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POULTRY MEAT ABNORMALITIES AND POST-MORTEM METABOLISM OF AVIAN MUSCLES

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“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

- Albert Einstein

ABSTRACT

Poultry is the most widely eaten type of meat all over the world. During the past decades, the continuous evolution of the market demand for poultry meat, along with the increased global population growth, established new selection criteria for broiler chickens. The substantial genetic progress made in the past 50 years has resulted in broiler being more sensitive to stress and more prone to develop metabolic-related defects, leading to significant changes in muscle structure and metabolism. Indeed, modern hybrids designed for meat production are more susceptible to the development of growth-related muscular abnormalities affecting P. major muscle (i.e. white striping, wooden breast and spaghetti meat), that currently deeply affect meat quality and whose incidence in recent years has reached worrying levels. Considering this scenario, this PhD project aimed at deepening the existing knowledge on the effects of muscular aberrations on poultry meat quality and carrying out a characterization of post-mortem muscle metabolism of fast-growing birds, in order to investigate the factors contributing to the intra and inter-specie variations in energy-generating metabolism and other biochemical processes. The first part of the present research permitted to establish the relationship between histidine-containing compounds and muscle post-mortem metabolism, highlighting that the greater concentration of anserine and carnosine of breast meat might provide resistance to the post-mortem pH decline, thus justifying the intra- and inter-specie differences existing between the pH_u values. Moreover, the results obtained in the second part concerning muscular abnormalities allowed to assess the peculiar effect of each growth-related abnormality on meat quality and provide information that might be valuable in managing abnormal meat for further processing, as well as offer new insights about the biochemistry controlling the early post-mortem metabolism of myopathic muscles. However, despite all the efforts conducted by researchers during the past decade, no effective solutions able to contrast the onset of myopathies have been found yet. Within this context, it seems unavoidable taking a step back, as further genetic improvements might be restrained by muscle biological potential and animal welfare concerns. In this scenario, since solving the issue at the root appears complex so far, further researches should be addressed at finding processing solutions able to reduce the negative implications of abnormalities on the quality and technological properties of meat.

List of publications originated from PhD research outcomes

Petracci M, **Baldi G**, & Soglia F (2019). Growth-related breast meat abnormalities in Broilers, L*ohmann Information International*, 53,12-18.

Baldi G, Soglia F, Laghi L, Tappi S, Rocculi P, Tavaniello S, Prioriello D, Mucci R, Maiorano, & Petracci M. (2019). Comparison of quality traits among breast meat affected by current muscle abnormalities. *Food Research International*, 115, 369-376.

Soglia F, **Baldi G**, Laghi L, Mudalal S, Cavani C, & Petracci M. (2018). Effect of white striping on turkey breast meat quality. *Animal*, 12, 2198-2204.

Baldi G, Yen C-N, Daughtry MR, Bodmer J, Bowker BC, Zhuang H, Petracci M, & Gerrard DE (2020). Exploring the factors contributing to the high ultimate pH of broiler Pectoralis major muscles affected by Wooden Breast condition. *Frontiers in Physiology*, submitted for publication.

Baldi G, Soglia F, & Petracci M (2020). Current status of poultry meat abnormalities. *Meat and Muscle Biology,* submitted for publication.

Chapter 1: Introduction and literature review

1.1 Meat as a food

1.1.1 Dynamics of poultry meat consumption and production

The public perception of the relationship between health and meat is often quite negative, especially in light of recent studies that classified the consumption of processed and red meat respectively as “carcinogenic” and “probably carcinogenic to humans”, on the basis of sufficient evidence for colorectal cancer (Bouvard et al., 2015). Contrariwise, poultry meat is usually considered nutritionally valuable by virtue of both its low-fat content and high-quality proteins, so much so that its regular consumption has been suggested and positively perceived (Bordoni and Danesi, 2017). Moreover, poultry meat is more affordable than red meats and faces up to religious and cultural restraints (Petracci et al., 2014). Indeed, chicken meat is one of the most widely eaten type of meat all over the world and plays an outstanding role as a protein source for a growing global population. Within this context, in the past decade, the global consumption of poultry meat has noticeably increased by 36%, while the per capita consumption increased by 20.6% in the same period (OECD, 2019). Actually, of all animal products, poultry sector showed the highest growth rates between 2007 and 2017, accounting for about 39% of total meat intake worldwide, followed by pork and beef (38.3 and 22.5%, respectively) (Figure 1.1).

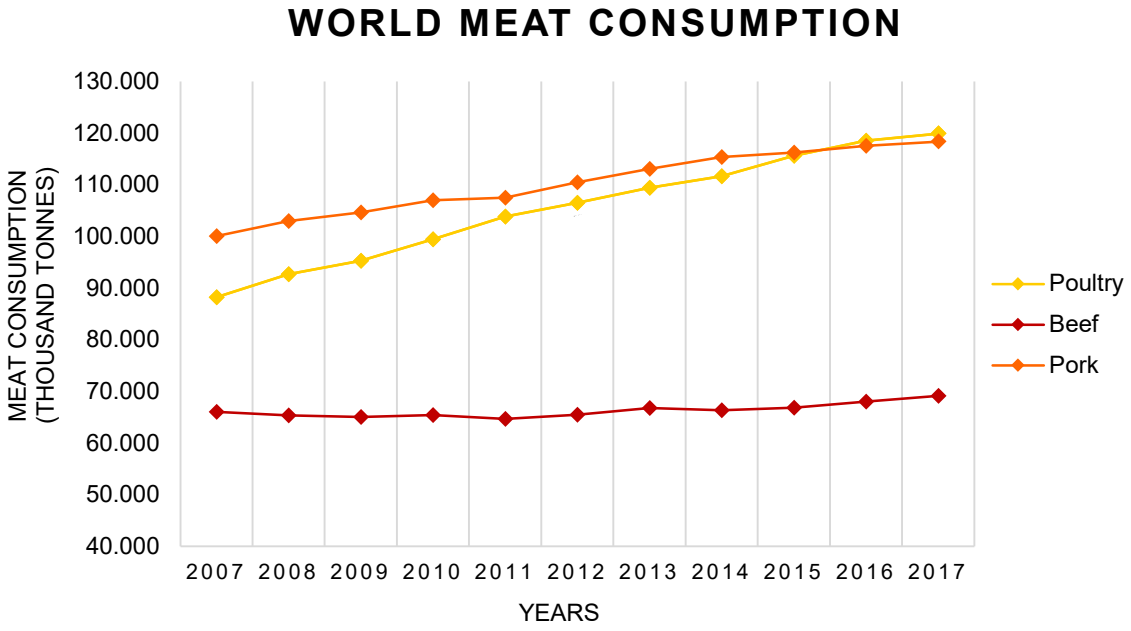


Figure 1.1. Global meat consumption from 2007 to 2017. Meat consumption is measured in thousand tons of carcass weight (except for poultry expressed as ready to cook weight) (OECD, 2019).

As a consequence, in order to meet the growing consumer demand, the meat market has undergone an intense upsurge in poultry meat production. In more detail, between 2007 and 2017 (Figure 1.2), global chicken meat production increased by 32.0 million tons or 41% and is expected to further grow (Windhorst, 2017; FAO, 2019).

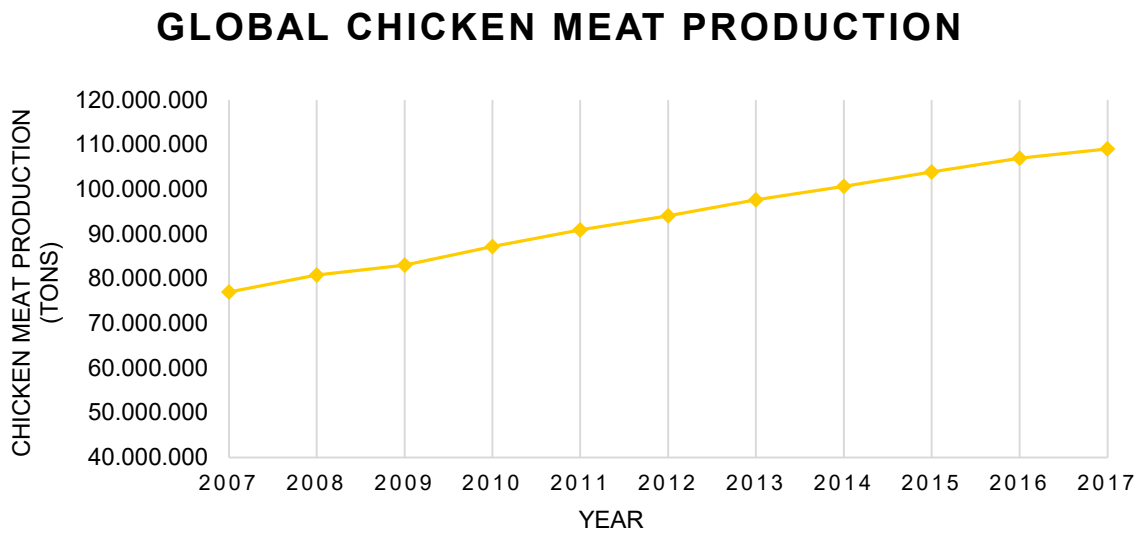


Figure 1.2. Global chicken meat production from 2007 to 2017, expressed in tons (FAO, 2019).

Indeed, a recent OECD-FAO projection highlighted that by 2024 global poultry meat production will further increase by 22.3%, with China, USA, Brazil, and Mexico being the most involved countries, while the absolute growth in the EU will be much lower (OECD-FAO, 2015). The same report showed that the production will increase faster in developing countries rather than in developed ones. Within this scenario, it is expected that poultry meat will be the main protein source also for the less developed countries, where small-size chickens, in contrast to pork and beef, can be consumed as a whole bird without the need of refrigeration (Windhorst, 2017).

1.1.2 Evolution of chicken meat market

The factors contributing to the worldwide success of chicken meat are found in the relatively low-cost, the absence of religious restraints as well as the sensory and nutritional properties (Petracci et al., 2019). Chicken meat is also distinguished for its ease of preparation and variety of commercialization: it is available as a whole bird or as cut-ups, fresh or frozen, bone-in or deboned, seasoned, raw or already cooked. However, even though the way of commercialization of chicken meat obviously differs by country, global consumers' attitudes over the years have been shifted from the consumption of the whole carcass to ready-to-eat and processed products (Mead,

2014). Table 1.1 shows the changes in meat consumption patterns over a 50-year period in U.S. and Italian markets.

Table 1.1. Changes in the marketing of broilers in the U.S. and Italy between 1968 and 2017 (data in %) (NCC, 2019; UNAITALIA, 2019).

	Marketing Forms					
	Whole Bird		***Cut ups***		***Further Processed***	
						
	USA	Italy	USA	Italy	USA	Italy
1968	78	100	19	0	3	0
1978	61	100	32	0	7	0
1988	29	45	53	53	18	2
1998	10	20	53	66	36	14
2008	11	15	43	65	46	20
2017	11	10	40	60	49	30

In the 1960s, the whole carcass represented the most common marketing form of chicken meat, consisting of about 80% and 100% of the U.S. and Italian market, respectively. On the contrary, nowadays the American market is mainly made up by the commercialization of processed products (i.e. sausages, nuggets, etc.), while the Italian market is still focused on cut-ups (60%) (Table 1.1). The changes in the way of poultry meat are commercialized is mainly driven by consumers' willingness to pay for the convenience of both ready-to-eat products and smaller portions already deprived of bone and skin (Fletcher, 2002). This evolution of the meat market has resulted in a change of meat quality attributes' importance. As an example, with increasing trends in further processing, the specific importance of meat functionality has increased because of its key role in determining the quality of ready-to-eat products. Indeed, as long as the meat market continues to evolve its characteristics, meat quality evaluation parameters will change consequently (Fletcher, 2002; Barbut, 2015).

1.1.3 Poultry Meat Quality

1.1.3.1 Poultry meat composition and nutritive value

The word "*meat*" refers to animal skeletal muscle and associated adipose and connective tissues as well as other edible tissues (i.e. offal) intended for human consumption. The primary components of meat are moisture (water), proteins, lipids, carbohydrates and inorganic matters (i.e. ash or minerals). Overall, skeletal muscle tissue is constituted of approximately 75% water, 19% protein, 2.5% lipid, 1% inorganic matter and 2.5% nitrogenous and non-nitrogenous components (Keeton and Eddy, 2004). The quantity of moisture, protein and ash are inversely related to the percentage of fat, while carbohydrate content is usually rather constant. However, meat composition can vary depending on many different factors, such as the species, animal's age and diet, the genotype, the farming system and the anatomical cut (Aberle et al., 2012).

Among all meat types, poultry meat is considered the most valuable in terms of nutritional characteristics, in light of its low energy concentration and its high nutrient density (Marangoni et al., 2015). Table 1.2 reports meat composition of some poultry species.

Table 1.2. Composition and nutritional values of different raw poultry meats. Values are expressed on a 100 g portion with or without skin. Adapted from Barbut (2015).

Species	Skin	Water (%)	Protein (%)	Fat (%)	Calories (kcal)
Chicken	+	68.6	20.3	11.1	186
	-	74.9	23.2	1.6	114
Turkey	+	69.8	21.6	7.4	159
	-	73.8	23.5	1.6	115
Duck	+	48.5	11.5	39.3	400
Goose	+	50.0	15.9	33.5	370

Because of the skin high fat content, the energetic value of poultry meat could vary up to 30% depending on the presence of skin. Overall, turkey meat is slightly lower in fat than chicken, while goose and duck meat have higher fat content. However, poultry meat is considered a very lean meat because, unlike red meats, most of the fat is deposited subcutaneously and it could be easily removed along with the skin (Barbut, 2015). Moreover, poultry meat presents a 1:3 saturated to unsaturated fatty acids ratio

and a high content of long-chain n-3 polyunsaturated fatty acids, whose nutritional benefits are well documented (Kris-Etherton et al., 2003).

Overall, poultry meat is a good source of proteins (20-23%): it is estimated that 100g of raw poultry meat could cover about 1/3 of the daily protein requirement (Bordoni and Danesi, 2017). Moreover, poultry meat protein composition is often defined as “high quality” in light of the low collagen content, making the meat more digestible.

Poultry meat is a source of several minerals, such as iron, sodium, zinc, and selenium. Although mineral concentration varies according to the animal’s diet and anatomical cuts, poultry meat does not significantly contribute to total mineral dietary intake. On the contrary, poultry is considered the ideal dietary source of vitamin B12 and other hydrophilic vitamins, with special regard to niacin (Marangoni et al., 2015; Bordoni and Danesi, 2017).

1.1.3.2 Poultry meat quality traits

Meat quality is a complex concept that can be simplified as “a set of properties that together identify what we appreciate about meat when we purchase it, eat it, or select it for use as a raw material for processing into meat products” (Purslow, 2017). The aforementioned “set of properties” used to define meat quality are those strictly linked with our sensory perception, such as appearance, color, tenderness, odor and so on. However, the relative importance of meat quality traits tightly depends on the form in which meat is marketed. As an example, if meat is marketed raw, the most important quality attributes are appearance and texture, since they most influence consumers’ selection and final satisfaction (Fletcher, 2002). On the other hand, extrinsic quality parameters related to meat functional properties such as pH and water holding capacity (WHC) are critical for processed products (i.e. sausages, breaded products, marinated fillets).

1.1.3.3 Color

Meat color is the primary factor leading customers purchase decisions. When light hits meat surface, it could be reflected, absorbed or scattered: meat color is essentially determined by the light which is reflected back to the eye (Cornforth and Jayasingh, 2014). The light reflecting from meat to the eye can be quantitatively measured using several systems such as chemical-spectral photometric methods and reflectance colorimetry, with the last being the most widely used because of its

accuracy, reproducibility, and objectivity (Hughes et al., 2014). Reflectance colorimetry describes human's perception of color through different color spaces. The most popular color space is the CIE LAB, which defines color as a combination of three parameters: i) lightness (L*); ii) redness (a*) and iii) yellowness (b*) (Petracci and Baéza, 2007).

Generally, several components are found to affect the color of raw meat. Among them, both concentration and chemical state of meat pigments (i.e. myoglobin, hemoglobin, and cytochrome C) as well as pH and muscle microstructure are considered the major contributing factors to poultry meat color (Fletcher, 2002; Carvalho et al., 2017).

Myoglobin is a complex molecule composed of a protein moiety (globin) and a non-protein portion consisting in a prosthetic group (i.e. heme group), having on its center an iron molecule which is responsible for binding compounds such as oxygen and water (Figure 1.3) (Barbut, 2015). The globin provides water solubility to heme and protects the heme iron from oxidation, so that the protein can maintain its functionality (Suman and Joseph, 2013).

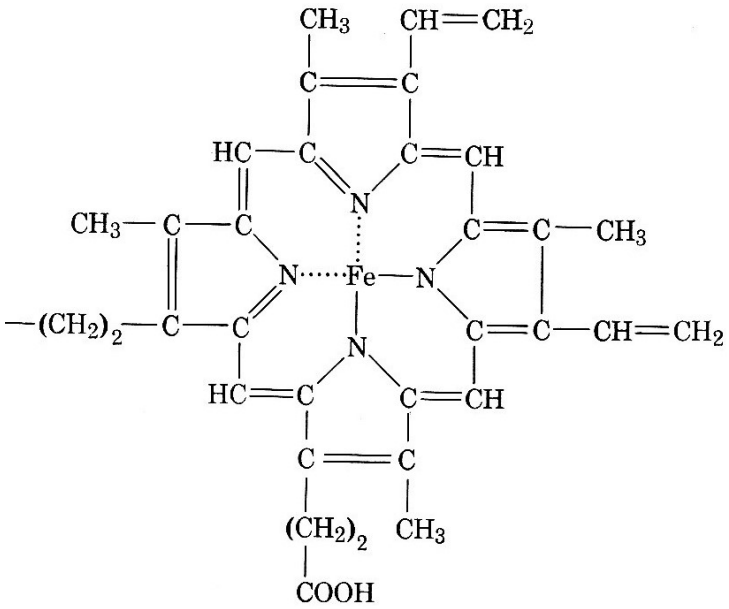


Figure 1.3. Heme group of myoglobin. The iron may be ferrous (myoglobin or oxymyoglobin) or ferric (metmyoglobin) (Cornforth and Jayasingh, 2014).

The iron's oxidation state along with its ligands significantly affects the color of meat. Indeed, when the iron molecule is in its reduced ferrous state (Fe^{2+}), it can combine with both oxygen or water. In the first case, the chemical state of the pigment is called **oxymyoglobin** and is responsible for the bright-red color of meat. In the second case, when the iron molecule reacts with water (i.e. in uncut meat), the meat

appears purple-red: this chemical form of myoglobin is called **deoxymyoglobin.** On the contrary, when the haem iron of myoglobin is oxidized to the ferric form (Fe^{3+}) it becomes unable to bind oxygen, thus resulting in the brownish pigment **metmyoglobin** (Aberle et al., 2012)**.**

While color attributes (i.e. a* and b*) are strongly associated with meat pigments, the parameter of lightness is mainly related to the structural attributes of muscle (i.e. fiber/myofibril size, presence of connective tissue, etc), which influence the extent of light that scatters within the muscle (Fletcher, 2002; Hughes et al., 2014). In other words, the less the light penetrates into the muscle, the more the light can be reflected thus making the meat bright to the human's eye. Moreover, muscle pH is found to affect the physical structure of meat and, as a consequence, its light reflecting properties. It is also well-known that many of the heme-associated reactions are pH-dependent (Fletcher, 2002).

1.1.3.4 pH

The pH is defined as the negative base 10 logarithm of the concentration of the reaction of H^+ with a water molecule to produce a hydroxonium ion (H_3O^+). With regard to meat, hydrogen ions result from dissociated lactic acid that accumulates in the muscle after the death of the animal (Honikel, 2014). The pH of meat is an important indicator of meat quality characteristics since it has been strongly linked with several quality attributes such as tenderness, water holding capacity, cook loss, juiciness and microbial stability (Qiao et al., 2001). Its measurement could be conducted both at 24h post-mortem (i.e. ultimate pH) or during post-mortem time, providing useful information about forthcoming meat quality attributes (Honikel, 2014). Indeed, the rate and the extent of pH falling that occurs at the death of the animal are key elements in defining meat quality (Scheffler and Gerrard, 2007).

The ultimate pH (pH_u) of meat reached at 24h post-mortem varies depending on species, muscle type, and pre-slaughter stress (Honikel, 2014). The normal pH_u value of broiler breast meat is around 5.8-5.9 and it is reached more than twice faster if compared to beef and pork (Aberle et al., 2012; Petracci and Berri, 2017). A pH falling different from normal could have severe implications on meat quality and color development. If muscle pH drops very quickly within the first hour post-mortem (i.e. when the carcass temperature is still high), many proteins (including myoglobin) denature and a defect known as pale, soft and exudative (PSE) syndrome can occur (Barbut, 2015). PSE-like meat characteristics are low pH_u values (<5.7) and pale color,

as a result of protein denaturation and the looser muscle structure that scatters more light back to the observer than meat with a normal pH (Barbut, 1997; Swatland, 2008) (Figure 1.4a). On the contrary, if glycogen storage has been depleted prior to the animal's death because of high-intensity activities or struggling, the pH drop is minimal because lactic acid production is reduced. This condition will result in dark, firm and dry (DFD) meat, which presents dark color and a dry appearance, resulting from a pH_u higher than 6.1 (Barbut, 2015) (Figure 1.4b).

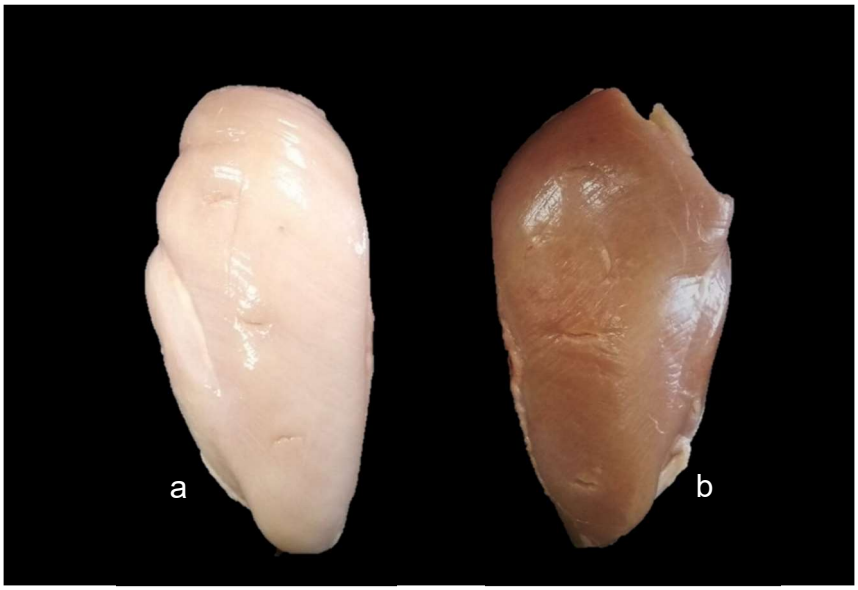


Figure 1.4. The appearance of PSE-like (a) and DFD (b) chicken breast meat.

Thus, pH measurements can be used to detect both meat quality defects and monitor manufacturing processes. Moreover, since muscle pH affects the water binding nature of the proteins, its measurement can provide useful information about meat technological properties.

1.1.3.5 Water-Holding Capacity

Composing about 75% of the total muscle mass, water is the major component of muscle tissue (Brewer, 2014). Water is distributed in several compartments within the tissue and generally exists in three main forms (Bowker, 2017) (Figure 1.5):

- Bound: water molecules which are strictly bound to muscle proteins by mono or multi-molecular adsorption;
- Immobilized: water molecules that are weakly attracted to both muscle proteins and bound water;
- Free: water molecules that are only held by weak capillary forces.

In other words, water molecules layers become successively more faintly bound as the distance from the reactive group on the protein increases (Aberle et al., 2012).

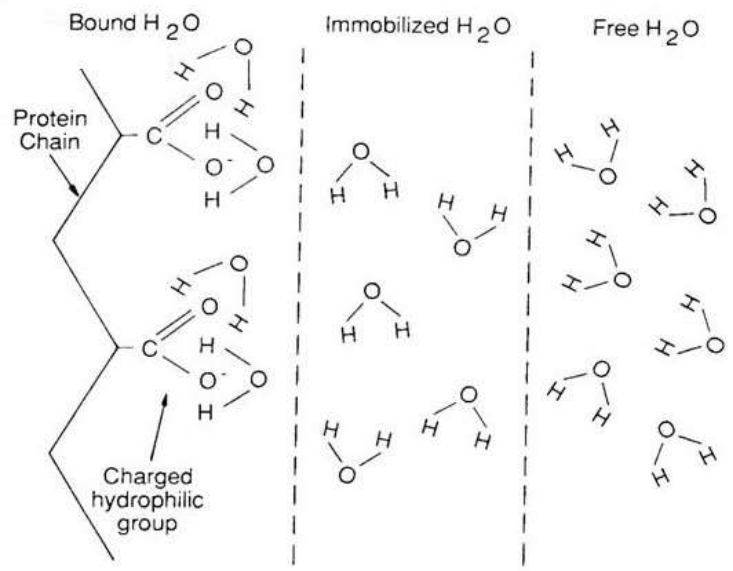


Figure 1.5. The three forms of water in the muscle (Aberle et al., 2012).

The majority of the water (85%) is located within the intra-myofibrillar compartment, held between the filaments of the myofibrils, while the remaining portion is located within the extra-myofibrillar spaces, specifically between the myofibrils, between myofibrils and sarcolemma or between myofibrils and muscle fiber bundles (Huff-Lonergan and Lonergan, 2005; Aberle et al., 2012).

Water-holding capacity (WHC) is the ability of meat to hold inherent or added water during processing and storage (Bowker, 2017). The release of water from meat is described as drip, purge, exudate or cook loss (Hughes et al., 2014). Poor WHC causes high drip loss that results in a loss of weight from the carcass, impaired appearance (because of the undesirable presence of exudate) and overall reduced meat quality (Woelfel et al., 2002).

Since the majority of water in the muscle is located in the intermolecular spaces, WHC especially resides in the water located between proteins, held by capillary forces (Brewer, 2014). As a consequence, any change in both the myofibril structure and the strength of reactive groups on muscle proteins will dramatically affect WHC. During the conversion of muscle to meat, several biochemical and structural changes occur. The main mechanisms affecting WHC during post-mortem are summarized as follows:

- Net charge effect: muscle proteins have a charge, whose repulsive nature allows the protein structure to expand and water to infiltrate (Aberle et al., 2012).

When pH declines during post-mortem, proteins' structure faints causing an overall reduction in their ability to bind water. Indeed, the net charge of muscle proteins decreases as long as the pH comes closer to the isoelectric point of muscle (i.e., the pH at which the overall net charge of the protein is zero), which is around 5.0-5.5 (Aberle et al., 2012). As a result, protein structure becomes compact and less capable to bind water (Bowker, 2017).

- Steric effect: the steric (or spatial) effect refers to the lack of space within the muscle fiber and the impossibility of the water to penetrate within the structure (Huff-Lonergan and Lonergan, 2005). This phenomenon is a direct consequence of the net charge effect and the transverse shrinkage of myofibrils occurring during the development of rigor mortis (Bowker, 2017).
- Proteolysis: during the conversion of muscle to meat, cytoskeletal proteins are degraded by endogenous proteases thus leading to the disruption of fiber organization. This process creates additional spaces for water to infiltrate within the muscle, thus leading to an improvement of WHC (Aberle et al., 2012).
- Exchange of ions: proteolytic degradation compromises protein membrane integrity and allows the diffusion of ions across the membrane, resulting in the replacement of divalent ions of protein chains with monovalent ions (such as sodium, Na^+). This exchange of ions results in an improvement of WHC since for every divalent cation replaced, one protein reactive group is capable to bind water (Aberle et al., 2012).

Besides these post-mortem-related mechanisms, WHC can also be improved by the addition of sodium chloride as a result of both protein solubilization and ion exchange (Barbut, 2015). However, the addition of above 5% of sodium chloride causes the so-called "salting out" undesired effect, which resides in protein aggregation and a severely reduced ability to bind water (Brewer, 2014).

The improvement of WHC can undoubtedly have many advantages on both meat quality and technological characteristics. Enhanced WHC of raw poultry meat leads to greater protein functionality, improved marinade absorption and retention, and greater processing and cooking yields (Bowker, 2017). Moreover, it is well established that the more the water is retained in the muscle, the higher sensory tenderness and juiciness are obtained (Morey and Owens, 2017).

1.1.3.6 Tenderness

Tenderness is one of the most important attributes involved in defining meat quality and influencing consumer's willingness to purchase. The texture of meat has been defined by Tornberg (1996) as "the composite of the structural elements of meat and the manner in which it registers with the physiological senses". The tenderness of cooked meat varies depending on intrinsic factors such as the structure of the original muscle (i.e., muscle fiber length and diameter, connective tissue, intramuscular fat), and extrinsic aspects, like deboning time and pre-slaughter conditions (Qiao et al., 2001; Aberle et al., 2012; Morey and Owens, 2017).

There are several tests used to assess meat tenderness, among those Warner-Bratzler (WBr) and Allo-Kramer (AK) shear force are the most used methods to detect the texture of poultry meat (Cavitt et al., 2005). If compared to a hedonic scale, both poultry meat WBr and AK shear forces are usually quite low: indeed, poultry meat is generally considered a tender meat (Table 1.3).

Table 1.3. Classification of tenderness intensity for Warner-Bratzler (WBr) and Allo-Kramer (AK) shear forces. Adapted from Morey and Owens, (2017).

Attribute	Hedonic scale	WBr shear force (kg)	AK shear force (kg/g)
Tenderness intensity	Extremely tough	> 16.0	> 16.7
	Very tough	14.1 - 16	14.9 - 16.7
	Moderately tough	12.2 - 14.1	13.1 - 14.9
	Slightly tough	10.3 - 12.2	11.2 - 13.1
	Neither tough nor tender	8.4 - 10.3	9.4 - 11.3
	Slightly tender	6.5 - 8.4	7.6 - 9.4
	Moderately tender	4.5 - 6.5	5.8 - 7.6
	Very tender	2.6 - 4.5	4.0 - 5.8
	Extremely tender	< 2.6	< 4.0

Aside from the above-mentioned traditional methods, many researchers are lately employing the razor blade (RB) methods (MORS and BMORS) for assessing tenderness in poultry meat. Indeed, the application of RB technique provides several advantages mainly related to its quickness (no sample cutting or weighing is required) and precision (Cavitt et al., 2005).

However, factors including deboning time, age of the animal as well as moisture content and cooking method can strongly influence meat tenderness. On the contrary, it has been demonstrated that connective tissue does not play a major role in determining chicken meat tenderness since broiler are slaughtered at very young ages, thus there are no mature collagen cross-links and collagen eventually melts during cooking (i.e., is not thermally stable) (Bruce and Aalhus, 2017).

1.2 Muscle structure and metabolism

1.2.1 Muscular tissue

The ultimate goal of poultry industries is producing high-quality meat protein sources by balancing meat yield, meat quality, and feed efficiency. These factors are bound together by cellular mechanisms governing the pre- and post-hatch muscle growth. There are three types of muscle tissue: cardiac, smooth and skeletal (or striated) muscle (Purslow, 2006). The skeletal muscle, being 35-65% of carcass weight, represents the major edible part of the carcass and it is considered the most important element for the quality of raw and processed meat (Aberle et al., 2012). Skeletal muscles vary depending on their shape, size, action, and function. However, they all share a common structure, which is highly defined by sheaths of connective tissue (Figure 1.6).

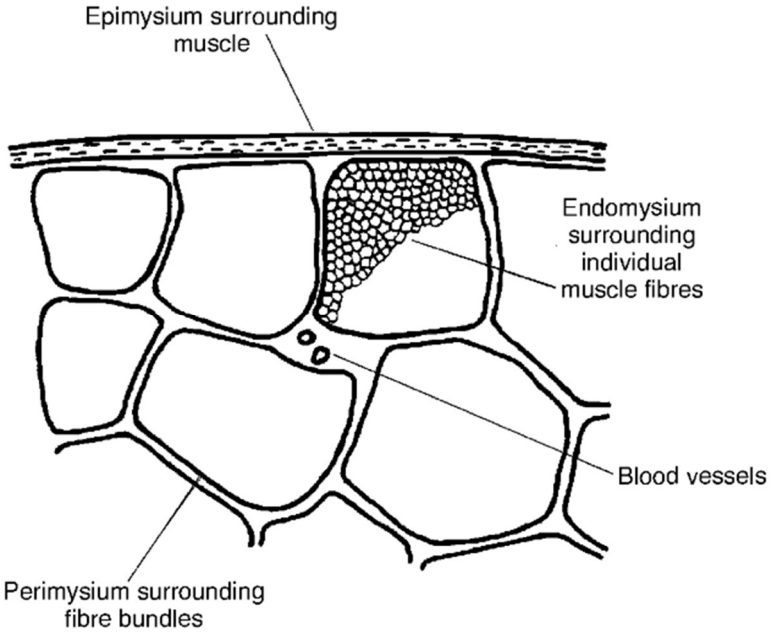


Figure 1.6. Connective tissue sheaths of a muscle (Warriss, 2000).

The whole skeletal muscle is contained within a dense layer of connective tissue called epimysium, which separates the muscle from the surrounding tissue. From a cross-sectional perspective, the muscle consists of numerous grouping of muscle fibers called bundles (or fascicles), which are separated from each other by a sheath of connective tissue known as perimysium. The muscle bundle is composed of smaller muscle fibers, that in turn are surrounded by a thinner network of connective tissue

called endomysium (Warriss, 2000; Aberle et al., 2012; Barbut, 2015). The structural unit of skeletal muscle is the muscle fiber (or myofiber) (Aberle et al., 2012). Myofibers are multinucleated cells with a diameter ranging from 10 to 100 μm and a length of many centimeters; muscle cells derive from myoblasts during muscle development, while some of them remain unfused and become satellite cells (i.e. undifferentiated cells involved in muscle repair mechanisms) (Holmes, 1987). Each myofiber consists of numerous functional subunits called myofibrils, which in turn are made up of smaller elements named myofilaments (or muscle filaments) (Holmes, 1987; Warriss, 2000). There are two sorts of myofilaments: the thick filaments (mainly composed of the protein myosin) and the thin filaments (mainly consisting of the protein actin) (Figure 1.7). The overlapping of thin and thick filaments results in the striated appearance of skeletal muscle when it is stained and viewed under a light microscope (Warriss, 2000; Barbut, 2015). This banding effect, which is due to alternating light and dark areas, explains the term “striated” muscle (Figure 1.8).

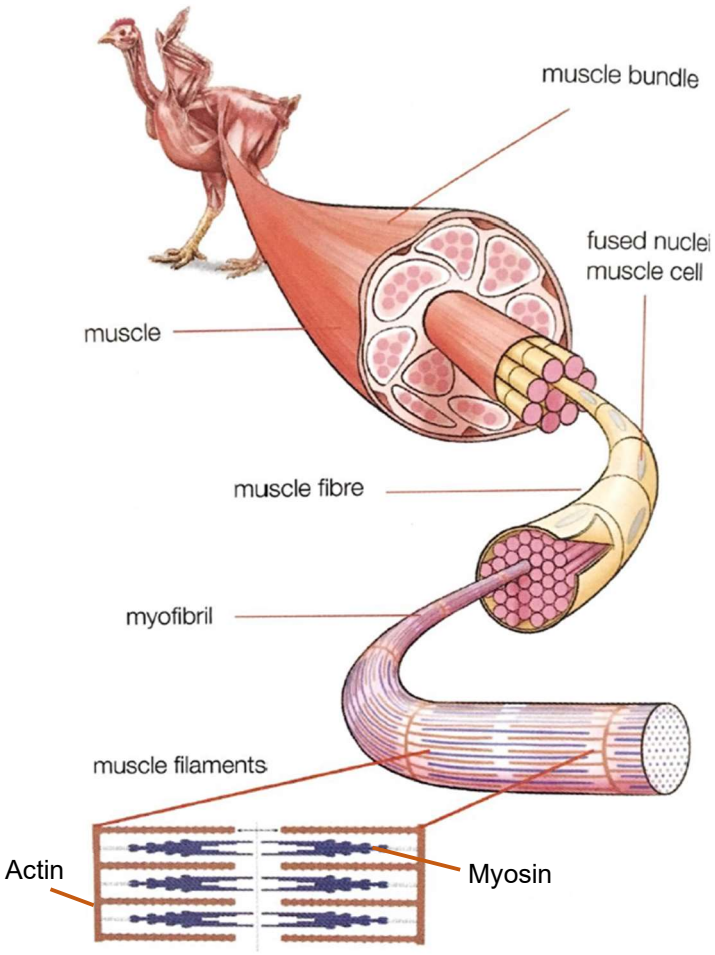


Figure 1.7 Diagram of the organization of skeletal muscle. Adapted from Tondeur and Simons, (2019).

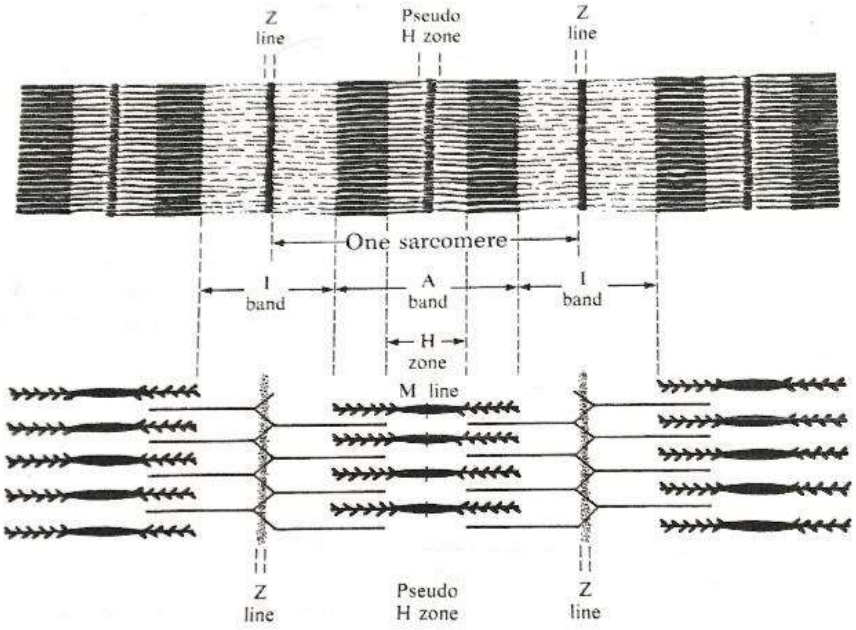


Figure 1.8. Schematic representation of a sarcomere (Aberle et al., 2012).

Since the dark bands alter the plane of polarized light (meaning that they do not have the same properties in all directions) they are defined as “anisotropic” (A-band), whereas the light bands appear to be singly refractive and are described as “isotropic” (I-band). The I band is bisected by a dark thin band names Z line (or Z disk): the section of the myofibril between two adjacent Z lines is called sarcomere and represents the fundamental contractile unit of the muscle (Holmes, 1987; Aberle et al., 2012; Dikeman and Devine, 2014) (Figure 1.8). Besides these elements, the H-zone is the region of the A-band where thin filaments do not overlap with thick ones, while M line is the structure running transversely across the sarcomere and whose function is to stabilize the thick filament (Purslow, 2006).

1.2.2 Muscle contraction

Muscle contraction is a phenomenon that involves a complex chain of events. In simple terms, muscle contraction is regulated by the concentration of calcium ions in the sarcoplasm and is based on sliding-filaments mechanism, made possible by the attachment/detachment of myosin heads along the actin filaments through the formation of cross-bridges, causing the thick filaments slide in between the thin ones (Holmes, 1987; Barbut, 2015). Thus, the movement of the thin filaments toward the M line causes the nearing of Z lines, thus decreasing the length of the sarcomere (i.e. the muscle shortens) (Holmes, 1987). In more detail, muscle contraction involves four proteins (Barbut, 2015; Ertbjerg and Puolanne, 2017) (Figure 1.9):

1) Myosin: represents the largest proportion of myofibrillar proteins (45%) and forms the thick filaments in muscle. The myosin molecule is divided into two functional units: the head and the rod. Its structure shows two heavy and two sets of light chains: each heavy chain is comprised of a globular head portion that has two associated light chains. The rod is formed by the two heavy chains, twisted to form an α-helical region, whose terminal portion is called light meromyosin. The heads can split ATP molecules into ADP and phosphate, which generates the energy needed for contraction (i.e. myosin is an ATPase). During contraction myosin heads form cross-bridges with the actin molecules and cause movement.
2) Actin: consisting the thin filaments, actin is formed by two chains of F-actin, assembled from individual G-actin molecules. The thin filament is made up of two strands of F-actin chains twisted together forming a double helix structure.
3) Troponin: globular protein positioned along the actin filament. There are three types of troponin, each of them exploiting a particular function:
    i. Troponin C: binds calcium ions;
    ii. Troponin I: binds to F-actin and inhibits actin-myosin interaction;
    iii. Troponin T: binds tropomyosin.
4) Tropomyosin: surrounds the actin helical structure.

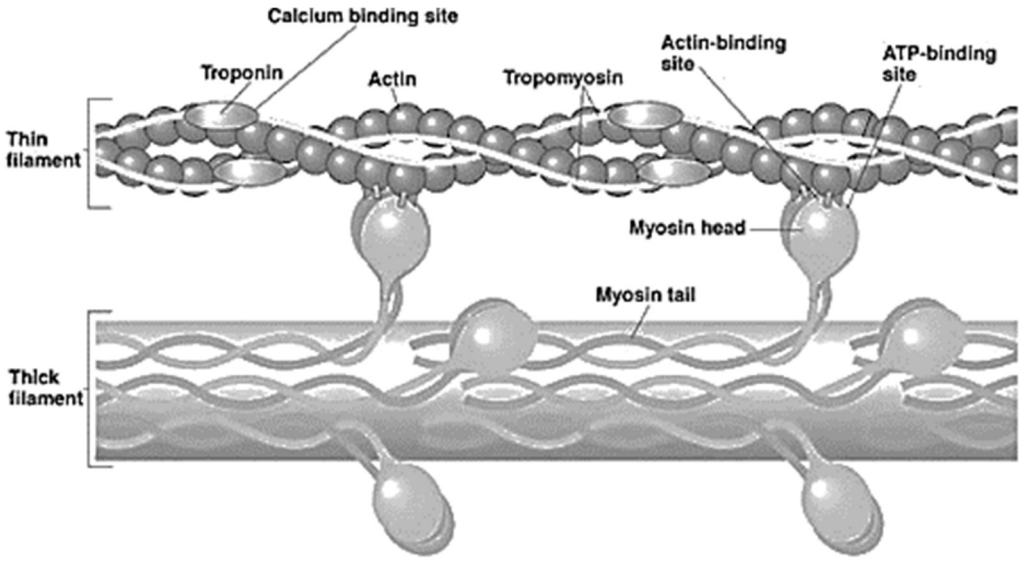


Figure 1.9. Protein involved in muscle contraction (Barbut, 2015).

Given these compulsory details, the muscle contraction mechanism can be simplified in four steps, reported in the figure below (Figure 1.10).

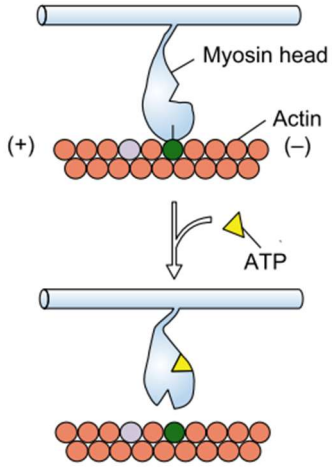
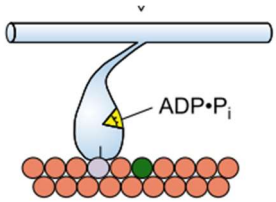
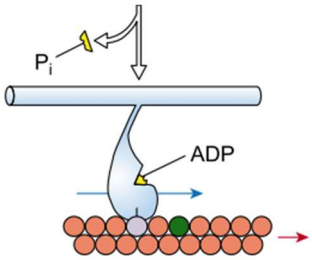
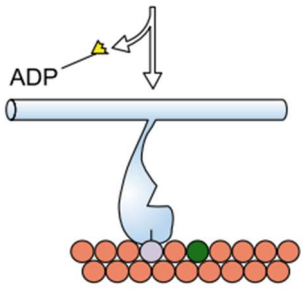
Nucleotide binding		ATP binds to the myosin head and releases it from binding to actin.
Hydrolysis of ATP		The ATP is hydrolyzed into ADP and inorganic phosphate (Pi): the myosin head shifts to the left. Interaction with actin is blocked by the tropomyosin-troponin complex. Myosin heads are “waiting” for the availability of a binding site on the thin filament. Sarcoplasmic reticulum releases Ca^{2+} which binds troponin complex, thus allowing the myosin head to bind to actin.
Pi release		The release of the Pi causes the power stroke: the myosin head pulls the thin filament towards the M line. The filaments overlap.
ADP release		The ADP is released. Myosin remains attached to actin until a molecule of ATP binds myosin head, causing its release from the actin and starting the cycle over again.

Figure 1.10. The mechanisms involved in muscle contraction. Adapted from Purslow, (2006).

Muscles contract in response to a nervous stimulation that causes a release of acetylcholine (i.e., a neurotransmitter that binds to a receptor located on the muscle cell surface), which produces depolarization of the myofiber membrane. Depolarization causes a release of Ca^{2+} from the sarcoplasmic reticulum, which is the structure that stores and releases calcium ions. These ions bind to troponin-C, which dislocates tropomyosin thus exposing the binding site of actin and allowing the ATP to react with myosin's heads (Purslow, 2006). The contraction process terminates when calcium is sequestered back into the sarcoplasmic reticulum.

1.2.3 Energy metabolism in living muscle

The major energy source for muscle metabolism is ATP (Holmes, 1987). Contraction is undoubtedly the most energetically demanding process, however, it has been estimated that the amount of ATP stored in the muscle (5-8 μmol/g) is sufficient to supply energy just for few seconds of high-intensity activity (Aberle et al., 2012; Barbut, 2015). Thus, in living muscle ATP must be generated continuously by several mechanisms such as: i) phosphagen system ii) aerobic and iii) anaerobic metabolism (Barbut, 2015) (Figure 1.11). Many of these mechanisms operate seamlessly during the conversion of muscle to meat, as muscle attempts to maintain homeostatic conditions (Ferguson and Gerrard, 2014; Matarneh et al., 2017) (see paragraph 1.3.1).

1.2.3.1 The phosphagen system

The most direct and immediate source for ATP re-synthesis is the direct phosphorylation of phosphocreatine (a high energy phosphate compound) by the reaction: **H^+ + ADP + phosphocreatine ⇄ ATP + creatine + H_2O**. This reversible reaction occurs in the sarcoplasm and it is catalyzed by the enzyme creatine kinase (Aberle et al., 2012). Phosphocreatine is more plentiful in muscle than ATP and can provide energy for contraction for about 15 seconds (Holmes, 1987). However, since muscle stores of phosphocreatine are limited, it needs to be replenished during resting periods through the re-phosphorylation of creatine (Aberle et al., 2012).

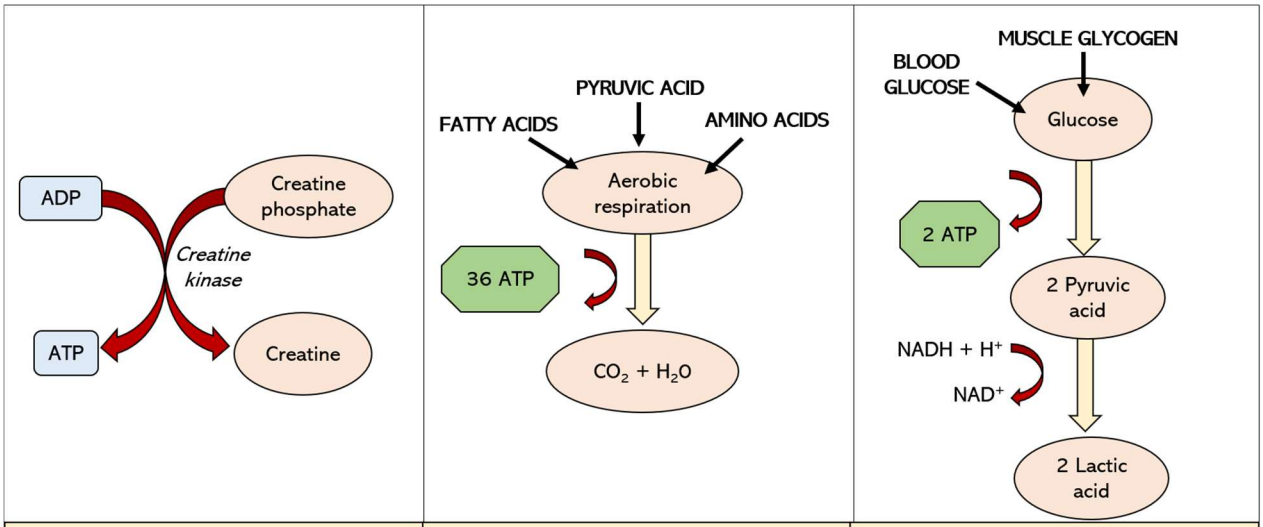
1.2.3.2 Aerobic metabolism

Aerobic metabolism is the most efficient way for ATP synthesis since it produces 36 moles of ATP for every 1 mole of glucose (Ferguson and Gerrard, 2014). Briefly, aerobic metabolism occurs in mitochondria (i.e. in presence of O_2) and relies on the degradation of nutrients (such as carbohydrates, proteins, and lipids) for the production

of carbon dioxide, water and ATP (Matarneh et al., 2017). During this process, muscle can get energy from both stored glycogen (broken down into glucose molecules through glycogenolysis) and blood-borne glucose, but also from pyruvic acid (through Krebs cycle), free fatty acids and some amino acids (Holmes, 1987). This metabolism is able to supply energy during moderate exercise.

1.2.3.3 Anaerobic metabolism

Under limited oxygen supplies for aerobic metabolism to sustain the energy requirements of the muscle, anaerobic metabolism is able to supply energy for a short period of time. The energy substrate for anaerobic metabolism is glucose, which is metabolized through glycolysis (a process that does not require oxygen) into 2 molecules of pyruvic acid (Aberle et al., 2012). During glycolysis, NAD^+ is reduced to $NADH + H^+$. In the absence of oxygen, muscle cells can oxidize NADH back to NAD^+ only by converting pyruvate in lactic acid (Holmes, 1987). Thus, lactic acid diffuses in the muscle and beckon through the circulatory system to heart, kidney or liver, where it can be converted back to glycogen (Holmes, 1987). During prolonged and intense exercise, muscles need oxygen to restore homeostasis, thus lactic acid accumulates in the muscle leading to muscle fatigue.



PHOSPHAGEN SYSTEM	AEROBIC METABOLISM	ANAEROBIC METABOLISM
Energy substrate is creatine phosphate	Energy substrates are glucose, pyruvate, fatty acids and amino acids	Energy substrate is glucose
No O_2, produces 1 ATP	Uses O_2, produces 36 ATP/glucose, CO_2 and H_2O	No O_2, produces 2 ATP/glucose and 2 lactic acids

Figure 1.11. ATP production in living muscle.

1.2.4 Types of muscle fibers

The metabolic pathway represents a criterion to classify muscle fibers. Indeed, fibers that rely on oxygen to generate ATP are classified as oxidative, vice versa muscle fibers that rely on anaerobic glycolysis are defined glycolytic (Holmes, 1987). Moreover, based on the rate at which ATP is split (i.e. the activity of myosin ATPase) muscle cells are classified as fast or slow (Barbut, 2015; Matarneh et al., 2017). Lastly, depending on myoglobin content, fibers are termed red or white. It is well established that in avian species there are three types of skeletal muscle fibers (Clark and Harding, 2017):

- **Type I (slow-twitch, oxidative)**: due to the low myosin ATPase activity, these fibers are designed for repetitive and endurance activities (e.g. maintaining the posture or flight) and produce energy through the oxidative pathway from glucose or fatty acids. For this reason, muscles with a high percentage of type I fibers are highly vascularized, rich in lipids, mitochondria, and myoglobin, that provides a red color (Taylor, 2004; Westerblad et al., 2010; Clark and Harding, 2017).
- **Type IIa (fast-twitch, oxidative/glycolytic):** having both oxidative and glycolytic metabolic capability, these fibers are often called “intermediate” and are pink in color. They can sustain rapid activities (e.g. sprinting or walking) and fatigue slowly (Warriss, 2000; Taylor, 2004).
- **Type IIb (fast-twitch, glycolytic):** these fibers generate energy through anaerobic metabolism and for this reason are minimally vascularized, poor in mitochondria and myoglobin. The low myoglobin content is responsible for their pale color, so much so that they are referred to as “white fibers”. Due to their high myosin ATPase activity (i.e. fast contraction ability), they are involved in high-intensity activities but they also fatigue quickly (Westerblad et al., 2010; Clark and Harding, 2017).

The main characteristics of muscle fibers types are summarized in table 1.4. Generally, muscles are a mosaic of different fiber types, even if in some cases one type could predominate (e.g. chicken Pectoralis major is 99% composed of glycolytic fibers) (Barbut, 2015). However, fiber composition greatly differs between species, muscles, and regions of the same muscle and also depends on animal activity (Taylor, 2004). For instance, a study conducted by Branciari et al. (2009) showed that the percentage of aerobic fibers grows from 0.5 to 3.8% in slow-growing *P.major* of

chickens reared in organic systems if compared to the ones reared in conventional systems, likely due to the increase in activity.

Table 1.4 Characteristics of muscle fibers. Adapted from Aberle et al. (2012).

Characteristic	Type I	Type IIa	Type IIb
Redness	++++	+++	+
Myoglobin content	++++	+++	+
Fiber diameter	+	+	+++
Contraction speed	+	+++	++++
Fatigue resistance	++++	+++	+
Mitochondria	++++	+++	+
Lipid content	++++	+++	+
Glycogen content	+	+	++++
Capillary density	++++	+++	+

The living muscle is an extremely complex system. Understanding its structure and the mechanisms involved in muscle contraction in the living animal is essential for the comprehension of the changes that occur during the conversion of muscle to meat.

1.3 Biochemistry controlling meat quality development

1.3.1 Post-mortem energy metabolism and conversion of muscle to meat

During the post-mortem period, a complex set of events takes place in the muscle, resulting in its conversion to meat. When the animals are harvested, cessation of blood circulation occurs and results in hypoxia and accumulation of the waste products of metabolism (Pearson and Young, 1989). However, muscle tissue does not cease functioning immediately after death and, becoming strictly anaerobic, continues to synthesize and utilize ATP in a futile attempt to maintain its ante-mortem homeostatic balance (Matarneh et al., 2017). Indeed, during the early post-mortem, muscle ATP concentration remains stable due to the utilization of both oxygen bound to myoglobin and phosphocreatine, which serves as an immediate energy source (Pösö and Puolanne, 2005). However, when phosphocreatine concentration becomes lower than 4μmol/g of muscle and the phosphagen system is unable to replenish ATP levels, the leading pathway for energy production becomes anaerobic glycolysis (Bendall, 1973). Glycogen, the main storage form of carbohydrates in muscle tissue, is degraded in glucose 1-phosphate through a process called glycogenolysis. This process is enzymatically mediated by glycogen phosphorylase, which cleaves off the terminal glucose, leaving a glycogen molecule without one glucose in its structure (Pearson and Young, 1989; Aberle et al., 2012).

$$\textbf{Glycogen}_n + P_i \rightarrow \textbf{Glycogen}_{n-1} + \textbf{Glucose 1-phosphate}$$

At this point, glucose 1-phosphate molecules can be readily converted to glucose 6-phosphate by the action of phosphoglucomutase.

$$\textbf{Glucose 1-phosphate} \rightarrow \textbf{Glucose 6-phosphate}$$

Free glucose molecules are likewise converted by the enzyme hexokinase to glucose 6-phosphate.

$$\textbf{Glucose + ATP} \rightarrow \textbf{Glucose 6-phosphate + ADP + } H^+$$

Now both glucose 6-phosphate molecules generated from glycogen or stored in the muscle can enter the glycolytic pathway (Figure 1.12).

Glycolysis consists of a sequence of 10 reactions where each six-carbon glucose moiety is mobilized into two three-carbon pyruvate molecules, along with the production of ATP, NADH, $H^{+,}$ and water. During post-mortem, pyruvate produced during this process is then converted by lactate dehydrogenase (LDH) to lactate, which accumulates in the muscle.

Pyruvate + NADH + H^+ → Lactate + NAD^+

It is often wrongly believed that lactate alone is responsible for the drop in pH. Actually, lactate production is essential to regenerate NAD^+, which is needed by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in order to continue glycolysis in anaerobic conditions (Matarneh et al., 2017). Moreover, for every pyruvate molecule converted into lactate, LDH consumes one H^+ thus acting as a buffer, by limiting the accumulation of free H^+ ions and prevent muscle acidification (England et al., 2017). Rather, the hydrolysis of ATP, which yields H^+ that accumulates in the muscle, is mainly responsible for lowering the pH (Pösö and Puolanne, 2005; Aberle et al., 2012; Scheffler et al., 2013; Ferguson and Gerrard, 2014; Matarneh et al., 2017).

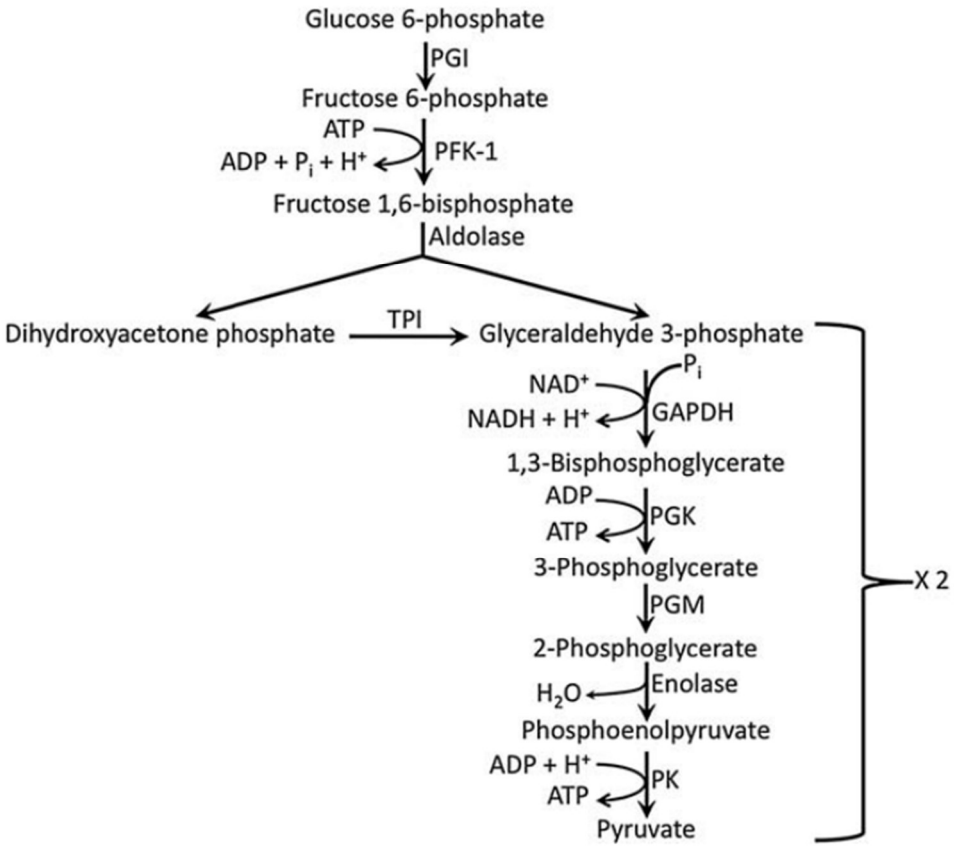


Figure 1.12. The glycolytic pathway (Matarneh et al., 2017).

Anaerobic metabolism is less efficient at regenerating ATP than aerobic metabolism. When ATP content declines (i.e. ATP hydrolysis exceeds its generation) and reach about 1 mmol/kg, it triggers the onset of rigor mortis (Pösö and Puolanne, 2005; Matarneh et al., 2017). Indeed, as long as ATP is available, the muscle remains extensible because there is sufficient energy to form and break actomyosin bonds. Then, the complete depletion of energy sources causes the permanent formation of myosin and actin cross-bridges and results in the inextensibility and toughening of the muscle, signaling the onset phase of rigor mortis (Scheffler and Gerrard, 2007; Barbut, 2015). The delay time before the onset of rigor mortis depends on species: usually, in poultry, it occurs within one hour post-mortem, while in beef it can take even 12 days (Aberle et al., 2012). However, during post-mortem storage, muscle tension eventually decreases (resolution of rigor mortis) as a result of several biochemical events such as the proteolytic degradation of specific myofibrillar proteins, that results in the increase of meat tenderness (Aberle et al., 2012).

1.3.1.1 The factors controlling the rate of post-mortem metabolism

The glycolytic rate during the conversion of muscle to meat is strongly related to the intensity of post-mortem metabolism. Typically, in poultry pH gradually declines from a value of 7.0 in the living muscle to an ultimate pH of about 5.8-5.9 (Petracci et al., 2017). However, the rate of post-mortem metabolism is influenced by several factors such as the animal species, fiber types, peri-mortem conditions, pre-slaughtering stress and so on (Matarneh et al., 2017). As an example, the acidification process normally takes 4-8 h in pigs and 15-36 h in cattle, while in poultry it is very rapid: breast muscle pH could fall to 6 within 30 min post-mortem and reach ultimate pH value within 6 h from the death of the animal (Warriss, 2000). Moreover, the rate of muscle acidification can also significantly differ intra-specie depending on the fiber composition of the muscles. Indeed, muscles with a higher proportion of type I fibers show slower rates of pH decline if compared to glycolytic muscles, since oxidative fibers have less glycolytic enzymes abundance and activity (Bowker et al., 2017). Moreover, muscles mainly composed of glycolytic fibers have a greater concentration of glycogen in resting muscle, being better equipped and prone to metabolize glycogen under anaerobic conditions. Differences in the rate of post-mortem metabolism among muscles with a different fiber type composition could also be explained by the increased buffering capacity of type IIb fibers and their higher concentration of

histidine-containing compounds (e.g. anserine and carnosine) (Aalhus and Price, 1991; Peiretti et al., 2011; Jung et al., 2013).

Besides all, it is well established that the rate of post-mortem metabolism is driven by the rate of ATP hydrolysis (Bendall, 1973; Scopes, 1974). Several enzyme systems present in the muscle (e.g. myosin ATPase, sarcoplasmic reticulum Ca^{2+} ATPase, mitochondrial ATPase, etc.) require the hydrolysis of ATP to perform cellular functions (Matarneh et al., 2017). Thus, since myosin is the most abundant protein in the muscle, there is increasing evidence that myosin ATPase is the major ATPase accountable for ATP hydrolysis post-mortem (Scopes, 1974; Ferguson and Gerrard, 2014). Myosin ATPase activity varies across fiber types, with glycolytic fibers possessing a higher ATPase activity than oxidative fibers (Bowker et al., 2017). Moreover, ATP splitting rate is influenced by both temperature and cytosolic Ca^{2+} concentration (Bendall, 1973). The rate of pH acidification is faster at 38°C, while ATP depletion significantly slows with the temperature being under 15°C (Scopes, 1973). On the other hand, high sarcoplasmic concentration of Ca^{2+} significantly increases the rate of post-mortem glycolysis by allowing the interaction between myosin and actin active sites (Matarneh et al., 2017). In this way, the onset of rigor mortis will occur earlier and faster. Indeed, electrical stimulation is widely used in red meat to accelerate post-mortem metabolism since, causing a massive liberation of Ca^{2+}, it hastens the development of rigor mortis and improves meat tenderness (Hammelman et al., 2003; Aberle et al., 2012; Matarneh et al., 2017).

1.3.1.2 The factors controlling the extent of post-mortem metabolism

The amplitude of muscle acidification is defined by the initial pH measured at 15 min after the death of the animal and the pH reached at 24 h post-mortem (i.e. pH_u) (Debut et al., 2003). The extent of post-mortem metabolism is a function of several factors. Mainly, it depends on the quantity of glycogen available in the muscle at death (i.e. its glycolytic potential) (Berri et al., 2005b; Scheffler and Gerrard, 2007). Indeed, glycogen content at the time of slaughter is considered to be directly proportional to the extent of post-mortem acidification, in other words, it is expected that muscle pH should drop as long as glycogen is available in the muscle (Matarneh et al., 2017). Rather, recent studies found that glycogen levels beyond 53 μmol/g are not linked with any further pH decline, affirming that when glycogen concentration is above this threshold, post-mortem acidification is controlled by other factors (England et al., 2016; Matarneh et al., 2017, 2018). The same authors explain that when glycogen is not

limiting, post-mortem metabolism could be controlled by the activity of the glycolytic key-regulatory enzyme phosphofructokinase (PFK). This enzyme, which during glycolysis catalyze the conversion of fructose 6-phosphate in fructose 1,6-biphosphate, is extremely susceptible to post-mortem drop in pH since it begins to be inactive at pH 5.9: in this way, as long as pH declines, PFK could become limiting and influence the extent of post-mortem acidification (England et al., 2014). Moreover, PFK is stimulated by AMP and ADP: increased concentrations of these adeno-nucleotides activate glycogenolysis and glycolysis, allowing greater amounts of substrates to pass through PFK and thus lowering the pH (England et al., 2014; Bowker et al., 2017; Matarneh et al., 2017). However, in some cases, the complete conversion of adenosine nucleotides to IMP could arrest glycolysis while PFK is still functioning (England et al., 2016).

Besides, the ultimate pH of a muscle also depends on the buffering systems of the muscle itself (Laack et al., 2001). Buffering capacity is defined as the ability of the muscle to maintain pH levels that allow the function of vital enzymes (Pösö and Puolanne, 2005). If muscle had had no buffers, the simultaneous production of lactate and protons by anaerobic glycolysis would have resulted in a very rapid pH drop. To support this, muscle mainly composed of glycolytic fibers have a greater buffering capacity, since they are adapted to prevent excessive drops in pH during fast movements in vivo (Pösö and Puolanne, 2005). The same compounds which regulate buffering capacity in the living muscle also control it in post-rigor muscle. In more detail, muscle buffering capacity is due by half to myofibril proteins, while lactate, phosphate compounds as well as histidine-containing dipeptides (e.g. anserine and carnosine) contributed to the other half (Matarneh et al., 2017). Davey (1960) and colleagues reported that carnosine and anserine (pKa= 6.83 and 7.04, respectively) can together contribute to 30% of the total buffering capacity of the muscle. The distribution of these dipeptides is specie-specific: while carnosine is predominant in beef and pork meat, anserine is the prevalent histidine dipeptide in poultry meat (Abe and Okuma, 1995). As a matter of fact, anserine is found to act as a physico-chemical buffer against proton production by glycolysis in breast muscle (Jung et al., 2013). Moreover, the content of histidine compounds is found to be closely related to the fiber type. As an example, oxidative muscles usually have a significantly lower content of histidine dipeptides, so much so that carnosine content is believed to represent a good indicator of the glycolytic activity of a muscle (Mora et al., 2008).

1.3.2 Implications of post-mortem energy metabolism on the development of meat quality

During the conversion of muscle to meat, the physico-chemical changes that occur in muscle tissues have a great impact on the quality of the forthcoming meat. A great number of factors such as pre-slaughter stress, stunning method, diet, and muscle type can influence muscle energy initial level and its utilization during post-mortem (Scheffler and Gerrard, 2007). The rate and the extent of the acidification process are able to influence color, texture as well as water holding capacity and shelf-life of meat (Ferguson and Gerrard, 2014).

If the extent of the acidification is limited and meat pH remains high, proteins maintain their functionality and WHC is improved. On the contrary, intense acidification that results in a low ultimate pH (i.e. near to the isoelectric point of muscle proteins) reduces the solubility of a large number of proteins and causes a reduction in WHC (Huff-Lonergan and Lonergan, 2005; Matarneh et al., 2017). Moreover, during post-mortem time and rigor development, a shrink in the diameter of muscle fiber occurs, thus causing an increase in extracellular space around the fibers (Bowker, 2017). Consequently, water migrates from inside the muscle fibers to the extracellular spaces, increasing light reflectance (Swatland, 2008; England et al., 2017). Indeed, post-mortem energy metabolism significantly affects also fresh meat color. Very low pH_u values are usually associated with a paler meat color, while darker meat cuts are related to a poor acidification resulting in an ultimate pH higher than 6.0 (Aberle et al., 2012; England et al., 2017).

It is well documented that post-mortem events deeply impact on textural properties of fresh meat. Once ATP is depleted, myosin irreversibly binds actin leading to the development of rigor mortis and muscle stiffness. Eventually, rigor mortis disappears and the meat becomes tender thanks to the action of endogenous proteases (Aberle et al., 2012). Within this context, a rapid drop in pH provokes protein denaturation thus decreasing WHC and resulting in a soft texture. On the contrary, a feeble acidification results in a greater WHC which gives the meat a firm and rigid texture (England et al., 2017).

1.4 Poultry meat abnormalities

1.4.1 Post-mortem related abnormalities

As stated in the previous paragraph, it is well established that post-mortem energy metabolism is highly relevant to meat quality. An unproper muscle acidification can severely compromise muscle protein integrity, having severe implications on the main functional and qualitative traits of fresh meat. Specifically, alterations in the extent and/or the rate of glycolysis, combined with other factors such as stress and temperature, could result in muscular abnormalities. The main post-mortem related abnormalities affecting poultry meat are pale, soft and exudative (PSE-like) and dark, firm, and dry (DFD) conditions, which are briefly introduced in paragraph 1.1.3.4 and better explained in the following paragraph.

1.4.1.1 Pale, Soft, and Exudative meat

With an incidence of up to 40%, PSE-like has become one of the biggest issues facing pork and poultry meat industries (Matarneh et al., 2017). The term “PSE” was originally coined to describe pork meat with light color, flaccid texture as well as impaired ability to hold water (Woelfel et al., 2002). Indeed, this syndrome was first observed in swine and it is generally believed to be associated to a genetic mutation in a sarcoplasmic reticulum’s receptor regulating calcium release and uptake, combined to a higher stress-susceptibility of animals due to genetic selection for muscle development (Rempel et al., 1995). During the past decades, PSE-like syndrome was observed also in poultry (Barbut, 1997; Woelfel et al., 2002; Strasburg and Chiang, 2009). This defective condition is likely due by an accelerated or excessive post-mortem metabolism immediately after slaughter, when carcass temperature is still high: indeed, PSE-like syndrome is a pH-temperature dependent phenomena (Bowker et al., 2017). Moreover, short or long-term pre-slaughter stress causes the release of hormones and enzymes that catalyze glycogen mobilization and utilization, thus leading to an accelerated post-mortem glycolysis and pH decline (pH<6 at 45 min post-mortem) (Warriss, 2000; Matarneh et al., 2017). Thus, the combination of low pH (caused by pre-slaughter stress conditions within the first hour post-mortem) (Figure 1.13) and the high temperature of the muscle, leads to the denaturation of sarcoplasmic and myofibrillar proteins. As a result, PSE-like meat has a pale and

unattractive color, soft texture, reduced WHC, higher drip losses and lower protein solubility (Barbut, 1997; Van Laack et al., 2000; Petracci and Cavani, 2012). As a consequence, PSE-like meat has very low processing yields, and the texture of products formulated with this kind of meat is brittle and grainy (Huang and Ahn, 2018).

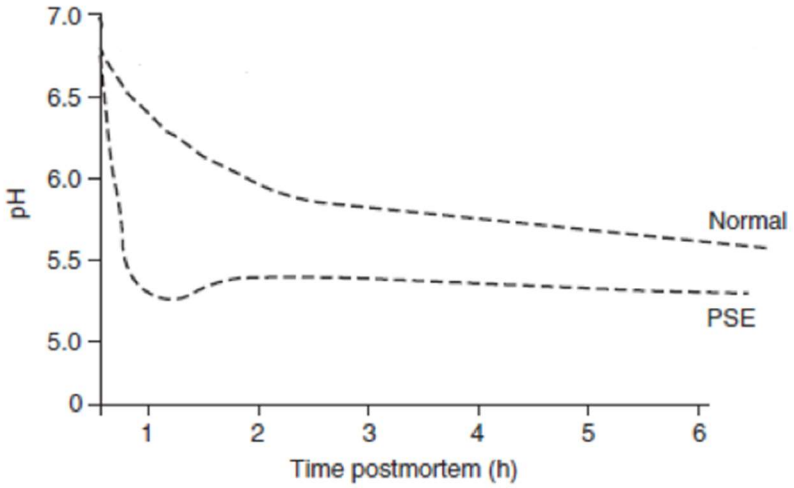


Figure 1.13. Normal and PSE-meat pH decline curves. Adapted from England et al. (2017).

Several studies aimed at establishing the underlying mechanism responsible for PSE-like syndrome in poultry have been conducted. Briefly, it has been evidenced that genetic selection for fast-growing and high-breast yield hybrid birds might play a role in the appearance of post-mortem related abnormalities since myofiber hypertrophy and inadequate vascular supply were cited as the factors contributing to PSE-like condition (Petracci and Cavani, 2012). Moreover, fast-growing birds seem more susceptible to heat stress due to their increased body temperature and metabolic heat production, thus leading to an accelerated post-mortem metabolism (Petracci et al., 2017).

Since PSE-like in poultry is mainly associated to ante-mortem stress, reducing stressing conditions during handling and transport along with the optimization of the chilling operations could reduce the incidence of PSE-like condition in poultry industry (Matarneh et al., 2017). Moreover, PSE-like meat could be used in further processed products, by adding defective meat to special formulations that allow restoring meat color and protein functionality, in order to improve process yields and meat texture (Lesiów and Kijowski, 2003).

1.4.1.2 Dark, Firm, and Dry meat

While short-term stress is the main factor leading to the development of PSE condition, long-term stress plays a key role in defining the appearance DFD syndrome, whose global incidence ranges from 10 to 20% (Lesiów and Kijowski, 2003). Muscle acidification occurring post-mortem is due by glycogen depletion into lactic acid; however, if glycogen storages are depleted ante-mortem because of long-term stress conditions, meat does not acidify properly and the pH_u remains high (>6.0) (Figure 1.14) (Warriss, 2000).

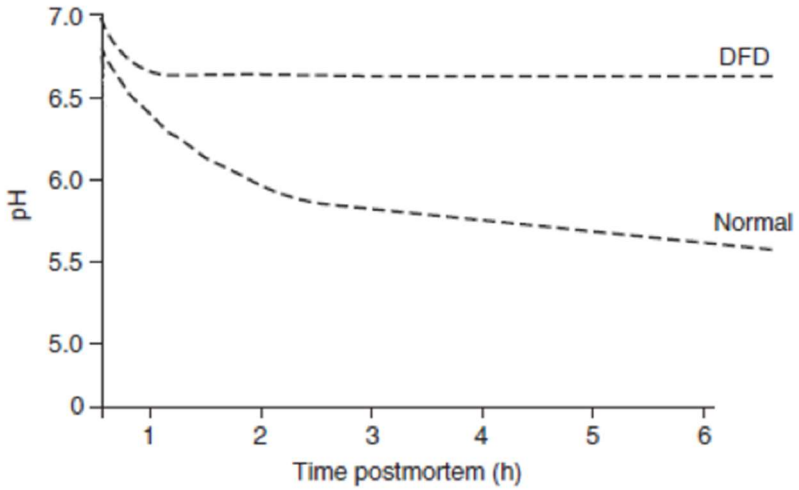


Figure 1.14. Normal and DFD meat pH decline curves. Adapted from England et al. (2017).

Thus, DFD syndrome is developed when the animal fails to restore muscle glycogen prior to slaughter: this condition results in abnormal dark color of meat, along with a very firm texture and dry, sticky surface (Matarneh et al., 2017). As a consequence of the high ultimate pH being very further from the isoelectric point, a little denaturation of muscle proteins occurs and water is still tightly bound to myofibrils (i.e. enhanced WHC), thus explaining the dry, sticky appearance and firm consistency of DFD meat (Tornberg, 1996; Kim et al., 2014). Albeit the higher WHC makes DFD meat a good raw material for processed meat products, the high pH value of this meat promotes bacterial growth and defines a very short shelf-life of breast fillets (Warriss, 2000). Moreover, the meat's high pH minimizes pigment loss, thus increasing light absorbance and giving the meat a darker appearance (Swatland, 2008).

Due to its reduced microbiological stability, DFD meat can be used in the formulation of heat-processed products (Lesiów and Kijowski, 2003). However, as for PSE, limiting stressful conditions during farming and pre-slaughter operations could reduce the incidence of DFD syndrome.

1.4.2 Growth- related abnormalities

In order to meet the growing consumer demand, the meat market has undergone an intense upsurge in poultry meat production, which since 1960 has increased fivefold and is expected to further grow by 3.6% per annum from today to 2030 (Steinfeld et al., 2006). In this regard, poultry industry has been inevitably forced to apply intensive selection procedures aimed at accelerating the growth rate and enhancing the muscle mass of animals (Petracci and Berri, 2017). Currently, chickens designed for meat production usually reach market weight (2.8 kg) within 47 days, namely about half the time compared to 40 years ago, while chickens' average daily weight gain is doubled in the past 50 years (Figure 1.15) (NCC, 2019).

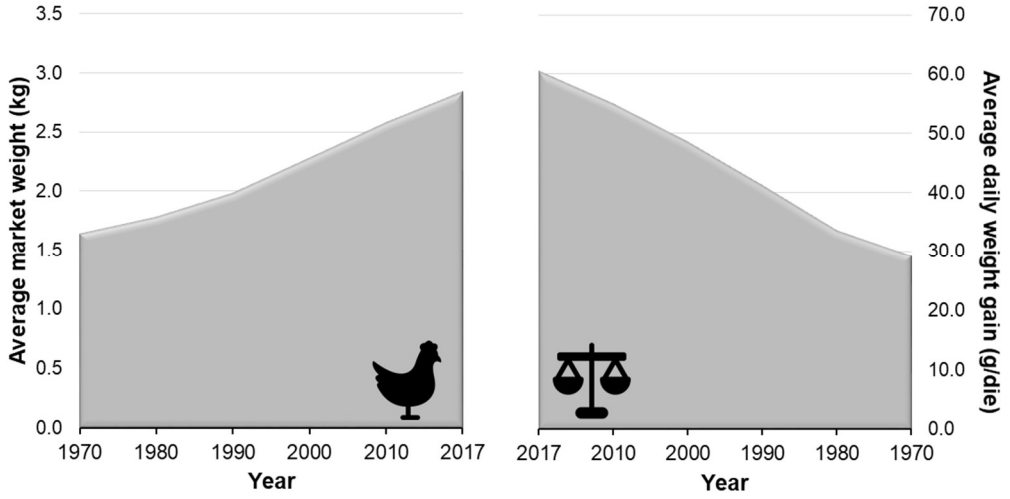


Figure 1.15. Development of average broiler market weight (kg) and daily weight gain (g/die) in the U.S. from 1970 to 2017. Data from NCC, 2019.

The substantial genetic progress of the past few decades has resulted in an increased size and meat yield of the breast muscle, which currently exceeds one-fifth of the weight of the bird and represents the most valuable part in broiler industry (Petracci et al., 2015). Within this context, the improvement in broiler meat production has coincided with the development and expansion of muscular defects affecting the Pectoralis major muscle of fast-growing broiler chickens. Among these, White Striping, Wooden Breast and Spaghetti Meat are the main myopathies that, alone or combined, currently affect breast muscles and negatively influence both visual aspect and technological properties of raw and processed meat, causing relevant economic troubles for the poultry industry.

White Striping (WS) is a widespread muscular abnormality frequently observed in both chicken and turkey breast muscle, macroscopically characterized by the presence of white striations of variable thickness and parallel to muscle fiber direction (Figure 1.16) (Kuttappan et al., 2012b).

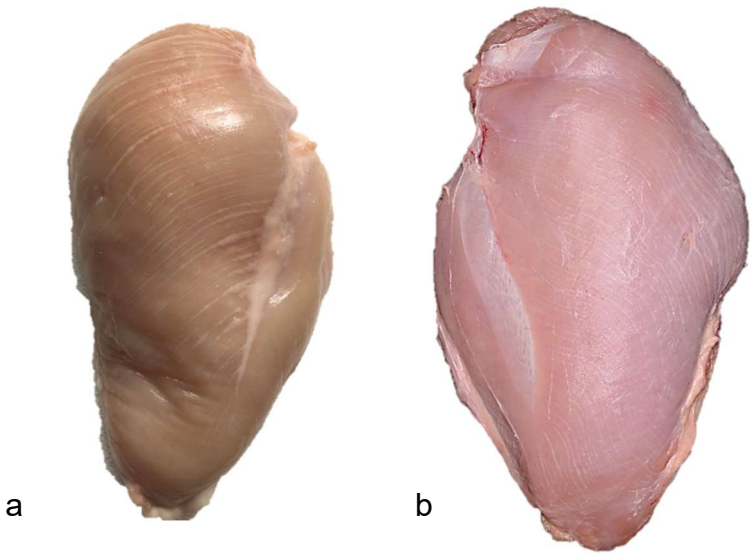


Figure 1.16. Chicken (a) and turkey (b) P. major muscle affected by WS myopathy.

White stripes usually cover the cranial part of the breast muscle and, depending on the severity grade, might extend until the caudal region of the fillet. The striations have been mainly identified as an accumulation of lipids (lipidosis) and connective tissue (fibrosis) (Kuttappan et al., 2013a). Indeed, microscopically, the histopathological changes associated with WS are mainly an abnormal deposition of adipose tissue at perymisial level, necrosis and lysis of fibers, variable cross-sectional area (sign of the existence of degenerating and regenerating fibers) and lymphocytes and macrophages infiltrations (Sihvo et al., 2014; Petracci et al., 2019)

Wooden Breast (WB) myopathy occurs as a focally or diffusely hardened consistency of P. major muscle, which appears pale, rigid, swollen and may have, in the most severe cases, viscous exudate and hemorrhages on its surface (Figure 1.17) (Sihvo et al., 2014). WB and WS often occur concurrently within the same muscle and share common histological features (Petracci et al., 2019). In more detail, WB fillets are characterized by the abnormal proliferation and thickening of the perimysial network (fibrosis). Indeed, the stiffness of WB muscles is likely caused by the extreme collagen deposition along with the accumulation of cross-linked collagen fibrils (Soglia et al., 2017; Velleman et al., 2017).

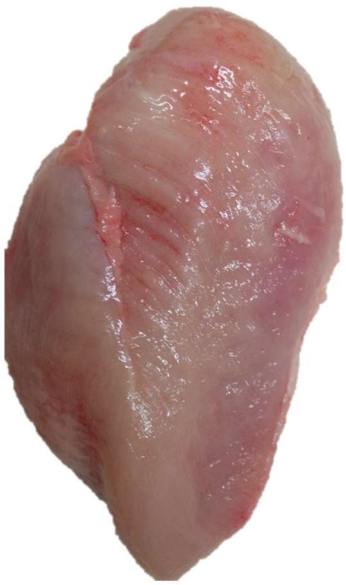


Figure 1.17. P. major muscle affected by WB myopathy.

Spaghetti Meat (SM) condition gets its name from the threadlike and detached muscle fiber bundles composing P. major muscle, which appears mushy, sparsely tight and similar to spaghetti pasta (Figure 1.18). Microscopically, SM fillets show inflammatory cells infiltrations and a rarefaction of the endo- and peri-mysial connective tissue which appears loose (immature), thus leading to poor muscle cohesiveness and the detachment of muscle fibers from each other (Baldi et al., 2018): as a consequence, the fillet appears mushy and undesirable.

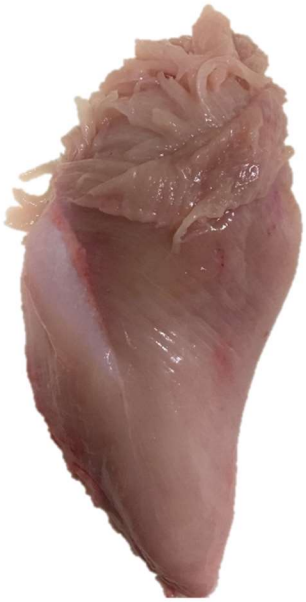


Figure 1.18. P. major muscle affected by SM myopathy.

SM abnormality shares similar histopathological and morphological features to what observed in turkey by Swatland (1990), who attributed these changes to the outgrowth of muscle fiber to the connective tissue.

Moreover, an emerging quality issue affecting the Pectoralis minor of fast-growing birds has emerged. The muscular defect is termed “gaping” because of the separation of the fiber bundles of the external portion of the bipinnate Pectoralis minor muscle (Soglia et al., 2019) (Figure 1.19).

Figure 1.19. P. minor muscle affected by the gaping defect.

Albeit the findings suggest that this condition is strictly related to peri-mortem as well as slaughtering procedures, the gaping defect is found to negatively impact the quality traits of P. minor meat, thus representing a relevant issue for broiler industries. Moreover, muscles affected by gaping seem to share common traits with the ones with PSE-like condition (i.e. lower pHu, pale color, impaired WHC) (Soglia et al., 2019).

1.4.2.1 Underlying mechanisms of growth-related abnormalities

Although these muscular defects are easily discernible by several macroscopic distinctive traits, microscopic observations have shown shared histological features (Soglia et al., 2018). Thus, it might be hypothesized a mutual underlying mechanism responsible for their occurrence, even though the precise etiology is yet to be elucidated.

Recently, it has been demonstrated that the occurrence of the aforementioned myopathies is not related to the existence of a specific gene (Pampouille et al., 2018),

rather hypoxia seems to play a key role in promoting these muscular defects (Mutryn et al., 2015; Abasht et al., 2016). The intensive selection practices carried out during the past decades extremely impacted on broiler muscle metabolism (i.e. shift toward glycolytic pathway) and architecture (i.e. increased muscle fiber diameter and number, reduced capillary density, etc.) (Petracci et al., 2019). Thus, it is reasonable to hypothesize that muscles become hypertrophic and, due to an impaired muscular oxygenation system and disposal of waste metabolic products, reactive oxygen materials accumulate in the muscle and likely lead to inflammatory processes (Petracci and Berri, 2017). From there on, muscle cells try to prevent inflammation but, sooner or later, myodegeneration overtakes the regenerative capacity of the muscle and results in the appearance of muscular issues (i.e. accumulation of fat and/or collagen tissue), peculiar traits of the aforementioned myopathies (Petracci et al., 2019).

1.4.2.2 Implications of growth-related abnormalities on meat quality

Meat affected by growth-related myopathies is usually considered harmless for human nutrition since no specific biological or chemical hazards have been found to be related to its consumption. However, WS, WB and SM myopathies were found to negatively affect both quality traits and technological properties of raw and processed meat.

Depending on the severity of the defect and the eventual co-existence within the same muscle, muscular abnormalities cause important modifications on meat chemical composition, since their occurrence is generally associated to an overall higher amount of moisture, fat and collagen to the detriment of proteins (Kuttappan et al., 2012a; Mudalal et al., 2015; Soglia et al., 2016b). These alterations in chemical composition are mainly due to the degenerative processes taking place in the muscle during the injury, as the replacement of fibers by both adipose tissue and collagen and the increase of extra-cellular water because of the inflammatory processes (i.e. edemas) (Clark and Velleman, 2017). As a result of the increased fat content and the elevated collagen-to-total-protein ratio, abnormal meats are characterized by a significantly lower nutritional value (Petracci and Berri, 2017).

In spite of the higher pH_u, meat affected by myopathies also exhibits reduced technological properties (Bowker and Zhuang, 2016). In detail, several authors reported their poor ability to hold water (Mudalal et al., 2015; Tijare et al., 2016; Tasoniero et al., 2017). In detail, among abnormal meats, WB fillets display a particularly remarkable impaired ability to hold both added (i.e. lower marinade uptake

and higher cooking loss) and constitutional water (i.e. higher drip loss) (Bowker et al., 2018; Dalgaard et al., 2018), as a result of the severe degeneration of muscle tissue. It has been also speculated that the reduced water holding and binding capacities of abnormal meat could be due to an overall reduction in protein functionality since myopathic muscles show a higher concentration of oxidized protein (Utrera and Estévez, 2012; Soglia et al., 2016a).

The appearance of muscular abnormalities exert an important effect also on the textural properties of raw meat, with a special reference to WB condition that causes significantly higher shear and compression forces of the fillets (Chatterjee et al., 2016; Soglia et al., 2017), while WS condition just sparsely affect texture of both raw and cooked meat (Kuttappan et al., 2013b).

Beyond the detrimental effect of muscular abnormalities on meat quality traits and technological properties, WS, WB and SM raise concerns over the consumer acceptance of meat, since their occurrence remarkably impairs the visual appearance of fillets and reduces the consumer´s willingness to buy (Kuttappan et al., 2012b; Huang and Ahn, 2018). Severely affected fillets are usually discarded or downgraded for the manufacture of further processed products (i.e., nuggets, sausages, hamburger), while moderate cases are marketed for fresh retailing (Petracci et al., 2014). Considering the high and unsustainable incidence of these worldwide-spread myopathies, it has been estimated that these defects result in $ 200 million loss per year in the US (Kuttappan et al., 2017b). The economic damage is related not only to poultry processors because of meat downgrading or discarding, lower processing yields and so on, but also to retailers, who have to deal with numerous complaints about meat quality.

1.4.2.3 Actual strategies for mitigation

It is commonly recognized that the incidence of myopathies boosts with increasing growth rate, slaughter age and weight (Lorenzi et al., 2014; Kuttappan et al., 2017b; Radaelli et al., 2017). Thus, attempts have been made in the field of animal nutrition to reduce the occurrence of abnormalities through the modulation of both feed formulation (i.e. dietary supplementation of antioxidants, organic minerals, vitamins and aminoacids) or dietary intake through feed restriction. However, the implementation of these strategies under commercial conditions might be challenging and does not result in any significant mitigation effect, because a reduction of the incidence of breast abnormalities could be attributed as an indirect consequence of

decreased slaughter weight and breast size of the animals (Petracci et al., 2019). Despite it was recently assessed that an increased arginine:lysine ratio can have significant mitigation effect on breast meat abnormalities (Zampiga et al., 2018), further researches are needed to confirm these outcomes. Within this context, the most efficient solution seems the incorporation of downgraded meat into the formulation of processed products. Finely or coarsely minced WB meat could be included in the formulation of hamburger and meatballs without detrimental effects on finished product quality (Brambila et al., 2017; Xing et al., 2017). Since it has been proved that muscular abnormalities mainly affect the superficial section of breast muscles (Baldi et al., 2018), one possible approach could be to separately process superficial and deep layers of fillets to limit the effect of meat downgrading (Petracci et al., 2019).

However, no really efficient solutions aimed at inhibiting the onset of myopathies or at least alleviating the symptoms and consequences on raw and processed meat quality have been found yet. Furthermore, it seems that genetic selection for broilers' growth has reached a plateau and further improvements might be restrained by muscle biological potential and animal welfare concerns (Tallentire et al., 2018). In this scenario, it has been recently suggested that particular attention should be given on the modulation of embryonic formation of additional myofibers, instead of relying on post-hatch selection aimed at increasing muscle mass accretion (Velleman, 2019).

Chapter 2: Materials and methods

2.1 Scientific experimentation

2.1.1 Research activities

The quality of poultry meat is the result of the physical and biochemical phenomena that occur in the muscle after the death of the animal. Among these, the rate and extent of post-mortem acidification play a determinant role in defining the final quality of the resulting meat. In this regard, the genetic progress made over the past 30 years and aimed at obtaining fast-growing genotypes has led to significant changes in muscle metabolism. From a morphological-functional point of view, genetic improvement has caused muscle fibers' hypertrophy as well as profound changes in their structural, functional and metabolic characteristics. Within this scenario, improvements in meat production have allowed a remarkable increase in muscle yield and growth rate, but on the other hand, modern hybrids are more prone to develop muscle disorders, whose incidence in recent years has reached worrying levels for the poultry industry. Although muscle abnormalities represent a significant problem for the meat industry, effective strategies aimed at reducing their magnitude and severity have not been identified yet.

Within this context, the research activities carried out during the PhD project mainly concerned the evaluation of the effect of muscular aberrations on poultry meat quality and the study of post-mortem metabolism of avian muscles in fast-growing genotypes. In more detail, the main activities could be divided into three research lines, as reported in Table 2.1.

Table 2.1. Research activities (A) carried out during the PhD.

Research Activities	
A1	a) Implications of growth-related muscular abnormalities (white striping, wooden breast, and spaghetti meat) on chicken meat quality b) Implications of white striping on turkey breast meat quality
A2	a) Post-mortem acidification patterns in chicken breast and leg muscles b) Post-mortem metabolism of different chicken muscles as affected by histidine-containing dipeptides content
A3	Post-mortem metabolism of broiler P. major muscle affected by Wooden Breast myopathy

2.1.2 Materials and Methods

2.1.2.1 pH

Raw meat

The pH was assessed following the iodoacetate method proposed by Jeacocke (1977). In more detail, 2.5 g of meat were manually minced and homogenized by Ultra-Turrax T25 basic (IKA-Werke, 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 of the homogenate was then assessed by a Jenway 3510 pH-meter previously calibrated at pH 4.0 and 7.0. This technique is suitable for the determination of early post-mortem muscle pH because iodoacetate stops the acidification in meat samples (Petracci and Baéza, 2007). This method has been applied in A1a, A1b and A2a research activities.

Powdered meat

With the aim of studying muscle acidification during post-mortem time, samples from chicken muscles were collected at different times after slaughter, immediately frozen in liquid nitrogen and stored at -80°C until processing. Then, frozen samples were powdered under liquid nitrogen using mortar and pestle (Figure 2.1) and aliquots of approximately 0.1 g were collected. Powdered muscle samples were lysed using a Tissue Lyser (Qiagen, USA) in 0.8 ml of ice-cold pH solution, containing 5 mM sodium iodoacetate and 150 mM KCl (pH 7.0). Samples were then centrifuged at 17,000 × g for 5 min and pH of the supernatant was measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, USA). This method has been applied in A2b and A3 research activities.

Figure 2.1. Meat sample powdered under liquid nitrogen.

2.1.2.2 Buffering capacity

Buffering capacity of meat samples was determined according to Matarneh et al. (2015). Briefly, about 1 g of meat was powdered under liquid nitrogen and homogenized in 10 ml of 5 mM sodium iodoacetate and 150 mM KCl solution (pH=7.0). After equilibration to 25°C, samples were titrated using 0.1 M NaOH. Samples pH was measured using an Orion Ross Ultra pH glass electrode (Thermo Scientific, USA) and buffering capacity (BC) was calculated as follows:

$$BC = \frac{\Delta B}{\Delta pH}$$

ΔB= increment of base expressed as μmol NaOH/g of tissue;

ΔpH= pH variation following the addition of NaOH.

This method has been applied in A3 research activity.

Buffering capacity of meat samples was determined according to Matarneh et al. (2015) with slight modifications. About 2.5 g of meat was weighed and homogenized with Ultra-Turrax (26.000 rpm for 15 s) in 25 ml of 5 mM sodium iodoacetate and 150 mM KCl solution (pH=7.0). After equilibration to 25°C, the homogenate was transferred into a 25 ml beaker with a stir bar and the initial pH (pH_i) was measured while stirring. The pH of homogenate was adjusted to pH 6.0 by adding HCl or NaOH and then titrated to 7.0 using 0.5 M NaOH. Samples pH was measured using a Jenway 3510 pH-meter (previously calibrated at pH 4.0 and 7.0) and buffering capacity (BC) was calculated as follows:

$$BC = \frac{\Delta B}{\Delta pH}$$

ΔB= increment of base expressed as μmol NaOH/g of tissue;

ΔpH= pH variation following the addition of NaOH.

This method has been applied in A2b research activity.

2.1.2.3 Color

Color of meat samples was assessed through a reflectance colorimeter equipped with an illuminant source C and previously calibrated with a reference color standard white ceramic tile (Y = 93.9, x = 0.3130, and y = 0.3190). Color measurement was performed, in triplicate, on the bone side (ventral) surface of breast muscles after 24 h post-mortem, in order to avoid color surface defects such as bruises and

discolorations. In detail, meat color was assessed using a Chroma Meter CR-400 (Minolta Corp., Italy) and expressed, according to the CIE LAB system, as lightness (L*), redness (a*) and yellowness (b*). This method has been applied in A1a and A1b research activities.

2.1.2.4 Total heme pigments

The procedure to assess total heme pigments consists in a spectrophotometric method based on the extraction of the heme-group from pigments by using acetone and hydrochloric acid as proposed by Hornsey (1956). In detail, 5 g of meat were homogenized (30 s at 13,000 rpm) in 20 ml of an acetone:water solution (20:1) and, after the addition of 0.5 ml of 37% HCl, samples were incubated in a dark place for 1 h. After filtration through a Whatman #1 filter paper, the absorbance of samples was read at 640 nm (against blank).

Total heme pigments were expressed as µg hematin/g of meat and calculated as follows:

$$Ematin = \frac{679.2\ x\ Abs\ 640\ nm\ x\ 5}{sample\ weight}$$

This method has been applied in A2a research activity.

2.1.2.5 Water holding capacity

Water holding capacity is referred as the ability of meat to retain its liquid during storage, processing and cooking. Its measurement has been assessed by using different methods.

2.1.2.5.1 Drip loss

The term “drip loss” refers to the fluid released by raw meat through passive exudation during its refrigerated storage (Petracci and Baéza, 2007). Samples of 8 x 4 x 2 cm dimensions were excised from each P. major muscle at 24h post-mortem, individually weighted and stored at 4°C for 48 h in plastic boxes. Then, each sample was cleaned from eventual superficial liquid accumulation and re-weighed in order to assess drip loss, calculated according to the formula:

$$Drip\ loss\ (\%) = \frac{(initial\ weight - final\ weight)}{initial\ weight} x\ 100$$

This method has been applied in A1b research activity.

2.1.2.5.2 Marinade uptake

Marinade uptake is the amount of marinade solution retained by raw meat during the marinade process and it is calculated as the percentage of weight gained by the sample in relation to its initial weight:

$$Marinade\ uptake\ (\%) = \frac{(marinated\ meat\ weight - raw\ meat\ weight)}{raw\ meat\ weight} x\ 100$$

In more detail, samples of 8 × 4 × 2 cm dimension and weighing about 60 g were excised from the cranial section of each P. major muscle, individually labeled and vacuum tumbled using a small-scale vacuum tumbler (Vakona Qualitat, Germany) with a 20% (wt/wt) aqueous solution containing sodium tripolyphosphate (1.8%) and sodium chloride (6%). The tumbler was 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 this process, samples were re-weighted and the difference in weight before and after tumbling was used to calculate marinade uptake. This method has been applied in A1b research activity.

2.1.2.5.3 Cooking loss

Cooking loss is the amount of fluid released by both raw and marinated meat after heat treatment. Raw and marinated samples were weighed, individually packaged under vacuum (99%) in heat-resistant plastic bags and cooked at 80°C in a water bath. Cooking time was defined, by inserting a temperature probe in a meat sample, as the time necessary to reach a 80°C temperature within the inner core (Petracci and Baéza, 2007). After the cooking process, samples were cool down at room temperature and re-weighed. The cooking loss was assessed according to the formula:

$$Cooking\ loss\ (\%) = \frac{(fresh\ weight - cooked\ weight)}{fresh\ weight} x\ 100$$

This method has been applied in A1b research activity.

2.1.2.5.4 TD-NMR relaxation properties

Time Domain Nuclear Magnetic Resonance (TD-NMR) is an analytical technique used to deeply investigate water content and behavior in muscles. A meat sample weighing about 600 mg with a height not exceeding 1 cm was collected from the cranial section of each P. major muscle with the aid of a specific tool and inserted into a glass tube for TD-NMR assay (Figure 2.2).

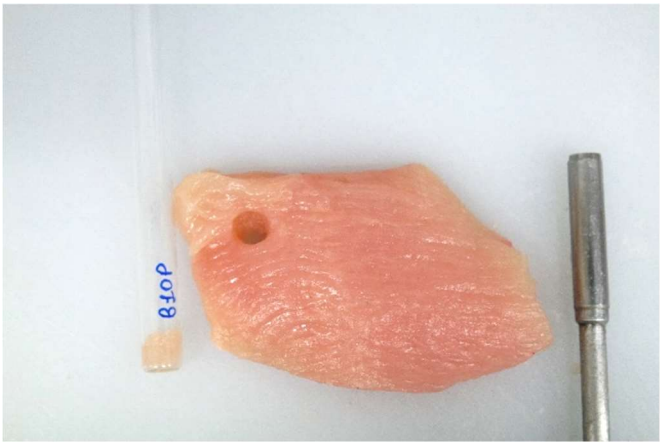


Figure 2.2. Meat sample for TD-NMR analysis.

Proton transverse relaxation (T_2) decay curves in meat samples were recorded at a constant temperature of 24°C and at the operating frequency of 20 MHz with a Bruker Minispec PC/20 spectrometer using the Carr-Purcell-Meiboon-Gill (CPMG) pulse sequence, as described by Petracci et al. (2013). The CPMG decays were then normalized by the sample weight and transformed into relaxograms (i.e. continuous distributions of relaxation times) through the program UPEN. As showed in Figure 2.3, each relaxogram was interpreted in terms of bound, intra-myofibrillar and extra-myofibrillar water proton pools. Then, in order to separately observe these protons populations, the relaxograms were fit to the sum of four exponential curves and the two with intermediate T2, describing the behavior of intra-myofibrillar protons, were combined. This method has been applied in A1a and A1b research activities.

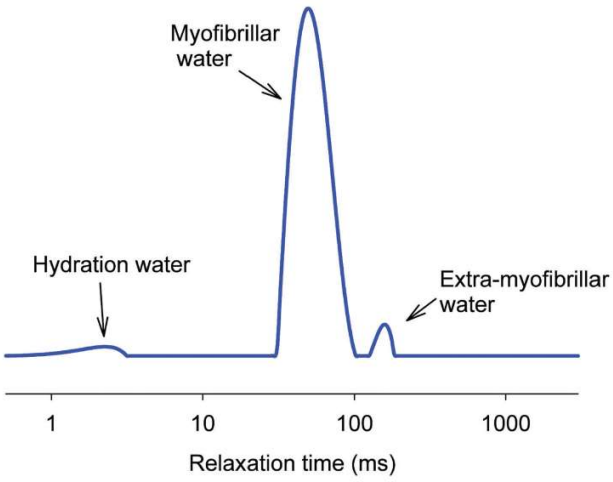


Figure 2.3. Transverse relaxation time spectra (T2) obtained for raw meat samples.

2.1.2.6 Tenderness

2.1.2.6.1 Warner-Bratzler shear test

Warner-Bratzler shear test was performed on 4 × 2 × 1 cm cooked samples cut parallel to the muscle fibers direction (Figure 2.4a), by using a TA-HDi Heavy-Duty Texture Analyzer (Stable Micro Systems Ltd, UK), equipped with a 25 kg loading cell and a Warner-Bratzler shear blade (Figure 2.4b). The shear values were expressed in kg. This method has been applied in A1b research activity.

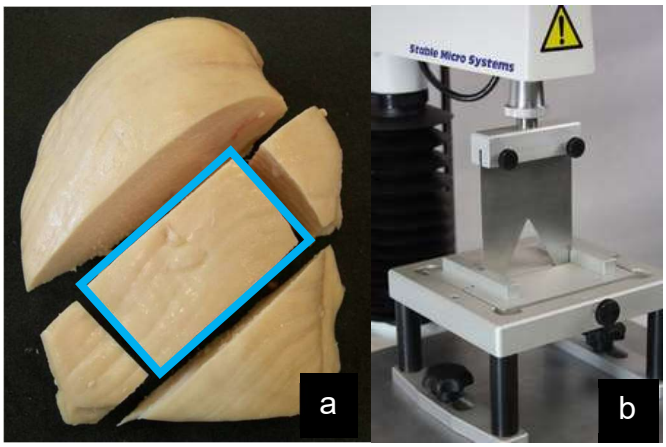


Figure 2.4. Meat sample (a) and Warner-Bratzler shear blade (b) used for shear force analysis.

2.1.2.6.2 Compression test

Compression force test was performed in order to evaluate the myofibril contribution to meat texture. In detail, 1.5 ×1 cm cylindrical samples were taken from the cranial section of each P. major muscle and compressed to 40% of their initial height by using a TA-XT2i Texture Analyzer (Stable Micro Systems Ltd., UK) set to

compress the samples with a speed of 3mm/s applying the force perpendicular to the muscle fibers, as described by Soglia et al. (2017). This method has been applied in A1a research activity.

2.1.2.7 Chemical composition

2.1.2.7.1 Moisture

The moisture content of raw meat was assessed according to AOAC official method, which is oven drying. Briefly, about 5 g of finely minced meat were accurately weighted in aluminum pans (previously heated for 1 h at 105°C and cooled to room temperature in a desiccator in order to achieve stable weight) and dried in a conventional oven for 16 h at 105°C. After allowing to equilibrate to room temperature in a desiccator, the samples' residue was weighed, and moisture content calculated according to the following formula:

$$Moisture\ (\%) = \frac{(initial\ weight - dry\ weight)}{initial\ weight}\ x\ 100$$

This method has been applied in A1a and A1b research activities.

2.1.2.7.2 Proteins

Protein content was determined according to the Kjeldahl method, based on the quantification of the amount of ammonia ions neutralized by sodium hydroxide. The method consists of three steps involving an acid digestion of the sample, distillation and titration of the ammonia complex. In detail, 0.5 g of meat samples were weighed and added to 12 ml of 96% sulfuric acid and 4% ortho-phosphoric acid solution and half Kjeltab, containing 3.5 g of K_2SO_4 and 3.5 g of selenium. Then, samples were gradually digested by increasing the temperature from 80 to 420°C for 3 h, allowing the generation of ammonium sulfate. Subsequently, distillation and titration were performed by using a Vapodest 50 s distill-titrator unit. After the addition of 50 ml of water, the excess acid solution was neutralized with 30% NaOH solution. After being condensed in the distillation unit, ammonia gas was captured by boric acid. Then, after the titration of distilled ammonia with 0.2 N HCl, protein content was assessed by multiplying the amount of organic nitrogen by a conversion factor of 6.25.

Protein content was then expressed following the formula:

$$Protein\ content\ (\%) = nitrogen\ content\ (\%)\ x\ 6.25$$

$$Nitrogen\ content\ (\%) = \frac{(S - B)x\ N\ x\ 1.4007}{sample\ weight}$$

S= ml of HCl required to titrate the sample;

B= ml of HCl required to titrate the blank;

N= normality of the acid solution.

This method has been applied in A1a and A1b research activities.

2.1.2.7.3 Lipids

Lipid content was assessed following the method proposed by Folch et al. (1957) with slight modifications. About 10 g of frozen meat were weighed and, after the addition of 50 µL of a BHT-methanol solution (1.2 mg/ml), homogenized by UltraTurra×at 21,500 rpm for 2 min with 100 ml of chloroform:methanol (1:1, v/v) solution in a 500 ml glass bottle with screw-cap. After being placed in an oven at 60°C for 20 min, 50 ml of chloroform were added to the bottles and homogenized at 21,500 rpm for 2 min. The homogenate was then filtered under vacuum and, after the addition of 50 ml of 0.88% KCl solution, samples were transferred into separating funnels and left overnight in order to separate the upper aqueous phase from the lower chloroformic one. The lower phase was then collected into a flask after filtration through anhydrous sodium sulfate. Following incubation for 4 h with sodium sulfate, samples were then filtered by filter paper and dried using a Rotavapor (Heidolph, Germany). Lipid content was determined as follows:

$$Lipid\ content\ (\%) = \frac{(flask\ final\ weight - flask\ initial\ \ weight)}{sample\ weight}\ x\ 100$$

This method has been applied in A1a and A1b research activities.

2.1.2.7.4 Fatty acids

After lipid extraction, fatty acids were quantified as methyl esters using a gas chromatograph GC Trace 2000 (EC instruments, Sweden) equipped with a flame ionization detector and a fused silica capillary column (100 m × 0.25 mm × 0.25 µm film thickness) and helium as carrier gas. The oven temperature was 100°C for 5 min, then increasing at a rate of 4°C/min up to 240°C, where it was kept for 20 min. The

individual fatty acids peaks were identified by comparison of retention times with those of methyl esters authentic standards, run under the same operating conditions. Then, results were expressed as percentage of the total fatty acid identified. This method has been applied in A1a research activity.

2.1.2.7.5 Ash

Ash content was assessed following AOAC official method. In detail, 5 g of finely minced meat were placed into ceramic crucibles, previously heated in a muffle furnace at 525°C for 1 h, allowed to cool at room temperature in a desiccator, and weighed. Samples were then placed in an air oven at 105°C for 2 h and moved into a muffle furnace at 200°C for 1 h and then incinerated at 525°C for 4 h. After cooling, the crucibles containing the ashes were equilibrated to room temperature in a desiccator and ash content was expressed as follows:

$$Ash\ (\%) = \frac{(sample\ initial\ weight - sample\ final\ weight)}{sample\ initial\ weight} x\ 100$$

This method has been applied in A1a and A1b research activities.

2.1.2.7.6 Total collagen

Collagen content was determined by using a spectrophotometric method proposed by Kolar (1990) and based on hydroxyproline assay. About 4 g of finely minced meat were carefully weighed 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, samples were heated in an oven at 105°C for 16 h in order to digest collagen. The hydrolyzed samples were diluted with distilled water into a 500 ml volumetric flask and, after filtering about 50-70 ml of sample with Whatman #1 filter papers, 5 ml of the filtrate were diluted with distilled water into a 100 ml volumetric flask. Subsequently, 2 ml of the final diluted sample were transferred to a test tube in with 1 ml of oxidizing agent (chloramine T). Samples were incubated in the dark for 20 min and after adding 1 ml of colorimetric solution, the tubes were placed in a water bath for 15 min at 60°C. The absorbance was read at 558 nm by using a UV/Vis Scanning Spectrophotometer (Jenway, UK) and the hydroxyproline content (HYP %) of each sample calculated following the formula:

$$HYP\ (\%) = \frac{(h\ x\ 2.5)}{(sample\ weight\ x\ V)}$$

h= hydroxyproline content (mg/ml), derived from the calibration curve;

V= ml of filtrate diluted to 100 ml.

This method has been applied in A1a and A1b research activities.

2.1.2.8 Hydroxylysylpyridinoline concentration

Hydroxylysylpyridinoline (HLP) is the principal non-reducible cross-link of muscle collagen. About 100 g of meat was lyophilized for 48 h, weighed and hydrolyzed in Duran tubes in 5 mL of HCL 6N at 110°C for 20 h. The determination of HLP was conducted after concentration and separation from other amino acids by selective elution from a CF 1 cellulose column, using the HPLC procedure as proposed by Eyre et al. (1984). To this purpose, a Kontron HPLC (Kontron Instruments, Italy), equipped with a Luna C18 column (250 cm × 4.6 mm × 5 μm) was used. Pyridoxamine 2HCl was used as internal standard to the eluent containing HLP. The identification of HLP in meat sample hydrolysates was performed by comparison with a purified HLP standard, that was prepared from bovine cartilage hydrolysates following the procedure proposed by Eyre et al. (1984). HLP concentration was calculated based on the concentration of collagen (m.w.= 300,000 g/mol) in each hydrolysate, assuming that the molar fluorescence yield of pyridoxamine was about 3.1 times that of HLP. Finally, HLP was expressed as moles of HLP per mole of collagen. This method has been applied in A1a research activity.

2.1.2.9 Thermal properties

Innovative techniques such as differential scanning calorimetry was used to investigate meat functional and structural properties. A differential scanning calorimeter DSC Q20 (TA Instrument, Germany), equipped with a low-temperature cooling unit Intercooler II (Perkin-Elmer Corporation, USA) was used to assess total protein denaturation enthalpy of meat samples. Temperature and melting enthalpy calibrations were performed with ion exchanged distilled water (mp 0.0°C) and indium (mp 156.60°C), while heat flow was calibrated using the heat of fusion of indium (ΔH = 28.71 J/g). For the calibration, the same heating rate and dry nitrogen gas flux of 50 ml/min used for the analysis were applied. Each muscle sample was weighed (about 25 mg) into a 50-μL aluminum pan, sealed hermetically and then loaded into the DSC instrument at room temperature. The heating rate of DSC scans was 5°C/min over a

range of 20 to 90°C, as proposed by Wattanachant et al. (2005). Empty aluminum pans were used as reference and for baseline corrections. Eight replications for each sample were performed and results were elaborated through a PeakFit Software (SeaSolve Software Inc., USA) and expressed in terms of total protein denaturation enthalpy (J/g) and the relative stromal proteins denaturation enthalpy (%). Generally, five endothermic peaks were seen in the thermograms (Figure 2.5).

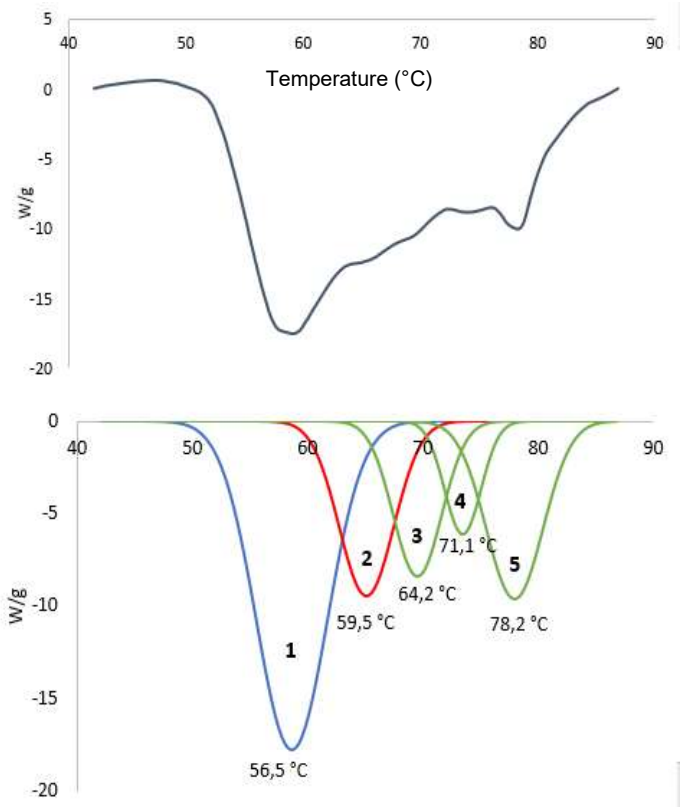


Figure 2.5 Endothermic peaks detected in the thermograms obtained through DSC.

Last, the five endothermic peaks were attributed to the denaturation of the myofibrillar (peak 1), stromal (peak 2) and sarcoplasmic proteins (peaks 3, 4 and 5). Their relative protein denaturation temperatures are reported in Table 2.2.

Table 2.2 Myofibrillar, stromal and sarcoplasmic proteins denaturation temperatures.

	Temperature (°C)	Proteins
Peak 1	56.9 ± 1.05	Myofibrillar
Peak 2	60.9 ± 1.46	Stromal
Peak 3	64.5 ± 1.37	Sarcoplasmic
Peak 4	70.1 ± 1.03	Sarcoplasmic
Peak 5	78.9 ± 0.60	Sarcoplasmic

This method has been applied in A1a research activity.

2.1.2.10 Histidine-containing compounds

The concentration of histidine-containing compounds in chicken meat samples was performed through H^1-NMR, as previously described by Marcolini et al. (2015) with modifications. About 0.5 g of finely minced meat was homogenized in 3 ml of distilled water by Ultra-Turrax T25 basic (IKA-Werke, Germany) (20 s at 11,000 rpm). Then, 1 ml of homogenate was transferred into a new tube and centrifuged at 14,000 rpm for 10 min at 4°C. An aliquot of 700 μL of supernatant was added into a new tube with 800 μL of chloroform, vortexed and centrifuged as before. Then, 500 μL of the supernatant were added to 200 μL of phosphate buffer/deuterium oxide/3-trimethylsilyl-propanoic-2,2,3,3-d4 acid sodium salt. Samples were centrifuged at 14,000 rpm for 10 min and then 700 μL of the supernatant was transferred into NMR tube. H^1-NMR spectra were then recorded at 25°C with a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Germany). Spectra were collected with a 90° pulse of 14 μs with a power of 10 W, a relaxation delay of 5 s, and an acquisition time of 2.28 s. This method has been applied in A2a and A2b research activities.

2.1.2.11 Post-mortem metabolism

2.1.2.11.1 R-Value

R-value represents the ratio of inosine:adenosine and its measurement gives information about the depletion of ATP within the muscle during the post-mortem time. To this purpose, 2 g of finely minced meat was homogenized in 25 ml of 60% perchloric acid solution by Ultra-Turra×T25 basic (IKA-Werke, Germany) (30 s at 11,000 rpm). The homogenate was filtered through Whatman #1 filter paper and 0.1 ml of filtrate was added with 4.9 ml of sodium phosphate buffer (pH = 7.0). Subsequently, R-value was calculated as the ratio of absorbance between adenine nucleotides (260 nm, ATP + ADP + AMP) and inosine (250 nm, inosine + inosine monophosphate + hypoxanthine metabolites of AMP). This method has been applied in A2a research activity.

2.1.2.11.2 Glycolytic metabolites

<u>Lactate</u>

After being powdered under liquid nitrogen with mortar and pestle, 0.1 g of frozen ground sample was lysed with a Tissue Lyser (Qiagen, USA) in 1 ml of ice-cold 0.5 M perchloric acid and incubated on ice for 20 min. Homogenates were centrifuged

at 17,000 × g for 5 min, then supernatants were transferred into new tubes and neutralized with 2 M KOH. Lactate was then determined using an enzymatic method modified for a 96-well plate as described by Hammelman et al. (2003). Briefly, 1.5 ml of buffer solution (35% hydrazine, glycine, NAD, 5M HCl) was added in clean glass tubes, along with 25 μL of sample and 75 μl of water (1:4 dilution). The absorbance of samples was read at 340 nm in order to assess the amount of substrate before the enzymatic reaction occurs. After the addition of 35 μl of 1:10 lactate dehydrogenase to each glass tube, samples were incubated for 1 h and 30 min. Following incubation, the absorbance of samples was read again at 340 nm. Lactate concentration was then calculated based on the calibration curve prepared using serial dilution (5000 μM – 156.25 μM) of lactate stock standard solution (5 mM) and expressed as μmol/g meat.

Glucose and glucose-6-phosphate

On samples prepared according to the determination of lactate, glucose and glucose-6-phosphate (G6P) were then determined using an enzymatic method modified for a 96-well plate as described by Hammelman et al. (2003). Briefly, 1.8 ml of buffer solution (triethanolamine, 200Mm EDTA, 1M $MgCl_2$, NADP) was added in clean glass tubes, along with 150 μl of sample and 150 μl of water (1:1 dilution). The absorbance of samples was read at 340 nm in order to assess the amount of substrate before the enzymatic reaction occurs. After the addition of 35 μl of 1:10 G6P dehydrogenase to each glass tube, samples were incubated for 20 min. Following incubation, the absorbance of samples was read at 340 nm. Subsequently, 40 μL of 11mg/ml ATP and 21 μl 1:10 hexokinase were added to all glass test tubes and, after 20 min of incubation, the absorbance of samples was read at 340 nm. Glucose and G6P concentrations were then calculated based on the calibration curve prepared using serial dilution (2400 μM – 150 μM) of G6P stock standard solution (10 mg/ml) and expressed as μmol/g meat.

Glycogen

After being powdered under liquid nitrogen with mortar and pestle, 0.1 g of frozen ground sample was lysed with a Tissue Lyser (Qiagen, USA) in 1 ml of ice-cold 1.25 M HCl. After heating at 90° C for 2 h, homogenates were centrifuged at 17,000 × g for 5 min, then supernatants were transferred into new tubes and neutralized with 1.25 M KOH. Glycogen was then determined using an enzymatic method modified for a 96-well plate as described by Hammelman et al., (2003). Briefly, 2 ml of buffer solution (triethanolamine, 200 Mm EDTA, 1M $MgCl_2$, NADP) was added in clean glass

tubes, along with 25 μL of sample and 75 μl of water (1:4 dilution). The absorbance of samples was read at 340 nm in order to assess the amount of substrate before the enzymatic reaction occurs. After the addition of 35 μL of 1:10 hexokinase, 35 μl of 1:10 G6P dehydrogenase and 11 mg/ml ATP to each glass tube, samples were incubated for 20 min. Following incubation, the absorbance of samples was read again at 340 nm. Glycogen concentration was then calculated based on the calibration curve prepared using serial dilution (4.800 μM – 150 μM) of glucose stock standard solution (5 mM) and expressed as μmol/g meat.

Glycolytic potential

Glycolytic potential (GP) was calculated as suggested by Scheffler et al. (2013), following the equation:

$$GP = 2\,x\,(glucose_{15min} + G6P_{15min} + glycogen_{15\mathrm{min}}) + lactate_{15min}$$

Adeno-nucleotides

Adenine nucleotides were quantified through reverse phase HPLC, using HP Agilent 1100 HPLC system (Agilent Technologies, USA), equipped with a Unisol C18 column (4.6 × 50 mm, 3 μm). In detail, after being powdered under liquid nitrogen with mortar and pestle, 0.1 g of frozen ground sample was lysed in 1 ml of ice-cold 0.5M perchloric acid and incubated on ice for 20 min. Homogenates were centrifuged at 17,000 × g for 5 min, then supernatants were transferred into new tubes and neutralized with 2M KOH. After filtration using 10 ml syringe equipped with nylon syringe filter, an aliquot of 50 μL of the filtered sample was transferred into a glass vial with permeable lid. HPLC standards (1 mM ATP, ADP, AMP, and IMP) were placed into glass vial as well. Adenine nucleotides concentration was then calculated and expressed as μmol/g of meat.

These methods have been applied in A2b and A3 research activities.

2.1.2.11.3 Phosphofructokinase activity

Phosphofructokinase activity of chicken P. major muscles was determined according to the procedures described by England et al. (2014). Briefly, after being powdered under liquid nitrogen with mortar and pestle, 0.1 g of ground muscle sample collected at 15 min post-mortem was homogenized at 1:10 (wt/vol) in ice-cold 100 mM K_2HPO_4 solution (pH= 7.4). Subsequently, aliquots of tissue homogenate were added to a reaction buffer containing 120 mM MES, 3.2 mM MgSO4, 2mM ATP, 1mM NADH,

3mM fructose-6-phosphate, 2U/ml triosephosphate isomerase, 1U/ml glycerol-3-phosphate dehydrogenase and 1U/ml aldolase (pH= 7.0). Enzymatic activity was then measured spectrophotometrically at 340 nm and reported as µmol NADH × min^{-1} × g^{-1}. This method has been applied in A3 research activity.

2.1.2.11.4 pH measurement using an in vitro glycolytic model

An in vitro system designed to simulate muscle post-mortem glycolysis was used to study muscle acidification during post-mortem time. The in vitro system composed of a buffer containing all metabolites required for glycolysis in addition to muscle tissue to serve as the source of glycolytic enzymes (Scopes, 1973). After being powdered under liquid nitrogen with mortar and pestle, 0.8g of ground muscle sample collected at 15 min post-mortem was homogenized at 1:10 (wt/vol) in a glycolysis buffer containing 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 60 mM KCl, 5 mM Na-ATP, 0.5 mM ADP, 0.5 mM NAD^+, 25mM carnosine, 30mM creatine, 40 mM glycogen, and 10mM sodium acetate (pH 7.4) (Scopes, 1974). Reaction vessels were incubated at 25°C for the duration of the trial. Following the diagram reported in Figure 2.6, aliquots (400 µl) for pH determination were removed from reaction vessels at 0, 15, 30, 120, 240 and 1,440 min and added with 100 µl of a 25 mM sodium iodoacetate and 750 mm KCl solution (pH=7.0). Samples were then centrifuged at 17,000 × g for 5 min, equilibrated to 25°C and pH was measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, USA).

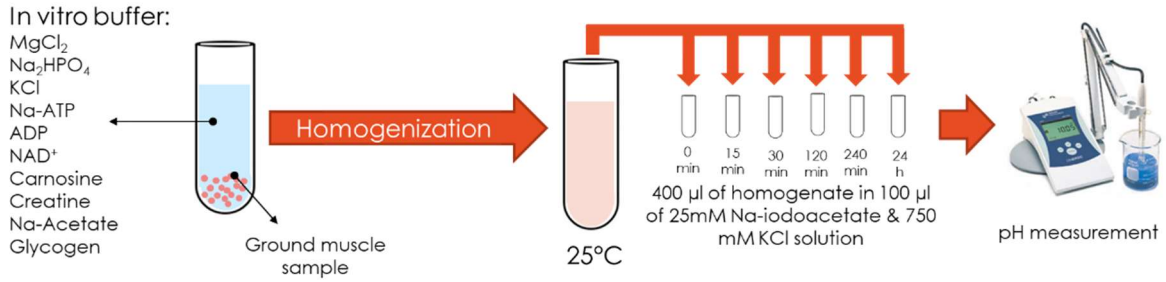


Figure 2.6 Processing samples for in vitro pH measurement.

This method has been applied in A3 research activity. All the above-mentioned biochemical assays have been performed in triplicate.

2.1.2.12 Histological evaluations

2.1.2.12.1 Fiber morphology

Muscle samples of approximately one cm^3 and oriented along the muscle fibers were removed from de-boned chicken P. major muscles at 24 h post-mortem. Meat samples were placed in 10% (vol/vol) buffered formalin fixative (pH=7) and stored at 4°C. Subsequently, samples were dehydrated in a graded series of ethanol, oriented for cross-sectional fiber sectioning and paraffin-embedded. Paraffin blocks were cut at 6µm with a cryostat, mounted on saline-coated microscopes slides and hematoxylin and eosin stained. In more detail, muscle sections were stained with hematoxylin for 6 min, rinsed with running deionized water and then submerged in eosin for 2 s and rinsed again with deionized water. Slides were then rinsed in 50% and 70% ethanol ten times, then in 95% ethanol for 30 s, then in 100% ethanol for 60 s. Muscle sections were then rinsed in xylene 7 times, dried with a Kimwipe and mounted. Digital photomicrographs were taken using a Nikon ECLIPSE microscope (Nikon Instruments Inc., USA) equipped with a 40× objective and processed using ImageJ software (NIH). This method has been applied in A3 research activity.

2.1.2.12.2 Sarcomere length

Muscle samples of approximately one cm^3 and oriented along the muscle fibers were removed from chicken de-boned P. major muscles at 24 h post-mortem. Meat samples were placed in 10% (vol/vol) buffered formalin fixative (pH=7) and stored at 4°C. Subsequently, samples were dehydrated in a graded series of ethanol, oriented for longitudinal fiber sectioning and paraffin-embedded. Paraffin blocks were cut at 3 µm with a cryostat, mounted on saline-coated microscopes slides and hematoxylin and eosin stained. Digital photomicrographs were taken with Nikon ECLIPSE microscope (Nikon Instruments Inc., USA) equipped with an oil-immersion 100× objective and processed using ImageJ software (NIH). For each section (n=6/sample), 15 myofibrils at least 5 sarcomeres long were selected for the assessment of sarcomere length, which was evaluated as the ratio between the total length of the myofibril (i.e. the distance between A bands) and the number of sarcomeres. This method has been applied in A3 research activity.

2.1.2.12.3 Fiber typing

Samples for fiber typing assessment were excised from the muscles, quickly frozen in isopentane (cooled with liquid nitrogen) and stored at -80°C until processing. Serial cross-sections (10 µm-thick) were cut on a cryostat microtome at -20°C and mounted on poly-L-lysine coated glass slides (Sigma-Aldrich, USA). The oxidative metabolism of the fibers was assessed according to the procedure described by Novikoff (1961) by reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) staining. This method has been applied in A2a research activity.

2.1.2.13 Statistical analysis

Details of statistical evaluations for each research activity are provided in Chapter 3.

Chapter 3: Research outcomes

3.1 Implications of muscular abnormalities on chicken meat quality

Research activity A1a

Scientific publication originated from this research activity

Baldi G, Soglia F, Laghi L, Tappi S, Rocculi P, Tavaniello S, Prioriello D, Mucci R, Maiorano G, & Petracci M. (2019). Comparison of quality traits among breast meat affected by current muscle abnormalities. *Food Research International*, 115, 369-376.

3.1.1 Introduction and aim of the study

Because of the efficiency of the production, the health claims attributed to its consumption, the relatively low-cost and the absence of religious constraints, chicken meat is actually the most widely eaten type of meat all over the world (Windhorst, 2017; Petracci et al., 2019). In order to keep up the increasing consumer demand for chicken meat, intensive selection practices along with the optimization of feeding and housing conditions have been accomplished during the past decades. Nowadays chicken meat production is based on fast-growing hybrids birds, solely designed for meat production and usually reaching market weight (2.8 kg) within 47 days, namely about half the time compared to 40 years ago, while chickens average daily weight gain is doubled in the past 50 years (Tallentire et al., 2016; NCC, 2019). Although the production performances resulted remarkably improved, the increased pressure on muscle growth and development rate has coincided with the expansion of muscular defects which, alone or combined, currently affect the Pectoralis major muscle of fast-growing broilers (Petracci et al., 2019). Among these, White Striping (WS), Wooden Breast (WB) and Spaghetti Meat (SM) are the main growth-related myopathies that are causing relevant economic troubles for the poultry industry (Kuttappan et al., 2016). Indeed, the occurrence of these myopathies not only impairs the visual appearance of breast meat, but also significantly affects quality traits and functional properties of the meat intended for processed products (Bowker and Zhuang, 2016; Soglia et al., 2016b; Petracci et al., 2019). Moreover, while the detrimental effects of the occurrence of WS and WB on meat quality traits have been widely established, no studies have been conducted yet to deeply investigate Spaghetti Meat condition and concurrently examine the three main growth-related abnormalities. Thus, this study aimed at evaluating the implications of WS, WB and SM abnormalities on raw meat color, pH, proximate composition, fatty acid profile, collagen, thermal properties as well as water mobility and texture.

3.1.2 Materials and methods

A total of forty-eight boneless and skinless Pectoralis major muscles were obtained from the same flock of broiler chickens (Ross 308 strain, females, 46 days of age, 2.8 kg body weight) reared and harvested under standard commercial conditions. In detail, before slaughter, animals were subjected to a total feed withdrawal of 10 h, including a 3 h lairage time at the processing plant. Subsequently, birds were 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 for 150 min until reaching 2- 3°C at the core. Then, fillets were selected at 3 h post-mortem in the deboning area of the commercial processing plant and classified, according to the occurrence of muscular abnormalities, into four experimental groups as follows: 12 Normal (N), 12 WS, 12 WB and 12 SM. During the selection, fillets showing the concomitant occurrence of multiple abnormalities were discarded. After being collected, fillets were transported under refrigerated conditions to the laboratory and stored at 4°C until 24 h post-mortem. Following the elimination of superficial fat and connective tissue, each fillet was weighed and cut in order to separate the superficial layer from the deep one. In more detail, after the removal of the first 4 mm of muscle tissue, the superficial section was obtained from about 0.4 to 1.5 cm below the breast muscle's surface, while the deep layer about 1.5 cm below the superficial one (Figure 3.1).

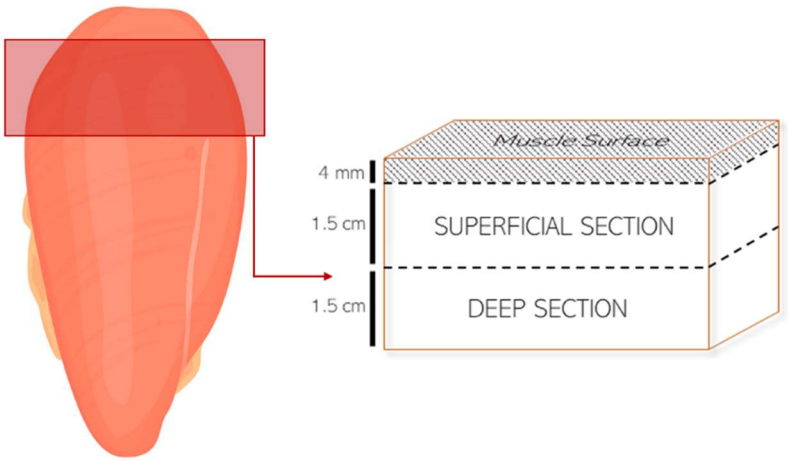


Figure 3.1 Representation of the sampling method to obtain superficial and deep sub-samples.

Sub-samples thus obtained were used to assess:

- Color (chapter 2, paragraph 2.1.2.3);
- pH (chapter 2, paragraph 2.1.2.1);
- Transverse relaxation time (T_2) by Time-Domain nuclear magnetic resonance (TD-NMR) (chapter 2, paragraph 2.1.2.5.4);
- Thermal properties (chapter 2, paragraph 2.1.2.9);
- Texture (chapter 2, paragraph 2.1.2.6.2).

The remaining parts of the raw breast fillet were finely ground with a blender for 1 min at 1-4°C and stored at -20°C for the assessment of proximate composition, fatty acid composition, collagen (chapter 2, paragraph 2.1.2.7) as well as its HLP cross-links (chapter 2, paragraph 2.1.2.8).

Data were then analyzed using the factorial ANOVA option of the GLM procedure present in SAS software (SAS Institute Inc., USA) testing the main effects of muscular abnormalities (N, WS, WB and SM) and sampling position (superficial and deep), as well as the interaction term on meat quality traits. Means were separated using Tukey's honestly test multiple range test of the GLM procedure.

3.1.3 Results and discussion

The results for meat pH_u and color are reported in Table 3.1. Albeit no significant differences were found within the deep section of the muscle, if compared to N, WB superficial samples showed a significantly higher pH_u ($P<0.05$), while WS and SM fillets exhibited intermediate values. These findings are consistent with previous studies, revealing that the occurrence of breast myopathies exerts a significant effect on breast pH_u (Mudalal et al., 2015; Bowker and Zhuang, 2016; Baldi et al., 2018), with a special reference to WB abnormality. The higher ultimate pH found within myopathic fillets is ascribed to the abnormal muscles reduced glycolytic potential combined with an altered energetic status and metabolism, as previously found for WS and WB myopathies (Abasht et al., 2016; Beauclercq et al., 2016).

As for meat color, lightness (L*) and yellowness (b*) were found to be significantly higher ($P<0.001$) in superficial WB samples, while the occurrence of muscle abnormalities did not affect meat color in the depth layer of breast muscle. The higher L* and b* values are respectively ascribable to the muscle tissue modifications following the histological degeneration in affected muscles and the increased lipid content found in WB fillets (Mudalal et al., 2015). Moreover, although redness was not

affected by sampling position, both superficial and deep sections of WS exhibited lower values than their counterparts. In addition, it seems that the influence of breast abnormalities on meat pH and color is narrowly restricted to the superficial section of P. major muscle, as previously found by for WS and SM (Baldi et al., 2018).

Table 3.1. Effect of breast abnormalities on ultimate pH and color (L, a*, b*), assessed on both the superficial (S) and deep (D) sections of P. major muscle. SEM= standard error of means. a-c: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). * = P<0.05; *** = P<0.001.*

	Parameters							
	pH		L*		a*		b*	
Group (G)	S	D	S	D	S	D	S	D
N	5.76^{b}	5.79^{ab}	54.8^{bc}	53.9^{c}	2.72^{ab}	2.36^{ab}	3.86^{bc}	3.05^{c}
WS	5.82^{ab}	5.79^{ab}	56.5^{abc}	54.2^{c}	2.37^{ab}	1.97^{b}	4.83^{ab}	3.27^{c}
WB	5.87^{a}	5.80^{ab}	58.4^{a}	55.8^{abc}	3.41^{a}	2.34^{ab}	5.66^{a}	3.63^{bc}
SM	5.83^{ab}	5.78^{ab}	57.3^{ab}	55.5^{abc}	2.71^{ab}	3.00^{ab}	4.90^{ab}	4.26^{bc}
sem	0.01		0.27		0.11		0.14	
	P-value							
G	*		***		*		***	
Position (P)	*		***		ns		***	
G × P	ns		ns		ns		ns	

Table 3.2 reports the results concerning chemical composition. If compared to control, both superficial WB and SM samples showed higher and lower (P<0.001) moisture and protein content, respectively, while WS fillets displayed intermediate values. In addition, moisture was found to be remarkably higher (P<0.001) in WB fillets superficial layer rather than in the deep one, while a lower protein content was observed within the surface of abnormal breasts, with only WB samples showing significant differences (P<0.001). Moreover, the interaction term between experimental groups and sampling position was found to be significant for moisture content (P<0.05). As for lipid and ash content, no significant differences were recovered within the muscle breast deep section, while considerably higher (P<0.05) values were found in the superficial layer of WB fillets if compared to their N counterpart. However, if the effect of the sampling position is considered, no differences were detected between the experimental groups. Thus, the occurrence of breast muscle abnormalities exerted an outstanding effect on the raw meat chemical composition, albeit it was found to be profoundly modified only within the surface of the muscle, which usually is the section most affected by the aforementioned myopathies (Baldi et al., 2018; Bowker et al., 2018). Overall, muscular abnormalities are mainly associated with a significant

increase in moisture and fat level, to the detriment of protein and ash content, with WB showing the most relevant effect on meat proximate composition. Overall, these results are consistent to what reported in previous study and corroborates the hypothesis that the detrimental effect of muscular abnormalities on meat chemical composition is likely due to the outstanding alterations in muscle structure observed after histological evaluations (Sihvo et al., 2014; Mazzoni et al., 2015; Velleman and Clark, 2015; Soglia et al., 2016b). In more detail, the higher moisture content is due to the presence edema (i.e. fluid accumulation) as a result of the inflammatory processes taking place within the muscle, while the increased lipid and collagen content are explained by the replacement of muscle fibers with adipose and connective tissues (i.e. lipidosis and fibrosis, respectively) (Kuttappan et al., 2013b; Sihvo et al., 2014). As a consequence of the myodegenerative processes, the reduced protein content is ascribable to the aforementioned changes in muscle architecture (Soglia et al., 2016b).

*Table 3.2. Effect of breast abnormalities on proximate composition of both the superficial (S) and deep (D) sections of P. major muscle. SEM= standard error of means. a-d: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). * = P<0.05; *** = P<0.001.*

	Parameters							
	Moisture (%)		Protein (%)		Lipid (%)		Ash (%)	
Group (G)	S	D	S	D	S	D	S	D
N	75.0^{d}	74.9^{d}	22.9^{a}	23.0^{a}	1.51^{c}	1.59^{bc}	1.58^{a}	1.49^{ab}
WS	75.3^{cd}	75.1^{cd}	22.0^{abc}	22.5^{a}	2.05^{ab}	1.84^{abc}	1.59^{a}	1.51^{a}
WB	77.1^{a}	75.8^{cd}	20.5^{d}	21.9^{abc}	2.12^{a}	1.80^{abc}	1.26^{b}	1.48^{ab}
SM	76.7^{ab}	75.9^{bc}	21.0^{d}	21.6^{bcd}	1.84^{abc}	1.83^{abc}	1.38^{ab}	1.59^{a}
sem	0.10		0.13		0.05		0.03	
	P-value							
G	***		***		*		*	
Position (P)	***		***		ns		ns	
G × P	*		ns		ns		ns	

The results for total fatty acid composition and nutritional indexes are reported in Table 3.3. Overall, the occurrence of myopathies did not significantly affect the total fatty acid composition, with the only exception of MUFA content (P<0.05), while the sampling position affected (P<0.05) both MUFA and SFA weight percentages. In particular, if compared to N deep samples, WS superficial fillets had greater (P<0.05) percentages of MUFA, essentially due to a higher (P<0.05) content of oleic acid. These results corroborate what previously found by Kuttappan et al., (2012a), who found that severe WS muscle showed remarkably higher MUFA content. Albeit no significant effects were observed for individual SFA contents, if compared to N, eicosapentaenoic

(EPA, C20:5 n-3) and docosahexaenoic (DHA, C22:6 n-3) acids were found to be lower in abnormal fillets superficial sections (data not shown), in agreement to what observed by Soglia et al. (2016a), and might be explained with the differential expression of the genes encoding for Δ5 and Δ6 desaturase. Regarding the nutritional indices (P/S, n-6/n-3, AI, TI), no significant effect of experimental group and sampling position was detected.

*Table 3.3. Effect of breast abnormalities on total fatty acid composition (%) and nutritional indices assessed on both the superficial (S) and deep (D) sections of P. major muscle. SEM= standard error of means. a-b: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). *= P<0.05.*

	Position (P)	Group (G)				sem	P-value		
		N	WS	WB	SM		G	P	G×P
SFA	S	34.09^{ab}	31.10^{b}	33.83^{ab}	32.72^{ab}	0.29	ns	*	ns
	D	33.20^{ab}	34.41^{ab}	34.87^{a}	34.13^{ab}				
MUFA	S	26.34^{ab}	28.82^{a}	25.56^{ab}	28.35^{ab}	0.30	*	*	ns
	D	24.80^{b}	25.91^{ab}	25.48^{ab}	26.75^{ab}				
PUFA	S	39.56	40.08	40.60	38.93	0.34	ns	ns	ns
	D	41.99	36.68	39.64	39.13				
∑ n-3	S	4.10	4.25	4.12	3.85	0.07	ns	ns	ns
	D	3.83	4.18	4.07	3.94				
∑ n-6	S	34.46	35.82	36.49	35.08	0.31	ns	ns	ns
	D	38.16	35.50	35.57	35.19				
n-6/n-3	S	8.68	8.46	8.88	9.31	0.20	ns	ns	ns
	D	10.49	8.56	8.78	9.13				
P/S	S	1.17	1.30	1.20	1.20	0.02	ns	ns	ns
	D	1.28	1.15	1.14	1.15				
Atherogenic index	S	0.38	0.35	0.37	0.37	0.01	ns	ns	ns
	D	0.38	0.39	0.39	0.37				
Trombogenic index	S	0.78	0.68	0.77	0.75	0.01	ns	ns	ns
	D	0.76	0.78	0.80	0.79				

Table 3.4 shows the results concerning intramuscular collagen (IMC) analysis. Variations in the amount, structure and composition of IMC can deeply impair breast meat quality since collagen plays a key role in determining meat toughness (McCormick, 1999). Moreover, thermal and mechanical stability of IMC is related to the chemical nature of the principal non reducible cross-link of muscle collagen, HLP, whose content gives information about collagen maturation (McCormick, 1999).

Overall, IMC amount was found to be remarkably higher in WB superficial samples if compared to both other experimental groups (P<0.001) and its deep counterpart (P<0.05). This result is consistent with the WB affected muscles' peculiar stiffness, which is also linked to the structural characteristics of collagen itself, such as cross-linking, fibril density etc. (Velleman et al., 2017). However, surprisingly, WB fillets did not show an increased level of HLP. Nevertheless, collagen was significantly less cross-linked (i.e. less mature) (P<0.05) in SM superficial samples compared to the other superficial samples, corroborating previous findings that revealed a progressive rarefaction of the the endo- and peri-mysial connective tissue of SM affected breasts (Baldi et al., 2018).

*Table 3.4. Effect of breast abnormalities on intramuscular collagen (IMC) and hydroxylysylpyridinoline (HLP) concentration assessed on both the superficial (S) and deep (D) sections of P. major muscle. SEM= standard error of means. a-b: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). ** P< 0.01; *** = P<0.001.*

	Parameters			
	IMC (µg/mg)		HLP (mol/mol of collagen)	
Group (G)	S	D	S	D
N	14.7^{b}	13.8^{b}	0.049^{a}	0.048^{a}
WS	14.0^{b}	13.4^{b}	0.053^{a}	0.050^{a}
WB	18.2^{a}	13.3^{b}	0.059^{a}	0.046^{ab}
SM	13.7^{b}	12.9^{b}	0.026^{b}	0.042^{ab}
sem	0.19		0.002	
	P-value			
G	***		**	
Position (P)	***		**	
G × P	***		ns	

The results regarding the peak forces (kg) recorded during the compression of both raw and cooked meat samples are shown in Table 3.5. As for raw meat samples, regardless of the sampling position, WB group exhibited the highest compression forces (P<0.001), while WS and SM samples showed intermediate values. These results corroborate the higher IMC content found in WB samples, that makes WB samples more resistant to the application of the compression force. In addition, if the sampling position is considered, all the experimental groups showed higher peak forces in P. major superficial section rather than in the deep one, with only WS and WB samples exhibiting significant differences (P<0.001). Surprisingly, after the cooking process, overall WB samples did not differ from other experimental groups, likely because of the denaturation and solubilization of the thermally-labile collagen

cross-links, which occur at temperatures between 53 and 63°C (Soglia et al., 2017). Moreover, the considerably lower compression force (P<0.05) observed in the superficial layer of SM, if compared to the deep portion, might be explained by considering the reduced HLP content found in the present study within the surface of SM affected breasts that might exert an effect on cooked meat tenderness.

Table 3.5 Effect of breast abnormalities on the raw and cooked meat compression force, assessed on both the superficial (S) and deep (D) sections of P. major muscle SEM= standard error of means. a-c: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05).
** = P<0.05; *** = P<0.001.*

	Parameter			
	Compression force (kg)			
	Raw		Cooked	
Group (G)	S	D	S	D
N	2.95^{bc}	2.01^{c}	3.36^{a}	2.85^{ab}
WS	3.53^{ab}	2.88^{bc}	3.41^{a}	3.26^{ab}
WB	4.45^{a}	3.41^{b}	2.95^{ab}	3.20^{ab}
SM	3.55^{ab}	2.92^{bc}	2.65^{b}	3.49^{a}
sem	0.11		0.08	
	P-value			
G	***		ns	
Position (P)	***		ns	
G × P	ns		*	

Advanced analytical techniques such as DSC are widely used to deeply investigate meat structural properties through the assessment of protein denaturation enthalpy. Protein denaturation studied by DSC shows the presence of different endothermic peaks in the range 50-80°C. Generally, three endothermic regions are found at 53-58, 61-68 and 72-80°C and are respectively ascribed to myofibrillar, stromal and sarcoplasmic proteins (Bircan and Barringer, 2002; Ali et al., 2015). However, the exact number of identified peaks can vary depending on animal type, age, sex, and also storage of chicken meat. In the present study, five endothermic peaks have been identified in the thermal profile at 56.5 ± 1.1, 60.4 ± 1.5, 65.3 ± 1.4, 71.4 ± 1.0 and 77.9 ± 0.6°C, respectively, corroborating what observed by Wattanachant et al., (2005). Thus, stromal proteins have been associated with the second peak (60.4 ± 1.5°C) and its contribution to the total denaturation enthalpy has been calculated through deconvolution of the peaks. The results for total and stromal protein denaturation enthalpy are reported in Table 3.6. As regards the surface of P. major muscle, total protein denaturation enthalpy was found to be significantly lower (P<0.001) in WB samples if compared to N group, according to the overall decreased

protein content of WB samples found within this study. Moreover, our study showed that denaturation enthalpy of stromal protein, expressed as percentage of total protein denaturation enthalpy, was remarkably higher in WB superficial samples (P<0.001), if compared to both N and abnormal fillets, while no differences were detected in the depth of breast muscle. These results are consistent with the increased IMC content found in the abovementioned WB fillets, due to the higher peri- and endo-mysial collagen deposition (Velleman et al., 2017). Moreover, although the sampling position effect was not significant (P>0.05), SM superficial samples showed a lower stromal protein denaturation enthalpy if compared to their deep counterpart.

*Table 3.6. Effect of breast abnormalities on total (ΔH, J/g) and stromal (ΔH, %) protein denaturation enthalpy assessed on both the superficial (S) and deep (D) sections of P. major muscle SEM= standard error of means. a-c: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). * = P<0.05; *** = P<0.001.*

	Parameter	
	Total protein denaturation enthalpy (ΔH, J/g)	
Group (G)	S	D
N	3.89^{a}	3.92^{a}
WS	3.30^{ab}	3.64^{ab}
WB	2.41^{c}	3.20^{abc}
SM	3.00^{abc}	2.87^{bc}
sem	0.19	
	P-value	
G	***	
Position (P)	*	
G × P	ns	
	Stromal protein denaturation enthalpy (ΔH, %)	
Group (G)	S	D
N	16.4^{b}	19.0^{b}
WS	16.9^{b}	18.2^{b}
WB	27.0^{a}	23.3^{ab}
SM	16.3^{b}	19.8^{ab}
sem	0.32	
	P-value	
G	***	
Position (P)	ns	
G × P	ns	

TD-NMR is a technique frequently used to investigate meat functional properties. The results concerning the relative intensity and T_2 relaxation times for the three proton populations ascribed to bound, intra- and extra-myofibrillar water are reported in Table 3.7. Overall, the occurrence of muscular abnormalities deeply affects both the distribution and mobility of the water fractions within the superficial layer of the muscle tissue, with the WB defect exerting the most outstanding effect. In detail, if compared to the superficial section of the other experimental groups, WB exhibited a remarkably increased proportion of bound and extra-myofibrillar water ($P<0.001$) to the detriment of the intra-myofibrillar fraction ($P<0.001$). Concurrently, the relaxation times observed for these proton populations were significantly increased in WB affected muscles. These findings contribute to explain the severely impaired water holding capacity of WB affected fillets. The higher T_2 relaxation times of the extra-myofibrillar water portion highlight that this water compartment is less tightly bound, according to the severe degenerative processes taking place in the muscle and resulting in a complete re-organization of the skeletal muscle architecture (Sihvo et al., 2014; Sundekilde et al., 2017). This corroborates the presence of exudate frequently observed in the superficial portion of WB fillets. Moreover, considering the degeneration processes typically observed in WB, the increased proportion of molecules ascribed to bound water might not be representative of the hydration water of proteins but might result from a kind of interaction between water molecules and connective tissue components, abundant in WB affected cases. This hypothesis is corroborated by the significantly higher T_2 relaxation times recorded for bound water in WB demonstrating that, as a consequence of their interaction with connective tissue, the water molecules ascribed to bound water in WB affected muscles are actually less tightly bound in comparison with those observed in the other experimental groups.

*Table 3.7. Effect of breast abnormalities on the relative intensity and T_2 relaxation time of the protons populations identified through TD-NMR assessed on both the superficial (S) and deep (D) sections of P. major muscle. a-d: Mean values within the same parameter followed by superscript letters significantly differ (P<0.05). ** P< 0.01; *** = P<0.001.*

	Parameters					
	Relative intensity (%)					
	Bound water		Intra-myofibrillar water		Extra-myofibrillar water	
Group (G)	S	D	S	D	S	D
N	3.35^{b}	3.51^{b}	87.1^{a}	89.4^{a}	9.50^{cd}	7.07^{d}
WS	3.33^{b}	3.32^{b}	82.3^{b}	87.3^{a}	14.31^{b}	9.36^{cd}
WB	8.50^{a}	3.27^{b}	73.6^{c}	87.4^{a}	17.88^{a}	9.29^{cd}
SM	3.36^{b}	3.34^{b}	85.7^{ab}	89.4^{a}	10.90^{c}	7.23^{d}
sem	0.26		0.59		0.43	
	P-value					
G	***		***		***	
P	**		***		***	
G × P	***		***		***	
	T_2 (ms)					
	Bound water		Intra-myofibrillar water		Extra-myofibrillar water	
Group (G)	S	D	S	D	S	D
N	2.33^{c}	1.88^{c}	42.7^{c}	41.8^{c}	145.9^{b}	142.5^{b}
WS	8.55^{b}	2.47^{c}	48.3^{b}	43.7^{bc}	158.0^{ab}	145.7^{b}
WB	15.35^{a}	2.21^{c}	54.1^{a}	43.3^{bc}	178.4^{a}	146.4^{b}
SM	3.04^{bc}	1.92^{c}	43.6^{bc}	41.8^{c}	146.2^{b}	149.5^{b}
sem	0.65		0.59		2.06	
	P-value					
G	***		***		**	
Position (P)	***		***		**	
G × P	***		***		**	

3.1.4 Conclusions

Over the past decades, as a result of the increasing growth rate and body size of modern hybrid birds, the poultry industry has been facing up the occurrence of many breast meat abnormalities, whose incidence has recently reached alarming levels. The findings of the present study reveal that, among all the myopathies, the occurrence of WB abnormality exerts the most profound and prominent effect on meat quality traits. Furthermore, overall results display that the manifestation of muscular abnormalities mainly affects the superficial section of P. major muscle, while the deep section was poorly affected. As for WB, we found a greater collagen content associated to an increased stromal protein denaturation enthalpy, while collagen cross-linking degree was not affected. Thus, the distinctive hardness of raw WB can likely due to an

increased amount of collagen without any difference in its maturation degree. In addition, a greater proportion of both extra-myofibrillar and bound water fractions associated with a higher mobility was observed. With regard to SM, the most interesting finding was the lower collagen cross-linking degree which can explain the typical tendency towards the separation of the fiber bundles composing the muscle tissue itself.

3.2 Implications of white striping on turkey breast meat quality

Research activity A1b

Scientific publication originated from this research activity

Soglia F, **Baldi G**, Laghi L, Mudalal S, Cavani C, & Petracci M. (2018). Effect of white striping on turkey breast meat quality. *Animal*, 12, 2198-2204.

3.2.1 Introduction and aim of the study

The forceful selection practices carried out during the past decades profoundly altered the animals' muscle physiology and thus led to the development of muscular abnormalities. Among these, the PSE-like condition has been extensively studied in turkeys (Woelfel et al., 2002; Strasburg and Chiang, 2009), as well as the focal and deep pectoral myopathy, frequently observed skeletal muscles of fast-growing turkeys (Siller and Wight, 1978; Sosnicki and Wilson, 1991). Lately, a new group of growth-related abnormalities (i.e. white striping, wooden breast and spaghetti meat) appeared in broilers, displaying profound degenerative myopathic lesions and occasional regenerative processes, along with a complete reorganization of the muscle architecture (e.g. proliferation of connective tissue and increased fat deposition) (Petracci et al., 2019). However, macroscopic and microscopic examinations of marketable turkeys revealed the presence of a diffuse hardening of the Pectoralis major muscles (analogous to wooden breast), as well as the presence of focal necrosis, and the proliferation of connective and fat tissue within the endo- and peri-mysial spaces (Grey, 1986; Sosnicki and Wilson, 1991). Albeit these degenerative changes have not been defined with specific terms, they were found to affect the quality of turkey breast meat and to overlap with the muscular abnormalities currently observed in broilers (Dutson and Carter, 1985). Moreover, the occurrence of white striations on the Pectoralis major muscle of heavy turkey hybrids has been observed since several years, however it has never been considered a quality issue by the poultry industries. Indeed, because of their big size, heavy male turkey breasts are usually sold sliced or as ready-to-eat products, thus white striations on turkey breast meat do not affect consumers purchases. Thus, while the effect of white striping (WS) on broiler meat quality traits has been deeply investigated, existing literature is lacking information concerning the implications of WS abnormality on breast meat quality traits. Thus, this study aimed at evaluating whether the occurrence of WS on Pectoralis major muscles of heavy male turkeys affects the quality traits and technological properties of meat in the same way observed for broilers.

3.2.2 Materials and methods

The study was performed in two separate trials on two flocks of heavy male turkeys (BUT BIG 6) reared and slaughtered under commercial conditions. In more detail, birds were fed *ad libitum* during the whole growing period using a feeding plan of 6 phases, depending on the age of the birds. Temperature was gradually decreased

from 32°C to 18°C on 4 weeks and was kept constant thereafter. The lighting program was 23L:1D in the first week and 18L:6D from 2 weeks to slaughter. Both flocks were processed at 137 days of age in the same commercial abattoir when reaching the most common slaughter weight of 20 kg used in the EU for heavy male turkeys. Birds were exposed to carbon dioxide for stunning, bled for 180 s and then, after being conveyed through scalding tanks (52°C for 180 s), plucked by rotating rubber fingers. After evisceration, carcasses were air-chilled passing through a two-step cold-air flow tunnel for 12 hours (until reaching 2 to 3°C at the core), breasts were deboned and cold stored until 48 h *post-mortem*. For the study, a total of 72 boneless and skinless Pectoralis major muscles were selected 48 h post-mortem and classified into three experimental groups (12 samples/group/trial), depending on the presence and severity of the myopathy:

- Normal (NORM): muscles that did not exhibit neither white striations nor any other defects;
- Moderate WS (MOD): thin (up to 1-mm) white-striations within the cranial part of the Pectoralis major muscle;
- Severe WS (SEV): medium-to-thick (from 1 to few mm) white striations within the cranial part of the Pectoralis major muscle.

The severity level of samples was identified according to the thickness of the white striations observed within the ventral surface of the pectoral muscles. In addition, muscles affected by other defects (i.e. hemorrhages, toughening, paleness, etc.) were not considered within this study.

Samples were transported under refrigerated conditions to the laboratory and, trimming from visible fat, cartilage and connective tissues, the Pectoralis major muscles were weighed, and color was measured (chapter 2, paragraph 2.1.2.3) on the bone-side surface of each muscle. Following, a first sub-sample was excised from the cranial portion of each pectoral muscle and used to assess ultimate pH (chapter 2, paragraph 2.1.2.1) and quality traits of non-marinated meat:

- drip loss (chapter 2, paragraph 2.1.2.5.1);
- cooking loss (chapter 2, paragraph 2.1.2.5.3);
- Warner-Bratzler shear force (chapter 2, paragraph 2.1.2.6.1).

Following, another sub-sample was excised from the same portion of the muscle and used to determine the quality traits after the marinade process. Thus, marinade uptake

was evaluated (chapter 2, paragraph 2.1.2.5.2) as well as drip loss, cooking loss and Warner-Bratzler shear force. In addition, TD-NMR relaxation properties (chapter 2, paragraph 2.1.2.5.4) and proximate composition (moisture, protein, lipid, ash and collagen; chapter 2, paragraph 2.1.2.7) were assessed on meat subsamples belonging from the same portion of the muscle.

Data were then analyzed using the two-way ANOVA option of the GLM procedure present in SAS software (SAS Institute Inc., USA) testing the main effects of the myopathy's severity grade (NORM, MOD and SEV) and replications, as well as the interaction term on meat quality traits. Means were separated by Tukey's HSD test at a level of $P<0.05$.

3.2.3 Results and discussion

The results for breast size (Figure 3.1) evidenced that, if compared to their NORM counterpart, both MOD and SEV muscles exhibited significantly higher weights (P<0.001), corroborating the hypothesis of a growth-related origin for the appearance of white striations that were frequently found to affect heavier broilers.

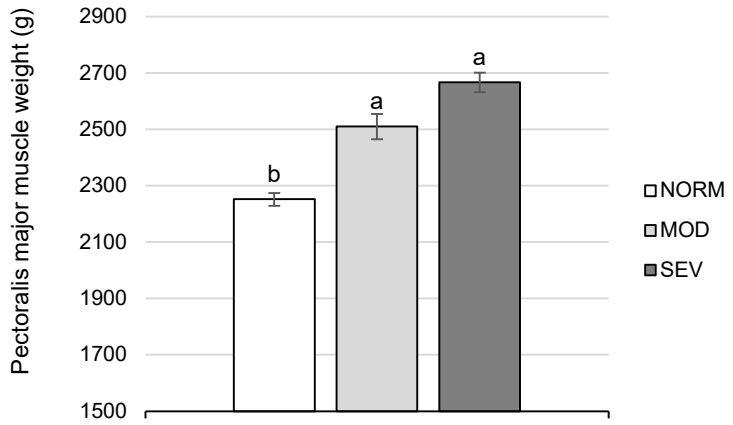


Figure 3.1 Pectoralis major muscle weight as affected by the occurrence of WS abnormality with different grade of severity. Error bars indicate standard error of means. n=72 (24 samples/group).
a,b: mean values followed by different superscript letters significantly differ among the groups (P< 0.05).

Breast fillets affected by WS exhibited a 20% increased weight in agreement with previous findings obtained on fast-growing broilers (Kuttappan et al., 2012a, 2017b; Mudalal et al., 2015). The same studies suggest a strong association between breast muscle development, slaughter age and the occurrence of muscular abnormalities. Similarly, an association between muscle degeneration and growth rate of the birds was also hypothesised in turkeys (Wilson et al., 1990).

The results concerning the effect of WS abnormality on turkey breast meat chemical composition are reported in Table 3.8.

Table 3.8 Pectoralis major muscle chemical composition as affected by the occurrence of WS abnormality with different grade of severity. n=72 (24 samples/group). a,b= P<0.05.

Parameter	Experimental Group			s.e.m.	P-value
	NORM	MOD	SEV		
Moisture (%)	74.5	74.9	74.5	0.1	ns
Protein (%)	23.2	22.6	22.9	0.1	ns
Lipid (%)	1.04b	1.22ab	1.38a	0.05	<0.05
Ash (%)	1.32a	1.21b	1.22b	0.01	<0.001
Collagen (%)	1.06	1.05	1.11	0.01	ns

If compared to NORM, lipid and ash contents of both MOD and SEV groups were significantly different. In detail, if compared to NORM muscles, SEV group exhibited higher lipid content (1.38 *vs.* 1.04%; *P*<0.05), whereas MOD fillets showed intermediate values. Otherwise, regardless the severity of WS abnormality, both MOD and SEV cases showed remarkably lower ash content if compared to control group (1.21 and 1.22 *vs.* 1.32%; *P*<0.001). No significant differences were observed for moisture, collagen and protein content among the groups. These findings were partially in agreement with the results of previous studies performed on white-striped broiler breast meat (Petracci et al., 2014). In detail, the different proximate composition of abnormal fillets was explained by the complete re-organization of the skeletal muscle tissue, involving the abnormal proliferation of connective and accumulation fat tissue (Mudalal et al., 2015). However, the occurrence of WS in turkeys resulted in a less noteworthy increased lipid content within the muscle tissue in comparison with that previously observed in broilers. Thus, since WS only partially affected the proximate composition of turkey meat, it might be hypothesised that WS does not imply a profound muscle fibre degeneration.

Table 3.9 showed the results concerning the mean quality traits of both raw and marinated meat as affected by WS.

Table 3.9 Effect of WS abnormality affecting turkeys Pectoralis major muscles with different levels of severity (MOD and SEV) on quality traits of non-marinated and marinated meat. n = 72 (24 samples/group). a,b = P<0.05

Parameter	Experimental Group			s.e.m.	P-value
	NORM	MOD	SEV		
Raw meat					
pHu	5.75	5.75	5.73	0.01	ns
L*	51.7	52.1	51.8	0.3	ns
a*	5.05	4.92	5.63	0.16	ns
b*	1.57	1.81	2.09	0.11	ns
Drip Loss (%)	1.1a	0.8b	1.0a	0.1	<0.05
Cooking Loss (%)	16.6	17.1	16.2	0.3	ns
WB shear force (kg/mm^2)	4.22	4.01	4.46	0.13	ns
Marinated meat					
Uptake (%)	12.0	10.6	11.1	0.3	ns
Cooking Loss (%)	18.4	18.6	19.1	0.2	ns
WB shear force (kg/mm^2)	2.62	2.57	2.59	0.07	ns

Overall, the main quality traits of raw (pH, colour, cooking loss and shear force) and marinated meat (uptake, cooking loss and shear force) were not affected by the occurrence of WS. However, the drip loss content significantly differed among the groups, with MOD fillets exhibiting the lowest values (0.8%; P<0.05), while NORM and SEV muscles did not differ between each other. Thus, in order to deeply investigate water distribution within the muscle, we applied the TD-NMR technique. Results showed that both the relative intensities and the T_2 relaxation times recorded for each proton population did not differ among the experimental groups (Table 3.10).

Table 3.10.Effect of WS abnormality affecting turkeys Pectoralis major muscles with different levels of severity (MOD and SEV) on relative intensity (R.I.) and T_2 relaxation time of the three proton populations (ascribed to the bound, intra- and extra-myofibrillar water fractions) identified through TD-NMR. N = 72 (24 samples/group).

Parameters		Experimental Group			s.e.m.	P-value
		NORM	MOD	SEV		
Bound water	R.I. (%)	3.7	3.9	3.6	0.3	ns
	T_2 (ms)	2.8	2.9	3.4	0.3	ns
Intra-myofibrillar water	R.I. (%)	87.0	85.4	85.8	3.1	ns
	T_2 (ms)	41.7	43.1	42.9	0.4	ns
Extra-myofibrillar water	R.I. (%)	9.3	8.7	10.4	2.1	ns
	T_2 (ms)	153.0	158.5	157.6	3.0	ns

Considering that no differences in the water mobility, cooking losses and marinade performances were found between NORM and WS fillets, it seems reasonable to hypothesize that both water holding and binding capacities of turkey breast meat were not affected by the occurrence of WS. These findings were quite unexpected since heavier muscles belonging to modern poultry hybrids generally showed an increased fibers' cross-sectional area that usually is negatively linked with the water holding ability of the muscle, because of the lower packing density of the fibers themselves (Remignon et al., 2000; Updike et al., 2005). Generally, the absence of adverse effects on breast meat quality resulting from the presence of WS in turkeys if compared to broilers, might be clarified with the differences in breast muscle morphology. Indeed, in broilers it has been highlighted a strong association between breast thickness and yield, as well as a greater impact of the pectoral muscle's weight on muscle depth in respect with length and width dimensions (Mudalal et al., 2015). Moreover, it has been recently found by Griffin et al. (2018) that the more the pectoral muscle's depth and yield increase, the higher the probability to observe severe myopathic changes within the muscle tissue. Considering these aspects, the absence of adverse effects of WS on turkey meat quality might be attributed to an overall different specie-specific development of the pectoral muscle. Indeed, while severe WS chicken fillets usually exhibit remarkable increased depth measured in the cranial

position, a more harmonious muscle development in white-striped turkey's pectoral muscle might result in a less pronounced myopathic alteration within the muscle tissue.

3.2.4 Conclusions

Since WS myopathy predominantly affects heavy turkey hybrids exhibiting a more pronounced development of the pectoral muscles, we suggest the hypothesis of a growth-related origin for the WS condition also in turkeys. However, the occurrence of WS only partially affected the proximate composition of meat and the main quality traits of both raw and marinated meat were not altered as well. Thus, since quality and technological properties of white-striped turkey muscles were comparable to those of the unaffected cases, the downgrading of breast meat affected by WS can be avoided, allowing to reduce the economic losses that are currently faced by the broiler industry. Thus, it seems reasonable to suggest a diverse specie-specific physiological response to the pressure in muscle tissue induced by the selection in turkeys that, although analogously led to the occurrence of WS, results in limited effects on meat quality.

3.3 Post-mortem acidification patterns in chicken breast and leg muscles

Research activity A2a

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3.3.1 Introduction and aim of the study

Muscle tissue is an extremely complex apparatus that guarantees the movement and sustenance for the living animal. In vivo, through hundreds of nervous stimuli, all the organs work in harmony and promptly reacts to maintain the homeostasis of the whole body (Astruc, 2014). However, after the killing of the animal, when the influx of oxygen and energetic substrates is interrupted, homeostatic mechanisms become compromised and muscle cells convert their metabolism from aerobic to anaerobic in a futile attempt to maintain muscle's ante-mortem homeostatic balance. The main catabolites of this process are lactic acid and H^+ which, due to the cessation of blood flow, accumulate in the muscle and lead to a drop in muscle pH (Matarneh et al., 2017). The rate and the extent of muscle acidification during post-mortem time are of fundamental importance in terms of the quality of the forthcoming meat (Scheffler and Gerrard, 2007). The acidification kinetics varies greatly depending on both the species and the muscle type considered. However, acidification patterns and ultimate pH can significantly vary even between muscles belonging to the same species. The inter- and intra-species pH variations are attributed to the different types and distribution of muscle fibers as well as the glycolytic potential of the muscle (Debut et al., 2003; England et al., 2016). However, intra-species differences in pH might be also attributed to the amino acid composition of muscle proteins, which results in a different ability of the muscle to contrast changes in pH (i.e. buffering capacity). In more detail, the content of two dipeptides, anserine and carnosine, was found to significantly affect buffering capacity in both living and post-rigor muscles (Puolanne and Kivikari, 2000). However, while several studies have been conducted on swine and cattle, actual research is lacking information about the factors involved in chicken muscle acidification. Therefore, this study aimed at exploring the acidification kinetics of three different chicken muscles characterized by a different in vivo energy metabolism, in order to investigate the aspects implicated in the intra-species pH variations as well as increase the understanding about post-mortem metabolism of chicken muscles.

3.3.2 Materials and methods

To perform this study, chicken muscles were needed that met three criteria: first, they must be supposedly characterized by a different in vivo energy metabolism, they must be belonging to the main commercial interest cut-ups (breast, thigh, and drumstick) and, lastly, be readily available for easy sampling post-mortem. Generally, in light of their locomotory function in the live animal (i.e. support rapid activities like

sprinting or walking), leg muscles should be composed by both type I and IIa fibers, while the pectoral muscle totally presents type IIb fibers (Wang et al., 2017). Thus, we selected the following three muscles (Figure 3.2):

- *Pectoralis major* (PM; breast): composed by glycolytic fibers;
- *Extensor iliotibialis lateralis* (EIL; thigh): supposedly characterized by an intermediate fiber composition;
- *Peroneus longus* (PL; drumstick): supposedly composed by intermediate and oxidative fibers.

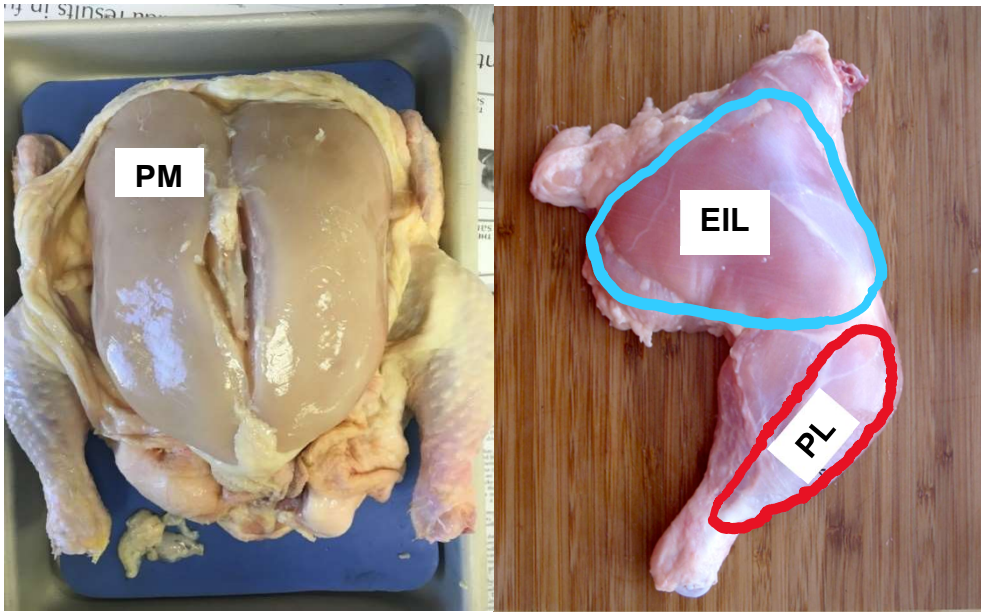

Figure 3.2 Chicken muscles selected for the experiment (PM=Pectoralis major, EIL=Extensor iliotibialis lateralis, PL=Peroneus longus).

A total of twelve carcasses were obtained from the same flock of broiler chickens (Ross 308 strain, males, 49 days of age, 3.0 kg body weight) farmed and harvested under standard commercial conditions. In detail, before slaughter, animals were subjected to a total feed withdrawal of 10 h, including a 3 h lairage time at the processing plant. Subsequently, birds were 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. Immediately after evisceration, each carcass was individually selected and samples from each bone-in muscle from the right side of the carcass were collected at 15, 120, 240, 480 and 1,440 min post-mortem with a scalpel. Carcasses have been kept at refrigerated temperature (4°C) for the whole duration of the trial. After the collection, samples were instantly frozen in liquid nitrogen and stored at -80°C in order to evaluate pH (chapter 2, paragraph 2.1.2.1) and R-value (chapter

2, paragraph 2.1.2.11.1). Then, at 24h post-mortem, fiber typing (chapter 2, paragraph 2.1.2.12.3), total heme pigments (chapter 2, paragraph 2.1.2.4) and concentration of histidine compounds (chapter 2, paragraph 2.1.2.10) were also assessed.

Data concerning pH and R-value were analyzed using the ANOVA for repeated measurements option of the GLM procedure present in SAS software (SAS Institute Inc., USA) testing the effect of the sampling time (15, 120, 240, 480 and 1,440 min). Furthermore, for each sampling time, overall data were processed with the One-Way ANOVA option of the same software in order to test the effect of the muscle type. Means were separated by Tukey's HSD test at a level of $P<0.05$.

3.3.3 Results and discussion

The muscles used within the study were intentionally selected based on the different function and metabolism in vivo thus, as a consequence, we hypothesized that the aforementioned muscles should possess different fiber types in light of their different energy metabolic characteristics. Figure 3.3 shows the representative images of PM, EIL and PL muscles stained for NADH-TR activity, a method used to highlight the oxidative metabolism of fibers. The PM muscle was entirely composed by type IIb fibers (grey), while EIL muscle simultaneously presented type I (dark blue), IIb and IIa fibers (light blue) (i.e. it is characterized by an intermediate fiber composition), corroborating what previously found by Wang et al. (2017) and Cong et al., (2017), who reported that EIL muscle is composed by 60.2% type IIb fibers, 20.4% type IIa fibers and 19.2% type I fibers. On the other hand, PL muscle was found to possess an intermediate fiber composition as well, but with a higher proportion of oxidative fibers if compared to EIL muscle. Albeit there is very limited number of published studies concerning chicken PL fiber type composition, PL muscles of cat and rat were found to be mainly composed by type IIa and I fibers (Ariano et al., 1973).

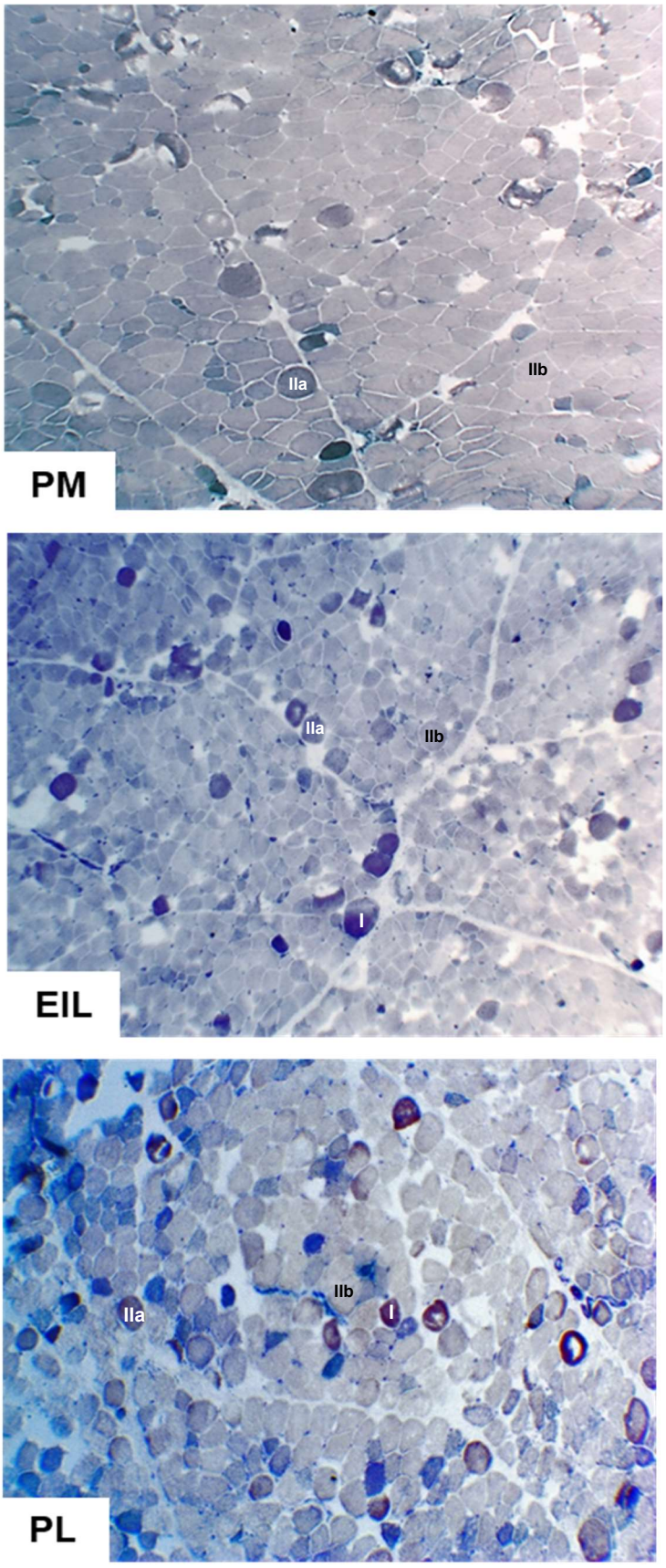

Figure 3.3 Representative images of PM, EIL and PL muscles stained for NADH-TR activity. Type I fibers appear dark blue, while type IIa and IIb fibers present light blue and grey color, respectively. (PM=Pectoralis major, EIL=Extensor iliotibialis lateralis, PL=Peroneus longus).

Results concerning muscle's total heme pigments are shown in figure 3.4. Among the factors contributing to meat color, the concentration and chemical state of meat pigments play a central role (Fletcher, 2002). On the other hand, the concentration of pigments is strictly dependent on muscle fiber composition (i.e. on the fibers' myoglobin content) (Suman and Joseph, 2013). As expected, the PL showed a significantly higher total heme pigment content if compared to EIL and PM muscles (67.4 vs. 48.8 and 40.5 mg hematin/kg meat; P <0.005), supporting the findings regarding its higher proportion of oxidative fibers. Indeed, the type I and IIa fibers that mainly constitute this oxidative muscle generally have a high myoglobin content, which confers to the muscle a darker color. On the contrary, PM (consisting almost exclusively of glycolytic fibers) and EIL (consisting of type IIa and IIb fibers), showed a limited content of total heme pigments, that gives the reason for the brighter color of the aforementioned muscles.

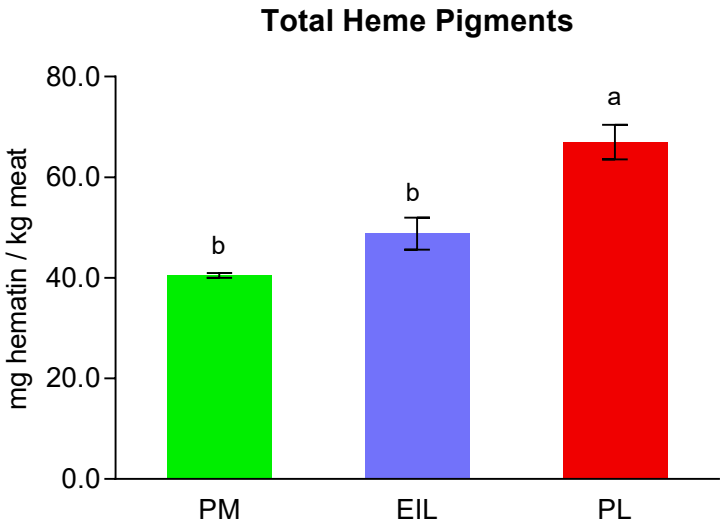

Figure 3.4 Mean total heme pigments values (expressed as mg hematin/kg of meat) in broiler PM, EIL and PL muscles assessed 24h post-mortem. a-b: mean values followed by different letters significantly differ (P<0.05). Error bars indicate standard error of means.

Results concerning muscle acidification during post-mortem time are shown in figure 3.5. Considering their different fiber composition, as expected, the muscles selected for the study showed a different rate and extent of the pH drop during post-mortem time. PM muscle exhibited the highest initial pH value (pH_{15}), being significantly different from those of leg muscles (thigh and drumstick), that however showed analogous pH_{15} values (6.64 vs. 6.44 and 6.40, respectively; P<0.001).

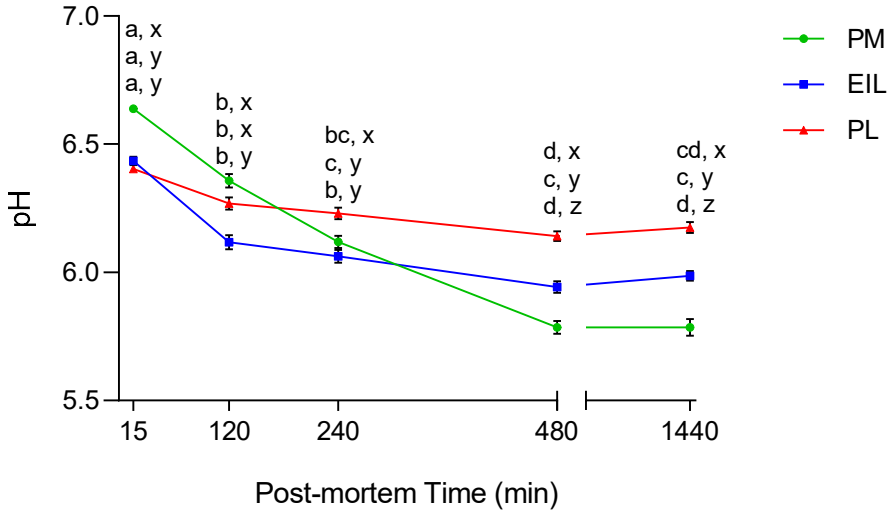

Figure 3.5 Mean pH values in broiler PM, EIL and PL muscles during post-mortem time. a-d: mean values followed by different letters significantly differ among the sampling times within the same muscle (P<0.05). x-z= mean values followed by different letters significantly differ among the muscles within the same sampling time (P<0.05). Error bars indicate standard error of means.

This result corroborates what previously found by Berri et al. (2005a) and might be attributed to the different muscle glycogen content at death. At 120 min post-mortem, PM and EIL muscles already exhibited a wider pH decline if compared to PL that, having a bigger proportion of oxidative fibers, is characterized by a slower acidification. Indeed, because the lower ATPase activity of the oxidative fibers along with its lower glycolytic potential, PL muscle reached ultimate pH values already at 4h post-mortem, while PM and EIL reached a plateau value 4h post-mortem later. As expected, after 24h from the death of the animal, PL muscle showed the highest pH value (6.18), while the PM the lowest (5.79) and EIL muscle an intermediate pH_u value (5.99). Albeit the acidification rates of EIL and PL was similar, the extent of the pH drop was found to be greater in EIL. However, overall, PM muscle exhibited the greatest extent of the pH drop (ΔpH=0.84 units), while PL showed the lowest (ΔpH = 0.23). The pH_u values recorded within this study corroborate what found in previous researches carried out on the same muscles (Liu et al., 2004; Karakaya et al., 2009; Zhang et al., 2012) and are essentially ascribable to the differences in glycolytic potential in relation to the different fiber composition of the muscles themselves. Indeed, PL muscle, being characterized by a higher proportion of oxidative fibers, exhibited a slower and slighter acidification, since the reduced glycolytic potential along with weaker ATPase activity

of its fibers are responsible for arresting post-mortem glycolysis, leading to meat having a relatively high ultimate pH (England et al., 2016).

Figure 3.6 shows the results concerning the onsets of the R-value parameter, which is an index of ATP depletion within muscle tissue and gives information about the biochemical kinetics that occurs in the muscle after the death of the animal. This parameter is calculated through the ratio of between adenine nucleotides and inosine metabolites: R-value close to 1.10 indicates the onset of rigor mortis, while values around 1.27 indicate its completion (Petracci and Baéza, 2007).

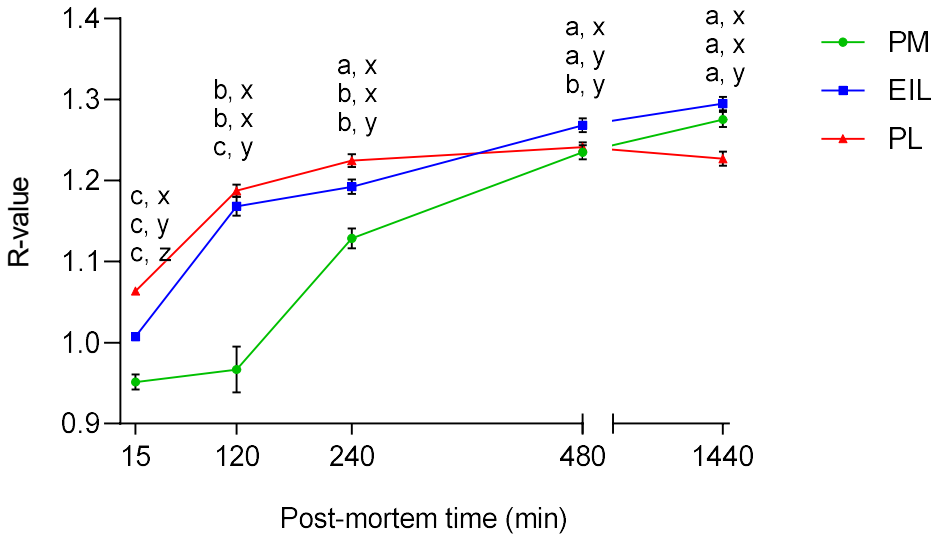

Figure 3.6 Mean R-values in broiler PM, EIL and PL muscles during post-mortem time. a-c: mean values followed by different letters significantly differ among the sampling times within the same muscle (P<0.05). x-z= mean values followed by different letters significantly differ among the muscles within the same sampling time (P<0.05). Error bars indicate standard error of means.

ATP depletion was found to be rather different within the muscles, especially during the first hours post-mortem. Indeed, the PM muscle initially presented a significantly slower ATP consumption if compared to leg muscles. This trend was rather unexpected considering its composition in glycolytic fibers, endowed with a high ATPase activity. However, the values achieved at 24 hours post-mortem showed that overall ATP consumption was greater in PM muscle (Δ=0.323), rather than in the EIL (Δ=0.287) and PL muscles (Δ=0.163). These results corroborate the pH trend, displaying that the extent of acidification was greater in PM compared to leg muscles. On the other hand, the achievement of rigor mortis in PL just after 240min post-mortem might be due to the lower amount of glycogen present in muscles predominantly

characterized by an oxidative metabolism such as Peroneus longus. Furthermore, EIL showed a trend of ATP depletion similar to PL in the first 4 hours post-mortem to then reach final values analogous to PM muscle, confirming to possess intermediate characteristics.

Anserine and carnosine are two imidazole dipeptides widely distributed in vertebrate organisms and particularly abundant in skeletal muscle (Gil-Agustí et al., 2008). These compounds are involved in several biological mechanisms, however they are particularly important for their ability to contrast pH changes both in vivo and during the conversion of muscle to meat, i.e. they are the major skeletal muscle buffers (Decker, 2001). Their content greatly varies with species, albeit muscle fiber type also influences their concentrations, with glycolytic fibers generally having higher anserine and carnosine concentrations compared to oxidative fibers (Boldyrev and Severin, 1990). Figure 3.7 shows the results for anserine and carnosine concentrations within PM, EIL and PL muscles. If compared to leg muscles, PM has a significantly higher concentration of both anserine and carnosine, confirming what previously found by Crush (1970) and Jung et al. (2013).

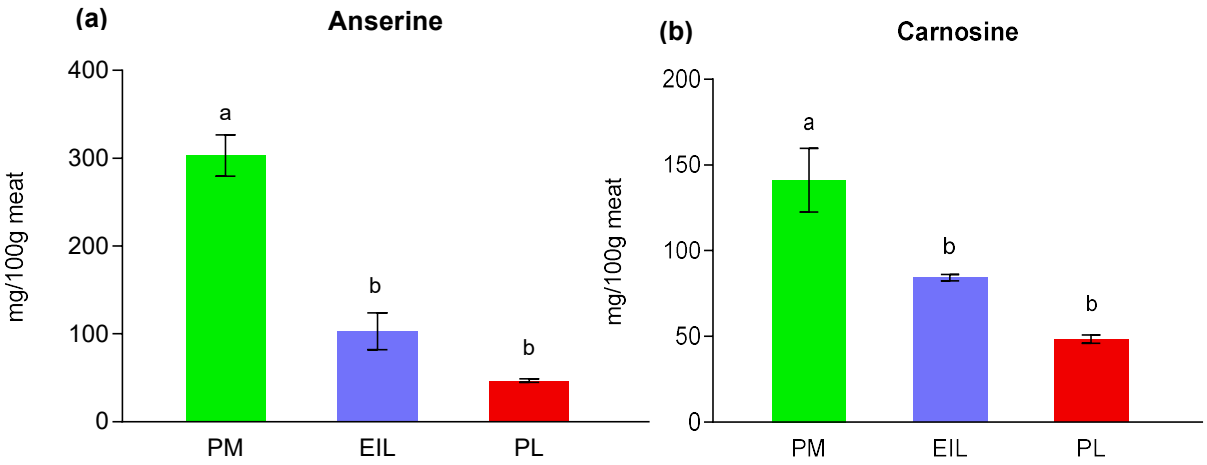

Figure 3.7 Anserine (a) and carnosine (b) content in broiler PM, EIL and PL muscles, expressed as mg/100g meat. a-b: mean values followed by different letters significantly differ among muscles (P<0.05). Error bars indicate standard error of means.

The reason for the higher concentration of histidine compounds in the pectoral muscle might be found in its higher proportion of fast-twitch glycolytic fibers, that mainly relies on anaerobic glycolysis to produce energy for short but intense movements. Indeed, buffering capacity is usually greater in those muscles characterized by poor capillarization because, during high-intense activities, the lactic acid produced through

glycolysis is completely dissociated at physiological pH (pKa= 3.86) and could provoke an excessive acidification in the living muscle, inhibiting the activity of enzymes and thus compromising muscle fibers functionality (Pösö and Puolanne, 2005). Indeed it has been estimated that anserine and carnosine contribute to 30-40% to upkeep muscle pH at the physiological value (Davey, 1960).

3.3.4 Conclusions

This study allowed to deepen the knowledge about post-mortem acidification kinetics of three different chicken muscles characterized by a different metabolic pathway, providing further information about the correlations between fiber type, muscle acidification, buffering capacity and metabolism in chicken meat. Overall results showed that muscles exhibit different acidification kinetics depending on their fiber type and metabolism. As expected, PM was characterized by a faster and greater muscle acidification if compared to leg muscles. The differences observed between the pH_u values of muscles corroborate what expected, although the magnitude of these differences appeared to be lower than what was initially hypothesized. Indeed, the pH_u of the pectoral muscle (5.80), seemed to be quite high if compared to that of meat of other species with a similar fiber composition (e.g. turkey breast). This phenomenon could be partially explained with the different concentration of anserine and carnosine, that possess a strong buffering capacity. Indeed, the pectoral muscle, in light of its marked glycolytic metabolism, should have exhibited relatively lower ultimate pH. Thus, it can be assumed that the greater concentration of anserine and carnosine found in PM muscle played a role in limiting extent of post-mortem acidification. Therefore, the different amino acid composition of proteins and, in particular the content of histidine dipeptides, might partially justify the differences existing between the pH_u values of meat belonging to different species.

3.4 Post-mortem metabolism of different chicken muscles as affected by histidine-containing dipeptides content

Research activity A2b

Scientific publication originated from this research activity

Baldi G, Soglia F, Laghi L, Babini E, & Petracci M. (2020). The role of histidine dipeptides on post-mortem metabolism of broiler breast and leg muscles, under preparation.

3.4.1 Introduction and aim of the study

Skeletal muscles are composed of various fiber types, which mainly differ in their contractility, metabolic activity and other distinguishing properties (Talbot and Maves, 2016). Metabolic characteristics of skeletal muscle deeply influence the pattern of energy metabolism in live animal, as well as during the conversion of muscle to meat occurring during post-mortem time (Chauhan and England, 2018). Furthermore, the metabolic type of the muscle is one of the focal factors associated to the variability of meat quality (Lee et al., 2016). In the past decades, several authors have used myoglobin concentration and lactate dehydrogenase activity to respectively distinguish the oxidative or glycolytic energy formation pathways of muscles (Flores et al., 1996; Hernández et al., 1998). However, more recently, Mora et al. (2008a) reported that the content of several aminoacids is closely related to the metabolic type of fibers. In more detail, the authors stated that carnosine content represents a good indicator of muscle glycolytic metabolism, since it has been widely established that its muscular concentration increases with the glycolytic activity of the muscle (Boldyrev and Severin, 1990; Aristoy and Toldra, 1991; Intarapichet and Maikhunthod, 2005; Gil-Agustí et al., 2008). Carnosine (β-alanyl-L-histidine) is a histidine-containing dipeptide widely abundant in skeletal muscles of mammals and other vertebrates, exploiting several biological functions (Barbaresi et al., 2019). Its content greatly varies depending on the specie and the muscle considered (Gil-Agustí et al., 2008). However, both carnosine and anserine, being the most abundant histidine-containing dipeptides in chicken meat (Tinbergen and Slump, 1976), have been the object of several poultry science-based studies because of their biological importance. Indeed, these compounds act as metal ion chelators, free radical scavenging and, in virtue of their pK_a, they possess the ability to buffer the acidic end-products (e.g. lactic acid and hydrogen ions) generated during periods of anaerobic metabolism (Castellini and Somero, 1981).

During post-mortem time, as a result of the hydrolysis of ATP to ADP, hydrogen ions are produced and buffered until their concentrations exceed the buffering capacity of muscle cells and the pH gradually declines (Aberle et al., 2012). Thus, it is reasonable to assume that the muscular concentration of histidine-containing compounds might provide resistance to the post-mortem pH decline, thus exerting an effect on muscle metabolism during early post-mortem. Within this scenario, the main objective of the present study was establishing the relation between the content of

histidine-containing compounds and muscle post-mortem acidification, examining the metabolic pathways of three different chicken muscles chosen on the basis of their carnosine content to represent the main metabolic types.

3.4.2 Materials and methods

Preliminary study

For the purpose of the study, three different chicken muscles were needed representing the three main metabolic types (oxidative, glycolytic and intermediate), in order to investigate the relation between the content of histidine-containing compounds and muscle post-mortem metabolism. Moreover, selected muscles needed to meet the following criteria: first, on the basis of their carnosine content, they must be supposedly characterized by a different in vivo energy metabolism, they must be belonging to the main commercial cut-ups (i.e. breast, thigh, and drumstick) and, lastly, be readily available for easy sampling post-mortem. Thus, in order to select the muscles representing the best compromise among the aforementioned criteria, ten different muscles (Table 3.11) from one single bird (Ross 308 strain, female, 49 days of age, 2.8 kg live weight) reared and slaughtered under commercial condition were selected at 24h post-mortem and used to evaluate ultimate pH (chapter 2, paragraph 2.1.2.1) and carnosine content (chapter 2, paragraph 2.1.2.10).

Table 3.11 Chicken muscles selected for the preliminary study.

Muscle	Anatomical region
Pectoralis major	Breast
Pectoralis minor	Breast
Triceps humeralis	Wing
Flexor perforans et perf.	Drumstick
Peroneus longus	Drumstick
Gastrocnemius pars int.	Drumstick
Biceps femoris	Thigh
Extensor iliot. lat.	Thigh
Semimembranosus	Thigh
Sartorius	Thigh

Main study

A total of eight carcasses were obtained from the same flock of broiler chickens (Ross 308 strain, females, 49 days of age, 2.8 kg body weight) farmed and harvested under standard commercial conditions. In detail, before slaughter, animals were subjected to a total feed withdrawal of 10 h, including a 3 h lairage time at the processing plant. Subsequently, birds were 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. Immediately after evisceration, each carcass was individually selected and samples from *Pectoralis major* (PM), *Extensor iliotibialis lateralis* (EIL) and *Gastrocnemious pars interna* (GI) bone-in muscles were collected at 15, 60, 120 and 1,440 min post-mortem, instantly frozen in liquid nitrogen and stored at -80°C in order to evaluate pH (chapter 2, paragraph 2.1.2.1) and glycolytic metabolites (chapter 2, paragraph 2.1.1.2.11.2) for each sampling time. Then, at 1,440 min post-mortem, the concentration of histidine-containing compounds (chapter 2, paragraph 2.1.2.10) as well as buffering capacity (chapter 2, paragraph 2.1.2.2) were also assessed.

Data concerning pH and glycolytic metabolites were analyzed using the ANOVA for repeated measurements option of the GLM procedure present in SAS software (SAS Institute Inc., USA) testing the effect of the sampling time (15, 60, 120 and 1,440 min). Furthermore, the same dataset was processed with the One-Way ANOVA option of the same software in order to test the effect of the muscle type (PM, EIL and GI) on pH and glycolytic metabolites for each sampling time (15, 60, 120 and 1,440 min post-mortem). Finally, data concerning buffering capacity and histidine-containing compounds evaluated at 1,440 min post-mortem were analyzed using One-Way ANOVA option as well, considering the muscle type as a main effect. Means were then separated by Tukey's HSD test at a level of $P<0.05$. Further, in order to establish the relationship between buffering capacity and histidine-containing compounds concentration, the correlation analysis has been conducted using the correlation matrices procedure present in SAS software (SAS Institute Inc., USA).

3.4.3 Results and Discussion

Preliminary study

Because of their biological importance, a number of studies have been carried out in order to examine the content of histidine-containing compounds in different type of meats through several analytical techniques (Davey, 1960; Wu and Shiau, 2002; Peiretti et al., 2011). The concentration of anserine and carnosine widely varies among animal species, so much so that the ratio of histidine compounds has been used to discriminate meat species in processed meat products (Abe and Okuma, 1995). As concern poultry meat, it has been widely demonstrated that anserine and carnosine content is affected by the breed, the gender, the age as well as the muscle type (Mora et al., 2008; Barbaresi et al., 2019). However, albeit it has been proved that breast

meat possesses higher content of histidine-containing compounds rather than thigh meat (Intarapichet and Maikhunthod, 2005), reference values about anserine and carnosine content of specific chicken muscles are not available in the literature, since the concentration of these compounds is often measured on a batch of mixed muscles belonging to the same anatomical region. Within this scenario, the preliminary study allowed to provide reference values about carnosine content and ultimate pH of 10 chicken muscles belonging to different anatomical regions (Table 3.12).

Table 3.12 Carnosine content (mg/100g meat) and ultimate pH of 10 different chicken muscles.

Muscle	Anatomical region	Carnosine (mg/100g meat)	pHu
Pectoralis major	Breast	133.1	5.84
Pectoralis minor	Breast	131.6	5.97
Triceps humeralis	Wing	108.2	6.06
Flexor perforans et perf.	Drumstick	100.8	6.57
Extensor iliot. lat.	Thigh	84.2	6.38
Biceps femoris	Thigh	82.1	6.36
Peroneus longus	Drumstick	78.2	6.33
Semimembranosus	Thigh	78.0	6.45
Sartorius	Thigh	72.3	6.29
Gastrocnemius pars int.	Drumstick	64.7	6.57

Thus, based on these preliminary results, three muscles supposedly characterized by a different in vivo energy metabolism have been selected on the basis of both carnosine content and ultimate pH (Figure 3.8):

i) *Pectoralis major* (PM; breast): chosen to represent the glycolytic-type muscle;

ii) *Extensor iliotibialis lateralis* (EIL; thigh): chosen to represent the intermediate-type muscle;

iii) *Gastrocnemious pars interna* (GI; drumstick): chosen to represent the predominantly oxidative-type muscle.

Thus, considering that a link exists between carnosine concentration and muscle glycolytic activity and that ultimate pH of a muscle greatly depends on its energy-generating pathway (Mora et al., 2008; Westerblad et al., 2010), these muscles seems to meet the criteria needed to pursue the objectives of this study.

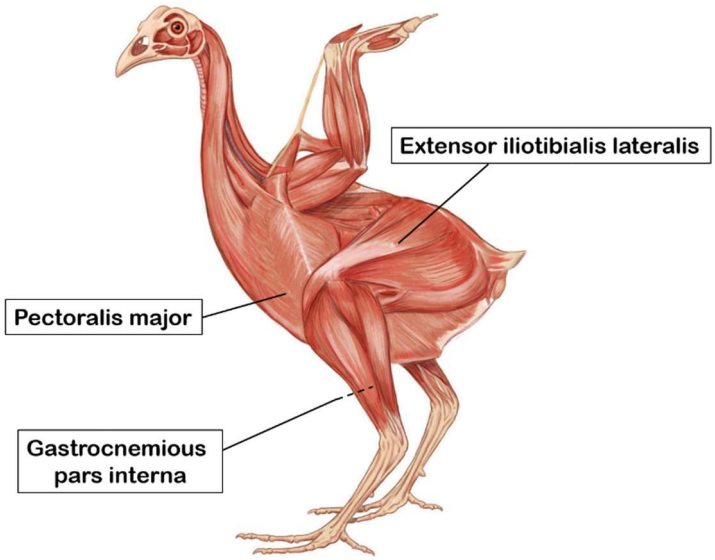

Figure 3.8 Anatomic location of muscles selected for the experiment.

Main study

The study aimed at establishing whether a relationship exists between histidine-containing compounds and post-mortem acidification of muscles belonging to different metabolic types. The results concerning histidine-containing concentrations (figure 3.9) revealed a large divergence in the dipeptide content between the three metabolic types of muscle. PM muscle showed the highest contents of both anserine and carnosine, which resulted to be 3.4- and 3.0-fold higher compared to what detected in GI muscles (409.0 vs 118.1 and 136.5 vs 45.6 mg/g meat, respectively), while EIL exhibited intermediate values for both the dipeptides. Overall, these data suggested that breast meat contains higher amounts of histidine dipeptides than does leg meat, confirming what found in previous studies (Intarapichet and Maikhunthod, 2005; Jung et al., 2013; Barbaresi et al., 2019). However, to our knowledge, no research ever aimed at evaluating the content of dipeptides on specific thigh or drumstick muscles, since most of the authors assessed anserine and carnosine content on a batch of muscles collected from chicken leg.

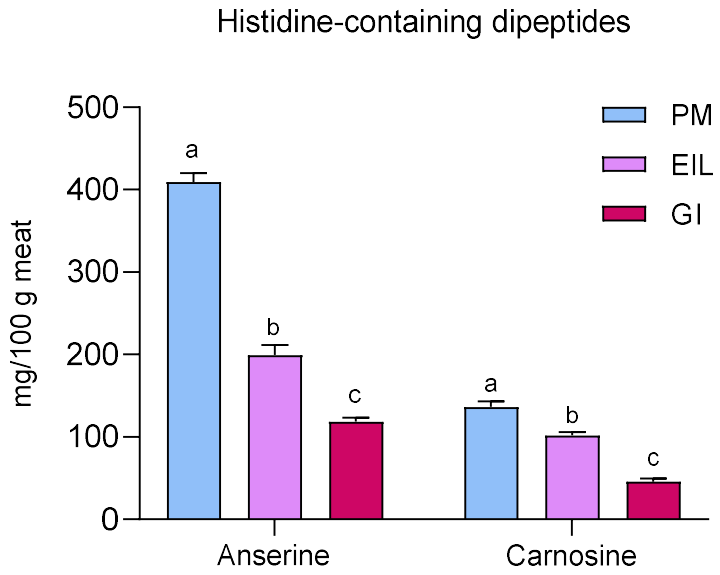

Figure 3.9 Mean values of anserine and carnosine content in broiler PM, EIL and GI muscles, expressed as mg/100g meat. a-c: mean values of anserine and carnosine content followed by different letters significantly differ among muscles (P<0.05). Error bars indicate standard error of means.

PM, being entirely composed by type IIb fibers, showed the highest values of the aforementioned dipeptides, in contrast to GI muscle that being mainly composed by type IIa and I fibers (Wang et al., 2004) showed the lowest values of both anserine and carnosine; on the other hand, EIL muscle, characterized by an intermediate fiber composition (Cong et al., 2017), exhibited intermediate values of dipeptides between PM and GI muscles. Thus, the results of the present study seemed to suggest that the content of histidine-containing dipeptides varies according to the different fibers composition of the muscles (i.e. to the different muscle metabolic types). These findings corroborate what previously suggested by Mora et al. (2008b) in a similar study conducted on pork muscles of different metabolic types. Further, it is well established that both anserine and carnosine, due to their great buffering capacity, play a key role in contrasting rapid changes in pH in both living and post-mortem muscles (Decker, 2001). Indeed, the higher content of anserine and carnosine in chicken breast meat might be explained with its in vivo metabolic behavior that makes the muscle more needy of physico-chemical buffer compounds able to contrast the protons produced through anaerobic glycolysis, resulting in a buildup of histidine compounds in the muscle. According to this hypothesis, leg muscles, that mainly relies on oxidative energy-generating pathway, exhibited a lower content of buffering compounds

because they do not necessitate to contrast large amount of acidic end products in vivo.

The results concerning muscle acidification during post-mortem time are shown in figure 3.10. Intriguingly, within the first 2 hours post-mortem, PM and GI muscles showed the same acidification rate and extent, while EIL outpaced, showing the lowest pH values in the same time frame. However, both GI and EIL pH values did not show significant differences between 120 and 1,440 min post-mortem, meaning that the acidification process of these muscles reached a plateau at 120 min, while PM muscle's pH continued to drop until 24 h post-mortem.

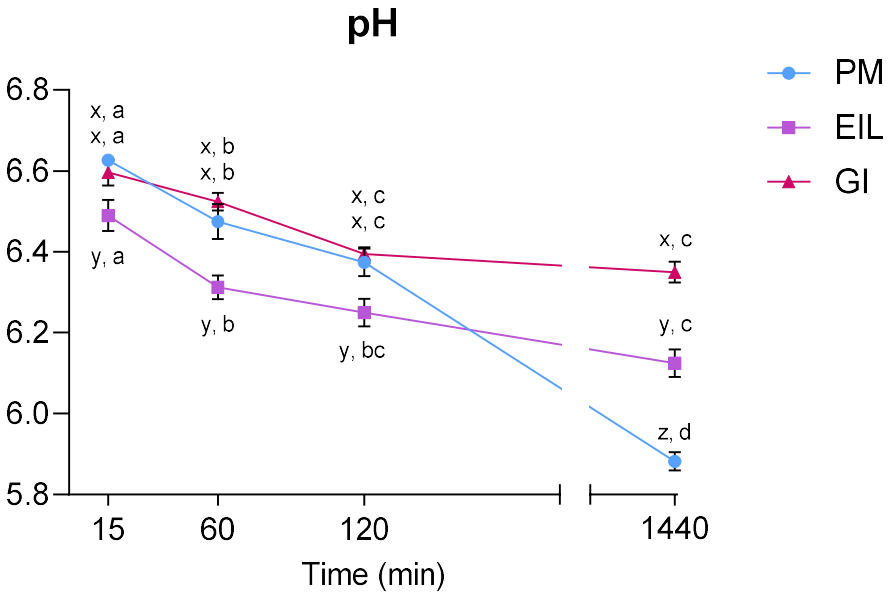

Figure 3.10 Mean pH values of broiler PM, EIL and GI muscles during post-mortem time.
a-d: mean values with different letters significantly differ among the time points within the same muscle (P<0.05). x-z= mean values with different letters significantly differ among the muscles within the same time point(P<0.05). Error bars indicate standard error of means.

Indeed, after 24 h post-mortem, PM reached the lowest pH value (5.88), quite the opposite to GI that exhibited the highest pH_u (6.35), while EIL muscle displayed an intermediate value between PM and GI (6.12). Thus, results suggested that the extent of muscle acidification was greatly different between muscles, with PM showing a ΔpH of 1.02 units, EIL of 0.37 and, lastly, GI muscle of 0.26. These divergences are ascribable to several factors, including the different metabolic type of fibers composing the muscles themselves and, consequently, the amount of muscular glycogen at death available to enter the glycolytic pathway (Pösö and Puolanne, 2005; Lefaucheur,

2010). Indeed, leg muscles, being primarily composed by type I and type IIA fibers, likely presented a reduced glycolytic potential that led to higher ultimate pH, as it will be examined later in this assay. Moreover, it is interesting to highlight that within the first 2 h post-mortem, PM and GI muscles, whose metabolic pathways should be opposite, showed a similar acidification process. Indeed, it would have be reasonable to expect that PM muscle, in virtue of its glycolytic metabolism, should have exhibited a faster pH decline. This unexpected trend might be explained with PM remarkably higher content of histidine compounds that might have prevented an excessive muscular acidification by buffering the acidic end-products of glycolysis. Further, muscle fibers composing GI muscle (i.e. type I fibers), being characterized by a low contraction speed and myofibrillar ATPase activity (Lefaucheur, 2010), are not accustomed to produce energy through anaerobic pathways in vivo, thus explaining the slower acidification rate detected during post-mortem time. Indeed, GI muscle reached ultimate pH values already at 2 h post-mortem, while PM muscle, whose fibers possess high-speed rate of contraction and presumably a greater carbohydrate content entering the glycolytic pathway, continued to acidify due to its compliancy to metabolize energy through the anaerobic pathway.

For better understanding of post-mortem metabolism, glycolytic metabolites were measured in broiler PM, EIL and GI muscles. Patterns of lactate formation followed pH decline and confirmed the differences in both acidification rate and extent detected among the muscles of different metabolic type (figure 3.11a). Indeed, accordingly to pH results, at 24h post-mortem PM showed significantly higher ($P<0.05$) lactate concentrations if compared to leg muscles, in agreement with what previously found by Berri et al. (2005a). However, considering the lactate content of PM muscle detected at 15 min post-mortem, breast muscle should have exhibited a lower pH at the same time point. Thus, this result confirmed what previously assumed, namely that the remarkable content of histidine dipeptides has probably buffered an excessive acidification of the breast muscle in the first hour post-mortem. Further, this scenario confirmed that histidine compounds exert their buffering capacity not only in vivo, but also during the conversion of muscle to meat, at least in the first steps post-mortem where muscle's pH is still close to the physiological value (6.8-7.0) where, due to their pK_as, anserine and carnosine exert their maximum buffering capacity (Boldyrev and Severin, 1990).

Mobilization of muscle glycogen during post-mortem metabolism likely drives pH decline (Matarneh et al., 2018) and might provide useful information concerning substrate utilization in muscles of different metabolic types. Patterns of glycogen depletion during post-mortem time are shown in figure 3.11b. PM showed significantly ($P<0.05$) higher content of glycogen at 15 min post-mortem if compared to leg muscles, confirming what observed by Wang et al. (2017). This result suggests that breast muscle, according to its glycolytic fiber composition, possesses a greater substrate entering the glycolytic flux, justifying the achievement of a respectively lower and higher ultimate pH and lactate concentration if compared to leg muscles. Glycogen content was depleted in both EIL and GI muscles within the first 2 hours post-mortem, corroborating the termination of glycolysis (i.e. achievement of pH_u) at 120min after the death of the animal. This result might be likely due to the reduced presence of carbohydrate entering the anaerobic glycolysis combined with a faster glycogen turnover. Indeed, it is well known that muscle mainly composed by oxidative fibers, such in the case of GI, are poor in glycogen (Aberle et al., 2012) and that both glucose uptake and glycogen turnover are higher in slow-twitch type I than fast-twitch type II fibers (Villa Moruzzi et al., 1981). Indeed, leg muscles showed lower glycogen content at early post-mortem but faster glycolytic rates than muscles with higher glycogen, corroborating what found by Choe et al. (2008) in pork meat. Further, the absence of glycogen at 24 h in GI muscle further demonstrated that glycolysis stops because of a lack of substrate. On the contrary, residual glycogen found in PM and EIL muscles at 24 h post-mortem suggest that glycolysis would have proceeded. In more detail, the pectoral muscle, in light of its marked glycolytic metabolism, should have exhibited a relatively lower ultimate pH. Thus, it can be postulated that the greater concentration of anserine and carnosine found in PM muscle played a role in limiting the extent of post-mortem acidification.

Glycogen degradation yields non-phosphorylated glucose molecules and glucose 1-phosphate, which is isomerized to G6P and enters the glycolytic pathway, while free glucose molecules are either converted by hexokinase to G6P or accumulated in post-mortem muscle (England et al., 2017; Matarneh et al., 2018). Patterns of glucose utilization and glucose-6-phosphate (G6P) generation are shown in figure 3.11c and 3.11d, respectively. While EIL and PM muscles showed a similar pathway of glucose depletion and G6P generation, GI muscle diverged showing significantly ($P<0.05$) lower values of both the metabolites, at least within 120 min post-

mortem. From here on, no differences in GI muscle's glucose have been found, corroborating the absence of glycogen detected from 120 min post-mortem onwards. Moreover, the reduced glucose content detected at 15 min in GI muscle, once again supported its higher ultimate pH due to a smaller flux of substrate entering the post-mortem glycolysis. However, overall reduced G6P concentrations detected in GI muscle might suggest that G6P is generated at a rate comparable to its consumption, since hexokinase (i.e. the enzyme that catalyzes the conversion of glucose into G6P) activity is greater in muscle mainly composed by oxidative fibers (Lefaucheur, 2010). Apart from the muscle type, from 120 min post-mortem onwards G6P accumulates in the muscles, likely because most of the enzymes become inactivated with the progression of muscular acidification (Scheffler and Gerrard, 2007).

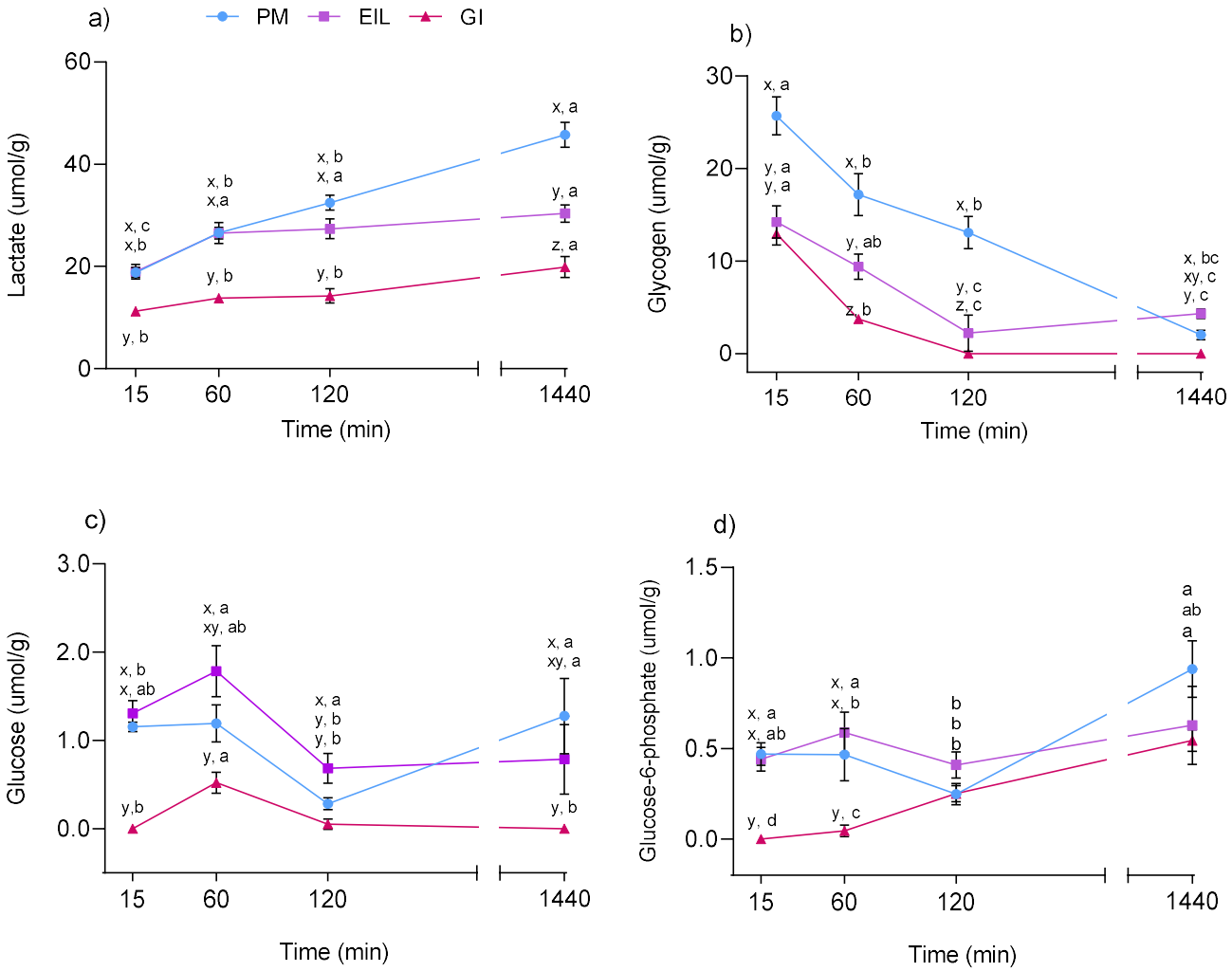

Figure 3.11. Average lactate (a, µmol/g), glycogen (b, µmol/g), glucose (c, µmol/g) and glucose-6-phosphate (d, µmol/g) of broiler PM, EIL and GI muscles (n=8/group) at 15, 60, 120 and 1,440 min post-mortem. a-c: mean values with different letters significantly differ among the time points within the same muscle (P<0.05). x-z= mean values with different letters significantly differ among the muscles within the same time point (P<0.05). Error bars indicate standard error of means.

Muscle glycolytic metabolites can be combined into a single measure termed as glycolytic potential (GP), useful to determine all the compounds present in the muscle that are capable to be converted into lactate, thus indicating the muscle's capacity to extend post-mortem glycolysis (Monin and Sellier, 1985). Hence, the determination of GP could somehow give information about post-mortem acidification, since it might play a limiting role in the extent of the pH decline (Mora et al., 2008; Chauhan and England, 2018). GP is closely linked to the muscles fiber composition (Shen et al., 2015). As an example, muscles composed by type IIb fibers usually have higher glycogen and glucose content than muscles made of type I fibers (Choe et al., 2008) and, consequently, a higher GP that will lead to a greater production of lactate and to a lower ultimate pH (Shen et al., 2015). Accordingly, GI and PM muscles showed the lowest and the highest GP, respectively, while EIL muscle exhibited intermediate values (figure 3.12). Thus, GP outcomes found within this study not only corroborate both pH and lactate content results, meaning that PM possess a higher content of substrate entering the glycolytic flux and leading to a lower ultimate pH, but also perfectly correlates the hypothesis that, on the basis of their carnosine content, PM, EIL and GI muscles are characterized by a different energy metabolism. In support of these outcomes, a similar study conducted on pork meat demonstrated that GP is closely related to meat ultimate pH and muscle metabolic type (Monin et al., 1987).

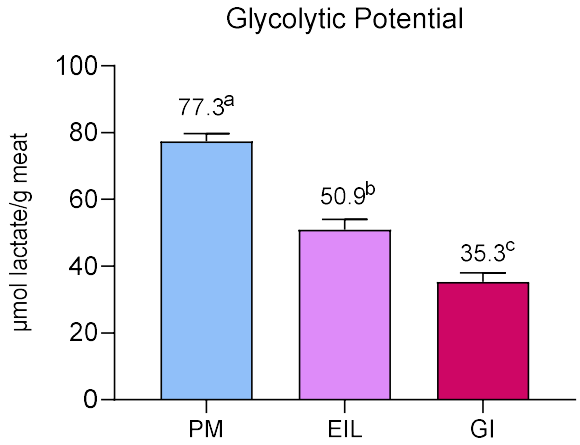

Figure 3.12. Average glycolytic potential (µmol lactate/g meat) of broiler PM, EIL and GI muscles (n=8/group). a-c: mean values with different letters significantly differ (P<0.05). Error bars indicate standard error of means.

Besides all, differences in the rate of post-mortem metabolism can be explored by investigating muscle buffering capacity (BC). In vivo, if muscle had no buffers, the simultaneous production of lactate and protons produced by anaerobic glycolysis during rapid movements would result in a very rapid pH drop. Indeed, muscle mainly composed by type IIb fibers possesses a greater BC, since they are adapted to prevent excessive drops in pH during fast movements in vivo (Pösö and Puolanne, 2005). Accordingly, at each pH range evaluated, PM muscle exhibited the highest BC, while on the contrary GI muscle showed the lowest and EIL displayed intermediate values (figure 3.13). These outcomes demonstrate that buffering capacity is greater in glycolytic muscles than in those more oxidative ones, confirming what reported in previous studies (Rao and Gault, 1989; Puolanne and Kivikari, 2000; Kylä-Puhju et al., 2004). These variations in buffering capacity between white and red muscle fibers have been mainly explained with differences in the contents of inorganic phosphate and histidine dipeptides, with anserine and carnosine being the major responsible for the aforementioned variations in BC among muscles of different metabolic types (Matarneh et al., 2015, 2017). Since the relationship that exist between muscle buffering capacity and histidine-containing compounds is widely established (Castellini and Somero, 1981; Decker, 2001), the higher BC of the breast muscle is explained by its higher concentration of histidine dipeptides. These results might clarify the slower acidification rate detected in PM muscle during the first post-mortem hours, which was quite unexpected.

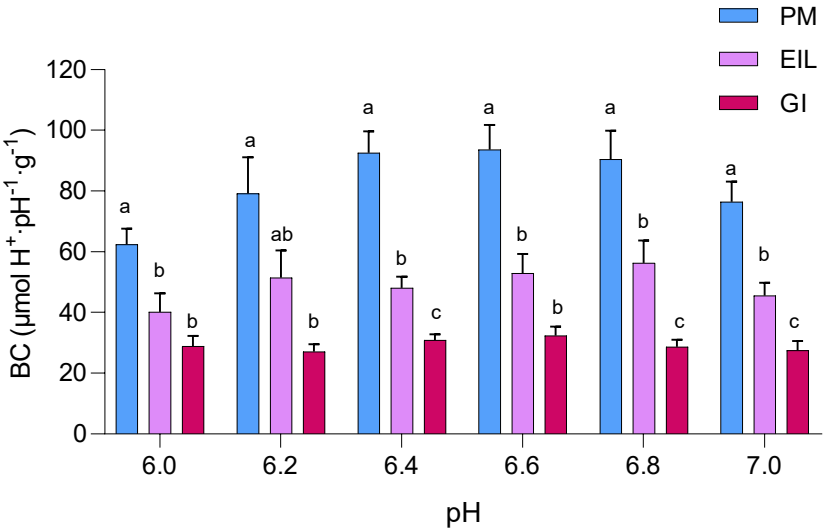

Figure 3.13 Buffering capacity (µmol $H^+ \cdot pH^{-1} \cdot g^{-1}$) (pH range 6.0-7.0) in PM, EIL and GI muscles. Data represent means ± SEM. a-c: mean values with different letters significantly differ among the same pH range($P<0.05$). Error bars indicate standard error of means.

Indeed, muscle mainly composed by type IIb fibers such as P. major, should exhibit a fast acidification due to the high contraction speed of the fast-twitch glycolytic fibers. Thus, in light of these outcomes, the slower acidification rate of the breast muscle within the first 120 min post-mortem (i.e. when muscle pH drops from 6.60 to 6.30) might be explained with the remarkably higher BC detected at pH 6.4 and 6.6. As previously discussed in this assay, this hypothesis is further supported by anserine and carnosine's pK_as values that, being respectively 6.38 and 7.04, guarantee the maximal buffering capacity at pH ranges included from 6.4 to 7.0 (Boldyrev and Severin, 1990).

In order to further establish the relationship between BC and histidine-containing compounds, analysis of correlation has been conducted in order to establish the connection between the aforementioned variables (Table 3.13). It has been found that both anserine and carnosine were positive correlated (P<0.001) with overall BC of muscles.

*Table 3.13 Correlations between overall muscles buffering capacity (BC) and anserine and carnosine content. ***=P<0.001.*

	BC	Anserine	Carnosine
BC	-	0.86***	0.79***
Anserine		-	0.87***
Carnosine			-

Within this scenario, it is reasonable to assume that the muscular concentration of histidine-containing compounds, having great outcomes on muscle buffering ability, might provide resistance to the post-mortem pH decline, thus exerting an effect on muscle metabolism during early post-mortem.

3.4.4 Conclusions

The main objective of this study was establishing the relation between the content of histidine-containing compounds and muscle post-mortem acidification, examining the metabolic pathways of three different chicken muscles chosen on the basis of their carnosine content to represent the main metabolic types. Albeit several studies have been conducted on pork meat due to the great intra-specie variability of muscle's metabolic type, to our knowledge no studies have been carried out aimed at evaluating post-mortem metabolism of chicken muscles characterized by a different pathway of energy production. However, results concerning pH curves and glycolytic intermediates suggest that the selection of the muscles for the experiment based on the carnosine content was accurate, since the carbohydrate metabolism of PM, EIL and GI muscles thoroughly reflects that of a glycolytic, intermediate and oxidative muscle, respectively. Moreover, this study allowed to establish that muscular concentration of histidine-containing compounds exert an important effect on muscle metabolism during early post-mortem. Indeed, being strictly correlated with muscle buffering capacity, histidine dipeptides might provide a resistance to the post-mortem pH decline, thus explaining the slower rate and extent of muscular acidification of P. major muscle during the first 2 h post-mortem. Further, residual glycogen found in PM at 24 h post-mortem suggest that glycolysis would have continued. In more detail, the pectoral muscle, in light of its marked glycolytic metabolism, should have exhibited a relatively lower ultimate pH. Thus, it can be postulated that the greater concentration of anserine and carnosine found in PM muscle played a role also in limiting the extent of post-mortem acidification.

3.5 Post-mortem metabolism of broiler P. major muscle affected by Wooden Breast myopathy

Research activity A3

Scientific publication originated from this research activity

Baldi G, Yen C-N, Daughtry MR, Bodmer J, Bowker BC, Zhuang H, Petracci M, & Gerrard DE (2020). Exploring the factors contributing to the high ultimate pH of broiler Pectoralis major muscles affected by Wooden Breast condition. *Frontiers in Physiology*, submitted for publication.

3.5.1 Introduction and aim of the study

In the interest of meeting the growing consumer demand for chicken meat, intensive selection practices have been carried out on broilers and, in conjunction, the incidence of metabolic disorders and muscular abnormalities affecting the P. major muscle has expanded during years (Malila et al., 2018). Among these, Wooden Breast (WB) is a myopathy whose occurrence has been reported in several plants all over the world including US, Italy, Brazil and Finland, with an incidence up to about 50% of affected individuals within the same flock (Griffin et al., 2018; Livingston et al., 2018). WB phenotypically is characterized by either a focally or diffusely palpably firm consistency of the breast muscle, which appears undesirable, pale, out-bulging and often displays superficial exudate and petechiae (Sihvo et al., 2014). From a microscopically point of view, WB affected muscles present a remarkably altered muscular architecture (Soglia et al., 2016b; Clark and Velleman, 2017), showing a diffuse thickening of the endo- and peri-mysial connective tissue associated with several degrees of fiber necrosis, immune cell infiltrations as well as extreme collagen deposition (fibrosis) (Sihvo et al., 2014; Mazzoni et al., 2015; Soglia et al., 2016b). Moreover, selection for enhancing P. major muscle mass played a major role in increasing muscle fiber size through post-hatch hypertrophic growth (Clark and Velleman, 2017) and reducing vessel density number (Sihvo et al., 2018), thus leading to a severe circulatory insufficiency (i.e., hypoxia) due to a reduced peri and endo-mysial connective tissue space.

The hallmark of WB myopathy is the elevated pH_u of meat near or up to 6.0 (Bowker et al., 2018; Baldi et al., 2019), which has been beforehand explained with an overall modified energetic status found in WB samples through metabolic studies (Mutryn et al., 2015; Abasht et al., 2016; Zambonelli et al., 2016). Having knowledge of post-mortem energy metabolism and glycolytic metabolites of WB muscles will provide new insights into this condition. Moreover, considering that the rate of post-mortem glycolysis can strongly influence the degree of myofibrillar contraction (Ferguson and Gerrard, 2014), it could be speculated if the extreme hardness of WB muscle could be partially due to an abnormal acidification process that might have caused an hypercontraction of sarcomeres. Within this scenario, this study aimed at deepening the knowledge about the effects of WB myopathy on glycolytic metabolites, muscle post-mortem metabolism, as well as the histological profile and muscle fiber

contraction degree, exploring the factors contributing to the higher ultimate pH of myopathic muscles.

3.5.2 Materials and Methods

A total of twenty-four birds were obtained from the same flock of broiler chickens (Ross 308 strain, males, 48 days of age, 3.0 to 3.5 kg live weight), reared and harvested using standard commercial procedures. Carcasses were selected immediately after evisceration from the line of a commercial broiler processing plant and categorized by two experienced people in unaffected (NORM) and WB (n=12/group), according to the presence or absence of WB myopathy. WB affected carcasses were selected based on palpable hardness and muscle rigidity throughout the whole P. major muscle, picking those ones showing the most severe myopathic conditions. Carcasses were stored in cold storage room at 4°C and samples of about one cm^3 were collected from cranial bone-in P. major muscles with the aid of a scalpel at both 15 and 1,440 min post-mortem, snap frozen in liquid nitrogen and stored at -80°C. Samples thus obtained were used for the assessment of pH (chapter 2, paragraph 2.1.2.1), glycolytic metabolites (chapter 2, paragraph 2.1.1.2.11.2), buffering capacity (chapter 2, paragraph 2.1.2.2), PFK activity (chapter 2, paragraph 2.1.2.11.3), and in vitro pH decline (chapter 2, paragraph 2.1.2.11.4).

Twenty-four additional deboned P. major muscle were collected from the deboning line of the same commercial broiler processing plant at 3h post-mortem and categorized into NORM and WB (n=12/group) following the same criteria described before. Fillets were stored at 4°C until 24h post-mortem and samples for sarcomere length evaluation (chapter 2, paragraph 2.1.2.11.2) and fiber morphology (chapter 2, paragraph 2.1.2.12.1) were taken from 4 different locations throughout the P. major muscle in order to evaluate the spatial effect on P. major morphological structure and sarcomere length. In more detail, samples were obtained from anteroventral (AV), anterodorsal (AD), posteroventral (PV) and posterodorsal (PD) regions of the P. major muscle affected by WB condition, while samples from NORM breast muscles were acquired from AV section (Figure 3.14).

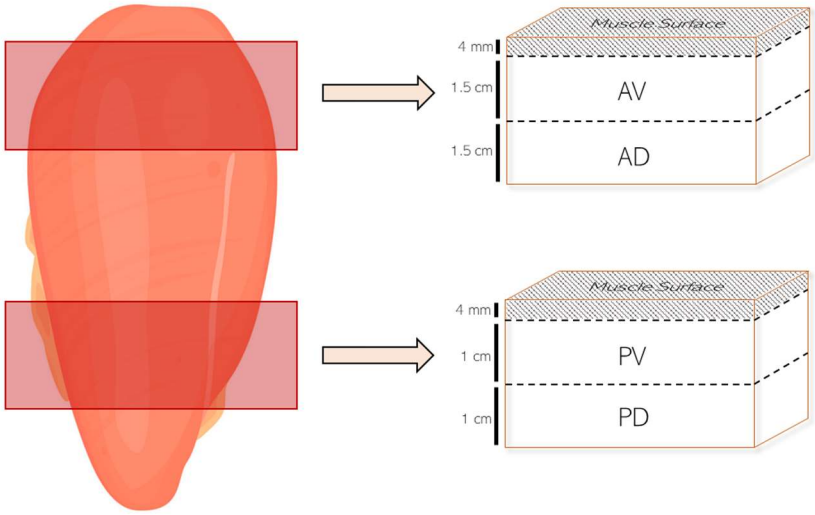

Figure 3.14 Schematic diagram of P. major muscle sampling location for the assessment of sarcomere length and fiber morphology (AV= anteroventral; AD= anterodorsal; PV= posteroventral; PD= posterodorsal).

After removing the first 4 mm of superficial muscle tissue, AV and PV samples were obtained from 0.4 to respectively 1.5 and 1 cm below breast muscle's surface. The AD and PD samples (1.5 and 1 cm thick, respectively) were then collected, being few millimeters distant from the AV and PV samples, respectively.

Data were then analyzed using the One-Way ANOVA option of the GLM procedure of SAS software (SAS Institute Inc, USA) by comparing affected (WB) and unaffected (NORM) groups within the same sampling time. Mean were evaluated using Tukey's multiple range test of the GLM procedure and considered significant at $P<0.05$. All data are expressed as means ± SEM.

3.5.3 Results and Discussion

Since selection programs have exerted significant consequences on muscle metabolism (Petracci and Berri, 2017), understanding post-mortem muscle glycolysis in WB affected birds may provide further knowledge into the disease. Results concerning the effect of WB myopathy on muscle pH and glycolytic metabolites are displayed in figure 3.15 and 3.16, respectively. While no differences were detected at 15 min post-mortem, a significantly higher ultimate pH of 6.32 ($P<0.001$) was observed in muscles affected by WB myopathy. Generally, normal P. major pH_u usually is

between 5.8-5.9, therefore the higher pH_u of WB meat found within this experiment and confirmed by previous studies (Kuttappan et al., 2017b) is commonly considered a hallmark for this myopathy.

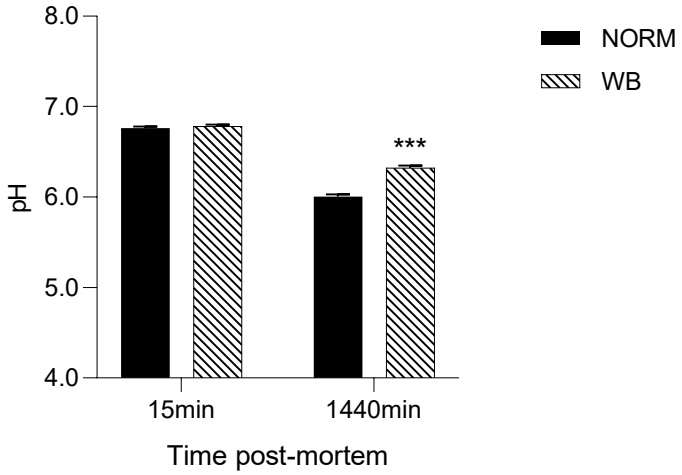

*Figure 3.15 Mean pH of NORM and WB broiler P. major muscle (n=12/group) at 15 and 1,440 min post-mortem. Data represent means ± SEM. Asterisks indicate significant difference within the time point (***=P<0.001).*

Accordingly, lactate contents followed pH decline, with WB muscles showing significantly lower values at 1,440 min (P<0.001; Figure 3.16A). The reduced lactate formation confirmed what found in severe WS+WB chickens by Malila et al. (2019) and might be explained with the loss of LDH enzyme from abnormal muscle fibers (Abasht et al., 2016). The higher pH_u of WB samples might suggest a premature cessation of post-mortem metabolism, which is usually attributed to the depletion of muscle glycogen and, indirectly, to its content at the death of the animal. Indeed, glycogen content was significantly lower in WB-affected muscles at 15 min (P<0.001; Figure 3.16B), corroborating the findings of Abasht et al. (2019) and Malila et al. (2019), whereas no differences were detected at 1.440min. It has been previously reported that glycogen storage in breast muscle decreases with the increase in muscle fiber size (Berri et al., 2007), thus suggesting that the artificial selection for genotypes with high growth rate and muscle yield (mainly achieved through fiber hypertrophy) might led to a reduced glycogen content in modern broiler breast muscles. On the other hand, G6P concentrations at both 15 and 1.440 min resulted to be remarkably lower (P<0.01 and P<0.001, respectively; Figure 3.16D) in WB meat as well, while glucose concentration was found to be higher (P<0.001; Figure 3.16C) in NORM samples at 1.440min. As a direct consequence of the diminished content of glycolytic metabolites, WB affected muscles exhibited a remarkably lower glycolytic potential (P<0.001),

which was found to be reduced by 30% if compared to one of unaffected samples (Figure 3.17).

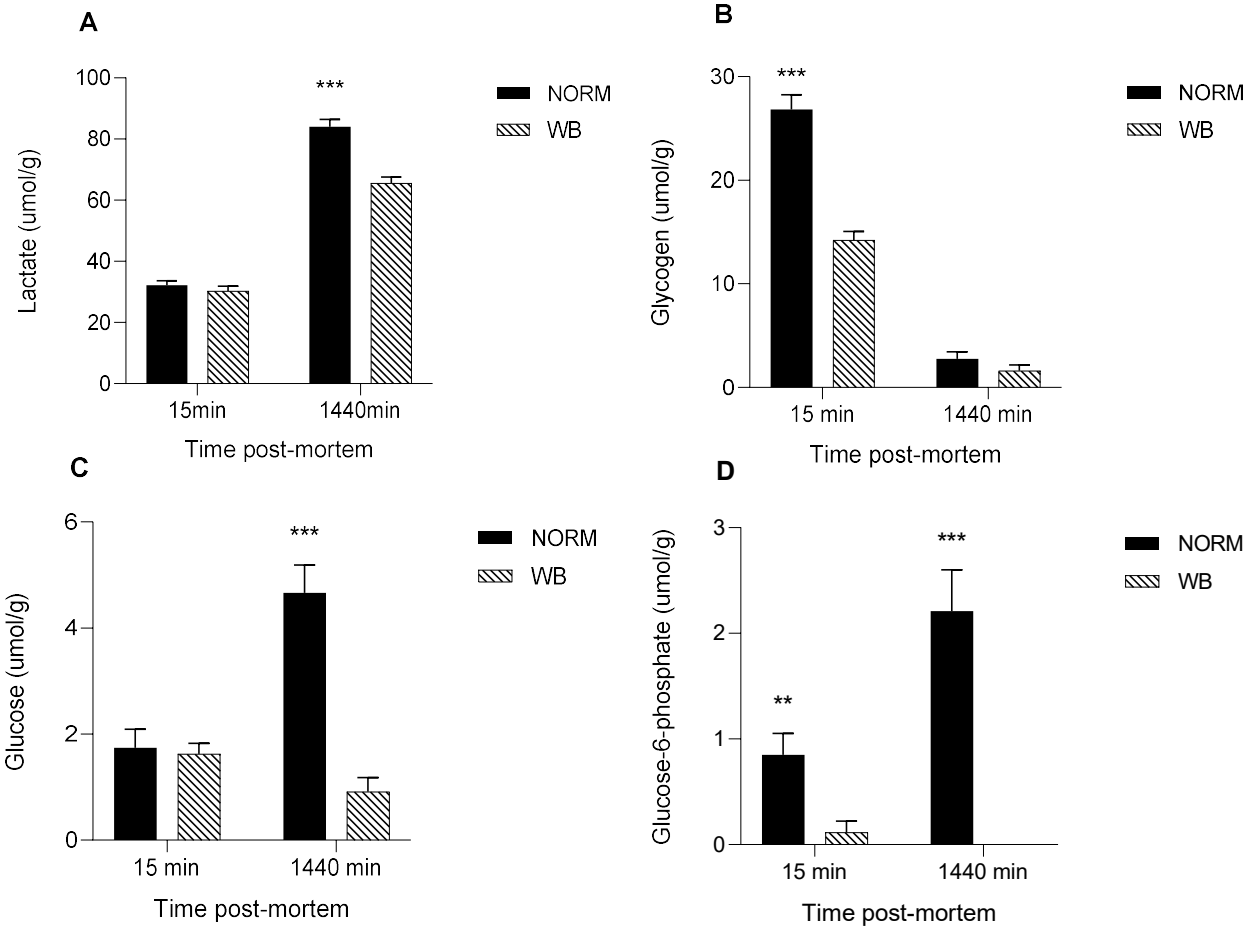

*Figure 3.16 Average lactate (A, µmol/g), glycogen (B, µmol/g), Glucose (C, µmol/g) and glucose-6-phosphate (D, µmol/g) of NORM and WB broiler P. major muscle (n=12/group) at 15 and 1.440 min post-mortem. Data represent means ± SEM. Asterisks indicate a significant difference within the time point (***=P<0.001; **=P<0.01*).

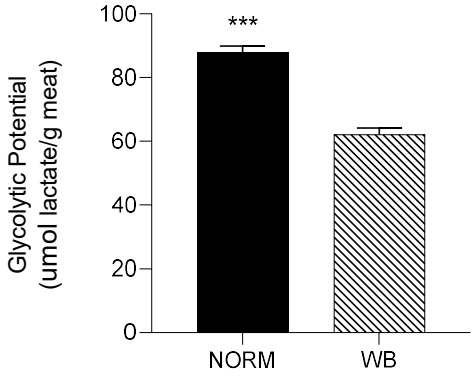

*Figure 3.17 Glycolytic potential (µmol lactate/g meat) of NORM and WB broiler P. major muscle (n=12/group). Data represent means ± SEM. Asterisks indicate a significant difference between experimental groups (***=P<0.001).*

The lower glycolytic potential found in WB samples suggests a reduced substrate flux through glycolysis and, therefore, a lower production of both H^+ and lactate, causing a higher ultimate pH of the forthcoming meat. Moreover, the absence of G6P at 1,440 min in WB meat might suggest that glycogenolysis is inhibited in myopathic muscles. These results confirmed what extensively reported in previous recent genetic and proteomic studies (Abasht et al., 2016; Zambonelli et al., 2016; Kuttappan et al., 2017a; Malila et al., 2019), suggesting that muscles showing severe myopathic lesions are usually characterized by down-regulated carbohydrate metabolism and, as a consequence, a reduced content of glycolytic metabolites. Indeed, overall data found within this study corroborate an altered glycogen and glucose metabolic pathway. Considering the extreme hypoxic conditions and inflammatory processes taking place in WB muscles, the reduced content of glycolytic metabolites could be explained by a rerouting of the carbohydrate flow from glycolysis to other metabolic pathways in order to contrast muscle inflammation (Abasht et al., 2016). Moreover, the residual glycogen found at 24h post-mortem suggests that glycolysis occurring after the death of the animal does not stop prematurely because of substrate deficiency.

In the presence of residual glycogen, Matarneh et al. (2018) suggested that pH_u is determined by the activity of phosphofructokinase (PFK), a key regulatory enzyme in the glycolytic pathway. Results concerning PFK activity are reported in Figure 3.18.

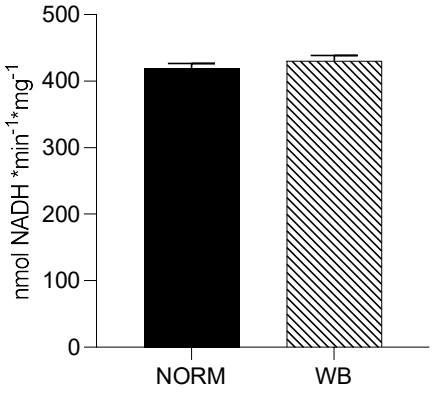

*Figure 3.18 Average phosphofructokinase activity (expressed as µmol NADPH * min^{-1} * g^{-1}) of NORM and WB P. major muscles (n=12/group). Data represent means ± SEM.*

Surprisingly, no differences were detected in PFK activity between affected and unaffected muscles, meaning that PFK is not responsible for arresting post-mortem glycolysis in WB-affected samples. Indeed, G6P content does not accumulate in myopathic muscles. However, England et al. (2014) reported that in some cases the depletion of adenosine nucleotides (ATP, ADP, and AMP) could arrest glycolysis while PFK is still functioning. Results concerning adenosine nucleotides are shown in Figure 3.17. ATP content (Figure 3.19A) was found to be remarkably lower in WB affected meat at 15 min post-mortem ($P<0.001$), with WB samples showing ATP concentration reduced by 50% if compared to NORM group (3.10 vs. 6.51 μmol/g). Intriguingly, WB affected samples showed significantly higher IMP content at 15 min post-mortem. No differences were detected in AMP concentrations (Figure 3.19C), while both ADP (Figure 3.19B) and IMP (Figure 3.19D) contents were found to be significantly lower in WB muscles at 1,440 min post-mortem ($P<0.05$ and <0.001, respectively). The lower IMP content found at 1,440 min within this study corroborates what found by Soglia et al., (2019, in press) and Abasht et al. (2016), who suggest that lower levels of adenosine nucleotides along with higher levels of catabolites such as xanthine and urate might explain a greater nucleotide degradation in myopathic muscles. On the other hand, lower ADP concentration of WB muscle at 1,440 min post-mortem might be the result of the attempt of WB muscles to combat the deficit in ATP concentrations, by generating ATP from 2 molecules of ADP. This reaction also produces one molecule of AMP, which in turn could be deaminated to IMP and NH_3. This might also explain the higher content of IMP in WB muscles at 15 min post-mortem, as a muscle attempt to contrast the lack of ATP in the first minutes after the death. However, the reasons behind the drastic reduction in ATP content might be different. The first hypothesis is related to creatine, a compound that plays a key role in muscular energy metabolism since it is directly involved in ATP synthesis in vivo (Mora et al., 2008). A study conducted by Sundekilde et al. (2017) revealed that dystrophic muscles present significantly lower creatine content, thus suggesting a perturbation in the energy-generating pathway. This result has also been confirmed by Soglia et al. (2019, in press), who found remarkably reduced creatine content in muscles affected by WB myopathy. Thus, being creatine present in lower concentrations in myopathic muscles, it is reasonable to speculate that the ATP-generating pathway might be compromised, resulting in reduced ATP concentrations in the early post-mortem. The second hypothesis concerns the impaired mitochondrial functionality of WB muscles found in

a recent study (Sihvo et al., 2018), where the authors reported that WB affected muscles exhibit mitochondrial swelling, vacuolation and cristae loss, clear indicators of osmotic imbalance and hypoxia. The same authors also suggest that experienced distress such as hypoxia during animal life might provoke higher susceptibility to develop post-mortem alterations. Considering these aspects, since in vivo ATP synthesis happens through mitochondrial respiration, it is logical to postulate that the reduced ATP content found within this study might be partially due to the impaired mitochondria functionality found in WB affected muscles. The drastic reduction in ATP concentrations in the early post-mortem period, along with the remarkably lower glycolytic potential of myopathic muscles, undoubtedly provoked a reduced extent of pH decline in WB samples (i.e. higher ultimate pH). However, ADP and AMP were not depleted by 1,440 min post-mortem, suggesting that adenine nucleotides alone were not responsible for arresting post-mortem glycolysis.

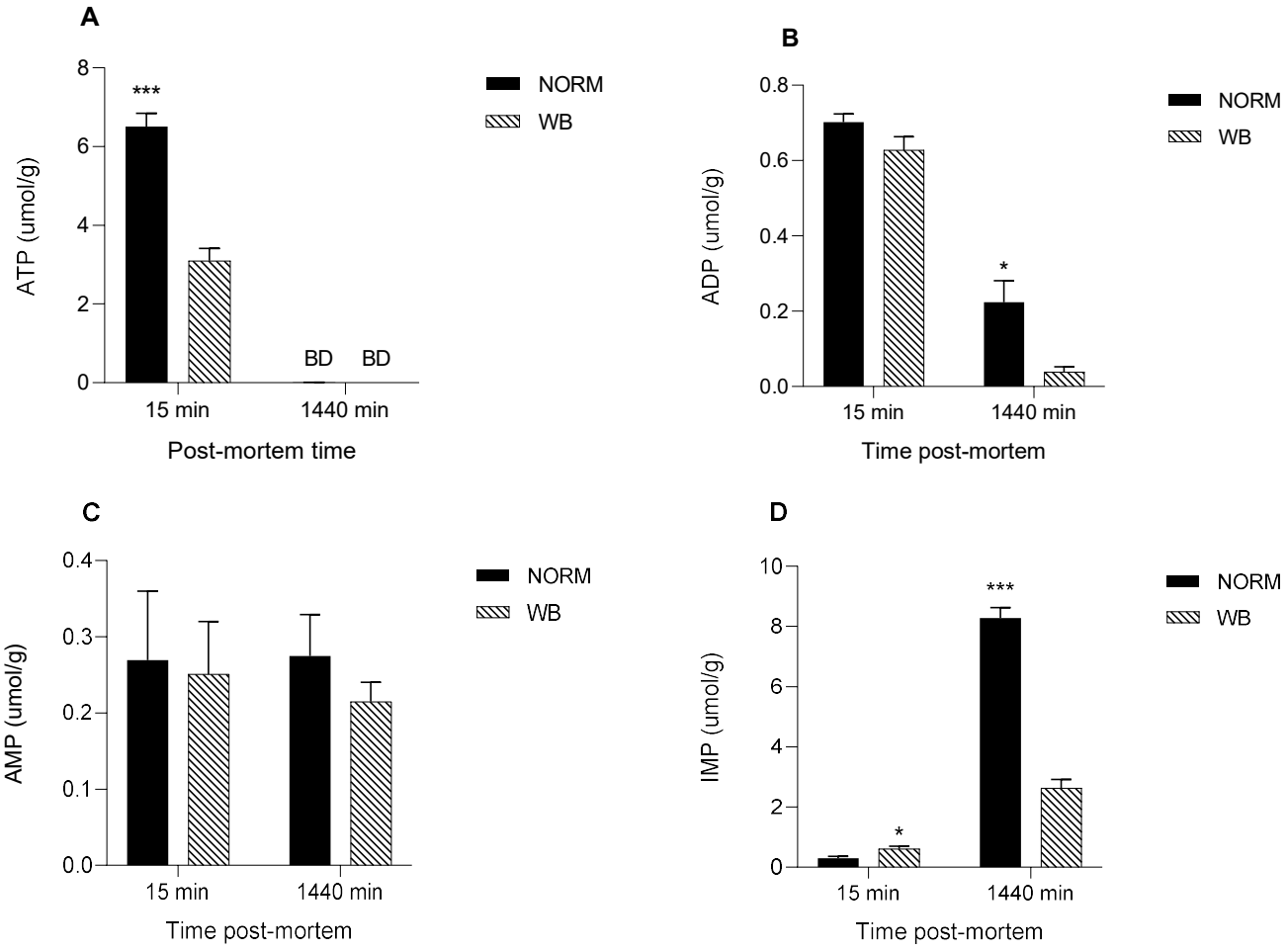

*Figure 3.19 Average ATP (A, µmol/g), ADP (B, µmol/g), AMP (C, µmol/g) and IMP (D, µmol/g) of NORM and WB broiler P. major muscle (n=12/group) at 15 and 1,440 min post-mortem. Data represent means ± SEM. Asterisks indicate a significant difference within the time point (***=P<0.001; *=P<0.05). BD= below limit of detection.*

Yet, during post-mortem, muscle ability to contrast sudden changes in pH (i.e. muscle buffering capacity) can also impact the ultimate pH of the muscle (Bendall, 1973). The results concerning muscles' buffering capacity are reported in figure 3.20.

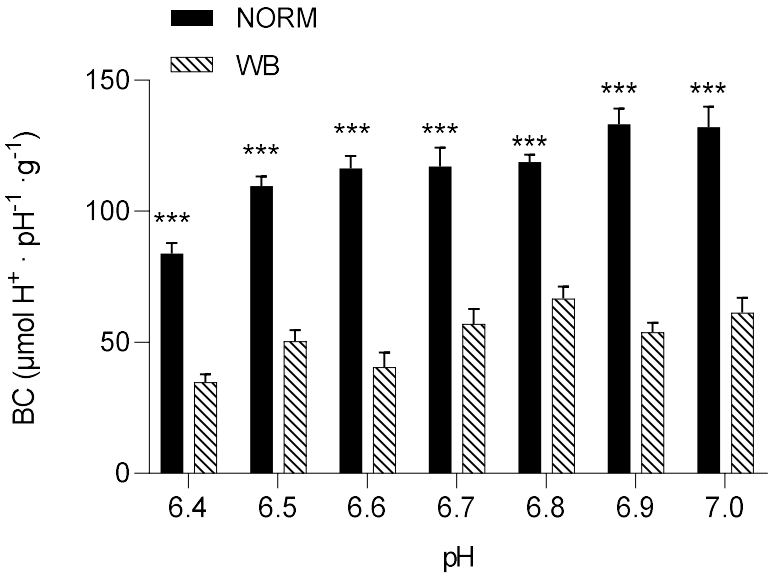

*Figure 3.20 Buffering capacity (µmol $H^+ \cdot pH^{-1} \cdot g^{-1}$) (pH range 6.4-7.0) in NORM and WB P.major muscles. Data represent means ± SEM. Asterisks indicate a significant difference within the same pH (***=P<0.001).*

Buffering capacity was significantly affected by the occurrence of WB myopathy. WB samples exhibited significantly lower buffering capacity (P<0.001) in the pH range of 6.4-7.0, meaning that WB affected muscles have a lower ability to buffer H^+ produced during post-mortem glycolysis. However, a reduced buffering capacity should imply the achievement of a lower ultimate pH, but this is not the case of WB meat. It is well known that muscle buffering capacity is due by half to myofibrillar proteins, while phosphate compounds, as well as histidine-containing dipeptides (e.g. anserine and carnosine), contributed to the other half (Matarneh et al., 2017).

In more detail, anserine and carnosine, in light of their pKa (6.38 and 7.04, respectively), are considered the most important compounds for the maintenance of homeostasis and prevent muscle damage (Davey, 1960; Jung et al., 2013). As in vivo, also during the conversion of muscle to meat anserine and carnosine play a determinant role in counteracting rapid changes in pH. However, a recent study conducted by Soglia et al. (2019, in press) revealed that the concentrations of these compounds were significantly lower in WB muscles compared to unaffected ones. In more detail, anserine and carnosine were found to be reduced respectively by 37.2 and 46.1% in myopathic muscles, corroborating what also found by Sundekilde et al.

(2017). Within this context, it is reasonable to assume that the lower buffering capacity of WB meat is due to its reduced content of buffering compounds.

Considering the overall data, these findings suggest that the reduced content of glycolytic metabolites only partially explains the higher ultimate pH detected in WB muscle. Indeed, residual glycogen found in WB samples at 1,440 min post-mortem, along with unaltered PFK activity, indicated that neither glycogen nor a deficiency of PFK activity was responsible for arresting glycolysis. On the other hand, the absence of G6P at 1,440 min raises the possibility that glycogenolysis was remarkably inhibited. However, the reduced content of G6P can be considered a direct consequence of the lower glycogen concentration detected in WB muscle at 15 min after death. Besides all, it is well established that post-mortem glycolysis is regulated by ATPase activity (Scopes, 1974). Thus, in order to further test the factors contributing to the higher ultimate pH in WB meat, an in vitro system was used to simulate muscle acidification with or without excess ATPase. Using this system, we were able to compare pH decline in affected and unaffected samples with or without 2 units of ATPase under the same environment (Figure 3.21).

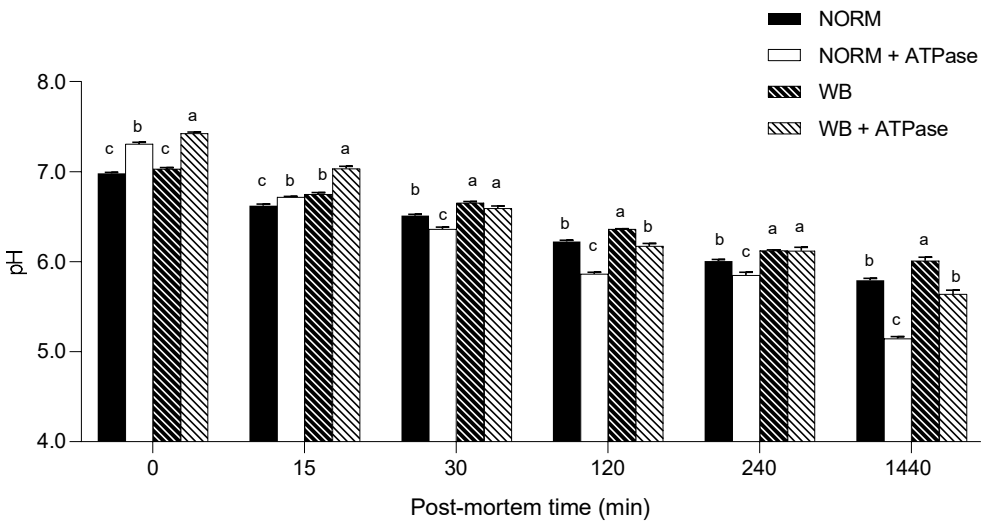

Figure 3.21 Mean pH of the in vitro model (n=6/group). Data represent means ± SEM. a, b, c, d means without a common letter significantly differ within a time point (P<0.05).

The pH of the in vitro system was significantly affected by the addition of ATPase in the buffer. Because muscle samples from both groups were homogenized in the exact same buffer, differences in pH decline should be a function of the incorporated muscle tissue. At both 0 and 15 min post-mortem, the addition of ATPase significantly raised muscular pH, indeed NORM+ATPase and WB+ATPase groups showed significantly higher pH values if compared to their counterparts without ATPase. This

trend is in agreement with a study conducted by Scopes et al. (1974), where the same in vitro system was used. The author explained that the increased muscle pH after the addition of ATPase to the buffer is due to the fact that, immediately before anaerobic glycolysis, H^+ ions are used along with ADP and phosphocreatine to produce creatine and ATP. Thus, for every phosphocreatine split, a certain amount of H^+ ions are absorbed, causing an increase of the pH. However, after the initial slower rate, the addition of ATPase increased the rate of pH decline of NORM+ATPase samples, which indeed exhibited the lowest pH values for each time point. This is likely due to the fact that an excess ATPase to a functioning muscle was added, thus increasing the glycolytic flux and, consequently, lowering the pH.

As regards WB samples, after 30 min post-mortem, the addition of ATPase seemed to increase the rate of pH decline, with the most interesting result being the one at the last time point. Indeed, at 1,440 min reaction vessels containing WB muscle with an excess of ATPase showed significantly lower values if compared to their counterparts without ATPase (5.67 vs. 6.01; $P<0.05$). Furthermore, no differences were detected among NORM and WB+ATPase groups at 1,440 min, meaning that an excess of ATPase can extend in vitro post-mortem glycolysis in WB muscles. Thus, considering the presence of damaged/abnormal myofibers linked to the occurrence of WB disease, it is reasonable to hypothesize that a deficiency and/or a disfunction of muscular ATPases might be accountable for arresting post-mortem glycolysis prematurely in WB muscles. Further, given the abundance of myosin ATPase in skeletal muscle, ATP hydrolysis by myofibrillar component likely drives post-mortem metabolism (Ferguson and Gerrard, 2014). Further studies are needed to investigate if post-mortem metabolism is repressed because of a deficiency rather than a reduced functionality of myosin ATPases.

Taken together, these data suggest that selection programs to improve growth rate have had significant effects on muscle metabolism, in addition to the repercussions exerted on muscular structure and histological profile of P. major muscle belonging to modern fast-growing broilers (Sihvo et al., 2014; Soglia et al., 2016b; Velleman, 2019). Although the effect of WB myopathy on muscle fibers and microstructure has been deeply investigated, just a few published studies took into consideration the evaluation of fiber morphology throughout different zones of P. major muscle (Clark and Velleman, 2017; Soglia et al., 2017). In this scenario, this study also aimed at evaluating the histological profile of 4 different regions of pectoral muscles

affected by WB myopathy. In more detail, both the surface and the depth sections of the cranial (anteroventral (AV) and anterodorsal (AD), respectively) and caudal zones (posteroventral (PV) and posterodorsal (PD)) have been considered. Figure 3.22 contains representative images showing the morphological structure of AV, AD, PV and PD regions of broiler P. major affected by WB myopathy. In agreement with previous studies (Sihvo et al., 2014; Clark and Velleman, 2017; Soglia et al., 2017), AV region exhibited severe myodegenerative lesions, including several extensive macrophages infiltrations, accumulation of fat (i.e. lipidosis) and connective tissue (i.e. fibrosis), as well as the presence of necrotic fibers surrounded by inflammatory cells infiltrates. Indeed, AV region seems to have the highest macrophages infiltrations if compared to all other locations.

Affected areas are interested by the replacement of damaged fibers with the deposition of connective tissue, showing a complete re-organization of the skeletal muscle structure. Moreover, according to Sihvo et al. (2014), a severe thickening in the interstitial tissue separating muscle fiber bundles as well as nuclei internalization have been widely found in AV area. The main causes associated with this severely altered histological profile have been attributed to the reduced microcirculation due to the excessive development of the pectoral muscle, which consequently led to impaired muscle fiber metabolism and oxygen supply (Kuttappan et al., 2013b). Among published studies, contrasting results are available about the effect of WB myopathy on the histologic characteristics of breast muscle's inner cranial region, probably depending on the severity grade of the myopathy. According to Soglia et al. (2017), the AD zone exhibited several myodegenerative traits, such as fibrosis, lipidosis, necrotic fibers surrounded by macrophages infiltrations, and the presence of giant and hypercontracted fibers. The thickening of the endomysial spaces with deposition of connective tissue was observed as well. Moreover, the existence of fibers with small diameters could be the result of fiber lysis, a process taking place in the muscle as a reparative and/or adaptive mechanism against fiber necrosis and degeneration. Indeed, also Clark and Velleman (2017a) found the presence of regenerating myofibers in the same region of P. major muscles affected by WB myopathy.

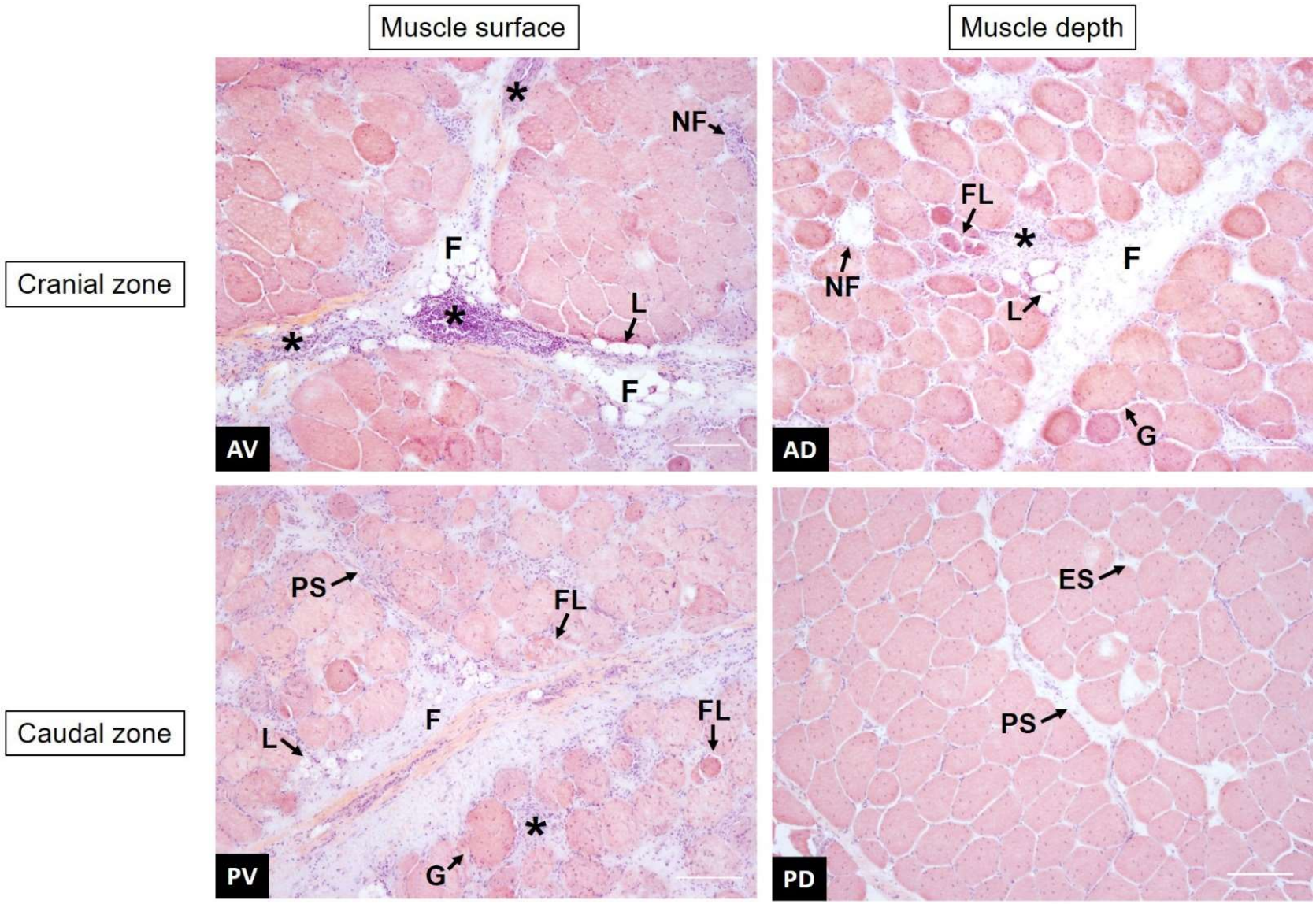

*Figure 3.22 Representative images of WB affected samples collected from anteroventral (AV), anterodorsal (AD), posteroventral (PV) and posterodorsal (PD) positions of pectoralis major muscle. *= inflammatory cells infiltrate; F= proliferation of connective tissue; L= fat tissue deposition; NF= necrotic fiber; FL= fiber lysis; PS= perimysial spacing; ES= endomysial spacing; G = giant hypertrophic fiber; Scale Bar = 150 μm*

In the most severe cases, WB myopathy affects both the cranial and the caudal regions of superficial pectoral muscle (Kuttappan et al., 2016). Indeed, from histological examinations conducted within this study, the PV region showed an overall compromised muscular structure architecture, comparable to what observed for AV region. In addition to fibrosis and lipidosis, also fiber lysis, thickening of the peri- and endo-mysial spaces and macrophages infiltrations surrounding necrotic fibers have been observed as well. These findings are quite conflicting with what observed in the same P. major region by Clark and Velleman (2017a), who reported that the histological profile of PV area was just slightly affected by the occurrence of WB myopathy. However, the divergence detected between the studies might be ascribable to the different severity grades of WB myopathy. Furthermore, based on the current knowledge, no studies have been conducted to investigate the histological traits of caudal deep section of P. major muscle affected by WB. Contrary to other locations, the PD region did not show a severely impaired histological profile. Indeed, neither infiltrating macrophages nor fiber necrosis have been found, while, on the other hand, endo- and peri-mysial spacing, as well as nuclei internalization and endomysial connective tissue proliferation, have been observed. The different degrees of myodegeneration between the upper and the inner caudal sections of the fillet might be attributed to the lower distance from the blood vessels responsible for oxygen translocation, as proposed by Soglia et al. (2017).

Aside from fiber morphology, sarcomere length represents a crucial measure for understanding muscle properties and functionality, to such extent that sarcomere patterns are often used as indicators of myofibril integrity and have been widely used to diagnose muscle pathologies (Moo et al., 2016). Since the rate and the extent of post-mortem muscular acidification influence the degree of myofibrillar contraction (Ertbjerg and Puolanne, 2017), it has been speculated if the extreme stiffness of WB muscles could be partially due to a hypercontraction of sarcomeres caused by an abnormal acidification process. Contrary to what expected, WB affected samples belonging to the AV region (superficial cranial) showed significantly ($P<0.001$) longer sarcomeres if compared to samples collected from the same area of NORM P. major muscles (1.86 vs 1.66 μm, respectively; figure 3.23).

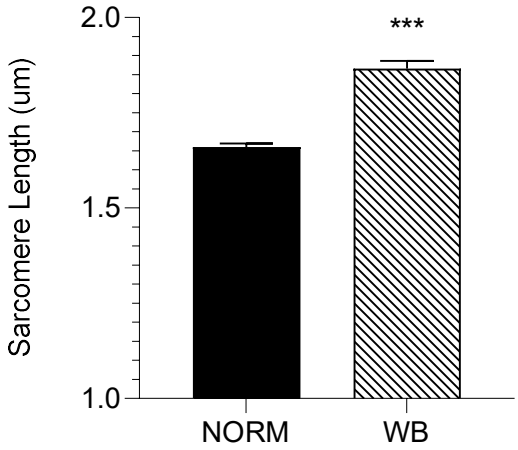

*Figure 3.23 Average sarcomere length (µm) assessed in the AV area 4 of both NORM and WB affected P. major muscles (n=6/group). Data represent means ± SEM. *** = P<0.001.*

This result corroborates what found in a recent study conducted by Sun et al. (2018) and a previous research carried out by Tijare et al. (2016), where the authors explained that the longer sarcomeres detected in the cranial region of WB muscle might be ascribable to the increased collagen content and the loss of muscle fiber that might prevent shortening. In light of the results collected within this study, it could be postulated that the overall reduced content of ATP detected among WB affected sample might result in a defective shortening process. In detail, since the force for shortening is ATP-driven (Ertbjerg and Puolanne, 2017), with a reduced content of ATP present in the muscle, the sarcomeres are less likely to shorten because there is not enough energy to allow muscle contraction (Owens and Sams, 1997). Moreover, a study conducted by Mutryn et al. (2015) suggested that the higher concentration of reactive oxygen species detected in WB muscles may impact calcium release from the sarcoplasmic reticulum, damaging the ability of muscle cells to contract. Thus, it is reasonable to hypothesize that the hypoxic conditions along with the compromised energy-generating pathway of myopathic muscles might have consequences on the degree of muscle contraction, as in vivo, also during post-mortem. Moreover, considering these outcomes, it can be postulated that the typical hardness of WB muscles is not due to muscle fiber hypercontraction, otherwise, it is linked to the excessive collagen deposition as suggested in previous studies (Sihvo et al., 2014; Soglia et al., 2016b). Further, considering that sarcomere length is not uniform across the muscle (Moo et al., 2016), the effect of WB myopathy on sarcomere length of different regions of P.major muscle (AD, AV, PV, and PD) has been investigated (Figure 3.24).

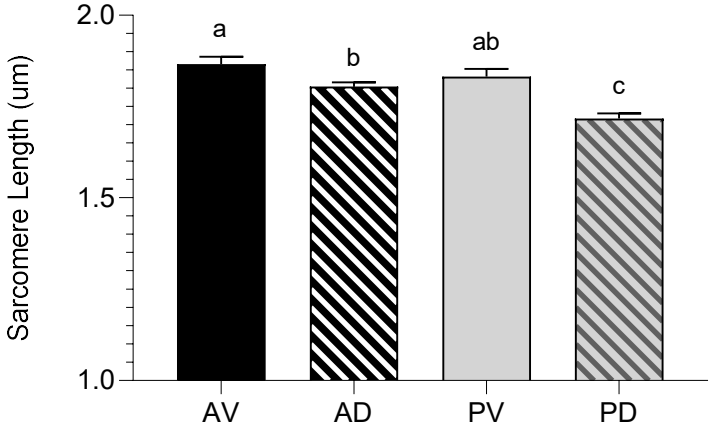

Figure 3.24 Average sarcomere length (μm) assessed in 4 different locations within P. major muscles affected by WB myopathy (n=6/group). Data represent means ± SEM. a-c means without a common letter significantly differ (P<0.05).

Intriguingly, samples belonging to the AV region of WB fillets showed the longest sarcomere length values (1.86 μm), while those belonging to the PD area exhibited the shortest (1.71 μm) and AD and PV displayed intermediate values (1.80 and 1.83 μm, respectively). Further, the superficial sections of both cranial and caudal regions (AV and PV, respectively) showed significantly longer (P<0.05) sarcomeres if compared to their deep counterparts. These divergences detected within muscle's thickness might be attributed to the different conformation and morphology of WB muscles, which usually show out-bulging and swollen areas in both cranial and caudal zones. In addition, considering fiber morphology outcomes, it can be assumed that AV and PV regions showed the most outstanding damages linked to WB disease (i.e. extreme collagen deposition, inflammatory cells infiltrations, endo- and peri-mysial spacing, etc.), might suggesting an overall defective myofiber contraction mechanism in vivo that led to longer sarcomeres.

3.5.4 Conclusions

This study aimed at deepening the knowledge about the effects of WB myopathy on glycolytic metabolites, muscle post-mortem metabolism, as well as the histological profile and muscle fiber contraction degree of four different regions within the P. major muscle, providing new insights about the factors contributing to the higher ultimate pH of myopathic muscles. The significantly higher pH_u of WB muscles was consistent with patterns of lactate formation, while buffering capacity was significantly lower in WB group. The findings suggest that WB reduced glycolytic potential only partially explains the higher ultimate pH, indeed residual glycogen found in WB samples at 24h post-

mortem, along with unaltered PFK activity, indicate that neither glycogen nor a deficiency of PFK activity was responsible for arresting glycolysis. On the other hand, the absence of G6P at 24h post-mortem raises the possibility that glycogenolysis was inhibited in WB muscles. Further, the dramatic reduction in ATP concentrations in the early post-mortem period suggests a defective ATP-generating pathway and might be responsible for the reduced extent of pH decline in WB samples. However, ADP and AMP were not depleted by 24h post-mortem, suggesting that adenine nucleotides alone were not responsible for arresting post-mortem glycolysis. Thus, muscle acidification with or without an excess of ATPase has been tested through an in vitro system, showing that excess ATPase can extend post-mortem glycolysis in WB muscles. Moreover, it has been investigated if the extreme stiffness of WB muscles could be related to a hypercontraction of sarcomeres caused by an abnormal acidification process. WB affected samples showed significantly longer sarcomeres if compared to samples collected from the same area of NORM P. major muscles, suggesting that the typical hardness of WB muscles is not due to muscle fibers hypercontraction but it is more likely linked to the excessive collagen deposition typical of this condition. The presence of long sarcomeres (>1.80 μm) in WB muscles could be explained with the compromised energy-generating pathway of myopathic muscles that might have consequences on the degree of muscle contraction, as in vivo, also during post-mortem. In conclusion, in light of all the mechanisms involved in the occurrence of WB defect, it might be complex to define a specific factor contributing to the higher ultimate pH of myopathic muscles. However, considering the overall reduced glycolytic metabolites and the myodegenerative processes associated with WB condition, data suggest that the higher pH_u of WB meat might be the outcome of a drastically impaired energy-generating pathways combined with a deficiency and/or a dysfunction of muscle ATPases, having consequences also on muscle fibers contraction degree.

Chapter 4: General Conclusions

Poultry is the most widely eaten type of meat all over the world. During the decades, the continuous evolution of the market demand for poultry meat along with the increased global population growth has resulted in new selection criteria for broiler chickens. Consumers preferences has shifted from the whole carcass to further processed products, leading to a strong selection toward birds characterized by high growth rate and breast muscle development. The substantial genetic progress made in the past 50 years has resulted in broilers being more sensitive to stress and prone to develop metabolic-related defects, leading to significant changes in muscle structure and metabolism. From a morphological-functional point of view, genetic improvement has caused muscle fibers' hypertrophy as well as profound changes in their structural, functional and metabolic characteristics. Modern meat-type hybrids are more susceptible to the development of muscle disorders, whose incidence in recent years have reached worrying levels for the poultry industry. Although growth-related muscle abnormalities (i.e. white striping, wooden breast and spaghetti meat) represent a significant problem for the meat industry, effective strategies aimed at reducing their magnitude and severity have not been identified yet.

Considering this scenario, this PhD project aimed at deepening the existing knowledge on the effects of muscular aberrations on poultry meat quality and early-post-mortem metabolism, in order to provide new insights useful to counteract the onset of these myopathies or, at least, to manage abnormal meat for further processing. In addition, since artificial selection played a determinant role also on muscle metabolic pathways, the second goal of this project was to investigate post-mortem metabolism of fast-growing birds, in order to deepen the existing knowledge about the factors contributing to the intra- and inter-specie variations in energy-generating metabolism and other biochemical processes having possible implications on meat quality.

Overall, the findings of this research can be summarized in the following relevant outcomes:

- on the contrary of what observed for broiler chickens, the occurrence of WS only partially affects turkey meat nutritional and technological properties. Therefore, turkey breast affected by WS should not be downgraded, thus allowing to reduce the economic losses that are linked to the occurrence of this myopathy. Considering the divergences found among chicken and turkey breast meats as affected by WS, it seems

reasonable to hypothesize a diverse specie-specific physiological response to the pressure in muscle tissue induced by the artificial selection that, albeit led to the occurrence of WS, exerts just limited implications on meat quality.

- The occurrence of muscular abnormalities in broiler chickens mainly affects the superficial section of P. major muscle, while the deep one is just poorly affected. This outcome represents an important clue for a possible strategy to reduce abnormal meat downgrading and discarding. Indeed, since the deep section is slightly damaged by the occurrence of abnormalities, one possible strategy could be to separate the ventral and dorsal layers of the abnormal fillets and distinctly handle them, in order to take advantage of their respective distinctive traits. As an example, the most affected section (i.e. ventral layer) could be used for the manufacturing of further processed products (i.e. sausages, nuggets, etc.), where the addition of ingredients along with the grinding processes could somehow mask meat impaired technological properties. On the other hand, the dorsal section could be used for the manufacturing of further products where the use of the whole muscle is required such as meat skewers, grilled sliced chicken breast, etc.
- Among all the muscular abnormalities, the occurrence of WB one exerts the most profound and prominent effect on broiler meat quality traits. WB meat is characterized by a massive collagen content associated to an increased stromal protein denaturation enthalpy, even if collagen cross-linking degree was not affected. Thus, it can be stated that the distinctive hardness of raw WB is due to an increased amount of collagen and not to a fibers' hypercontraction condition. Indeed, WB affected fillets show longer sarcomeres if compared to samples collected from the same area of unaffected P. major muscles. The presence of long sarcomeres might be linked to the overall compromised energy-generating pathway of myopathic muscles that might have consequences on the degree of muscle contraction, as in vivo, also during post-mortem.
- WB affected muscles show a remarkably higher ultimate pH, that can be considered a hallmark of this myopathy in broilers. In light of all the mechanisms involved in the occurrence of the defect, it might be complex

to define a specific factor contributing to the higher ultimate pH of myopathic muscles. However, considering the overall reduced glycolytic metabolites and the myodegenerative processes associated with WB condition, it can be assumed that the higher pH_u of WB meat might be the outcome of a drastically impaired energy-generating pathways combined with a deficiency and/or a dysfunction of muscle ATPases, having consequences also on muscle fibers contraction degree.

- When muscles acidification kinetics are taken into consideration, a great intra- and inter-specie variability has been noticed. Chicken P. major muscle shows a greater rate and extent of post-mortem acidification if compared to leg muscles, but a quite high pH_u if compared to that of meat of other avian and mammal species with similar fiber composition. The residual glycogen found in chicken breast muscle at 24h post-mortem further suggests that glycolysis could have continued. Thus, it can be assumed that the greater concentration of histidine compounds of breast meat might provide a sort of resistance to the post-mortem pH decline, thus explaining the slower-than-expected rate and extent of muscular acidification of P. major muscle and justifying the intra-and inter-specie differences existing between the pH_u values.

The present PhD thesis dealt with two relevant topics concerning poultry meat quality. As regards the study of chicken meat post-mortem metabolism, the research permitted to examine the intra-specie variations existing during post-mortem acidification and sharpen the current knowledge by assessing the role of buffering compounds in defining ultimate pH of muscle characterized by different in vivo energy metabolism. Since chicken breast meat usually shows an ultimate pH quite higher if compared to meat belonging to other species with a similar fiber composition (i.e. turkey breast, pork loin, rabbit loin), other studies should be addressed to the evaluation of muscular acidification of the aforementioned meats, in order to further establish and consolidate the relationship between histidine compounds and muscle post-mortem metabolism.

Overall, this thesis also allowed to deepen the existing knowledge about muscular abnormalities, a relevant issue affecting poultry industry worldwide due to their high incidence rates and negative implications on meat quality. The results obtained with standard and advanced techniques permit to highlight the peculiar effect

of each muscular abnormality on meat quality and provide information that might be useful in managing abnormal meat for further processing. Moreover, the study of post-mortem metabolism of WB muscles allowed to investigate the energy-generating pathways of fillets affected by muscular abnormalities, providing new knowledge about the biochemistry controlling the early post-mortem of abnormal muscles. Despite all the efforts conducted by researchers during the past ten years, the causative mechanisms responsible for the occurrence of these abnormalities are still uncertain and further investigation aimed at inhibiting the onset of abnormalities or at least alleviating the symptoms and consequences on meat quality should be carried out. Within this context, taking a step back seems unavoidable by now, as further genetic improvements might be restrained by muscle biological potential and animal welfare concerns. In this scenario, since solving the issue at the roots appears complex so far, further researches should be addressed at finding processing solutions able to reduce the negative implications of abnormalities on meat qualitative and technological properties. Within this context, it could be intriguing set up a study aimed at evaluating the effectiveness of the application of innovative and emerging technologies such as pulsed electric fields and ultrasounds in order to improve the qualitative traits of meat affected by muscular abnormalities.

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