mudalal *et al.*, 2015), but this study aims at evaluating the textural properties of meat keeping the superficial layer separated from the deep one. Among the cooked samples, compression values (measured both at 40 and 80% of compression) did not differ among the groups, indicating that the shearing properties of cooked meat were not affected by WB, with the only exception being the samples at 10 h *post-mortem*. In that case, the surface layer of the WB cases (both the superficial and the deep part)

exhibited lower values than at other time points, measured at 80% compression.

Table 4.1 Compression values measured on both raw and cooked samples excised from the superficial (s) and the deep (d) layer of NORM and WB fillets. The values (expressed in Newtons) were recorded after compressing the samples to 40% and 80% of their initial height following storage for 10, 24, 72, 120 and 168 h *post-mortem*.

Group		Storage time (*post-mortem* h) 10	24	72	120	168	sem
Raw meat		Compression Force (Newtons)					
40%	NORM s	6.9^{Y}	8.8^{XY}	8.8^{XY}	6.9	8.8^{XY}	0.9
	NORM d	6.9^{Y}	4.9^{Y}	6.9^{Y}	6.9	4.5^{Y}	0.9
	WB s	27.5^{X}	20.6^{X}	22.6^{X}	18.6	22.6^{X}	1.9
	WB d	11.8^{Y}	10.8^{XY}	10.8^{XY}	8.8	8.8^{Y}	0.9
80%	NORM s	17.7^{Y}	15.7^{XY}	14.7	14.7^{XY}	17.7	0.9
	NORM d	15.7^{Y}	12.8^{Y}	14.7	14.7^{XY}	14.7	0.9
	WB s	$32.4^{a,X}$	$24.5^{ab,X}$	20.6^{b}	$24.5^{ab,X}$	21.6^{ab}	1.9
	WB d	16.7^{Y}	12.8^{Y}	10.8	11.8^{Y}	11.8	0.9
Cooked meat		Compression Force (Newtons)					
40%	NORM s	55.9^{a}	44.1^{ab}	33.4^{ab}	32.4^{b}	36.3^{ab}	1.9
	NORM d	54.9	51.0	35.3	33.4	36.3	1.9
	WB s	52.0	49.1	45.1	42.2	34.3	2.9
	WB d	48.1	46.1	39.2	40.2	34.3	1.9
80%	NORM s	$105.0^{a,XY}$	69.7^{ab}	55.9^{b}	53.0^{b}	57.9^{ab}	5.9
	NORM d	$131.5^{a,X}$	95.2^{ab}	61.8^{b}	60.8^{b}	72.6^{b}	6.9
	WB s	69.7^{Y}	67.7	56.9	54.0	51.0	2.9
	WB d	89.3^{XY}	76.5	63.8	62.8	62.8	3.9

N = 120; 6 samples / storage time / group. ^{a,b} Mean values within the same row followed by different superscript letters significantly differ among the storage time ($P \leq 0.05$); X,Y Mean values within the same column followed by different superscript letters significantly differ within the compression groups; sem = standard error of mean.

On the other hand, a progressive decrease in hardness was found to occur during *post-mortem* storage of the NORM samples. In agreement with cooked meat compression results, the intra-fillet sampling position did not exert any relevant effect on Allo-Kramer shear force. An overall progressive tenderization process took place during storage ($P \leq 0.001$) of NORM and WB samples, the last ones exhibiting significantly higher ($P \leq 0.05$) shear

force values (data not shown). The findings from texture analysis revealed that the differences between the WB *vs*. NORM groups were mainly detected when raw meat rather than cooked was analysed. This result might be partly explained considering the findings from the histological observation of the WB cases. Thus, the increase of interstitial connective tissue resulting in fibrosis (Sihvo *et al.*, 2014), observed also in this study, and the increased deposition of extracellular matrix (glycosaminoglycans; Velleman and Clark, 2015) seen within the WB samples might exert a relevant effect on raw meat hardness. However, in spite of aforementioned increased amount of connective tissue components found in the WB cases, thermally labile cross-links may be the reason for the similar evolution of compression and shear force values measured on both WB and NORM cooked samples. Heat denaturation/solubilisation of collagen was thus found to occur readily at temperatures between 53 and 63°C (Martens *et al.*, 1982).

4.2.1.3.3 Particle size analysis

The typical shape of a particle size distribution curves for both NORM and WB samples are shown in **Figure 4.3**. In their previous study, Lametsch *et al.* (2007) extracted myofibrillar protein from porcine *Longissimus dorsi* muscles and found a mono-modal distribution curve that was related to the length of the myofibrils and their fragmentation products. In fact, the length of a purified myofibril was previously reported to be around 100 µm (Hopkins *et al.*, 2000). In this study, the distribution curve exhibited a tri-modal shape likely because of the structural differences between the species and muscles and that no purification of the myofibrillar protein was performed before analysing the particle size distribution of the samples.

This protocol was followed according to Lametsch et al. (2007) who found that, when using multi angle light scattering to estimate particle size, measurements can be performed on a meat homogenate and differences in the particle size distribution were more pronounced when a meat homogenate rather than partly purified myofibrils was analysed.

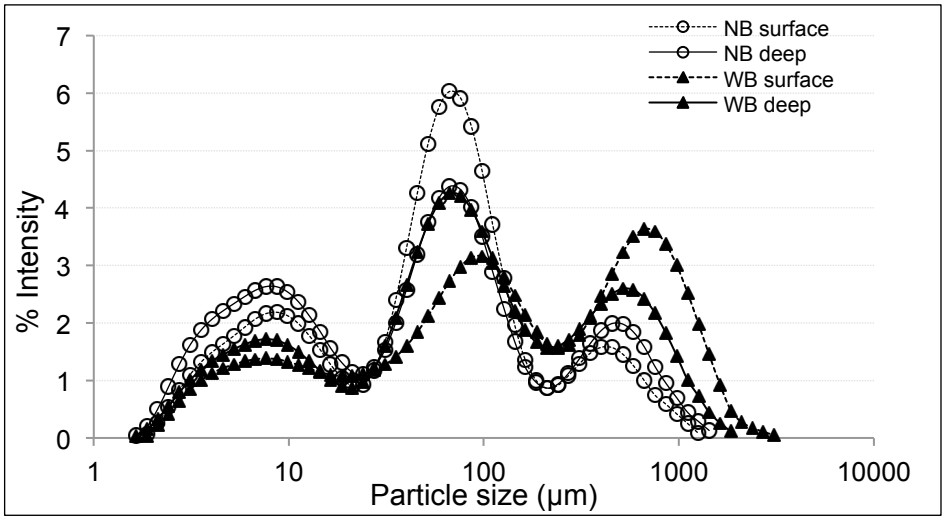


Figure 4.3 Example of a typical shape of the particle size distribution curves for both NORM (round-shaped, open) and WB (triangle-shaped, solid black) samples considered as surface (dotted line) and deep (solid line) layer.

Moreover, following this procedure, a difference in the fragmentation pattern between NORM and WB samples was observed. In comparison with NORM, the WB cases exhibited a higher overall volume of the fraction of particles characterized by a larger size. This effect was more evident in the superficial part than in the deep layer of the WB muscles, as shown in **Figure 4.4** where the evolution of the D10, D50 and D90 during refrigerated storage at 4 ± 1°C is reported. No significant differences were found either in the superficial or the deep layer of the NORM samples during a 7-days storage. On the other hand, both the superficial and the deep part of the WB cases exhibited remarkably higher ($P \leq 0.001$) D10, D50 and D90 indexes that significantly ($P \leq 0.001$) decreased following storage. The results concerning the evolution in D50 and D90 for both NB and WB samples are shown in Figures 4B and 4C. As to D50, a similar trend was observed for both the superficial and the deep layer of NORM samples without any relevant variations caused by the storage time. On the other hand, after increasing up to day 3, an overall decrease in D50 was observed in the WB fillets at day 7 (both in the superficial and the deep part). A similar trend was observed in D90 and D [4:3] except for the deep layer of the WB samples that exhibited a particle size distribution comparable to those found in NORM. This increase in intermediate and large size particles observed in the WB samples (mainly

the superficial layer) might be explained by considering the role of the extracellular matrix in maintaining the skeletal muscle structure. As previously reported, an accumulation of interstitial connective tissue and diffuse thickening in the interstitial fraction and deposition of variable amounts of loose connective tissue (fibrosis) was widely found in the WB samples.

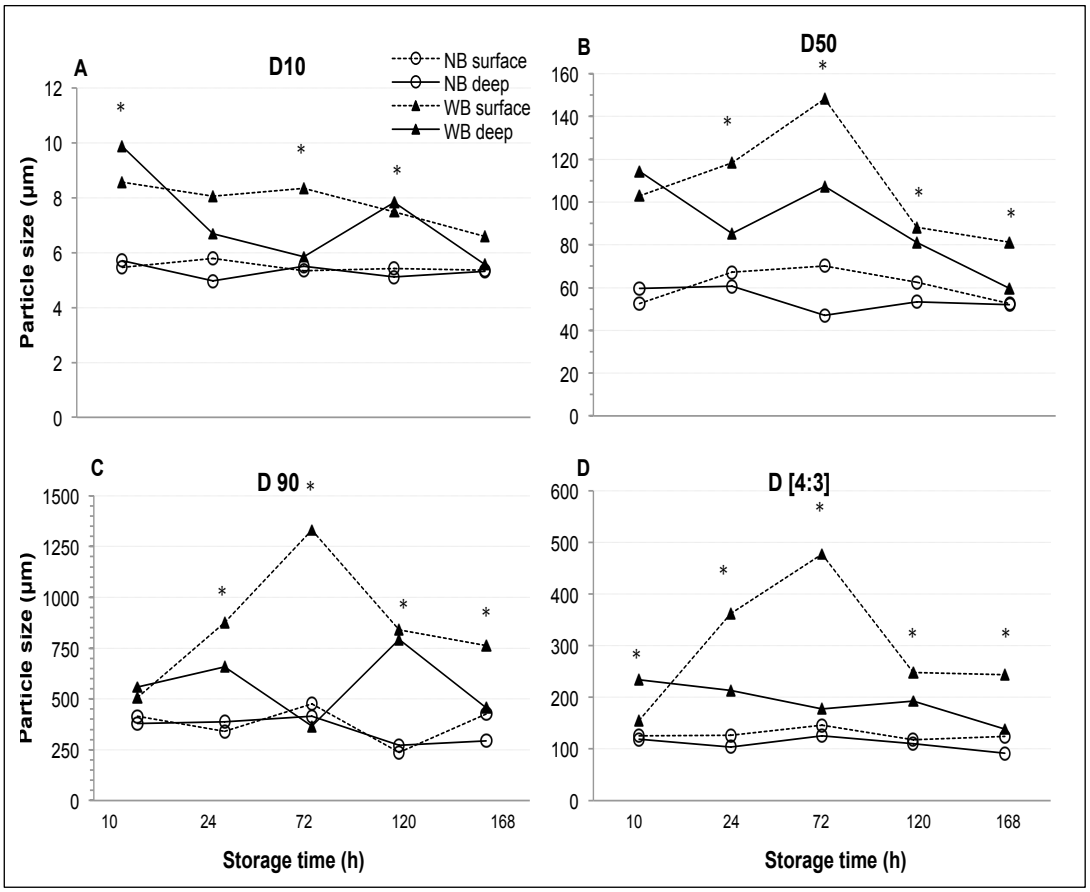


Figure 4.4 Results of the distribution parameters resulting from the multi angle light scattering during 7 days of refrigerated storage for both Normal (NORM) (round-shaped, open) and Wooden Breast (WB) (triangle-shaped, solid black) samples considered as superficial (dotted line) and deep (solid line) layer (N = 30). The parameters D10 (a), D50 (b), D90 (c) related to the particle size below which 10, 50, and 90% of the particle exist and D [4,3] (d) in which the proportion of particles for each size is weighted according to their volume when calculating the mean size were considered in this study. * = Within each storage time mean values significantly differ ($P \leq 0.001$) among the experimental groups (NB surface, NB deep, WB surface, WB deep).

Such collagenous structures provide the connective tissue with a high degree of inherent strength, which in turn, contributes to resistance to shearing of the muscle tissue during homogenization. The larger particle size

of WB muscles is likely a consequence of not only the connective tissue itself giving rise to larger particles, but also that it protects other structures, such as the myofibrils, against fragmentation during the homogenization. In agreement with our findings, Sihvo *et al.* (2014) observed a longitudinal fragmentation in degenerating fibers, but the results of this study indicate that the increase in connective tissue overcomes the longitudinal fragmentation in WB cases. Similarly, Feit and Domke (1982) demonstrated that the increased resistance to fragmentation observed in dystrophic chicken muscles might be attributed to an altered muscle structure. In addition to collagen, since the proteoglycans are another principal component of the extracellular matrix and are particularly stable at low temperatures, their accumulation around the skeletal muscle fibers and within the interstitial spaces (Serrano and Munoz-Canoves, 2010) might also be responsible for the larger fraction of larger particles and for the increase in particles with larger size found within the WB samples. On the other hand, the structures in the WB cases giving rise to the larger particles may have exhibited a degradation processes at the later stage of storage (from day 3 towards 7), leading to muscle tissue fragmentation including softening of the connective tissue and subsequently resulting in an overall decrease in larger particles.

4.2.1.3.4 Correlations

The correlations between the results from multi angle light scattering, compression test and shear force are shown in **Table 4.2**. Significant correlations ($P \leq 0.001$) were found between all the distribution parameters obtained from multi angle light scattering and the compression test (both 40 and 80% compression) assessed on raw meat. This suggests that the increased amount of connective tissue components observed in the WB samples provides a high degree of inherent strength which in turn result in modified textural properties and contribute to resistance of the muscle tissue during homogenization. On the other hand, no significant correlations were found between the distribution parameters and either the compression test or the shear force measured on cooked meat. This finding might be explained considering the thermally labile cross-links that can be speculated to be present in the newly deposed connective tissue found in WB. In detail, the

heat-induced changes in the extracellular matrix (leading to denaturation of collagen and likely of proteoglycans) may be the reason for the analogous evolution in textural properties assessed after cooking the NORM and WB samples.

Table 4.2 Correlations between the distribution parameters D10, D50, D90 and D[4:3] resulting from multi angle light scattering, compression test and shear force.

					Compression raw		Compression cooked	
	D10	D50	D90	D[4:3]	40%	80%	40%	80%
D10								
D50	0,655 ***							
D90	0,497 ***	0,868 ***						
D[4:3]	0,561 ***	0,920 ***	0,987 ***					
Compression raw 40%	0,323 **	0,447 ***	0,458 ***	0,466 ***				
Compression raw 80%	0,336 **	0,449 ***	0,523 ***	0,527 ***	0,516 ***			
Compression cooked 40%	0,307 *	0,223	0,196	0,213	-0,191	0,078		
Compression cooked 80%	0,039	-0,055	-0,197	-0,168	-0,195	-0,123	0,609 ***	
Shear force	0,050	-0,043	-0,128	-0,118	-0,058	0,101	0,327 *	0,563 ***

* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

4.2.2 Effect of a 7-days refrigerated storage on the main proteolytic indicators in broiler *Pectoralis major* muscles affected by Wooden Breast abnormality

4.2.2.1 Materials and Methods

A total of 60 boneless and skinless *Pectoralis major* muscles were randomly selected from the same flock of high-breast yield hybrids (males, Ross 508, 37-38 day-old) in the deboning area of a commercial processing plant (HKScan, Eura, Finland). Thirty WB cases (exhibiting diffused hardened areas and macroscopic changes) and thirty Normal fillets (NORM) were selected in two batches at 3 h *post-mortem* by manual palpation and visual appearance, kept at 0-2°C in ice and sampled at 10 h *post-mortem*. After trimming off any superficial fat, cartilage and connective tissue, both NORM and WB fillets were packed into a loose polyethylene bag and stored at 5 ± 1ºC for 10, 24, 72, 120 and 168 h *post-mortem*. At each time point three

NORM and three WB fillets were sampled and cut in order to separate the surface from the deep layer according to the sampling procedure adopted in our previous study. Then, sub-samples were excised from the superficial and deep layer of each sample, coarsely cut (to avoid an excessive fragmentation of the muscle tissue that could influence the analysis) by using a kitchen blender and used to assess

Free calcium	Par. 2.7.5
SDS-PAGE	Par. 2.7.2.2 (a)
Western-Blot against desmin	Par. 2.6.3
Calpain activity	Par. 2.6.2

Being the WB samples diffusely affected cases, our sampling protocol led us to analyse both the lesion (surface) and the non-lesion (deep layer) areas of the WB samples and compare them with their corresponding portion within the NORM.

4.2.2.2 Statistical analysis

The findings were statistically evaluated with the Two-Ways ANOVA option of the GLM procedure present in SAS software (1988). The main effects of meat abnormality coupled with the sampling position (NORM superficial, NORM deep, WB superficial, WB deep), the storage time and their interactions were evaluated and means separated using the Tukey's HSD test (multiple range test) of the GLM procedure (SAS Institute, 1988).

4.2.2.3 Results and Discussion

The *post-mortem* proteolytic processes taking place within the *Pectoralis major* muscles were studied in order to improve the current knowledge concerning the effect exerted by the Wooden Breast condition on proteolysis as well as proteolytic enzymes activity in breast meat.

Figure 4.5 shows the electrophoretic patterns of the myofibrillar proteins extracted from chicken *Pectoralis major* muscles at different *post-mortem* times (10, 24, 72, 120 and 168 h after slaughtering). Several myofibrillar protein bands including nebulin (500 kDa), MHC (200 kDa), α-actinin (103 kDa), actin (43 kDa) and tropomyosin (36 kDa) were identified

according to their molecular weight and migration patterns (Lowey *et al.*, 1969; Sakakibara and Yagi 1970; Samejima and Wolfe, 1976; Schiaffino and Reggiani, 1996; Tomaszewska-Gras *et al.*, 2011).

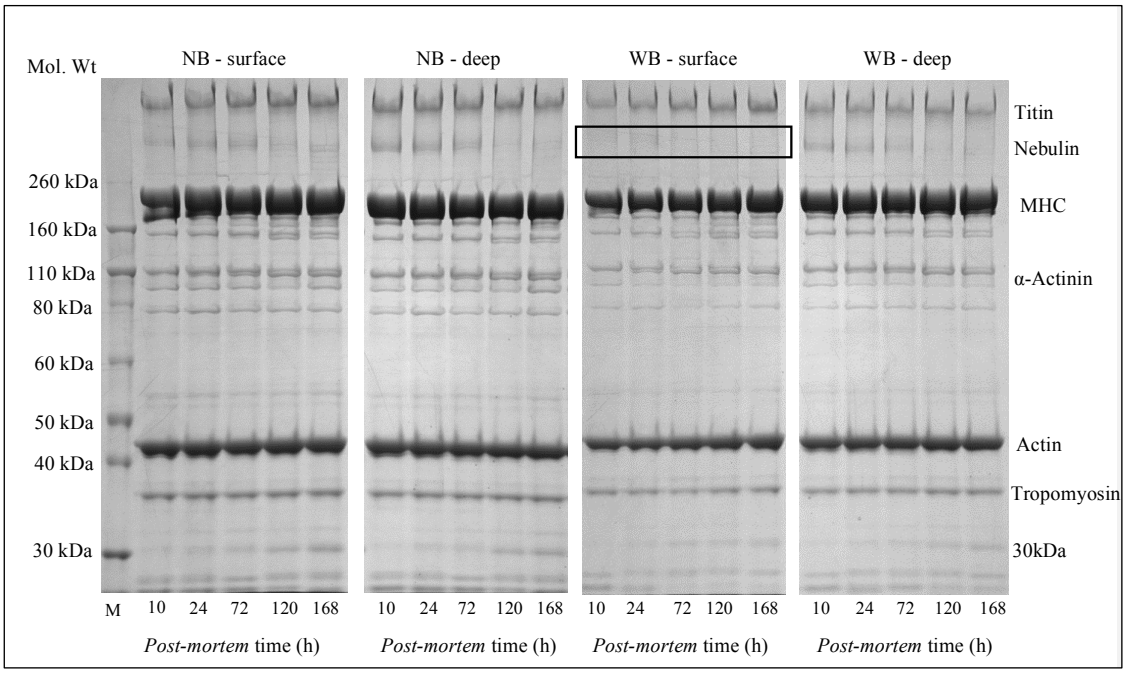


Figure 4.5 7% Tris-Acetate gel electrophoresis of myofibrillar proteins extracted from both the superficial and the deep layer of Normal (NORM) and Wooden Breast (WB) chicken *Pectoralis major* muscles at different *post-mortem* times (10, 24, 72, 120 and 168 h). M = Molecular weight marker.

An overall increase in the number of detectable bands was observed after aging both in the NORM and the WB samples as a result of the proteolytic processes affecting the myofibrillar protein fraction during storage. In agreement with previous studies performed on avian muscles, a 30-kDa degradation product presumably partly resulting from the hydrolysis of troponin T (Lee *et al.*, 2008) appeared and accumulated during aging of both the NORM and the WB fillets. In addition, according to previous studies myofibrillar proteins degradation in dystrophic chickens was found to occur 2-fold faster than within their unaffected counterpart (Hillgartner *et al.*, 1981; Ashmore *et al.*, 1986). On the other hand, although similar electrophoretic profiles were observed comparing the same sampling position (superficial vs. superficial and deep *vs.* deep layer) for NORM and WB samples, an evident lack of a high-molecular weight band, ascribed to nebulin, was found in the

superficial layer of the WB fillets at 10 h *post-mortem*. This finding might be explained considering that proteolysis of nebulin during aging was previously found to occur in chicken muscles starting from 3 h *post-mortem* by both SDS-PAGE analysis (Paxhia and Parrish, 1988; Chou *et al.*, 1994) and Western Blot (Tomaszewska-Gras *et al.* 2011). Nevertheless, conversely to what previously observed by Tatsumi and Takahashi (1992), the amount of free calcium measured within this study through a Ion Selective Electrode might not contribute in explaining the increased degradation rate for nebulin observed within the superficial layer of the WB samples. As shown in **Table 4.3**, no significant differences were found between the two experimental groups in the total amount of free calcium assessed at each storage time with the only exception being the amount measured at 168 h *post-mortem* in which the WB samples (both the superficial and the deep layer) exhibited a significantly higher free calcium content in comparison with the NORM (96.4 and 88.0 *vs.* 20.3 and 53.0 μM; $P \leq 0.001$). In addition, although the amount of free calcium measured in NORM was slightly affected by ageing, a remarkable increase was observed within the WB cases (both in the superficial and the deep layers) at 168 h *post-mortem*. The findings of the present study evidenced that, according to previous studies performed on normal chicken muscles, free calcium reached its ultimate concentration within the first 24 h *post-mortem* (Nakamura, 1973; Ji and Takahashi, 2006).

Table 4.3 *Post-mortem* changes in free calcium concentration (μM) measured through an Ion Selective Electrode on both the superficial and the deep layer of Normal (NORM) and Wooden Breast (WB) chicken *Pectoralis major* muscles at different times *post-mortem* (10, 24, 72, 120 and 168 h).

		Post-mortem time (h)				
		10	24	72	120	168
Group	NORM - surface	65^{a}	20^{b}	25^{b}	24^{b}	$20^{b,y}$
	NORM - deep	55	18	19	27	53^{xy}
	WB - surface	57^{ab}	51^{b}	45^{b}	19^{b}	$96^{a,x}$
	WB - deep	47^{b}	18^{b}	17^{b}	53^{ab}	$88^{a,xy}$

^{a,b} Mean values within the same row followed by different superscript letters significantly differ among the storage times ($P \leq 0.05$); ^{x,y} Mean values within the same column followed by different superscript letters significantly differ among groups ($P \leq 0.05$);

In contrast with a previous study performed by Hay *et al.* (1973) on normal

chicken breast muscles, the calcium sequestering properties of the sarcoplasmic reticulum of the WB cases might be impaired during *post-mortem* aging. As a result, a gradual leakage of calcium ions into the sarcoplasm during ageing might contribute to explain the presence of significant differences between NORM and WB cases at 168 h *post-mortem*. Similarly, an increased level of free calcium was previously observed in mdx mice (Turner *et al.*, 1988). Also the profound changes occurring within the *post-mortem* period involving the release of calcium ions as a result of the higher proteolytic degradation of calcium sequestering interfilament proteins (such as titin and nebulin) (Bond and Warner, 2007) might contribute in explaining the higher amount of free calcium observed in WB samples at 168 h *post-mortem*.

In the present study, casein zymography was perfomed on both the superficial and the deep layer of NORM and WB *Pectoralis major* muscles in order to assess the activity of both the native and the autolyzed calpain forms. As showed in **Figure 4.6**, two clear bands, ascribed to μ/m calpain and its autolyzed form migrated as a doublet into the casein gel whereas the μ-calpain band was not detected. These results are consistent with previous studies performed by Kitagaki *et al.* (2000) and Chang *et al.* (2016) in which only one calpain isoform was observed in *post-mortem* avian and ostrich muscles. This is likely due to the very limited μ-calpain activity present in most of the chicken tissues (Lee *et al.*, 2007) and might be partly explained considering that the sensitivity of zymography was previously found not to be high enough to detect all the μ-calpain activity present in *post-mortem* bovine muscles (Hopkins and Geesink, 2009). In addition, since the calcium concentration required for their activation is very low, μ-calpain might be mobilized very soon after slaughter leading to very low activity 12 hours *post-mortem* (Lee *et al.*, 2008). In agreement, μ/m calpain was identified as the dominant calpain form within the chicken *Pectoralis major* muscle accounting for the 90% of the total calpain activity (Lee et al., 2007).

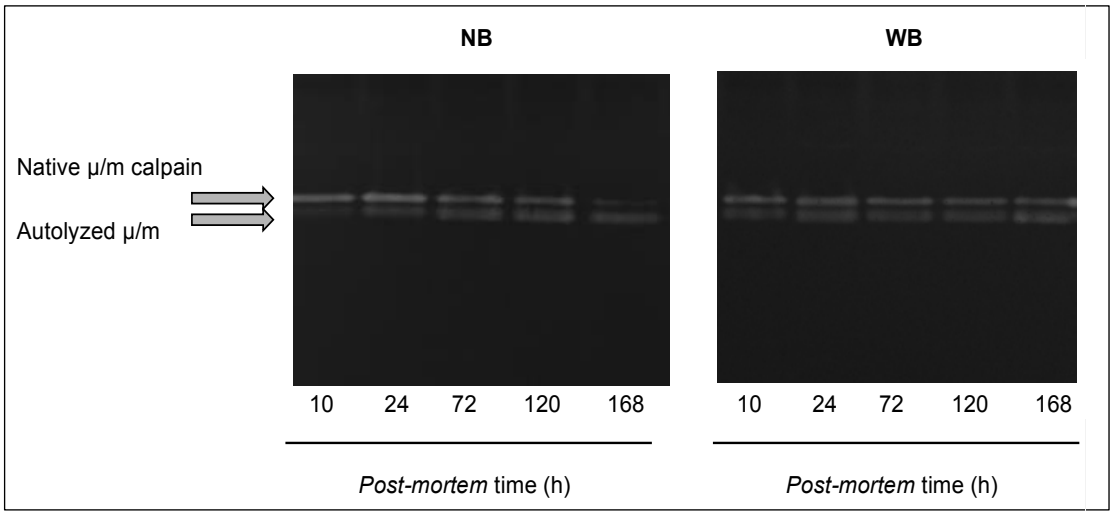


Figure 4.6 Zymograms showing activity of native (upper left side arrow) and autolyzed (lower left side arrow) μ/m calpain assessed on the superficial layer of both Normal (NORM) and Wooden Breast (WB) chicken *Pectoralis major* muscles at different times *post-mortem* (10, 24, 72, 120 and 168 h).

As shown in **Figure 4.7**, the unautolyzed μ/m calpain activity significantly decreased ($P \leq 0.05$) from 100% in 10 hours-samples to 37% within the *Pectoralis major* muscles stored up to 168 h *post-mortem*, regardless of the occurrence of the WB condition and the intra-fillet sampling position. Concurrently, a 2 to 3-fold increase ($P \leq 0.05$) in the autolyzed μ/m calpain activity was measured at longer *post-mortem* times with the superficial section exhibiting the highest values. Overall, the autolyzed μ/m calpain activity measured at 10 and 168 h *post-mortem* was 21 and 56% of the unautolyzed one measured in NORM samples 10 hours after slaughtering which was considered as 100%. Within this context, the absence of significant differences in μ/m calpain activity might contribute in explaining the similarities in textural properties of cooked NORM and WB cases observed by Mudalal *et al.* (2015).

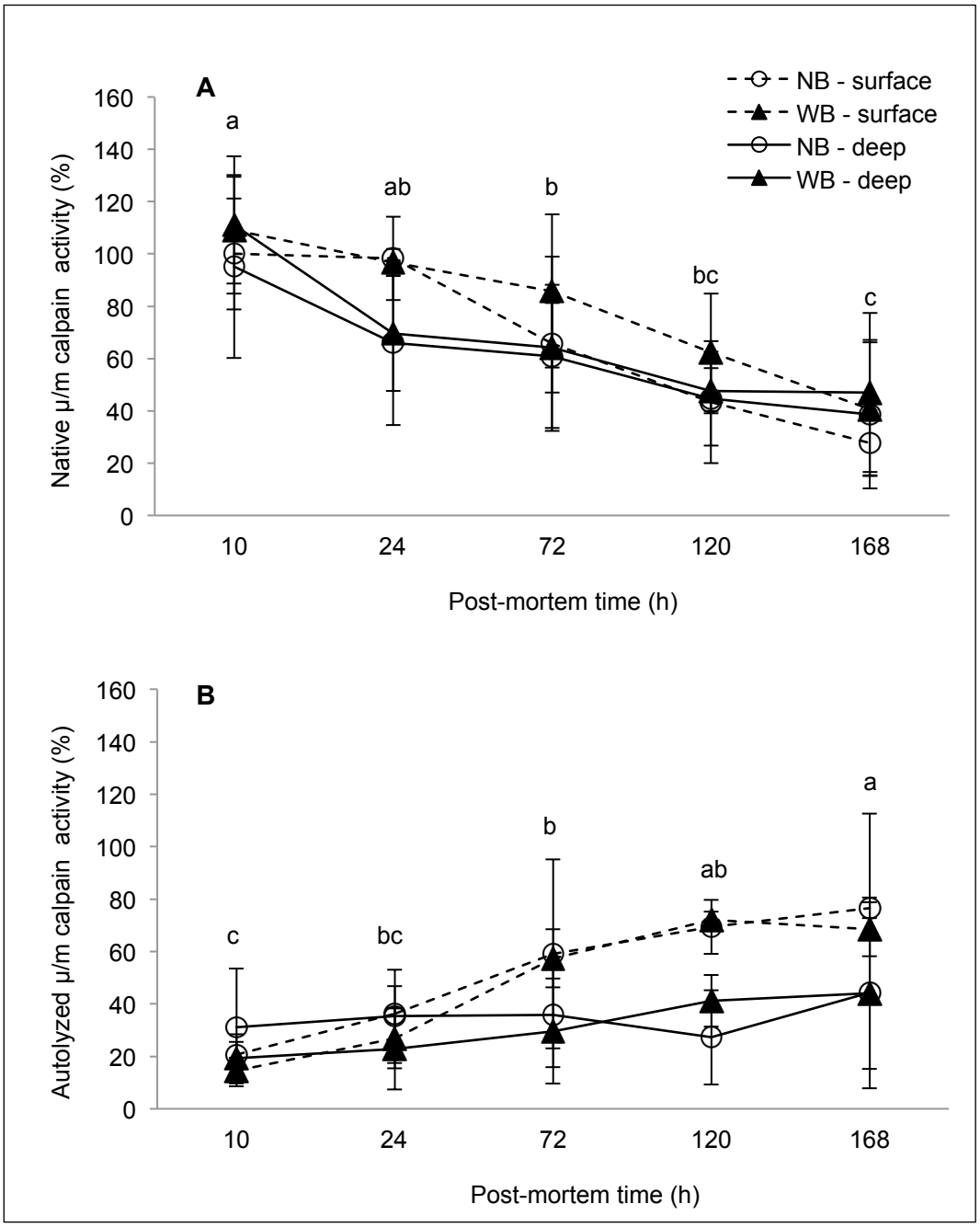
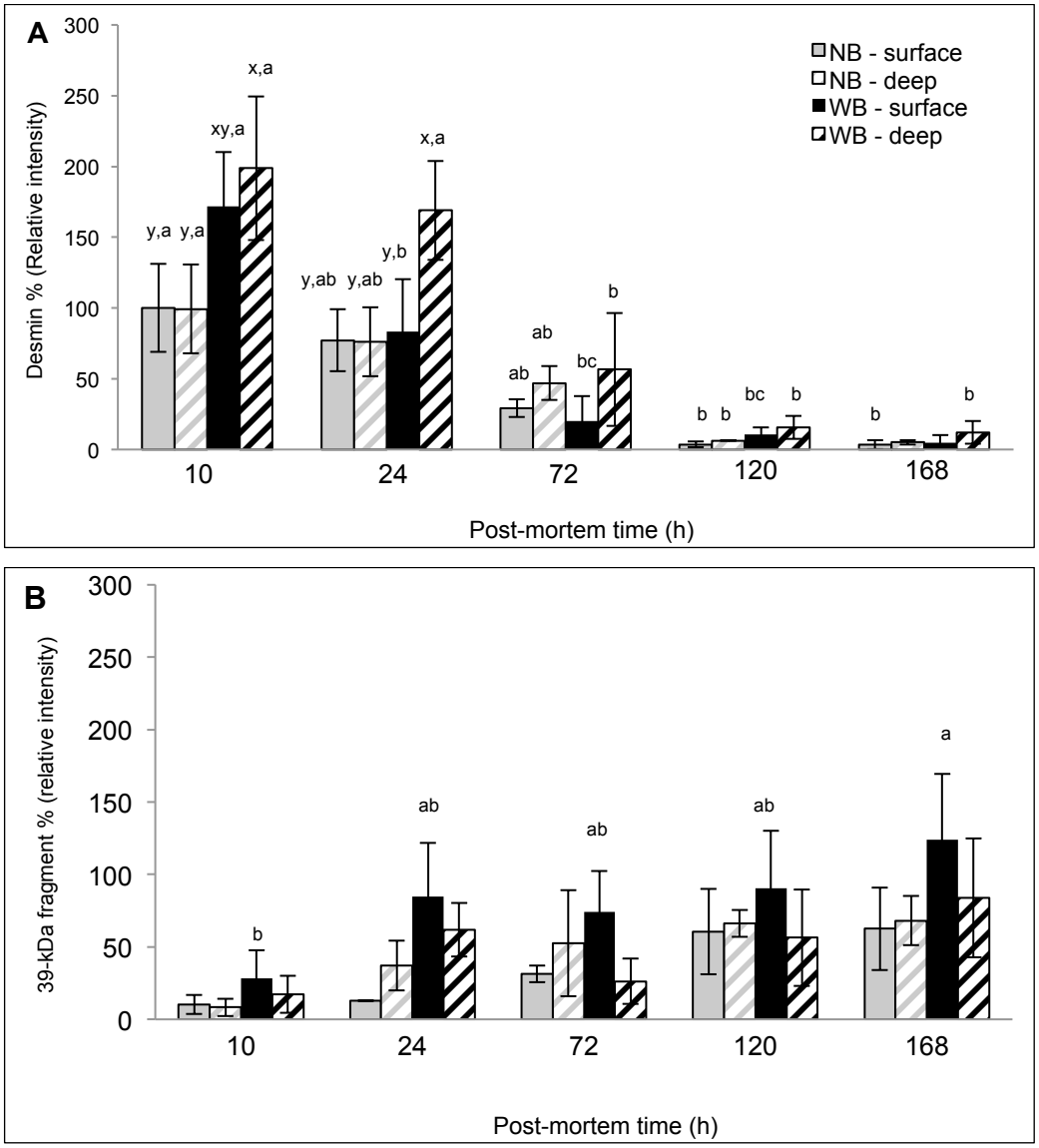


Figure 4.7 Effect of different *post-mortem* storage time (10, 24, 72, 129 and 168 h) on native (A) and autolyzed µ/m calpain (B) activity (expressed as %) assessed on both the superficial (dotted line) and the deep (solid line) layer of Normal (NORM) (round-shaped, open) and Wooden Breast (WB) (triangle-shaped, solid black) *Pectoralis major* muscles with casein zymography. Error bars indicate standard deviations. [a,b] Mean values within the same row followed by different superscript letters significantly differ among the storage times ($P \leq 0.001$);

Considering its importance in muscle structure integrity and its central role as a target to determine meat tenderness (Koohmaraie and Geesink,

2006) desmin degradation during storage was assessed in order to understand whether the occurrence of the Wooden Breast condition exerts any relevant effect on *post-mortem* proteolysis. The relative intensities of intact desmin band and its degradation fragments were quantified considering as 100% the intensity of the band assigned to intact desmin in NORM samples at 10 hours *post-mortem*. Only intact desmin (52 kDa) and its 39 kDa-intact rod domain (Baron *et al.*, 2004) were clearly detectable and thus, quantified. As shown in **Figure 4.8**, if compared to NORM, a significantly larger ($P \leq 0.05$) amount of desmin was observed in both the superficial and the deep layer of the WB samples at 10 h *post-mortem* (1.7 and 2.0 fold greater, respectively). This phenomenon might be partly explained considering that, since according to previous studies (Sihvo *et al.*, 2014) the WB cases exhibited severe histological lesions accompanied by fiber regeneration, a response mechanism involving an increased accumulation of desmin might be hypothesised in order to maintain muscle cyto-architecture and thus also affecting raw meat texture (Mudalal *et al.*, 2015). As the time *post-mortem* proceeded, a sharp decrease of the intact desmin band coupled with a progressive accumulation of its 39-kDa degradation fragment was observed without any significant difference among the groups.

Figure 4.8 Effect of different *post-mortem* storage times (10, 24, 72, 129 and 168 h) on intact desmin band (A) and its 39-kDa degradation fragment (B) (expressed as % considering as 100% the intensity of the band assigned to intact desmin in NORM samples at 10 hours *post-mortem*) assessed on both the superficial (solid bars) and the deep (diagonal striped) layer of Normal (NORM) (light grey) and Wooden Breast (WB) (black) *Pectoralis major* muscles by Western Blot. Error bars indicate standard deviations.



[a,c] Means having different superscripts differ among the *post-mortem* storage times ($P \leq 0.001$); [x,y] Means having different superscripts differ among the groups ($P \leq 0.001$).

4.2.3 Conclusions

The findings of the present study led to a deepened knowledge concerning the effect of the Wooden Breast abnormality on the histological features, texture, fragmentation pattern and evolution of the proteolytic indicators of meat during 7-days of storage. Considering the different degree

of myodegeneration observed within the WB muscles, in the present study the superficial and the deep part of the *Pectoralis major* muscles were analysed separately in order to investigate if the sampling position exerts a relevant role in determining the main features of the Wooden Breast fillets. Although no changes in the histological features were found after 10, 72 and 168 hours *post-mortem* either in NORM or WB samples, the latter exhibited severe myodegeneration accompanied by nuclear internalization, hyaline and vacuolar degeneration and accumulation of perimysial and endomysial connective tissue in both superficial and deep part. With regard to texture, the differences among the groups were mainly detected when raw meat rather than cooked was analysed. The hardness of raw WB was mainly observed in the superficial part of the fillet. As to particle size analysis, the increase of connective tissue (extracellular matrix) and fibrosis might account for the different fragmentation pattern observed between the superficial and the deep layer in the WB cases, with the superficial part exhibiting a higher amount of larger particles and an increase in particles with larger size during storage.

In addition, aside from the occurrence of the WB condition and the intra-fillet sampling position, similar electrophoretic profiles, exhibiting an overall increase in the number of detectable bands after aging, were observed. Nevertheless, an evident lack of nebulin was noticed within the superficial layer of the WB cases at 10 hours *post-mortem*. Two clear bands ascribed to native μ/m calpain and their autolyzed forms were detected through casein zymograms. As previously observed, although both the occurrence of the WB condition and the sampling position did not exert any relevant effect, native μ/m calpain activity significantly decreased during storage. Concurrently, an increase in the activity of the autolyzed μ/m calpain was measured. Interestingly, although no differences were found in the progressive accumulation of the 39-kDa degradation fragment, a significantly higher amount of desmin was observed within both the superficial and the deep layer of the WB cases at 10 hours *post-mortem*.

In conclusion, the findings of the present study evidenced that the increased hardness that typically affect the WB cases might not be exclusively attributed to the differences in the proteolytic processes taking

place within the *post-mortem* period. Thus, further studies are needed in order to investigate and identify the main mechanisms leading to the development of the typical hardened consistency exhibited by the WB samples.

CHAPTER 5
IMPLICATIONS OF WHITE STRIPING AND POOR COHESION ABNORMALITIES ON MEAT QUALITY AND HISTOLOGICAL FEATURES OF BROILERS *PECTORALIS MAJOR* MUSCLES

Recently, it has been observed that the WS defect is also associated with another muscle abnormality termed as Poor Cohesion (PC) and commonly referred to as "Spaghetti Meat", exhibiting an altered structural integrity of the *Pectoralis major* muscle perceivable by pitching its cranial surface (Bilgili, 2015; Sirri *et al.,* 2016). Some years ago, also Swatland (1990) observed an analogous abnormality affecting turkey breast muscles exhibiting a loose structure in which muscle fiber bundles could be pull away by fingers. Thus, since an increased cross-sectional area was observed in muscle fibers from disintegrated meat, Swatland (1990) suggested that these fibers have outgrown their connective tissue. As a result, PC defect seemed to mainly affect the connective tissue within the perymisial compartments leading to the formation of large intracellular spaces. Thus, the fluid lost by myofibrils during *post-mortem* period might result in muscle disintegration. In addition, more recently Ahn *et al.* (2010) observed that the perymisial *septa* of breast muscles in fast-growing birds was thinner than the slow-growing counterpart. As a consequence of their impaired appearance and quality traits, these abnormal fillets are normally downgraded by the poultry meat industry and used for further processing. Thus, there is serious interest in the meat industry to understand the effect exerted by the occurrence of these abnormalities on meat quality traits. Within this context, although both quality and histological traits of WS and wooden breast fillets have been extensively studied (Petracci *et al.,* 2015; Velleman and Clark, 2015; Kuttappan *et al.*, 2016), no information are available concerning the PCD. In addition, since the PC defect often appears in association with WS, this study aimed at evaluating the effect exerted by WS and PC abnormalities (occurring alone or

combined within the same *Pectoralis major* muscle) on muscle histology and meat quality traits (Submitted for publication to *Animal*).

5.1 Materials and Methods

The study was conducted on two flocks of broiler chickens reared and slaughtered under commercial conditions into two consecutive weeks. The birds were homogeneous in genotype (Ross 308 strain), gender (males), age and weight at slaughter (47 days and 2.8 kg) as well as feeding plan (ad libitum access to a wheat/sorghum-soybean multiphase diet). Prior to slaughter, broilers were subjected to a total feed withdrawal of 10 h, including a 3 h lairage time at the processing plant.

Subsequently, birds were processed under commercial conditions: exposed to carbon dioxide for stunning, bled for 180 s and then after being conveyed through scalding tanks (52°C for 220 s), plucked by rotating rubber fingers. After evisceration, carcasses were air-chilled passing through a cold-air flow tunnel (−6°C for 150 min) until reaching 2 to 3°C at the core. Then, a total of 96 *Pectoralis major* muscles (48 samples/each replication) were selected at 3 h *post-mortem* in the deboning area of the same commercial processing plant. The samples were classified by two experienced people according to the presence of WS and PC abnormalities. In detail, the *Pectoralis major* muscles were classified into four experimental groups as follow (by excluding those having signs of wooden breast condition):

- Normal (NORM): fillets exhibiting neither white striations nor tendency towards separation of the muscle fiber bundles composing the *Pectoralis major*.
- WS: exhibiting superficial medium-to-thick white striations in the cranial part of the fillet.
- PC: exhibiting an overall impaired integrity and tendency towards separation of the muscle fiber bundles composing the *Pectoralis major* muscle especially within the cranial part of the fillet.
- WS/PC: affected by both WS and PC abnormalities.

After being collected, the samples were bagged by group and brought to the laboratory under refrigerating conditions. Then, muscles were trimmed from superficial fat, visible cartilage and connective tissues and subsequently

stored at 2 to 4°C until 24 h *post-mortem*. Then, the following analytical determinations were performed:

Morphometric measurements	Par. 2.1
pH	Par. 2.2
Colour	Par. 2.3
Moisture	Par. 2.7.1
Proteins	Par. 2.7.2.1
Fat	Par. 2.7.3.2
Collagen	Par. 2.7.2.6
Ash	Par. 2.7.4
Protein solubility	Par. 2.7.2.3
Myofibril Fragmentation Index	Par. 2.6.1
Protein oxidation	Par. 2.8.2
SDS-PAGE	Par. 2.6.3.2 (a)
NMR-relaxation properties	Par. 2.4.5

5.2 Statistical analysis

Data were analysed using the ANOVA option of the GLM procedure present in SAS software (SAS Institute Inc., Cary, NC, USA) and testing the main effects for type of meat quality abnormality (NORM, WS, PC and WS/PC), position (superficial and deep) and replication, as well as the interaction terms on meat quality traits. Means were separated using Tukey's honestly test multiple range test of the GLM procedure (SAS Institute Inc., Cary, NC, USA).

5.3 Results

5.3.1 Histology

The findings of the histological observations performed on PC and/or WS fillets revealed that, if compared to their unaffected counterpart (NORM), all samples affected by muscle abnormalities exhibited degenerative processes affecting the *Pectoralis major* muscles. Notwithstanding, the microscopic histological features, displayed in **Figure 5.1**, were consistent with the macroscopic grading (classification) of the fillets. Indeed, myodegenerative fibers were not observed within the NORM samples whose fibers exhibited a regular polygonal profile, normal cross-striated architecture

as well as a structured endo- and peri-mysial connective tissue. With regard to the WS, the main histological features associated with the occurrence of this defect were nuclear internalization, loss of cross striations, vacuolar and hyaline degeneration, necrosis and lysis of the fibers, inflammatory cells infiltration, variable cross sectional area (degenerating and regenerating fibers), lipidosis and fibrosis. A distinctive features observed within the WS samples were the increased deposition of adipocytes within the connective tissue (lipidosis) and fat infiltrations attaining the amount of fibrillar components (longitudinal section). On the other hand, when palpated after chilling, the *Pectoralis major* muscles affected by PC defect were extremely soft and brittle (especially in the ventro-cranial portion). In general, this myopathy exhibited histological features reported for associated myopathies (e.g. white striping, wooden breast) including: extensive fibers degeneration and regeneration, hyalinization, poor fiber uniformity, compromised connective and fat tissue deposition. However, a progressive rarefaction of the endo- and peri-mysial connective tissue leading to muscle fibers detachment from each other was observed in PC muscles. In addition, thin and splitted fibers surrounded by loose (immature) connective tissue and abundant inflammatory cells infiltrations were found by examining the longitudinal sections. Interestingly, the samples affected by both WS and PC defect exhibited an alternation of the pathological traits previously described. In detail, some areas exhibited the typical features of the PC defect while others evidenced the pathological changes largely associated with white striping. Overall, the most severe histopathological lesions frequently affected only the superficial section of the fillet while the deep one often exhibited normal traits.

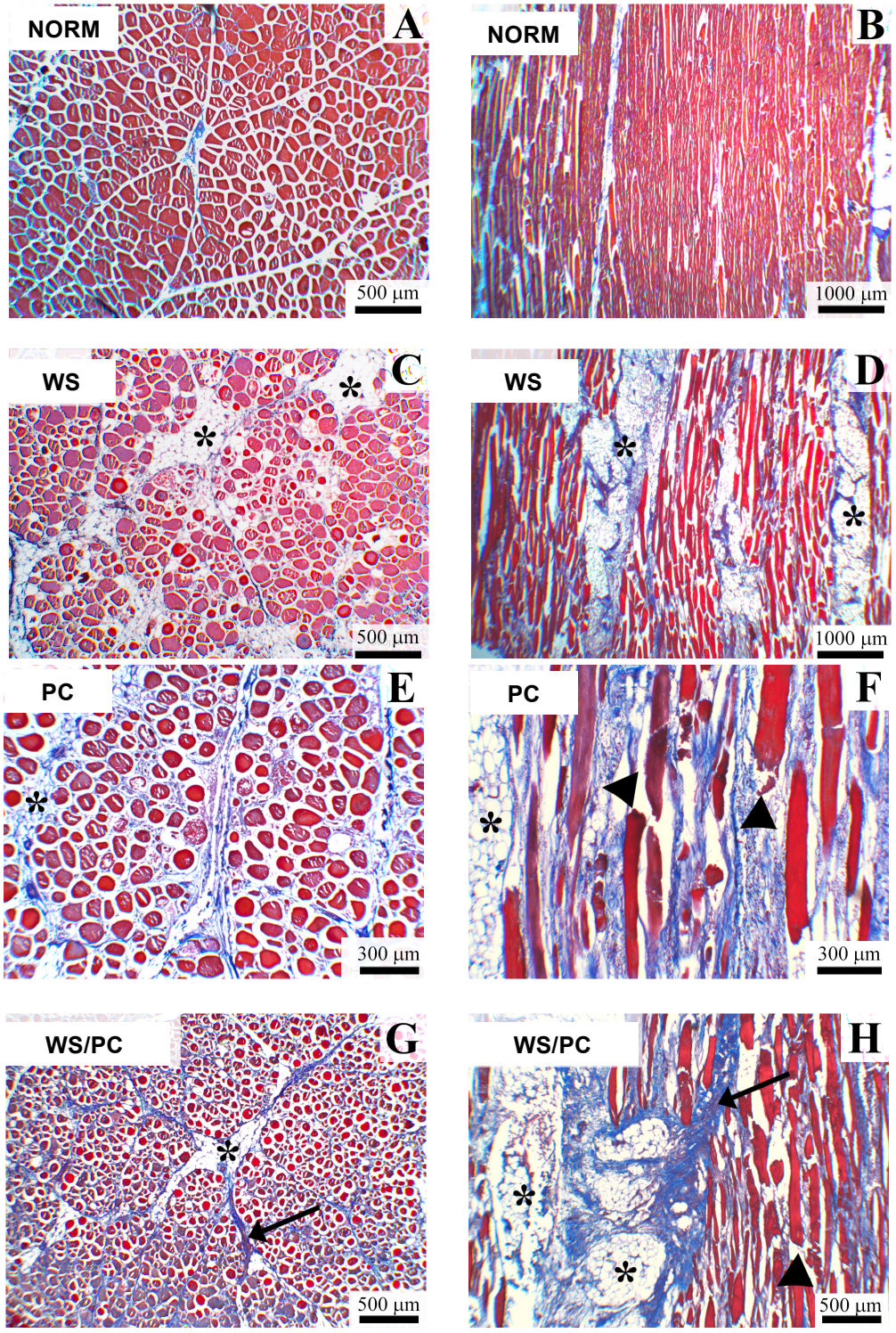


Figure 5.1 Chicken *Pectoralis major* muscle, Masson's trichrome. Representative images of transversal (A, C, E and G) and longitudinal (B, D, F and H) histological sections of normal (N), White Striping (WS), Poor Cohesion (PC) and fillets affected by both abnormalities (WS/PC). In normal samples (N) skeletal muscle fibers show a regular polygonal profile, compact and normal shape and size: also pery- and endo-mysial spaces are normal (A and B). In samples affected by WS is peculiar abundant endomysial fatty tissue infiltrated and, above all, perimysial (C and D, asterisks): fatty infiltration in some cases matches the fibrillar

part. Even in this case, there were observed rounded fibers of different dimensions. In the PC samples muscle fibers were reduced in number and spaced apart (sometimes almost isolated) from each other; such fibers have a rounded profile and very variable dimensions. The peri- and endo-mysial connective tissue is compromised (particularly rarefied around some fibers) (E and F), while adipose tissue infiltrates the space between the muscle fibers (E and F, asterisks). In the longitudinal section were observed numerous split fibers immersed in the rarefied endomysial connective tissue and infiltrated by inflammatory cells (F, arrowheads). WS/PC breasts muscle show histopathological features related to those previously described in the PC and WS. In this case, it is observed in some parts of the section an abundant proliferation of endomysial and perimysial connective (G and H, arrows). In addition, it is possible to observe split fibers (H, arrowhead) and adipose infiltration (G and H, asterisks).

5.3.2 Weight and dimension of raw fillets

The results for weight and dimensions of the breast fillets are shown in Table 1. Overall, if compared to NORM, all abnormal samples exhibited significantly higher weight ($P < 0.001$) and thickness ($P < 0.05$) measured at the top (H1), middle (H2) and bottom (H3) positions. As for length, WS and WS/PC samples revealed significantly higher ($P < 0.005$) values in comparison with both NORM and PC fillets, which did not differ between each other. None of the abnormalities significantly affects the width.

Table 5.1 Effect of WS and PC abnormalities on weight and dimension of raw *Pectoralis major* muscles.

	NORM	WS	PC	WS/PC	sem	*P*-value
Weight (g)	231.6^{b}	268.9^{a}	278.1^{a}	309.2^{a}	5.06	*<0.001*
Length (mm)	174.3^{b}	180.3^{a}	174.6^{b}	180.4^{a}	0.99	*<0.05*
Width (mm)	93.1	94.3	93.9	97.6	0.97	NS
Top height (H1)[1] (mm)	34.5^{d}	40.3^{b}	37.7^{c}	43.0^{a}	0.48	*<0.001*
Middle height (H2)[2] (mm)	23.9^{c}	27.4^{b}	28.7^{ab}	30.8^{a}	0.44	*<0.001*
Bottom height (H3)[3] (mm)	7.3^{b}	8.9^{ab}	10.1^{a}	8.7^{ab}	0.29	*<0.01*

N = 24 samples / group; $^{a-d}$ Means within the same parameter followed by different superscript letters significantly differ ($P<0.05$). NS = not significant; sem = standard error of means.
[1] H1 was measured at the thickest point in the cranial part. [2] H2 was measured at the half distance of the breast length. [3] H3 was measured far from the end of the caudal part by 1-cm toward a dorsal direction.

5.3.3 Proximate composition

The results for proximate composition are shown in **Table 5.2**. If compared to the deep section, a significantly higher ($P < 0.001$) moisture

content was found within the superficial layer of the *Pectoralis major* muscles belonging from all the experimental groups.

Table 5.2 Effect of WS and PC abnormalities on proximate composition of the *Pectoralis major* muscles.

Parameter	Position (P)	Experimental Group (EG)				sem	*P*-values		
		NORM	WS	PC	WS/PC		EG	P	EG×P
Moisture (%)	s	75.0^{bc}	75.2^{b}	76.3^{a}	76.1^{a}	0.17	*<0.001*	*<0.001*	NS
	d	74.7^{c}	74.4^{c}	75.2^{b}	75.1^{bc}				
Protein (%)	s	23.6^{ab}	22.5^{cd}	21.9^{d}	21.9^{d}	0.09	*<0.001*	*<0.001*	NS
	d	24.3^{a}	23.5^{b}	23.5^{b}	23.4^{bc}				
Lipid (%)	s	1.53^{d}	2.47^{a}	1.82^{bcd}	2.40^{a}	0.04	*<0.001*	*0.001*	*<0.05*
	d	1.58^{cd}	2.05^{ab}	1.59^{cd}	1.93^{bc}				
Ash (%)	s	1.20^{a}	1.15^{ab}	1.10^{b}	1.10^{b}	0.01	*<0.001*	*0.001*	NS
	d	1.21^{a}	1.16^{ab}	1.16^{ab}	1.19^{a}				
Collagen (%)	s	0.86^{ab}	0.92^{a}	0.94^{a}	0.92^{a}	0.01	NS	*<0.001*	NS
	d	0.82^{b}	0.84^{b}	0.83^{b}	0.84^{b}				

N = 24 samples / group. $^{a-d}$ Means within the same parameter followed by different superscript letters significantly differ (*P*<0.05). NS = not significant; sem = standard error of means.

In addition, PC and WS/PC meat exhibited significantly higher (P < 0.001) moisture values in both the superficial and the deep sampling position. With regard to protein, a significantly higher (P < 0.001) content was found in both the superficial and deep section of NORM fillets. In addition, if compared to the deep counterpart, a significantly lower (P < 0.001) protein content was observed within the superficial layer of all the abnormal samples with the major differences being measured in PC and WS/PC fillets (WS: 22.5 *vs*. 23.5%; PC: 21.9 *vs*. 23,5%; WS/PC: 21.9 *vs*. 23,4%). As for lipid content, both the superficial and deep sections of WS and WS/PC samples exhibited significantly higher (P < 0.001) values, the last displaying a higher lipid content on the surface layer rather than in the deep (2.40 *vs*. 1.93%; P < 0.05). Although no significant differences among all the experimental groups were found within the deep section, a significantly higher (P < 0.001) ash content was found in the superficial portion of NORM samples. In addition, if the effect of the sampling position is considered, a lower ash content was found within the superficial layer of WS/PC samples (1.10 *vs*. 1.19%; P <

0.05). At last, although no effect of the sampling position was found for NORM, an increase ($P < 0.05$) in collagen content was observed when comparing the deep and superficial sections of all abnormal fillets.

5.3.4 Functional properties and colour

The results for functional properties and colour of breast fillets are shown in **Table 5.3**. Overall, if compared to NORM, all abnormal fillets exhibited significantly higher ultimate pH on both the superficial and the deep sections, with the first exhibiting the highest values. As for raw meat colour, no significant differences were found among the samples with the only exception of both the superficial and the deep layers of the WS/PC fillets, which exhibited a significantly higher yellowness in comparison with NORM. In addition, although redness was not affected by sampling position, all the deep sections were lighter than their superficial counterparts.

Table 5.3 Effect of WS and PC abnormalities on colour and functional properties of *Pectoralis major* muscles.

Parameter	Position (P)	Experimental Group (EG)[1]				sem	*P*-value		
		NORM	WS	PC	WS/PC		EG	P	EG× P
pH	s	5.86^{c}	5.96^{a}	6.03^{a}	6.00^{a}	0.01	*<0.001*	NS	NS
	d	5.87^{c}	5.93^{ab}	5.95^{ab}	5.90^{bc}				
Lightness (L*)	s	54.4^{c}	54.5^{bc}	54.4^{c}	54.8^{abc}	0.20	NS	*<0.001*	NS
	d	56.4^{a}	56.2^{a}	56.2^{ab}	56.1^{ab}				
Redness (a*)	s	0.89	1.13	0.75	1.00	0.06	NS	NS	NS
	d	0.97	1.34	0.85	1.09				
Yellowness (b*)	s	10.3^{c}	12.6^{abc}	11.5^{abc}	13.0^{ab}	0.21	*<0.001*	NS	NS
	d	5.87^{c}	5.93^{ab}	5.95^{ab}	5.90^{bc}				
Protein Solubility (mg/g)	s	244.6^{abc}	219.9^{bc}	216.4^{c}	213.4^{c}	2.82	*<0.01*	*<0.001*	NS
	d	261.2^{a}	244.6^{abc}	240.3^{abc}	251.3^{ab}				
MFI (%)	s	24.4^{d}	35.5^{a}	31.4^{abc}	34.4^{ab}	0.65	*<0.001*	*<0.05*	*<0.05*
	d	27.0^{cd}	30.9^{abc}	29.5^{abcd}	27.4^{bcd}				
Carbonyls (nmol/mg)	s	0.59^{abc}	0.44^{bc}	0.71^{ab}	0.40^{c}	0.04	*<0.01*	*<0.05*	*<0.05*
	d	10.8^{bc}	13.2^{ab}	11.7^{abc}	13.3^{a}				

N = 24 samples / group; $^{a-d}$ Means within the same parameter followed by different superscript letters significantly differ ($P<0.05$). NS = not significant; sem = standard error of means.

With regard to total protein solubility, even if lower values were found in fillets affected by muscle abnormalities, significant differences were found

only when the deep layer of NORM fillets was compared with superficial section of samples affected by PC abnormality (PC and WS/PC groups). In addition, a significant increase in total protein solubility was observed moving from the superficial towards the deep layer of WS/PC samples. As for MFI, if compared to NORM, significantly higher ($P < 0.001$) values were found in the superficial section of all the abnormal groups. In addition, even if according to the statistical analysis, both the main effects and the interaction term were significant, no clear trends were found for carbonyls.

5.3.5 Electrophoretic analysis

The electrophoretic patterns of myofibrillar and sarcoplasmic proteins for both the superficial and deep sections of NORM, WS, PC and WS/PC samples are shown in **Figure 5.2**. Overall, no significant differences were found by comparing the deep sections of the four experimental groups. On the other hand, an increased number of high molecular-weight bands (ranging in size from 100 to 250 kDa) ascribed to both the myofibrillar and the sarcoplasmic protein fractions was found within the superficial layer of all abnormal samples.

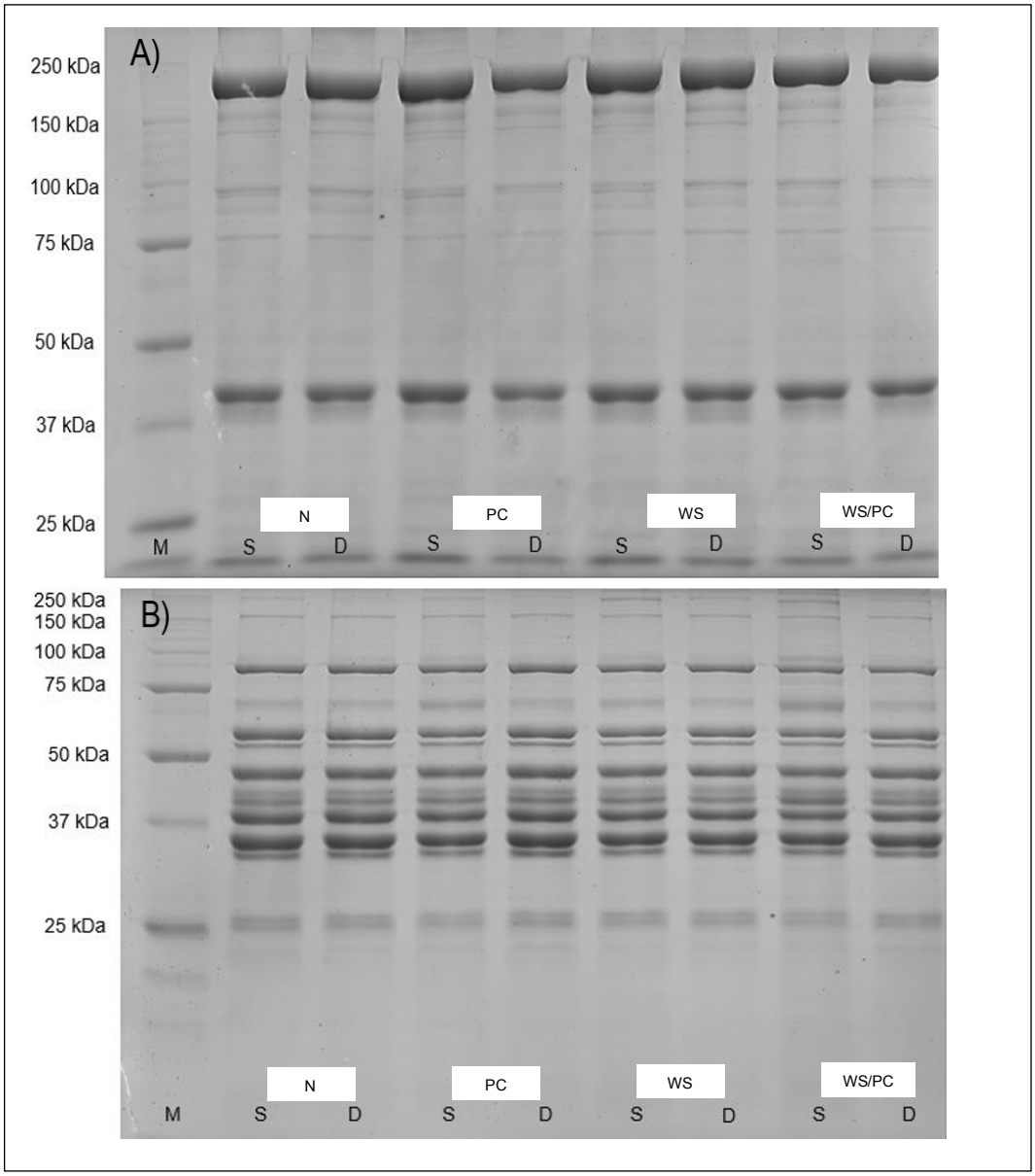


Figure 5.2 Poly-acrilamyde gel (7.5%) electrophoresis pattern of myofibrillar (A) and sarcoplasmic (B) proteins obtained from superficial (S) and deep (D) sections of chicken breast fillets belonging from different experimental groups (N, PC, WS and WS/PC). M = molecular weight marker.

5.3.6 NMR-relaxation measurements

A typical T_2 spectra obtained by NMR considering the superficial and the deep layer of a NORM *Pectoralis major* muscle is shown in **Figure 5.3**.

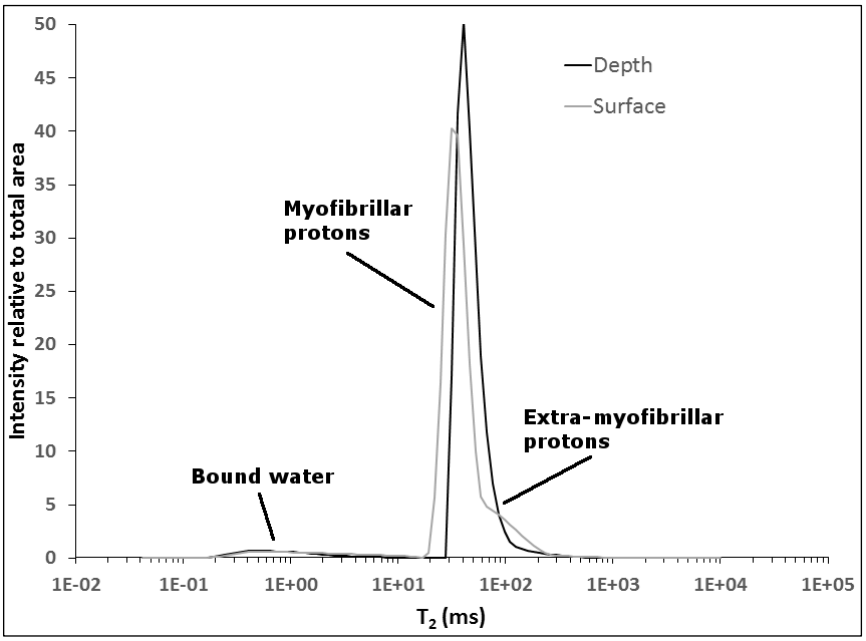


Figure 5.3 T_2 spectra obtained on the samples collected in depth (black solid line) and surface (gray solid line) on a breast from normal (N) group. To allow for a direct comparison, the intensities are scaled so that the total area equals 100.

The relative intensity (R.I.) and T_2 transverse relaxation time of the three protons populations observed in the present work are reported in **Table 5.4**. Water partition throughout meat compartments was influenced both by abnormality and position. Superficial samples having PC abnormality (PC and WS/PC groups) were characterized by higher percentages of extra-myofibrillar water, at the expenses of water located in the myofibrils spaces (intra-myofibrillar). In addition, superficial WS/PC samples had a lower percentage of bound water if compared with NORM and WS group, while PC showed intermediate values. These differences mostly disappeared in depth, with the exception of extra-myofibrillar water, which was higher in WS/PC group if compared with controls. The reduction of water in a single compartment was accompanied, with few exceptions, by an inversely proportional change in T_2.

Overall, the consequence of the different levels of distribution of water between intra and extra-myofibrillar compartments was that, compared to N samples, WS/PC samples showed higher differences in water intensities between samples collected on the surface and in depth.

Table 5.4 Effect of WS and PC abnormalities on relative intensity (R.I.) (%) and T_2 relaxation time (ms) of the protons populations identified through nuclear magnetic resonance.

Parameter		Position (P)	Experimental Group (EG)				sem	*P*-value		
			NORM	WS	PC	WS/PC		EG	P	EG×P
Bound Water	R.I. (%)	s	3.74^{ab}	3.59^{b}	3.47^{bc}	3.30^{c}	0.06	*<0.001*	*<0.001*	NS
		d	3.90^{a}	3.92^{a}	3.73^{ab}	3.69^{ab}				
	T_2 (ms)	s	1.17^{ab}	1.20^{ab}	1.25^{ab}	1.35^{a}	0.03	*<0.05*	*<0.001*	NS
		d	1.10^{b}	1.07^{b}	1.13^{b}	1.18^{bc}				
Intra-myofibrillar protons	R.I. (%)	s	90.5^{a}	90.1^{a}	87.5^{b}	85.5^{b}	0.36	*<0.001*	*<0.001*	*<0.01*
		d	92.2^{a}	91.4^{a}	91.6^{a}	90.2^{a}				
	T_2 (ms)	s	45.2^{bc}	47.3^{ab}	47.4^{ab}	50.0^{a}	0.44	*0.001*	*<0.001*	*<0.05*
		d	45.1^{bc}	44.8^{c}	44.9^{bc}	46.1^{ab}				
Extra-myofibrillar protons	R.I. (%)	s	5.73^{c}	6.31^{bc}	9.00^{ab}	11.2^{a}	0.38	*<0.001*	*<0.001*	NS
		d	3.92^{d}	4.66^{cd}	4.64^{cd}	6.09^{c}				
	T_2 (ms)	s	173.0	176.9	169.8	183.0	5.60	NS	NS	NS
		d	184.6	177.4	180.5	181.2				

N = 24 samples / group. $^{a-d}$ Means within the same parameter followed by different superscript letters significantly differ (P < 0.05). NS = not significant; sem = standard error of means.

In details, even if extra-myofibrillar T_2 was not modified by experimental factors, WS/PC group exhibited increased intra-myofibrillar T_2 in respect to NORM samples (50.0 *vs*. 45.2 ms; P < 0.05). Moreover, an increase in the T_2 relaxation time of the bound water fraction was observed by comparing the superficial and deep portion of the white striped samples (WS and WS/PC). Concomitantly, higher T_2 for the intra-myofibrillar proton population were observed in WS (47.3 *vs*. 44.8 ms; P < 0.05).

5.4 Discussion

The remarkable increase in growth rate and breast yield achieved in the past years have recently been associated with the higher incidence of several muscle abnormalities affecting chicken pectoral muscles (Petracci *et al.*, 2015; Velleman, 2015; Kuttappan *et al.*, 2016). The eventual alterations in muscle growth were tested by measuring fillets weight and dimensions. In general, the findings of the present study revealed that, if compared to normal, fillets affected by WS and PC abnormalities exhibited higher weights and increased thickness and length. Therefore, as previously observed for WS (Kuttappan *et al.*, 2012a; 2013b; Mudalal *et al.*, 2014) and WB (Mudalal

et al., 2015; Tasoniero *et al.*, 2016), birds displaying higher breast-size seem to be more prone to develop the PC abnormality.

In this study, the effect of the sampling position (superficial *vs*. deep) on the main quality parameters of the chicken breast meat have been considered. With regard to proximate composition, the findings revealed that PC occurrence is associated with a remarkable decrease in protein content coupled with a relevant increase in moisture level, which were observed also for the wooden breast condition. On the other hand, in agreement with previous studies (Kuttappan *et al.*, 2012a) fat content was affected only by the concurrent occurrence of WS. Notwithstanding, the occurrence of these muscle abnormalities seems to mainly affect the superficial portion of the *Pectoralis major* muscles rather than the deep one. In addition, since collagen was barely affected by the occurrence of WS and PC abnormalities, an increase in its content might be associated with the wooden breast condition (Tasoniero *et al.*, 2016).

The different modern myopathies described so far share some microscopic aspects. We described a range of microscopic lesions such as, internalization of nuclei, loss of cross striation, vacuolar and hyaline degeneration, necrosis and lysis of fibers, inflammatory cells infiltration, degenerating and regenerating fibers of variable size, lipidosis, proliferating connective tissue and fibrosis. In this regard, Kuttappan *et al.* (2016) reported that hereditary muscular dystrophies in the chicken, WS and wooden breast closely share some gross and/or histological lesions. Whether WS and wooden breast are different expressions of the same myopathy or not is yet to be demonstrated, as stated by Velleman (2015), but the histological lesions described are very similar. In a recent publication Radaelli *et al.* (2017) in chicken breast muscles affected by WS or wooden breast at 46 days of age not observed any difference regarding the histological characteristics. The histopathological features associated with the occurrence of PC abnormality are in agreement with those previously reported by Bilgili (2015). In addition, the same author states that PC histologically shows morphological changes similar to those reported for other myopathies (e.g., white striped, woody breast): extensive fiber degeneration and regeneration, hyalinization, poor fiber uniformity, increased

fat and connective tissue deposition. A particular characteristic observed in the PC sections were the progressive rarefaction of the endomysium and perimysium connective tissue. It is likely that the architecture and structural integrity is affected by the immaturity of the newly deposited collagen as previously described by Bilgili (2015). With regard to white striped meat, the microscopic lesions include vacuolar and hyaline degeneration, lysis, mild mineralization, fibers scattered in an abundant collagen-rich connective tissue and exhibited a high variability in size (degeneration and regenerating fibers) and interstitial inflammation along with fibrosis. However, the most severe histological lesions were observed within the samples concurrently affected by both WS and PC abnormality with a high connection existing between the occurrence of histopathological lesions, proximate composition and an increased proportion of both intra- and extra-myofibrillar water. Abundant adipose tissue deposition infiltrating the endo- and peri-mysial spaces were observed in agreement with Kuttappan *et al.* (2013a) and Radaelli *et al.* (2017). These results are also consistent with the findings of proximate composition. Indeed, the white striped samples exhibited the highest lipid content.

With regard to ultimate pH value, if compared to the normal, all abnormal fillets (apart from the type of abnormality) exhibited significantly higher values within the superficial portion of the *Pectoralis major* muscle. Thus, it seems that ultimate pH is altered to the same extent by the occurrence of both PC and WS conditions. Besides, previous studies evidenced that ultimate pH was remarkably altered following the occurrence of wooden breast (Mudalal *et al.*, 2015). In a recent study, a moderate positive genetic correlation was found to exist between pHu and WS condition. Hence, a link between the occurrence of the WS abnormality and the energetic status of the *Pectoralis major* muscle during life was hypothesised (Alnahhas *et al.*, 2016) and confirmed by Zambonelli *et al.* (2016). Indeed, the reduced muscular vascularization and glycogen reserves observed in modern heavy broilers may impair the energy supply to muscle fibers (Alnhhas *et al.*, 2016). In addition, a reduction in muscle capillary density leading to an inadequate oxygen supply and metabolic waste products displacement resulted from the selection for increased pectoral

muscle pH (Alnhhas *et al.*, 2015). An overall reduction in glycogen content within the wooden breast fillets was recently associated with a decreased amount of some glycolytic intermediates (i.e. glucose 6-phosphate and fructose 6-phosphate) (Abasht *et al.*, 2016). On the contrary, although an increased pHu was found in wooden samples as a result of in vivo reduction in muscular glycogen reserves, an intensified glycolytic activity was evidenced. Thus, the findings of these studies suggested that changes in glycolysis may arise from a different glucose utilization rather than from its availability (Abasht *et al.*, 2016; Zambonelli *et al.*, 2016).

The occurrence of muscle abnormalities did not exert any relevant effect on colour with the only exception of the yellowness, which displayed significantly higher values within the fillets affected by muscle abnormalities. In detail, in agreement with our previous study (Petracci *et al.*, 2013), as a consequence of the increased accumulation of intra-muscular fat and the consequent deposition of the yellow pigments introduced with a corn-based diet (Bianchi *et al.*, 2007), the WS samples exhibited the highest values.

As for the functional properties, if compared to their abnormal counterpart, normal fillets exhibited a higher protein solubility measured within the superficial portion. Overall, these findings are both consistent with the protein content observed in the present study and in agreement with previous studies performed on WS (Mudalal *et al.*, 2014; Bowker and Zhuang, 2016). Indeed, even if no relevant effect was observed within the dorsal portion, an overall reduction in sarcoplasmic protein solubility was found to affect the ventral surface of the cranial end in moderate and severe WS fillets (Bowker and Zhuang, 2016). On the other hand, myobrillar protein solubility was not affected in both the superficial and the deep sampling position. Protein solubility is normally used as an index to evaluate protein functionality, denaturation and their impact on water holding capacity of meat (Mudalal *et al.*, 2014). According to the findings of our previous study, the occurrence of muscle abnormalities (either alone or combined within the same *Pectoralis major* muscle) significantly affect the water holding capacity of meat (Mudalal *et al.*, 2015). Since the T_2 relaxation times were successfully used to study the interaction existing between water molecules and muscle structure (Bertram and Andersen, 2006), this study aimed at

evaluating the impact of WS and PC abnormalities onto water partition through NMR. According to our findings, the superficial section of all the samples affected by muscle abnormalities exhibited a higher proportion of extra-myofibrillar water, with the PC samples displaying the highest values. In addition, the lower proportion of bound and intra-myofibrillar water coupled with the increased intra-myofibrillar T_2 relaxation time observed within the abnormal samples revealed a greater mobility of water within the meat structure. Similarly, an increased proportion of extra-myofibrillar water was found in WS and wooden breast meat. This might be the result of the degenerative processes taking place within the muscle fibers and leading to an increased moisture within the extra-cellular spaces and, consequently, to an overall reduction in water holding capacity of meat (Mudalal *et al.*, 2015), in agreement with our histological observations. Indeed, if compared to the deep portion, several degenerating and necrotic fibers were found within the superficial layer of the affected fillets. Similarly, a clear gradual decrease in the histopathological lesions was previously found moving towards the deep portion (about 1 cm-deep) of the wooden breast muscles (Tasoniero *et al.*, 2016).

As for carbonylation level as an index for protein oxidation, no clear trends were observed. In agreement the previous study, protein carbonylation level was mainly affected by the occurrence of wooden breast rather than WS abnormality. However, the amount of carbonyls measured within the present study is slightly lower than those reported in the previous works. If compared to their normal counterpart, the superficial section of the fillets affected by muscle abnormalities exhibited a higher MFI. Thus, it seems reasonable to associate the occurrence of muscle abnormalities with a more intense proteolytic degradation of muscle tissue. Accordingly, the electrophoretic separation of the myofibrillar protein fraction belonging from the superficial section of the abnormal muscles evidenced an increased number of high molecular-weight bands. This is likely due to the proteolytic processes affecting the structural proteins (such as titin, nebulin and vinculin) composing the sarcomere. Similarly, an increased number of high molecular-weight bands was found within the water soluble fraction ascribed to sarcoplasmic proteins. Although unexpected, this result might be explained

considering that, as a consequence of proteolysis, the fragments resulting from the high molecular-weight myofibrillar proteins can exhibit a different solubility and thus being extracted together with the water-soluble fraction. Clearly these bands can not be ascribed to the protein fragments which typically result from the proteolytic processes occurring during the *post-mortem* period, but might be attributed to the degenerative processes associated with the occurrence of muscle abnormalities. Indeed, in a previous study performed by Lee *et al.* (2008) proteolysis generated fragments exhibiting a molecular weight ranging from 30 to 110 kDa.

5.5 Conclusions

This study reveals that both WS and PC abnormalities have adverse effects on quality traits and histological features of broiler meat. From histological comparison, WS abnormality shows a greater adipocyte deposition and fat infiltration, otherwise PC defect displays a progressive rarefaction of the endo- and pery-misial connective tissue, which likely is involved in an altered structural integrity of the *Pectoralis major* surface. Overall, proximate composition of abnormal samples was found to be significantly modified according the type of abnormality, especially within the superficial layer of the fillets. In fact, WS fillets have higher lipid content, whereas in PC samples was observed a remarkable decrease in total protein content coupled with an increased moisture level. From NMR investigation, PC fillets show a relevant increase of extra-myofibrillar water portion at the expenses of the intra-myofibrillar one and, as a consequence, display a reduced water holding capacity even in respect to WS fillets. Moreover, the occurrence of muscle abnormalities is associated with a more intense proteolytic degradation of muscle tissue leading to the formation of high molecular-weight protein fragments.

Overall results reveal that WS and PC myopathies primarily affect the superficial layer of *Pectoralis major* muscle and just mildly the deep section. Furthermore, the occurrence of PC abnormality generally leads to a more pronounced modification of the meat quality traits rather than the mere presence of WS myopathy.

General Conclusions

The remarkable accretion of the pectoral muscles size (achieved through a post-hatch hypertrophic growth of the existing fibers) and the increased growth rate achieved in the past few decades may have resulted in broiler hybrids being more vulnerable to oxidative stress and prone to develop inherent muscle fiber defects, insufficient capillarisation and growth-induced myopathies. Indeed, selection for increased body weight and pectoral muscles development has induced profound changes in muscle structure and its metabolic status that led to a remarkable increased incidence of several muscular myopathies and abnormalities mainly affecting the *Pectoralis major* muscles. In detail, being associated to various muscular defects such as reduced capillary density, impaired cations homeostasis and muscle damage, the remarkable increase in growth rate of broilers attained in the past few decades led to higher incidences of PSE-like condition and DPM. In addition, recently, the onset of a new group of emerging myodegenerative abnormalities of unknown aetiology (white-striping, wooden breast and poor cohesion defects) has been observed.

Within this context, the studies carried out during the PhD project aimed at deepening the current knowledge concerning histological features, quality traits and technological properties of broiler *Pectoralis major* muscles affected by emerging muscular abnormalities, clarifying their respective peculiarities and similarities as well as investigating the underlying mechanisms involved in their occurrence.

Overall, the findings of the present research evidenced that:

- a complex aetiology is associated with the occurrence of the white-striping, wooden breast and poor cohesion defects with the alterations being more pronounced within the superficial layer of the cranial portion of the *Pectoralis major* muscles when more than one abnormality coexist. In addition, a progressive reduction of the histopathological lesions and altered muscle architecture

were observed moving from the superficial towards the inner section of the muscles.

- The histological observations evidenced profound degenerative myopathic changes and a complete reorganization of the skeletal muscle structure within the *Pectoralis major* affected by muscular abnormalities. Abnormal rounded fibers and polyphasic myodegeneration often associated with lymphocytic infiltrations and occasional regenerative processes. More precisely, although white-striping, wooden breast and poor cohesion defects share similar gross and histological lesions including extensive fiber degeneration and regeneration, hyaline and vacuolar degeneration, inflammatory cells infiltration, proliferation and thickening of the endomysial and perimysial connective tissue, fibrosis and lipidosis, wooden breast and poor cohesion defect typically exhibited longer sarcomeres and a progressive rarefaction of the endomysial and perimysial connective tissue, respectively.
- With regard to meat quality, in spite of their higher ultimate pH value that should result in improved water holding capacity of meat, the profound histopathological lesions observed in *Pectoralis major* muscles affected by muscular abnormalities resulted in severely impaired quality traits and technological properties of meat. Indeed an overall increased relative intensity and T_2 relaxation time for the extra-myofibrillar water portion was observed in all abnormal samples. In addition, reflecting the main histopathological features, both the occurrence and the type of muscular abnormality remarkably affected textural traits and chemical composition of meat. In detail, the *Pectoralis major* muscles affected by wooden breast exhibited significantly higher amount of moisture and collagen coupled with a reduced oxidative stability. Furthermore, the findings of the present

research evidenced that the increased hardness that typically affects the wooden breast cases has a structural origin and might not be exclusively attributed to differences in the proteolytic processes taking place within the *post-mortem* period. On the other hand, white-striped meat revealed an increased lipid content, whereas an overall decrease in total protein coupled with remarkably higher moisture levels were observed within the muscles affected by poor cohesion defect.

- Overall, the occurrence of muscle abnormalities significantly affected both protein profile and gene expression levels. Indeed, concomitant occurrence of white-striping and wooden breast revealed 204 differentially expressed genes related to myodegeneration and muscle development, reactive oxygen species metabolism and oxidative stress, inflammatory processes, polysaccharide and glucose metabolism, proteoglycan synthesis as well as ion homeostasis and calcium signalling pathway.

Thus, according to the outcomes of the present research and the pathogenesis associated with the occurrence of muscle abnormalities, it is reasonable to hypothesise the existence of a complex network of biological changes that, acting simultaneously, are responsible for the phenotypic features and the consistent impairment of muscular metabolism of the abnormal muscles. Although the causative mechanisms responsible for the occurrence of these abnormalities are still uncertain, the findings of the present research (together with the information available in literature) suggest that, being involve in initiating the inflammatory mechanism, selection for high-growth rate and breast yield broiler hybrids might exert a relevant role in triggering the progression of the metabolic responses responsible for the occurrence of muscle abnormalities.

Overall:

- As a consequence of the impaired visual appearance, abnormal meats are normally downgraded by the poultry industry and diverted to further processing with considerable economic losses.
- However, considering that both quality traits and technological properties are remarkably affected, the poultry industry needs to identify proper strategies in order to minimize the negative effects resulting from the inclusion of these abnormal meats into processed products.
- With regard to consumers, although not currently aware of these issues, the occurrence of muscular abnormalities might result in disaffection towards the consumption of poultry meat.

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