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Effect of exercise on glycolytic potential in *M. pectoralis major* in normal and wooden breast (WB) broilers

Niina Kalmu

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HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

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Tiivistelmä – Referat – Abstract
Poultry meat has become popular in human diet and to secure the growing demand of poultry meat, breeders have developed broilers with fast growth and high breast muscle yield. Because of this genetic selection modern broilers are suffering from various quality defects in their breast muscles, one of which is called WB myopathy. The aim of the thesis was to study whether exercise, WB status or age have effects on ultimate pH and glycolytic potential (sum of total glucose and lactic acid content, given as lactate) in *M. pectoralis major* of modern broilers chickens. A total of 154 post-rigor breast muscle samples of male broilers of hybrid Ross 308 were used in this study.

Broilers were divided randomly into three groups (A-C). Group A birds were not subjected to any exercise during the experiment. Group B birds lived 25 days exactly like group A but after that the drinking water dispensers was elevated to a higher level. Group C birds had their drinking water dispensers elevated to a 5 cm higher level after three days, and it was risen stepwise to 25 cm during the study. Broilers were slaughtered randomly from different groups at 20, 30 or 41 days old. Group B results were disregarded due to technical problems. Initial and ultimate pH, total glucose, free and in glycogen, and lactic acid contents for glycolytic potential, were determined from the breast muscle samples.

The results showed that there were no differences between groups A and C ($p > 0.05$). There were differences between normal and WB cases in ultimate pH (pH_u; $p = 0.000$), total glucose content ($p = 0.000$), lactate content ($p = 0.007$) and glycolytic potential ($p = 0.000$), but the pH values 5 minutes after death showed no significance (pH_5; $p = 0.190$). There were differences in pH_u, total glucose and glycolytic potential ($p = 0.000$), and lactate ($p = 0.007$), between ages 20, 30 and 41 days old birds. As the severity of WB increased in the breast muscle, the pH_u increased and total glucose, lactate content and glycolytic potential decreased. The correlations showed significant relationships between WB score and all the above variables ($p < 0.000$).

The results of the current study show that exercise does not have an effect on the levels of carbohydrates in *M. pectoralis major* of modern broiler chickens, but WB status and increasing slaughter age result a decrease in glycolytic potential and increase in ultimate pH.

Avainsanat – Nyckelord – Keywords
Glycolytic potential, pH, wooden breast, exercise

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Tiivistelmä – Referat – Abstract

Siipikarjanlihasta on tullut suosittu ihmisten ruokavaliossa. Kasvavan kysynnän turvaamiseksi kasvattajat ovat kehittäneet nopeakasvuisia ja suuren lihassaannon tuottavia broilereita. Tämän vuoksi nykyaikaiset broilerit kärsivät rintalihasten erilaisista laatuongelmista, joista yhtä kutsutaan WB-myopatiaksi. Maisteritutkielman tarkoituksena oli tutkia liikunnan, WB: n vakavuusasteen ja iän vaikutuksia lopulliseen pH-arvoon ja glykolyyttiseen potentiaaliin nykyaikaisen broilerin Ross 308 hybridin *M. pectoralis major* -lihaksessa. Kokonaisuudessaan 154 post-rigor-näytettä tutkittiin.

Broilerit jaettiin satunnaisesti kolmeen ryhmään (A-C). Ryhmän A linnuille ei tehty mitään harjoitusta kokeen aikana. Ryhmän B linnut elivät 25 päivää täsmälleen kuten ryhmä A, mutta sen jälkeen juomaveden annostelijat nostettiin korkeammalle tasolle. Ryhmän C lintujen juomavesiannostelijat nostettiin 5 cm korkeammalle tasolle kolmen päivän kuluttua, ja se nousi tutkimuksen aikana vaiheittain 25 cm: iin. Broilerit teurastettiin satunnaisesti eri ryhmistä 20, 30 tai 41 päivän ikäisinä. Ryhmän B tuloksia ei otettu huomioon useimmissa analyyseissä teknisten ongelmien vuoksi. Rintalihasnäytteistä määritettiin alkuperäinen ja lopullinen pH, glukoosin kokonaismäärä sekä maitohappopitoisuus glykolyyttisen potentiaalin laskemista varten.

Tulokset osoittivat, että ryhmien A ja C välillä ei ollut eroja (p > 0,05). Normaalien ja WB-tapausten välillä oli merkitseviä eroja lopullisessa pH:ssa (pHu; p = 0,000), glukoosin kokonaispitoisuudessa (p = 0,000), glykolyyttisessä potentiaalissa (p = 0,000) ja laktaattipitoisuudessa (p = 0,007), mutta pH-arvoissa 5 minuuttia kuoleman jälkeen (pH_5) ollut eroja (p = 0,190). Teurastusiän välillä 20, 30 ja 41 päivän ikäisissä oli erot lopullisessa pH:ssa (pH_u), glukoosin kokonaispitoisuudessa ja glykolyyttisessa potentiaalissa (p = 0,000) sekä laktaattipitoisuudessa (p = 0,007). Kun WB:n vakavuus lisääntyi rintalihaksessa, pH_u nousi ja glukoosin kokonaispitoisuus, laktaattipitoisuus ja glykolyyttinen potentiaali laskivat. Korrelaatiot osoittivat riippuvuuden WB:n vakavuuden ja tutkittujen muuttujien välillä (p <0,000).

Tämän tutkimuksen tulokset osoittavat, että liikunnalla ei ole vaikutusta hiilihydraattipitoisuuksiin broilereiden rintalihaksessa, mutta WB:n vakavuusasteen ja teurastusiän kasvu johtavat glykolyyttisen potentiaalin laskuun ja lopullisen pH-arvon nousuun.

Avainsanat – Nyckelord – Keywords
Glykolyyttinen potentiaali, pH, puurinta, liikunta

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
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PREFACE

The master´s thesis is a part of wider study called *Wing Flapping project* that started in autumn 2019 in the Faculty of Agriculture and Forestry of University of Helsinki, Finland, led by Associate Professor Per Ertbjerg and Research Director, Professor Emeritus Eero Puolanne. Other researchers that participated in the study were Doc. Jere Lindén and ELL Minna Söderström from the Faculty of Veterinary Medicine and ETT Liisa Keto and Gabriel da Silva Viana from LUKE. The research was funded by Kyllikki and Uolevi Lehikoinen Foundation. Atria Oyj and HKScan Oyj also participated funding of the research. The aim of the main project was to study the effect of exercise (wing flapping) on the prevalence of WB myopathy in *M. pectoralis major* (*MPM*) muscle of broiler chickens. In the study, broilers were raised, terminated and the samples were collected in the premises of Large animals Research Unit (KEK) at University of Helsinki. Collected muscle samples were analysed at the premices of Helsinki University laboratory. The study was carried out from October to November 2019. Because of the Coronavirus pandemic, the analyses and finalizing the thesis took place between 2019 and 2021.

I want to express my gratitude to Per Ertbjerg for his support and advice. I am sincerely grateful to my supervisor Eero Puolanne for his kind support and supervision, and tremendous guidance. I especially want to thank also Yuemei Zhang for her guidance and encouragement with the chemical analyses and Taru Rautavesi for her technical assistance and guidance in the procurement of laboratory and chemical materials. I also want to thank Jere Lindén, Minna Söderström, Liisa Keto, Gabriel da Silva Viana, Jasmin Brandes, Xinyue Dong, Outi Brinck, Jian Lyu, Binbin Li, Minnamari Edelmann, Mikko Kangas and Antti Knaapila for their help and wonderful collaboration. I would like to express my profound gratitude to my family, friends, and co-students for their ultimate support, understanding and encouragement along this path.

Seinäjoki, March 2021

Niina Kalmu

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ABBREVIATIONS

ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BC	buffering capacity
Glucose-1-P	glucose-1-phosphate
Glucose-6-P	glucose-6-phosphate
MPM	*M. pectoralis major*
NAD^+	nicotine adenine dinucleotide oxidized form
NADH	nicotine adenine dinucleotide reduced form
pH_u	ultimate pH 24 hours *post mortem*
pH_5	pH 5 minutes *post mortem*
PFK	phosphofructokinase
SE	standard error of mean
VO_{2max}	maximal oxygen uptake
WB	wooden breast

1. INTRODUCTION

Poultry meat has become popular in human diet because of its low fat and high protein content (Petracci et al., 2019). To secure the growing demand of poultry meat and to keep the meat price low, poultry breeders have developed broilers with fast growth and high feed conversion (Norring et al., 2019). The genetic selection of fast-growing broilers is causing quality impairments called white striping (WS), wooden breast (WB) and spaghetti meat (SM) which are causing damages and losses in the poultry industry.

The size of broiler breast muscle, *M. pectoralis major* (*MPM*) has increased in the past decades to maximize the yield, so that the muscle may grow too large in relation to the size of broiler (Zuidhof et al., 2014). High breast muscle yield of modern broiler hybrids results in fiber hypertrophy that is compromising blood and oxygen supply to the muscle leading to the development of hypoxia (Sihvo et al., 2018; Petracci et al., 2019). Sihvo et al. (2014) suggest that the genetic selection towards high breast muscle yield together with the fast growth rate, has probably further increased the susceptibility of the modern broiler to oxidative stress.

As capillaries provide oxygen for the muscle and maintain the blood supply, they also remove by-products from muscle (Nelson & Cox, 2005). A decreased capillary density compromises the afore-mentioned functions. Capillary density in muscles, fiber type composition and the right ratio of heart size to the total muscle mass of the animal determine the availability of oxygen in muscles (Barbut, 2015; Pösö & Puolanne, 2005). Broilers with high breast muscle yield have been reported to have lower capillary density in the *MPM* muscle due to increased myofiber diameter (Hoving-Bolink et al., 2000). Sihvo (2019) found that in the breast muscle, the capillary number per unit area decreases with the age of birds. In the same study, it was found that the vessel number was significantly lower in WB cases in unaffected areas of breast muscles, compared to normal unaffected breast muscles of birds in the same age group.

Previous studies have suggested that the high growth rate and increased final weight of the broilers are linked to prevalence of WB myopathy (Sihvo et al., 2014; Petracci et al., 2015). The main contributing factor has been suggested to be the hypoxic state in muscle when there is lack of sufficient oxygen and the carbohydrate metabolism is not able to function properly, due to

decreased capillary density and increased myofiber diameter (Mutryn et al., 2015; Kuttappan et al., 2016; Petracci et al., 2019; Sihvo, 2019).

Energy metabolism in living animal is aerobic, but however, in stressful situation and in heavy exercise or rapid movements, anaerobic metabolism provides additional short-term fuel for muscle to function. Anaerobic metabolism is initiated alongside aerobic metabolism for muscle to overcome the high energy needs during intensive movements, like e.g. wing flapping (Nelson & Cox, 2005). After the stress or strenuous exercise, the lactate derived from anaerobic energy production in muscle fibers will be turned back to glycogen in liver by using aerobically produced adenosine triphosphate (ATP), or it will be used aerobically in aerobic muscle fibers, heart, brain and in other tissues (Juel, 1997; Pösö & Puolanne, 2005).

Phosphocreatine (creatine phosphate) in muscle provides energy by regenerating ATP from adenosine diphosphate (ADP), for about ten seconds lasting activities, and after that glucose is in blood and animal tissues the primary carbohydrate from which the energy is obtained (Meléndez-Hevia et al., 1993; Nelson & Cox, 2005). Glucose is stored as glycogen (up to tens of thousands of glucose units/molecule) that is deposited and stored in skeletal muscles and in liver. In liver it is mainly used for maintaining the blood glucose level. In skeletal muscles, glycogen remains untouched if blood circulation provides glucose and oxygen enough for muscle contraction, but if not, glycogen will be used for energy production.

Anaerobic metabolism takes place also after slaughter when muscle turns into meat. The muscle maintains most fiber functions after the animal death and the energy needed is derived from skeletal muscle glycogen since blood circulation cannot not provide glucose anymore. In living muscle, energy can be produced also from fatty acids (only aerobically), and from proteins. Relationships of the uses of fatty acids or carbohydrates varies between animal species, ruminants using relatively more fatty acids and pigs and poultry relay more on carbohydrates.

Glycolytic potential is the sum of glycogen, glucose (and glucose-1-P + glucose-6-P) and lactate *post mortem*, expressed in $2 * \text{glucose content} + \text{lactate content}$ [µmol/g]. Glycolytic potential acts as an indicator of antemortem glycogen level in muscle. (Monin & Sellier, 1985). It is generally known that there is strong negative correlation between the ultimate pH and glycolytic

potential (Sibut et al., 2011). The glycolytic potential has also been shown to be highly heritable (Le Bihan-Duval et al., 2008). Gene expression profiling has been used for characterization of differences in normal and WB-affected cases (Sibut et al., 2011; Velleman & Clark, 2015; Soglia et al., 2016; Abasht et al., 2016; Zambonelli et al., 2016; Malila et al., 2019; Zhao et al., 2020). Their studies confirm that between normal and WB cases, there are differences in the genes that control carbohydrate metabolism enzymes.

Previously, the effect of endurance exercise on pH and glycolytic potential has not been studied in broilers. In humans and rats, the effect of endurance exercise has been found to increase the size and number of mitochondria and mitochondrial enzyme activities in the skeletal muscles that are involved in the exercise, as well as to increase the GLUT4 glucose transporter activity (Ingjer, 1979; Howley et al., 1995; Carter et al., 2000; Holloszy, 2008). In WB-affected breast muscles compared to normal breast muscles the ultimate pH (pH_u) is higher and glycolytic potential is lower (Baldi et al., 2020b).

The thesis is a part of wider study called *Wing Flapping project* that started in autumn 2019 in the Faculty of Agriculture and Forestry of University of Helsinki, Finland, led by Associate Professor Per Ertbjerg and Research Director, Professor Emeritus Eero Puolanne. The aim of the main project is to study the effect of exercise (wing flapping) on the prevalence of WB myopathy in *MPM* muscle of broiler chickens.

This thesis focuses on carbohydrate metabolism of the Wing Flapping study. The literature part includes a short review of WB myopathy and effect of exercise on metabolism, focusing on anaerobic energy metabolism in muscle. The literature part focuses on the concepts of glycogen, glycogenolysis, glycolysis, glycolytic potential, buffering capacity and pH and makes an attempt to summarize their relationships. Carbohydrate metabolism in general, is described in great detail in every textbook of biochemistry, and therefore, will not be treated much here. However, material that directly targets the broiler muscle was collected.

The aim of the current thesis was to study whether exercise, WB status or age have effects on ultimate pH and glycolytic potential (sum of total glucose and lactic acid content, given as lactate) in *MPM* muscle of modern broilers chickens.

2. LITERATURE REVIEW

2.1 Wooden breast myopathy

In the past decade, quality defects termed white striping (WS), wooden breast (WB), and spaghetti meat (SM), have raised the attention among researchers as well as in industry due to their high incidence in fast growing broiler lines (Figure 1). These quality defects have raised many concerns regarding economic issues as well as animal welfare.

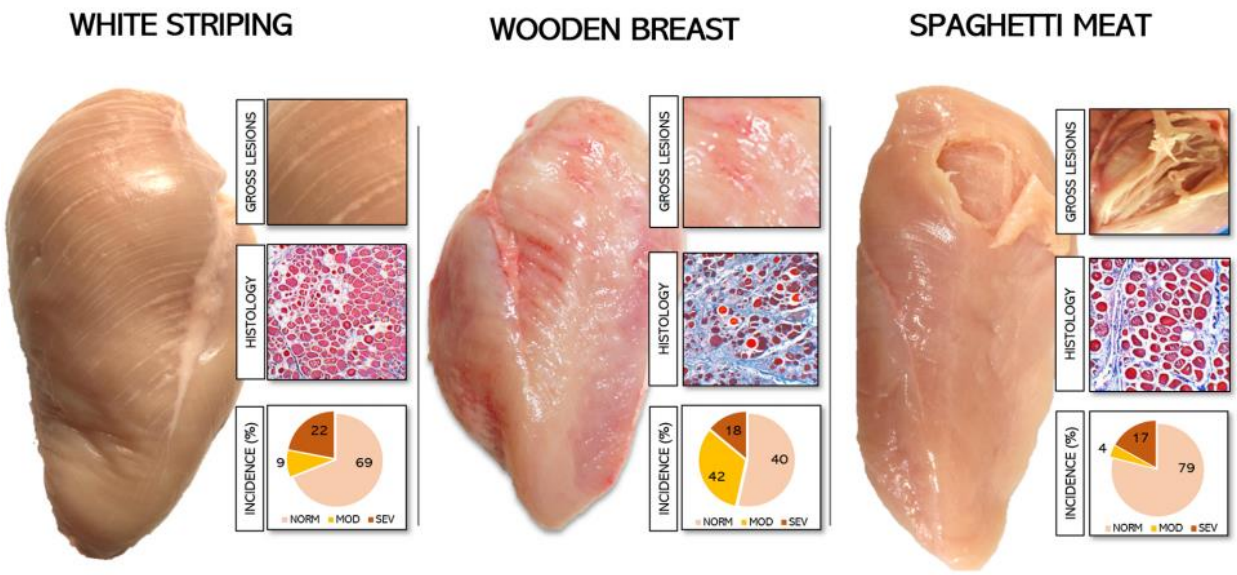

Figure 1. Muscular abnormalities affecting the *M. pectoralis major* (*MPM*) muscle of fast-growing broiler chickens (Baldi et al., 2020a).

WB myopathy was defined macroscopically and histologically by Sihvo et al. (2014). Studies of hardened consistency of broiler breast muscle *(MPM)* have been conducted since 2012 when first signs were observed (Kuttappan et al., 2012; Petracci., 2013; Sihvo et al., 2014). The myopathy starts to develop at the age of two weeks and is causing paleness and hardness that is sometimes accompanied with white striations (Sihvo et al., 2017). In some cases, a clear viscous fluid appears on the surface of the *MPM* muscle. Lesion begins focally and develops into a diffuse lesion affecting the whole breast muscle. WB myopathy is restricted to the *MPM* muscle of broiler chickens and is detected *post mortem* with no clear antemortem signs related to the condition.

2.2 Oxygen uptake capacity and effect of exercise

The effect of endurance exercise on aerobic metabolism has been studied in human athletes and some animals, mostly in rats (Howley et al., 1995; Holloszy, 2008; Carter et al., 1999). No such studies, however, have been performed in broilers, but the results can be anticipated to apply with broilers as well. Endurance exercise aims to improve maximal oxygen uptake, develop ability to tolerate changes in the cardiovascular respiratory system and in the neuromuscular system with a high intensity or long duration exercise. During endurance exercise, the previously mentioned systems are trained by upsetting a normal state of equilibrium. Maximal aerobic power (VO_{2max}) and blood lactate response to exercise are used as measures of the ability of cardiovascular respiratory system to carry oxygen and the ability of muscles to use it for energy production under extreme exercise (Howley et al., 1995). Endurance exercise has been found to improve the oxygen uptake capacity, increase the cardiac output, and the oxygen diffusion from blood. It has also been found to increase the size and number of mitochondria and mitochondrial enzyme activities in the skeletal muscles that are involved in the exercise, as well as to increase the GLUT4 glucose transporter activity (Ingjer, 1979; Carter et al., 2000; Holloszy, 2008).

2.3 Carbohydrate metabolism in broiler muscles

Aerobic capacity decreases in order cattle > pig > poultry (Pösö & Puolanne, 2005). The high ratio of heart size to total muscle mass of the animal and high capillary density in muscle thus guarantees the sufficient oxygen transport into the muscles. Animals having imbalance in the ratio of heart size to total muscle mass and low capillary density may suffer from an insufficient oxygen supply to muscles, especially to the white-type ones. Fiber type composition is a relevant factor in relation to the aerobic capacity of the muscle due to fact that white fibers have less myoglobin (for oxygen storage) and lower content of oxidative enzymes compared to red fibers (Barbut, 2015).

Papinaho et al. (1996) showed that broiler breast muscle consists entirely fast contracting type IIB white muscle fibers 100 % (myosin-ATPase method of Brooks & Kaiser, 1970). White muscle fibers are fast twitch and highly glycolytic by nature (Barbut, 2015). Muscles composed of these muscle fibers are capable of short burst of activity in “fight or flight” situations and are easily fatigued. The capillary density is low because they do not rely on fast nutrient transfer.

During intensive physical exercise and/or mental stress, these muscles rely on glycogen and anaerobic glycolysis for additional energy production. Based of the above, it can be concluded that broilers have a low aerobic capacity, which exposes their muscles easily to anaerobic status during intense movements.

2.3.1 Glycogen

A highly branched polysaccharide molecule glycogen may consist of up to approximately 55 000 α-D-glucose units which are linked to the core protein glycogenin (Meléndez-Hevia et al., 1993). These glucose units are linked together by α-1,4-glycosidic bonds and branches are generated by α-1,6-glycosidic bonds (Figure 2).

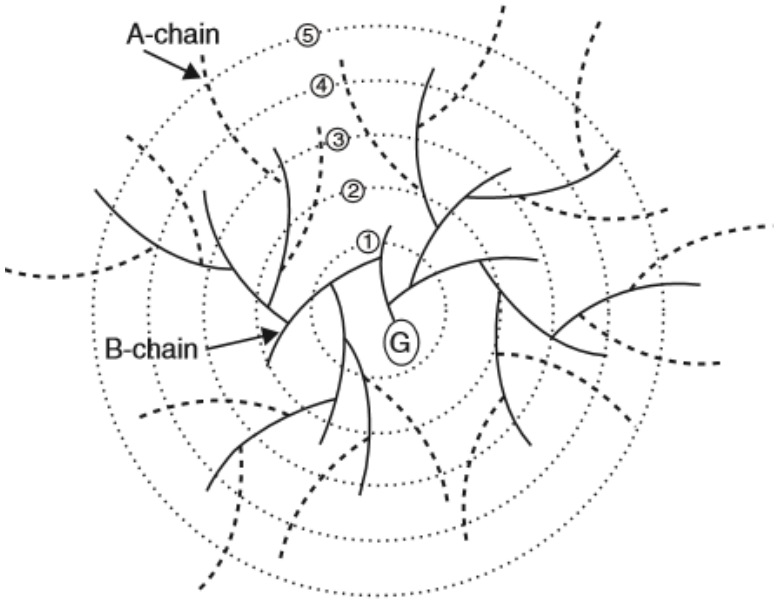

Figure 2. Glycogen molecule according to Meléndez-Hevia et al. (1993). Figure adopted from Immonen (2000).

The branched structure of the glycogen molecule provides many points of attack for glycogen phosphorylase, allowing more glucose to be released simultaneously (Meléndez-Hevia et al., 1993). Glycogen phosphorylase can release a maximum of 34.6% of glucose from outermost tier of glycogen. The full outermost tier of the glycogen molecule contains 50% of the total carbohydrate of the molecule, like all the remaining tiers of the rest when the previous outermost has been used.

Meléndez-Hevia et al. (1993) tested theoretical optimizations of molecular design of the structure of glycogen. They found that the volume is minimal, when there are 13 glucose units in linear chains in a tier, and each chain has two similar 13-unit branching sidechains forming the next tier. At the same time, this optimal structure maximizes the number of reducing ends of the outermost tier in each level of glycogen content. Structural studies of the actual molecule show that the theoretical optimum is the same than the real structure in muscles.

In skeletal muscle, glycogen exists in two forms, acid-soluble, high molecular weight macroglycogen (M.W. 10^7) and acid-insoluble, low molecular weight proglycogen (M.W. 400,000), which differ in the ratio of protein to carbohydrate (Graham et al., 2010). In pigs, proglycogen is utilized in severe stress as well as *post mortem* and macroglycogen is utilized during aerobic exercise, and less so *post mortem* (Rosenvold et al., 2003). The utilization of proglycogen and macroglycogen has not been studied in poultry. The existence of different glycogen pools has, however, been challenged.

Glycogen phosphorylase and glycogen debranching enzyme (GDE) are bound to the glycogen molecules, and both are responsible of glucose release (Graham et al., 2010). In addition, there are several other enzymes and proteins bound to the glycogen each having their own role (Figure 3).

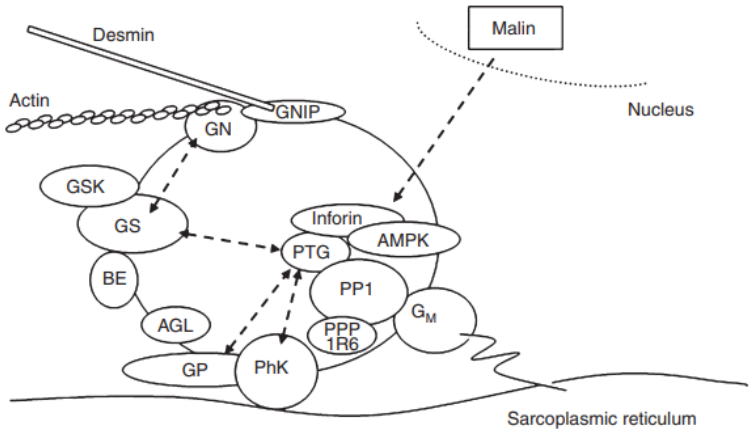

Figure 3. A schematic summary of proteins known to interact with glycogen. GDE, glycogen debranching enzyme; AMPK, AMP-activated protein kinase; GBE, glycogen branching enzyme; GM and PP1R6, regulatory subunits of protein phosphate 1 PP1; GN, glycogenin; GNIP, glycogenin-interacting protein; GS, glycogen synthase; GSK, glycogen synthase kinase; GP, glycogen phosphatase; PhK, phosphorylase kinase; PTG, protein targeting to glycogen (Graham et al., 2010).

Glycogen analyzes have described been as challenging, because glycogenolysis and glycolysis can proceed very rapidly during sample preparation and analysis (beef samples; Immonen & Puolanne, 2000). Immediate freezing with liquid nitrogen after obtaining fresh muscle samples, prevents the onset of glycogenolysis and glycolysis. Glycogen is hydrolyzed to glucose with strong acid (HCl) (Lowry & Passoneau, 1973) at 100 °C. Total glucose content is determined enzymatically based on NAD-linked assay catalyzed hexokinase and glucose-6-phosphate dehydrogenase to determine the total carbohydrate content as glycogen, glucose-1-P, glucose-6-P, and free glucose.

2.3.2 Glycogenolysis

Muscle phosphocreatine (creatine phosphate) provides energy as a form of adenosine triphosphate (ATP) for ten seconds instant explosive activities (Nelson & Cox, 2005). Creatine kinase breaks the phosphate bond and liberates the phosphate from phosphocreatine which is then added to adenosine diphosphate (ADP) to make ATP. The reaction produces only one ATP without any oxygen available, however, the reaction is not actually involved in carbohydrate metabolism but in the production of ATP. When phosphocreatine has run out, then glycogenolysis and subsequent glycolysis will begin.

In situation of hard physical exercise or mental stress, muscle is strongly activated by adrenaline and noradrenaline. Glycogen is preferred as the primary energy source of stress, triggered by adrenaline (Nelson & Cox, 2005; Puolanne & Immonen, 2014). When a cascade involving adrenaline release, starts the glycogenolysis, glycogen phosphorylase will be activated. Glycogen phosphorylase cuts glucose-1-P units from glycogen molecule with an enormous speed, and the reaction does not consume ATP. Action of glycogen phosphorylase limits to 4 residues before the branching point (limit dextrin).

GDE disassembles the branch point by degrading the 1,6-bonds, for enabling the further action of glycogen phosphorylase (Nelson & Cox, 2005). In one end of the GDE molecule there is transferase, which removes three glucose units and places them at the end of another chain. The remaining glucose unit is released by 1,6-glucosidase, located in the other end of GDE, as free glucose and, and the glucose will be phosphorylated immediately. GDE will then move further

away allowing glycogen phosphorylase to function again. Due to these series of reactions, muscle glycogen will be degraded to glucose-1-P. When glycogen is used as an energy source, phosphoglucomutase converts glucose-1-P to glucose-6-P. Consequently, muscle fiber provides glucose units without having a need to rely on glucose from the blood. Glucose cannot escape from muscle fiber because it is phosphorylated, thus being charged. However, if blood circulation provides glucose for energy production, hexokinase or glucokinase moves a phosphoryl group from ATP to glucose molecule to produce glucose-6-P and ADP, and the reaction consumes one ATP.

Kylä-Puhju et al. (2004) studied the activity of GDE in porcine muscles and found that the activity of GDE is not particularly affected by the pH in the pH range from 5.5 to 7 but the activity of GDE was close to zero at temperatures below 15 ºC. Ylä-Ajos et al. (2007) studied the activity of GDE and glycogen phosphorylase in chicken *pectoralis superficialis* (*M. pectoralis major, MPM*) and *quadriceps femoris* (*M. quadriceps femoris, QF*) muscle *post mortem*. They found that in white very glycolytic *MPM* muscle, glycogen phosphorylase activity was higher than in dark more oxidative *QF* muscle, while the GDE activity was lower in white *MPM* muscle than in dark *QF* muscle. This supports the hypothesis that low GDE activity might restrict the rate of rapid glycogen degradation and rapid pH decline in situation of hard physical or mental stress. Thus, fast chilling stops the rapid pH decline because otherwise, too much lactate and protons would accumulate in the muscle.

2.3.3 Glycolysis

In glycolysis, glucose-1-P derived from glycogenolysis is converted to pyruvate and three molecules of ATP are generated (Nelson & Cox, 2005). The reaction chain produces from nicotinamide dinucleotide the reduced form (NADH), 2 pyruvate and 2 protons. When lactate is formed, protons are absorbed by pyruvate and NADH is oxidized to NAD^+ which is required for the continuing of anaerobic glycolysis (Figure 4, Equation 1). ATP is formed as ADP molecule is phosphorylated. In aerobic conditions, NADH and protons are used in mitochondria to produce ATP.

$$2\ CH_3COCO^-{}_2 + 2\ H^+ + 2\ NADH \rightarrow 2\ CH_3CH(OH)CO^-{}_2 + 2\ NAD^+ \qquad (1)$$

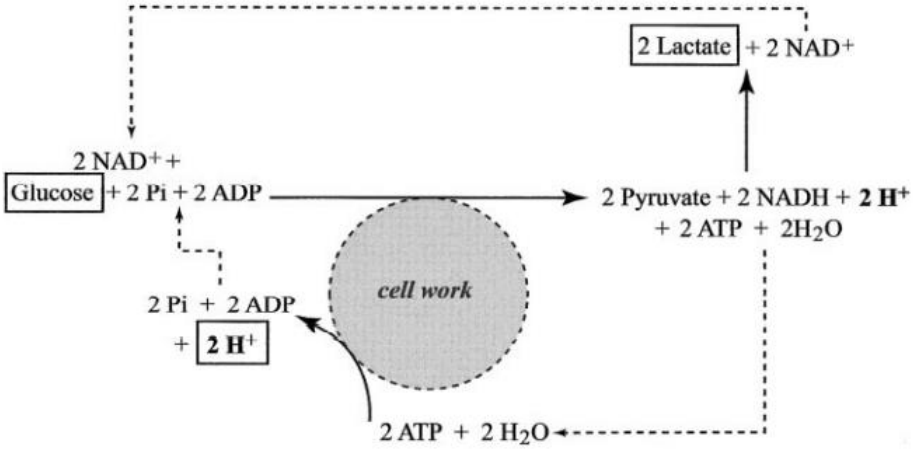

Figure 4. Glycolytic regeneration of ATP coupled to ATP hydrolysis as would be the case during skeletal muscle contraction with no ATP contribution from mitochondrial respiration. Source of the protons that can accumulate in the cytosol is ATP hydrolysis. Balance of these reactions leaves the molecules highlighted by rectangles (Robergs et al., 2004).

Lactate + proton (lactic acid) are the end products of anaerobic metabolism which lowers the pH of the blood and the muscle (Robergs et al., 2004; Nelson & Cox, 2005). One glucose molecule from blood results 2 lactate molecules (Equation 2).

$$\text{Glucose} \rightarrow 2 \text{ lactate} + 2H^+ + 2 \text{ ATP (or 3 when glucose from glycogen)} \quad (2)$$

Lactate and protons accumulate due to the anaerobic breakdown of glucose. In living animal, monocarboxylate transporters (MCTs) carry lactate and protons through the cell membrane from the higher concentrations towards the lower concentration (Pösö & Puolanne, 2005). When lactate is transported to the liver, it is used for aerobic syntheses of glycogen or glucose-6-P. After slaughter (death), the circulatory system no longer works, and so lactate, and protons will be trapped in blood and especially in muscle tissues.

Lactate dehydrogenase (LDH) enzymes turn pyruvate to lactate and vice versa. Lactate dehydrogenases occur as five tetrameric isoenzymes (LDH-1 ... LDH-5) in which four units are composed of muscle-type (M) or heart-type (H) chains (Markert, 1963; Nelson & Cox, 2005). M-type changes pyruvate to lactate and H-type changes lactate to pyruvate. LDH in broiler breast muscle has not been studied much, however it can be assumed that LDH-5 (M_4) is predominant in the white, anaerobic breast muscles, which facilitates a high capacity to produce lactate, thus allowing the continuation of glycolysis (Dawson et al., 1964). Abasht et al. (2016)

found that lactate content was reduced in WB-affected *MPM* muscle compared to normal *MPM* muscle. The same phenomenon was observed by Malila et al. (2019) and Baldi et al. (2020b) in samples taken 24 hours *post mortem*. However, Malila et al. (2019) found no differences in lactate levels in 20 minutes *post mortem* nor found Baldi et al. (2020b) 15-minutes *post mortem*.

The pKa of lactic acid is 3.86, which means that at the physiological pH range, it is almost completely dissociated to a lactate ion and a proton (Nelson & Cox, 2005). When pH increases with one unit, ratio of lactate to lactic acid increases tenfold (e.g., pH 3.86 1 lactate :1 H^+; pH 5.86 100:1; pH 6.86 1000:1; Equation 3).

$$log \frac{c_{lactate^-}}{c_{lactic\ acid}} = pH - pKa \quad (3)$$

According to this equation, when the pH has decreased to 5.86, lactic acid is no longer about 1 ‰ of total lactate, like in living muscle. Puolanne and Kivikari (2000) found that the lactate content of post-rigor broiler breast muscle is about 100 µmol/g.

Phosphofructokinase (PFK) is the enzyme in glycolysis that catalyzes the conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP (Scheffler & Gerrard, 2007). Fructose 1,6-bisphosphate is the molecule for continuing and speeding the glycolysis. England et al. (2014) found that glycolysis begins to slow down at pH 6 and stops near pH of 5.5. When pH decreases from 5.9 to 5.5 in white glycolytic muscles of porcine and broiler, the activity of PFK decreases, which may stop the glycolysis, but however, Baldi et al. (2020b) found no differences in PFK activity between normal and WB muscles, meaning that PFK is not responsible for arresting postmortem glycolysis in WB-affected samples. Instead, their data show that the reduced glycolytic potential of WB muscles only partially explains the higher pH_u of WB, as residual glycogen along with unaltered PFK activity suggests that neither glycogen nor a deficiency of PFK is responsible for arresting glycolysis prematurely. Baldi et al. (2020b) claim that the dramatic reduction in ATP concentrations in the early postmortem period suggests a defective ATP-generating pathway that might be responsible for the reduced pH decline in WB muscles.

Nakamura (1970) studied the effect of exercise on *MPM* and *M. biceps femoris* in White Leghorn female chickens. In that study the exercise was applied to the birds only just before slaughter. The results showed that preslaughter exercise increased the lactic acid content in blood to almost threefold (heparin solution method of Barker & Summerson, 1941), compared to that in non-exercised control group (Table 1).

Table 1. Lactic acid content from chicken blood µg/ml (Nakamura, 1970).

Control	210±100
Exercise	610±110

Glycogen content was determined from the muscles immediately after slaughter and 8 hours *post mortem* (anthrone reagent method of Roe & Daily, 1966). Immediately after slaughter, glycogen content was a half in exercised *MPM* muscle compared to that in control group (Table 2). In exercised *M. biceps femoris* muscle the difference was even higher, compared to control group. However, the glycogen contents of the muscles determined immediately after slaughter were surprisingly small. After 8 hours from slaughter, almost all glycogen was depleted from both muscles of both exercised and control group.

Table 2. Glycogen content (mg/g) and ultimate pH of breast and leg muscles (Nakamura, 1970).

		Glycogen content (mg/g)		Ultimate pH
		Immeditely after death	8 hours post-mortem	
Control	Breast	8.4±0.2	≒0	5.7±0.1
	Leg	1.2±0.5	≒0	6.0±0.1
Exercise	Breast	4.2±0.9	≒0	6.1±0.1
	Leg	0.3±0.3	≒0	6.5±0.1

Decreased glycogen content after exercise in both muscles elevated the pH_u values of both muscles. Exercised muscles reached their pH_u value more rapidly than muscles that were not exercised (Figure 5).

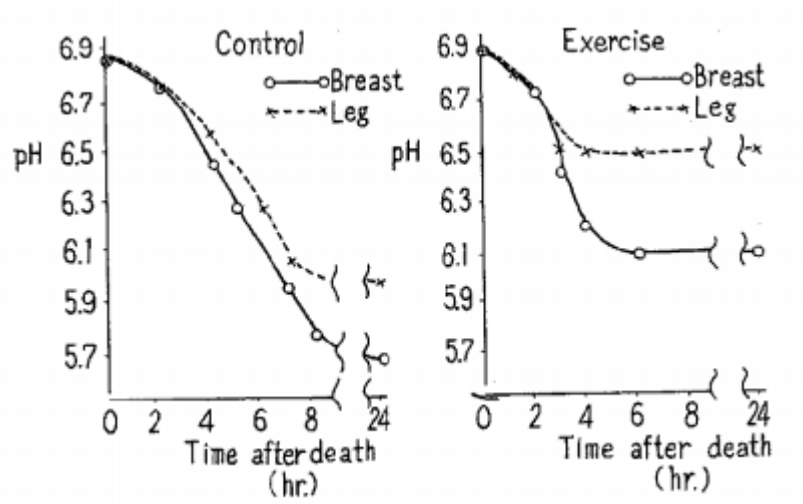

Figure 5. Changes in pH of breast and leg muscles during storage (Nakamura, 1970).

However, calculations of Nakamura's results show that the glycogen contents are in control breasts 47 / legs 7 µmol/g and in exercised breasts 23 / legs 2 µmol/g. Lactate contents of the muscles were not given. Surprisingly, at start, all pH values were 6.9, and pH_u values were 5.7 / 6.0 and 6.1 / 6.5, respectively. Taking the buffering capacity into the account, this indicates that the values did not fit well with calculational values, derived from buffer capacity, but the glycogen values are too low.

2.3.4 Glycolytic potential

Glycolytic potential is the sum of glycogen, glucose (and glucose-1-P + glucose-6-P) and lactate *post mortem*, expressed in 2 ∗ glucose content + lactate content [µmol/g]. Glycolytic potential acts as an indicator of antemortem glycogen level in muscle. (Monin & Sellier, 1985). Glycogen content in muscle of resting well fed porcine and bovine is approximately 80-100 µmol/g (1.5 to 1.8%). The glycolytic potentials have been studied more in cattle and pigs. For broiler, there is limited knowledge. However, Papinaho et al. (1996) found that glycolytic potential in broiler *MPM* muscle is 76.8 ± 2.9 µmol/g at 7 to 10 minutes *post mortem* (Table 3), but however, there is ambiguity in the calculation of glycolytic potential in the study.

Table 3. The biochemical and meat quality parameters for broiler breast fillets at 7–10 minutes *post mortem* (Papinaho et al., 1996).

	Mean ± SEM	N
Citrate synthase, μmol/(g min^{-1})	4.91±0.15	57
Lactate dehydrogenase, μmol/(g min^{-1})	1331±30	56
Glycogen, mg/g muscle	2.42±0.17	50
Lactate, μmol/g muscle	46.9±2.2	50
Glycolytic potential, μmol lactate equivalent/g muscle	76.8±2.9	50
pH	6.69±0.01	77
R-value	0.907±0.001	50
Color - L*	54.8±0.4	80
Color - a*	2.20±0.11	80
Color - b*	4.00±0.26	80
Cooking loss, %	18.3±0.3	80
Shear force, kg/g	9.67±0.33	80

Ylä-Ajos et al. (2007) studied the glycolytic potential of chicken pectoralis superficialis (*M. pectoralis major, MPM)* and quadriceps femoris (*M. quadriceps femoris, MQF*) muscles 25 min after stunning (*post mortem*) and found that glycolytic potential in *MPM* muscle was 116 ± 2.5 μmol/g (Table 4), which was higher than what Papinaho et al. (1996) found (Table 3).

Table 4. GDE and Phos activity, glycolytic potential, pH 25 min after stunning and ultimate pH in chicken *quadriceps femoris muscle* and *pectoralis superficialis* muscle (Ylä-Ajos et al., 2007).

	Quadriceps femoris				Pectoralis superficialis					
	Mean	Min	Max	*n*	Mean	Min	Max	*n*	SEM	*P*
GDE (Δabs/Δ min)	0.048	0.020	0.077	25	0.014	0.006	0.025	24	0.002	***
Phos (U g^{-1} muscle)	5.9	1.9	10.0	10	18.0	10.0	30.1	10	1.2	***
Glycolytic potential (mmol lactate equivalent kg^{-1} muscle)	56.2	33.1	89.9	25	116.6	90.1	134.7	25	2.5	***
pH_1	6.88	6.59	7.10	25	6.70	6.42	6.95	25	0.02	***
pH_u	6.89	6.14	7.24	25	6.03	5.77	6.43	25	0.04	***

SEM = standard error of mean, *** $P < 0.001$.

Recently however, Baldi et al. (2020b) studied the differences in glycolytic potential between normal and WB muscles 15 minutes and 24 hours *post mortem*. The results showed that the glycolytic potential was approximately from 90 to 100 μmol/g in normal unaffected *MPM* muscle, and from 60 to 70 μmol/g in WB-affected *MPM* muscle. Glycolytic potential values are calculated and approximated from Figure 7.

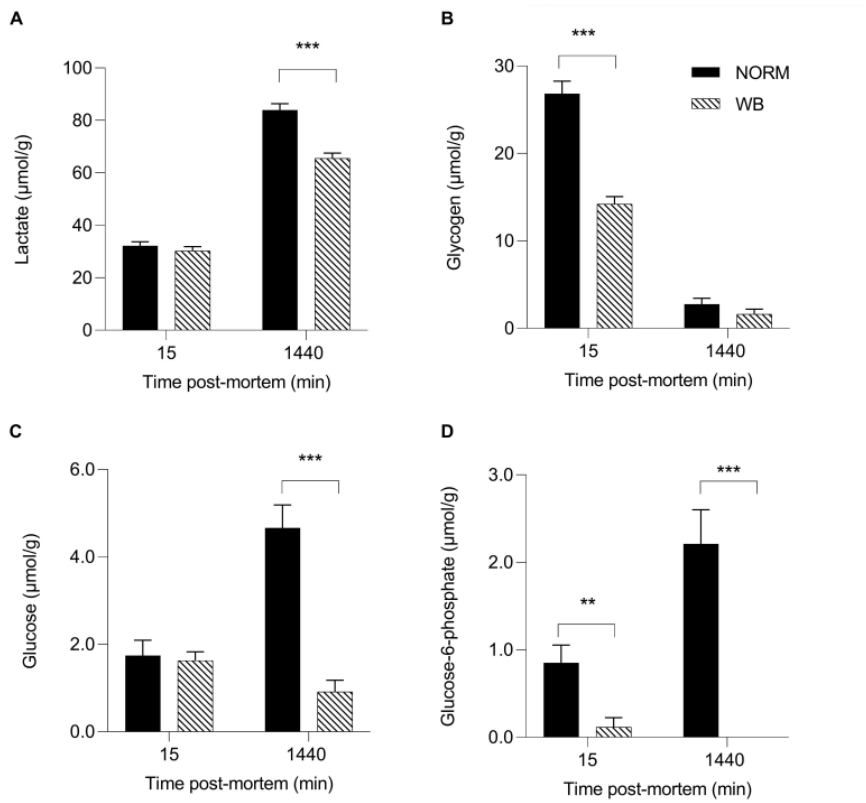

Figure 7. Average lactate (A, µmol/g), glycogen (B, µmol/g), glucose (C, µmol/g), and glucose-6-phosphate (D, µmol/g) of unaffected (NORM) and wooden breast (WB) broiler pectoralis major muscles (n = 12/group) at 15 and 1,440 min, *post mortem*. Data represent means ± SEM. Asterisks indicate a significant difference within the time point (***P < 0.001; **P < 0.01) (Baldi et al., 2020b).

The glycolytic potential of WB and normal cases (sum of 15 min and 1140 min combined), glycolytic potential was significantly decreased in WB-affected broiler *MPM* muscle compared to normal unaffected *MPM* muscle (Figure 8).

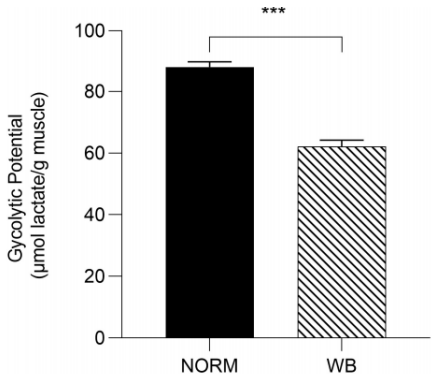

Figure 8. | Glycolytic potential (µmol lactate/g meat) of unaffected (NORM) and wooden breast (WB) broiler pectoralis major muscles (n = 12/group). Data represent means ± SEM. Asterisks indicate a significant difference between experimental groups (***P < 0.001) (Baldi et al., 2020b).

Variations between studies may be due to age of broilers, breed used in the study, feed withdrawal time (fasting) or sampling timing (Table 5). The differences may also be due to the fact, that as muscle fiber size increases, the muscle glycogen content decreases (Berri et al., 2007). Variations of muscle glycogen content depends aside nutrition and exercise on the predominant myofiber type, sex, species, and the stress level of animal before slaughter (Gregory, 1998; Barbut, 2015).

Table 5. Studies of glycogen (or total glucose), lactate, and glycolytic potential of broiler *M. pectoralis major (MPM)* muscle

Reference	Normal	WB
Papinaho et al. 1996		
Breed	Ross 208	
Age (d)	37	
Sex	Mixed sex	
Feed withdrawal time (h)	8 – 10	
Glycogen mg/g (7 –10 min *post mortem*)	2.42 ± 0.17 (26.87 µmol/g)	
Lactate µmol/g (7 –10 min *post mortem*)	46.9 ± 2.2	
Glycolytic potential µmol/g muscle	76.8 ± 2.9	
Ylä-Ajos et al. 2007		
Breed	Ross 508	
Age (d)	35	
Sex	n.g.	
Feed withdrawal time (h)	13.5–16	
Glycogen µmol/g (25 min after stunning)	n.g.	
Lactate µmol/g (25 min after stunning)	n.g.	
Glycolytic potential µmol/g	116.6 ± 2.5	
Baldi et al. 2020b		
Breed	Ross 308	
Age (d)	48	
Sex	Male	
Feed withdrawal time	n.g.	
Total glucose µmol/g (15 min *post mortem*)	29*▫	16*▫
Lactate µmol/g (15 min *post mortem*)	32*▫	28*▫
Glycolytic potential µmol/g (15 min *post mortem*)	90*▫	60*▫
Total glucose µmol/g (24 h *post mortem*)	9*▫	3*▫
Lactate µmol/g (24 h *post mortem*)	82*▫	64*▫
Glycolytic potential µmol/g (24 h *post mortem*)	100*▫	70*▫

*SEM values not given. ▫Calculated/approximated values from the Figure 7. n.g. Not given.

2.3.5 Buffering capacity

Buffering capacity (BC) is a system to resist the change of pH when acid or alkali is added. Muscle fibers have the characteristics of weak acids mainly because they contain phosphate compounds and imidazole groups (Kivikari, 1996). BC capacity keeps the pH at a certain level thus prolonging the time of effective fiber activity so that the muscle enzymes can function at their optimal pH range in anaerobic situations (Pösö & Puolanne, 2005). According to Puolanne and Kivikari (2000), white muscles need remarkably higher BC due to the high glycolytic capacity which may result a high content of lactate. Due of the high BC, white muscle fibers are able to resist pH change when lactic acid is formed in physical or mental stress.

BC in porcine and beef muscles varies from 40 to 60 mmol H^+/(pH*kg), in the range of pH 5.5 – 7.0 (Puolanne & Kivikari, 2000). In broiler, BC is at its maximum level, or especially high in the area, where the pH is high in living muscle as well as *post mortem*. In the study of Puolanne and Kivikari (2000), the mean BC values in pH range 5.5-7.0 for broiler breast muscle *MPM* is 58 mmol H^+/(pH*kg) and for broiler leg-thigh muscle *(QF*) 41 mmol H^+/(pH*kg) (Figure 8.). BC for beef *M. longissimus dorsi* muscle is 51 mmol H^+/(pH*kg), for pork *M. longissimus dorsi* 52 mmol H^+/(pH*kg), for beef *M. triceps brachii* 48 mmol H^+/(pH*kg) and for pork *M. triceps brachii* 45 mmol H^+/(pH*kg). Maximum values (dH^+/dpH) were at pH 6.9, in breast muscle 88 H^+/(pH*kg) and in thigh muscle 56 H^+/(pH*kg) (Figure 9). Variations in phosphate compounds, histidylimidazole residues of myofibrillar proteins and the dipeptides carnosine and anserine explain the differences in BC of different type of muscles as well as in different animal species. White muscle fibers have higher content of histidine compounds which explains the difference comparing to red muscle fibers (Olsman & Slump, 1981). Without this system, accumulation of lactate and protons would mean a very rapid drop in pH.

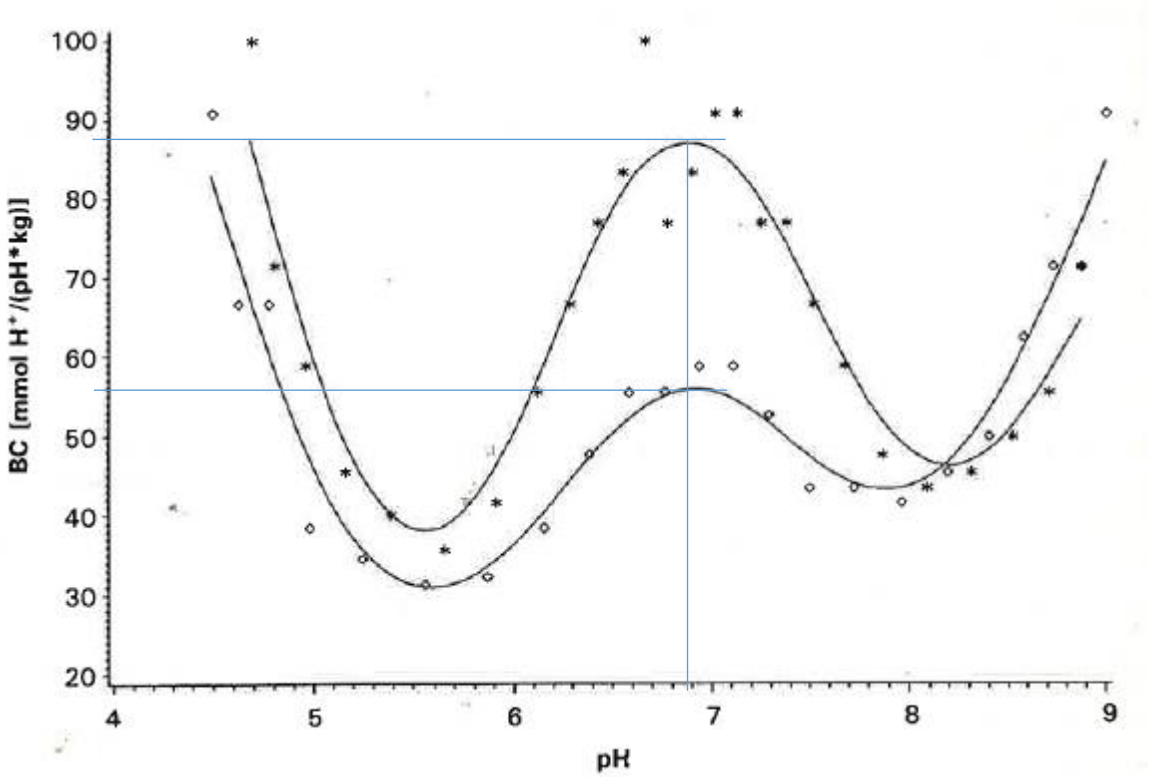

Figure 9. Buffering capacity curves of broiler breast (*) and thigh muscles (▫). Measurements made using a dilution ratio of 10/100 (Kivikari, 1996). Lines drawn in the figure are done to ease the approximations. Maximum values (dH^+/dpH) were at pH 6.9, in breast muscle 88 $H^+/(pH*kg)$ and in thigh muscle 56 $H^+/(pH*kg)$.

Baldi et al. (2020b) studied the BC of WB-affected *MPM* muscles and found that in affected muscles the BC was about half compared to that in normal unaffected muscles (pH range from 6.4 to 7.0, Figure 10). In the study, WB myopathy had a significant effect on the BC of broiler *MPM* muscle which indicates that WB-affected muscles show lower ability to buffer H^+ protons produced during postmortem glycolysis.

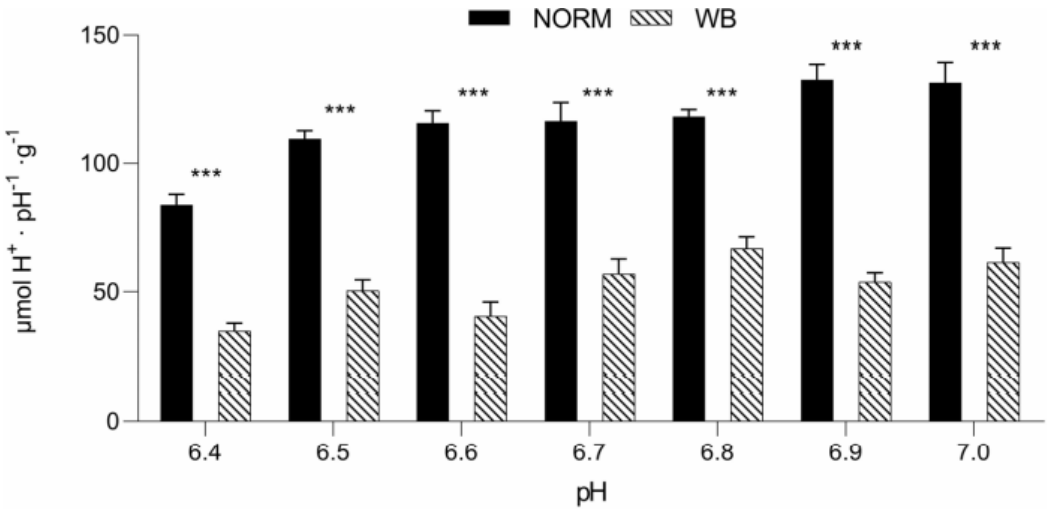

Figure 10. Buffering capacity (µmol H^+ ∗ pH^{-1} ∗ g $^{-1}$) (pH range 6.4–7.0) in unaffected (NORM) and wooden breast (WB) pectoralis major muscles (n = 12/group). Data represent means ± SEM. Asterisks indicate a significant difference within the same pH value (***P < 0.001) (Baldi et al., 2020b).

2.4 pH

The amount of lactate + protons produced from glycogen, determines the postmortem pH of the muscle. In living broiler, the pH is between 6.9 and 7.2 when lactate + proton content is less than 1 mmol H^+/(pH*kg). In heavy exercise, the pH value of living muscle may fall to 6.2-6.4 in less than 30 seconds (Pösö & Puolanne, 2005). The pH in a living muscle does not fall below 6.2 (Robergs et al., 2004). Fear, stress, or fights before slaughter deplete the glycogen storages of the muscle. This leads to low glycogen content at the time of slaughter and high lactate + proton content ultimately leading to low pH value (Gregory, 1998). The pH of pre-rigor muscle samples obtained can be determined by using Na-Iodoacetate method. Iodoacetate inhibits the enzyme glyceraldehyde-3- phosphate dehydrogenase and arrest glycolysis and the consequent pH fall which occurs in anaerobic tissue (Jeacocke, 1977).

Several researchers have studied the differences in pH values between normal and WB cases (Table 6). The ultimate pH value of broiler breast muscle seems to have increased during the years (Papinaho, 1996, vs. others). As well as the size of the broiler has increased (Zuidhof et al., 2014; Petracci et al., 2019). Recent studies show that in WB-affected cases, the pH is higher than in normal breast muscle.

Table 6. Studies of pH values of *M. pectoralis major (MPM)* muscle in normal and wooden breast (WB) broilers.

Reference	Normal	WB	Moderate WB	Severe WB
Papinaho, 1996				
MPM, pH_u	5.75 ± 0.01			
Mudalal et al. 2015				
MPM, pH_u	5.80 ± 0.01	5.87 ± 0.01		
Trocino et al. 2015				
MPM, pH_u	5.84*	5.85*		
Chatterjee et al. 2016				
MPM, pH_u	5.88 ± 0.12		6.04 ± 0.13	6.03 ± 0.14
Tasoniero et al. 2016				
MPM, pH_u	5.92 ± 0.04	6.04 ± 0.02		
Zambonelli et al. 2016				
MPM, pH_u	5.87 ± 0.03	6.06 ± 0.03		
Dalle Zotte et al. 2017				
MPM, pH_u cranial	5.91 ± 0.12			6.03 ± 0.12
MPM, pH_u caudal	5.86 ± 0.13			5.91 ± 0.13
Kuttappan et al. 2017				
MPM, pH_u (6wk age)	5.89 ± 0.03			6.04 ± 0.03
MPM, pH_u (9wk age)	5.93 ± 0.02			6.15 ± 0.04
Wold et al. 2017				
MPM, pH_u (day1 & 2) whole fillet	5.99 ± 0.12			
MPM, pH_u (day 3) upper 1 cm	6.3 ± 0.10		6.3 ± 0.16	6.3 ± 0.09
Xing et al. 2017				
MPM, pH_u	5.87 ± 0.02	6.00 ± 0.02		
Bowker et al. 2018				
MPM, pH_u	5.92 ± 0.16			6.07 ± 0.18
Cai et al. 2018				
MPM, pH_u	5.79 ± 0.02	5.98 ± 0.02		
Chen et al. 2018				
MPM, pH_{3h}	6.00 ± 0.04	6.00 ± 0.09		
MPM, pH_{24h}	6.00 ± 0.08	5.99 ± 0.04		
Dalgaard et al. 2018				
pH_u intramuscular				
day 0	5.94 ± 0.14		5.99 ± 0.12	6.06 ± 0.13
day 1	5.85 ± 0.17		5.92 ± 0.15	6.08 ± 0.10
day 2	5.82 ± 0.13		6.02 ± 0.07	6.01 ± 0.15
MPM, pH surface				
day 0	6.18 ± 0.11			6.28 ± 0.09
day 2	6.12 ± 0.08			6.24 ± 0.11
day 5	6.15 ± 0.08			6.34 ± 0.09
day 7	6.24 ± 0.05			6.39 ± 0.10
Baldi et al. 2019				
MPM, $pH_{surface}$ (24h)		5.87 ± 0.07		
MPM, pH_{depth} (24h)		5.80 ± 0.08		

Table 6. Continues

Reference	Normal	WB	Moderate WB	Severe WB
Baldi et al. 2020b				
MPM, pH (15min)	6.70*	6.80*		
MPM, pH (24h)	6.00*	6.32*		
de Almeida Assunção et al. 2020				
MPM, pH_u	5.87 ± 0.01		6.02 ± 0.01	6.03 ± 0.01
Xing et al. 2020				
MPM, pH_{3h}	6.22 ± 0.02	6.25 ± 0.02	6.28 ± 0.02	6.39 ± 0.02
MPM, pH_{24h}	5.86 ± 0.04	5.95 ± 0.04	5.98 ± 0.04	6.05 ± 0.04

*SEM values not given.

Baldi et al. (2020b) studied the pH of normal and WB broilers and found no differences in pH_{15} (15minutes *post mortem*) between WB-affected and normal *MPM* muscles. However, differences were detected in pH_u (24 hours *post mortem*) showing that the pH_u was higher in WB-affected muscles compared to that in normal muscles (Figure 11).

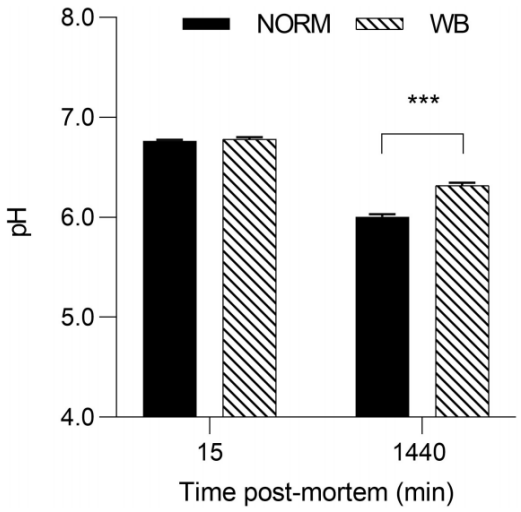

Figure 11. Average pH of unaffected (NORM) and wooden breast (WB) broiler pectoralis major muscles (n = 12/group) at 15- and 1,440-min *post mortem*. Data represent means ± SEM. Asterisks indicate significant difference within the time point (***P < 0.001) (Baldi et al., 2020b).

2.5 Summary of the literature review

Broiler breeding aims for fast growth and high muscle yield which is why the modern broilers are suffering from various quality defects in their breast muscles, one of which is called WB myopathy. High breast muscle yield of modern broiler hybrids results in fiber hypertrophy by increasing the myofiber size. On the other hand, because broiler *MPM* muscle consists of 100% of IIB white muscle fibers, the capillary density is low. This ultimately may lead to a development of hypoxia in the muscle: the energy metabolism of the muscle will not be able to function properly as capillaries cannot provide the necessary the blood supply to maintain the sufficient oxygen level and to remove the end-products of the metabolism from muscle.

The energy metabolism of the muscle is always aerobic in a long run, but however, in situation of high physical and/or mental stress the energy metabolism of the muscle turns glycogenolytic/anaerobic. Anaerobic metabolism is initiated alongside aerobic metabolism for muscle to overcome the high energy needs. Anaerobic metabolism takes place also after death of the animal when blood circulation stops, and blood does not provide glucose and oxygen for muscle to function anymore. Muscle maintains most fiber functions after death by with the help of ATP from muscle glycogen. Glycogen phosphorylase and GDE release glucose from glycogen molecule in series of enzymatic of reactions, ultimately leading to formation of pyruvate. To keep the glycolysis ongoing and to obtain ATP, NAD^+ is needed, and it is generated from the reaction of pyruvate to lactate. The formation and accumulation of lactate and protons lowers the pH of the muscle, but BC of the muscle prevents the pH going to a too low level.

Endurance exercise has been found to improve the cardiac output, increase the size and number of mitochondria and mitochondrial enzyme activities in the skeletal muscles that are involved in the exercise, as well as to increase the GLUT4 glucose transporter activity. WB myopathy has been found cause increase in pH_u values and decrease in the glycolytic potential in *MPM* muscle.

3. EXPERIMENTAL RESEARCH

3.1 Aims and objectives

The aim of the main project is to study the effects of long-term exercise (wing flapping) on the prevalence of WB myopathy in *MPM* muscle of broiler chickens. The aim of the current thesis is to study whether exercise, WB status or age have effects on ultimate pH and glycolytic potential (sum of total glucose and lactic acid content, given as lactate) in *MPM* muscle of modern broilers chickens.

The objectives of the study were to investigate:

1. Does exercise have an effect on pH_u and glycolytic potential.
2. Does the WB have an effect on pH_u and glycolytic potential.
3. Does the age have an effect on pH_u and glycolytic potential.

3.2 Materials and methods

3.2.1 Experimental design

In total 171 male broilers, breed Ross 308, arrived in University of Helsinki, Large animals Research Unit (KEK), at Viikki Farm on 14th of October 2019. Broilers were divided randomly into three groups (A-C) and eight pens (1-8). Group A birds were not subjected to any exercise during the experiment. Group B birds lived 25 days exactly like group A but after that the drinking water dispenser was elevated to a higher level. Group C birds had their drinking water dispenser elevated to a 5 cm higher level after three days and it was risen stepwise to 25 cm during the study. Birds were fed ad libitum. Group B results were disregarded from most analyses, due to technical problems.

3.2.2 Sampling

Equal number of broilers were taken from each pen and terminated at three different ages (Table 7). During the study, 17 broilers died between samplings.

Table 7. Termination sessions with varying ages.

Date of Termination	Termination	Age of the broilers (days)	Group	N
4th Nov 2019	1	20	A, C	40
14th Nov 2019	2	30	A, B, C	58
25th Nov 2019	3	41	A, B, C	56

On the termination day, sampling speed was approximately 5 minutes / bird. Pre-rigor breast muscle samples for initial pH (pH_5) analyses were collected within these 5 minutes. Birds were cut open from the middle of the thoracic cavity and examined by veterinarian. Left breast muscle was removed from the carcass, leaving the right breast muscle still attached. Three experienced evaluators did a visual examination of general appearance of the breast muscles, and palpation by fingers to determine the muscle consistency upon which numbers from 0 to 3 were assigned depending on the varying degree of consistency. Unaffected breast muscles assigned with grades 0 and 0.5 were considered normal, while grades from 1 to 3 assigned increasing severity of WB myopathy. Approximately, $2 * 2$ cm slices were cut from the cross-section of breast muscle for pH_5 (5 minutes *post mortem*) and carbohydrate analyses (Figure 12). Pre-rigor samples taken for carbohydrate analyses were wrapped in foil immediately after cutting, frozen with liquid nitrogen and stored in -70 °C freezer for further analyses, but were not analysed for the current thesis. Immeadiately after sampling, carcasses were packaged in plastic bags with feathers and skin on, in ice and transported from KEK to University laboratory for post-rigor sampling of next day.

Post-rigor sampling was done by removing the right breast muscle from the carcass. Again, approximately, $2 * 2$ cm slices were cut from the cross-section of breast muscle for pH_u (ultimate pH 24 hours *post mortem*) and carbohydrate analyses (Figure 12). Samples taken for carbohydrate analyses were wrapped in foil and stored in -70 °C freezer for carbohydrate analyses. A total of 154 post-rigor samples were collecteted during the study.

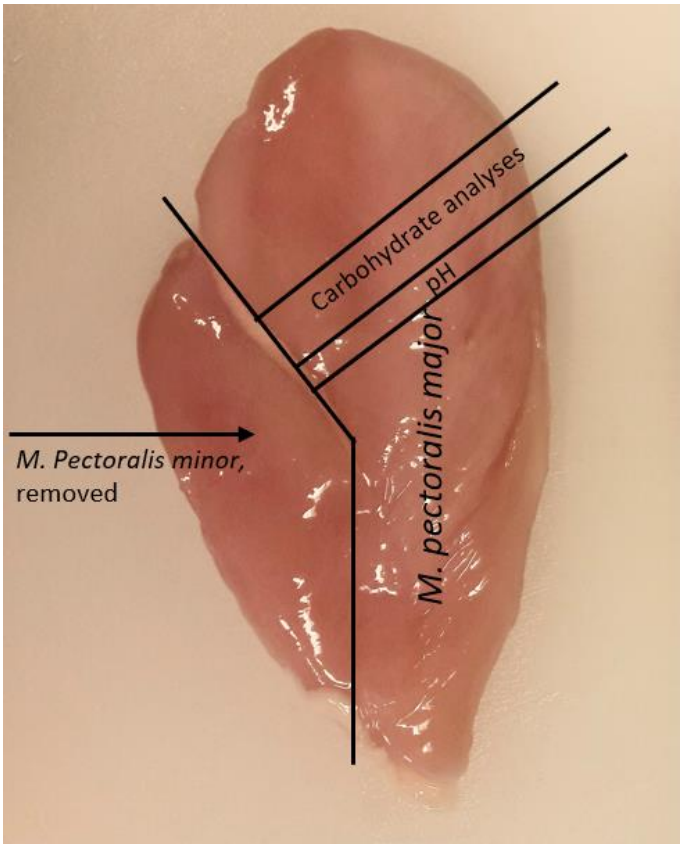

Figure 12. Slices cut from the *M. pectoralis major* (*MPM*) muscle for carbohydrate and pH analyses. *M. pectoralis minor* part was removed before sampling.

3.2.3 pH analysis

In total, 0.52 g Na-Iodoacetate and 5.592 g KCL were weight and diluted in 500 mL of Milli-Q water. Muscle sample of 0.5 g was mashed with metal stirrer inside the test tube containing 5 mL of Iodoacetate solution. Duplicate samples were made from each bird for pH_5 and pH_u analyses. Sampling spot from the muscle slice is shown in Figure 13.

The values for pH_5 and pH_u were determined at room temperature approximately at 22 °C by using pH meter (Seven GO Pro, Mettler-Toledo GmbH, Schwerzenbach, Switzerland) equipped with glass electrode (Mettler-Toledo Inlab 427, Mettler-Toledo GMBH, Schwerzenbach, Switzerland) during the same week as termination and sampling was done.

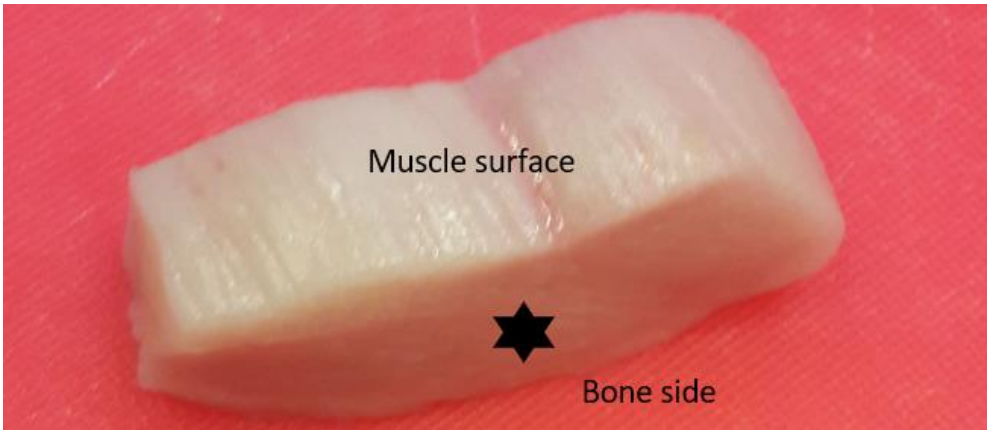

Figure 13. Slice taken from the *M. pectoralis major* (*MPM*) muscle. Samples for carbohydrate and pH analyses were taken from the close to the bone (dorsal) side of the muscle which was usually free from lesions. Sampling spot marked with an asterisk.

3.2.4 Carbohydrate analyses

Sample treatment

Approximately 0.5 g sample was taken from the muscle slice still frozen. Sampling spot from the muscle slice is shown in Figure 13. After weighing, 10 mL 1 M HCL was added inside the 50 mL falcon tube. Samples were homogenised with homogeniser (Ultra Turrax T25 Basic, Ika®-Werke GMBH, Staufen, Germany) at speed 13 500 rpm 2 ∗ 20 seconds. Duplicate samples were made from each bird for total glucose and lactic acid analyses.

Total glucose analysis.

After homogenising, 2 mL of homogenate was collected in 15 mL test tubes for total glucose and lactic acid analyses. The homogenate collected for total glucose analyses were hydrolysed in heating cabinet (Memmert 700, Memmert GMBH, Schwabach, Germany) of 100 °C for 2 hours. The heated samples were cooled to room temperature before adjusting the pH between 6.5 to 7.8 (Lowry & Passoneau, 1973) with 2 M NaOH containing Trizma-base. The volume used for adjusting pH was recorded.

From the tubes, after adjusting pH, 1 mL solution was taken and pipetted directly in 2 mL eppendorf tubes and centrifuged (Eppendorf Centrifuge 5424, Eppendorf AG, Hamburg, Germany) for 5 minutes at 4000 G. Supernatant was collected for analyses. Total glucose content was determined enzymatically using Roche diagnostic kit no. 20767131322 (Roche

Diagnostics GMBH, Mannheim, Germany). Pipetting was done in 70 µL microcuvettes (Brand® UV-Cuvette micro, Brand GMBH, Wertheim, Germany) according to Table 8.

Table 8. Pipetting of glucose kit reagents into70 µL microcuvettes.

	Volume µL
Sample or standard	40
R1	400
	After 10 minutes read the absorbance at λ=340 nm
R2	80
	After 15 minutes read the absorbance at λ=340 nm
	Total volume 520 µL

Absorbances were determined spectrophotometrically (UV-Spectrophotometer UV-1800, Shimadzu CO, Kyoto, Japan) at wavelength 340 nm. Amount of glycogen as total glucose was calculated by using glucose standard curve.

Lactic acid analysis.

The homogenate collected for lactic acid analyses were not hydrolysed in the heating cabinet as the homogenate used for total glucose analyses were. The pH of the homogenates used for lactic acid analyses were adjusted to pH of 10 with 2 M KOH. The volume used for adjusting pH was recorded.

After the adjusting of pH, 1 mL was taken from the tubes and pipetted directly in 2 mL eppendorf tubes and centrifuged (Eppendorf Centrifuge 5424, Eppendorf AG, Hamburg, Germany) for 5 minutes at 4000 G. Supernatant was collected for analyses. The lactic acid content was determined enzymatically by using Boehringer Mannheim diagnostic kit no. 10139084035 (R-Biopharm AG, Darmstadt, Germany) Pipetting was done in 70 µL microcuvettes (Brand® UV-Cuvette micro, Brand GMBH, Wertheim, Germany) according to Table 9.

Absorbances were determined spectrophotometrically (UV-Spectrophotometer UV-1800, Shimadzu CO, Kyoto, Japan) at wavelength 340 nm. Amount of lactic acid was calculated by using lactic acid standard curve.

Table 9. Pipetting lactate kit reagents into 70 µL cuvettes.

	Volume µL
Solution 1	250
Solution 2	50
Solution 3	5
Sample or standard	25
Milli-Q	225
	After 5 minutes read the absorbance at λ=340 nm
Solution 4	5
	After 30 minutes read the absorbance at λ=340 nm
	Total volume 560 µL

Glycolytic potential

Glycolytic potential was calculated according to Monin & Sellier (1985), (Equation 4).

$$GP = 2([glycogen] + [glucose] + [glucose\text{-}6\text{-}P]) + [lactate] \quad (4)$$

The contents of glycogen, glucose-1-P, glucose-6-P, and free glucose were determined as total glucose content of the sample.

3.2.5 Statistical analysis

All data were analysed using SPSS 25 Statistics (IBM, US, 2020), Means and significances were tested with ANOVA. Tukey test was done if ANOVA showed significances. Correlation analyses were carried out using Pearson´s correlation method.

4.1 Results

4.1.1 Tendency according to WB score and correlations

Breast muscles were evaluated and scored according to the increasing severity of WB myopathy. The results also include the results of group B (exercise after 25 days), which was excluded from the examination of other results due to technical problems. The results show that as the severity of WB increased, the pH_5 and pH_u value of the breast muscle increased and breast muscles total glucose, lactate content and glycolytic potential decreased (Table 10). The correlations show that there were significant relationships between WB score and the variables ($p < 0.000$). However, there were no interactions due to age and group. Therefore, the results below will be presented for one fixed variable at a time (Appendices 15 & 16). From this on, however, the results include only the groups A (no exercise) and C (exercise) and the separation of no WB (from scores 0 to 0.5) and WB (from score 1 to 3).

Table 10. Tendency according to WB score. Unaffected breast muscles assigned with grades 0 - 0.5 were considered normal, while grades 1 – 3 assigned increasing severity of WB myopathy. All groups included (A = no exercise, B = exercise after 25 days and C = exercise).

WB score	pH_5	pH_u	Total glucose (µmol/g)	Lactate (µmol/g)	Glycolytic potential (µmol/g)	N
0	6.58	5.67	6.99	106.62	120.61	56
0.5	6.59	5.71	6.01	103.96	115.99	26
1	6.62	5.70	5.29	98.16	108.75	25
1.5	6.63	5.79	4.52	97.81	106.86	11
2	6.64	5.80	3.87	96.14	103.90	22
2.5	6.68	5.80	1.33	95.87	98.62	5
3	6.66	5.86	1.46	90.28	94.47	9
Correlation	0.236	0.456	- 0.418	- 0.333	- 0.443	
Significance	0.003	0.000	0.000	0.000	0.000	

pH_5: pH 5 minutes after slaughter, pH_u; ultimate pH next day

4.1.2 Effect of exercise

There were no significant differences between non-exercised group A and exercised group C ($p > 0.05$), which shows that exercise had no effect in any of the parameters studied (Table 11). For group A, pH_5 was 6.63 ± 0.02 and for group C, pH_5 was 6.62 ± 0.02. The pH_u for group A was 5.74 ± 0.02 and for group C pH_u was 5.71 ± 0.02. For group C, total glucose content was 4.88 ± 0.43 µmol/g and for group A total glucose content was 5.41 ± 0.55 µmol/g. Lactate content was 99.76 ± 1.97 µmol/g for croup A and 103.03 ± 2.13 µmol/g for group C. Glycolytic potential was 110 ± 2.60 µmol/g for group A and 112 ± 2.36 µmol/g for group C.

Table 11. pH values and glycolytic potential in non-exercised (Group A) and exercised (Group C) broilers.

	Group A	Group C	Significance
N	59	55	
pH_5	6.63	6.62	0.658
SEM	0.02	0.02	
pH_u	5.74	5.71	0.237
SEM	0.02	0.02	
Total glucose (µmol/g)	5.41	4.88	0.455
SEM	0.55	0.43	
Lactate (µmol/g)	99.76	103.03	0.261
SEM	1.97	2.13	
Glycolytic potential (µmol/g)	110.73	112.85	0.549
SEM	2.60	2.36	

pH_5: pH 5 minutes after slaughter, pH_u; ultimate pH next day, SEM; standard error of mean.

4.1.3 Normal breast muscle and WB breast muscle

Significant differences were observed in almost all of the studied parameters between normal and WB cases ($p < 0.007$) (Table 12). The pH_5 for normal *MPM* muscle was 6.61 ± 0.02, while the value for WB-affected *MPM* muscle was 6.64 ± 0.02. There was no significant difference in pH_5, between normal and WB broilers ($p = 0.190$). However, significant differences were obtained in pH_u values between normal and WB broilers ($p < 0.001$). In WB-affected *MPM* muscle, the pH_u value was higher (5.77 ± 0.02) and in normal unaffected *MPM* muscle lower (5.68 ± 0.02).

Total glucose content was significantly decreased in WB-affect *MPM* muscle compared to normal *MPM* muscle ($p < 0.001$). Total glucose content was about half in WB-affected *MPM*

muscle (3.77 ± 0.43 µmol/g), compared to that in normal *MPM* muscle (6.45 ± 0.50 µmol/g). Reduced lactate formation was found in WB-affected *MPM* muscle (97.32 ± 2.23 µmol/g), compared to normal unaffected *MPM* muscle (105.8 ±1.75 µmol/g; $p = 0.007$). Glycolytic potential was lower in WB-affected *MPM* muscle (105.7 ± 2.55 µmol/g), while in normal *MPM* muscle the value was higher (117.98 ± 2.15 µmol/g; $p < 0.001$).

Table 12. pH values and glycolytic potential in normal breast muscle and WB breast muscle.

	Normal	WB	Significance
N	59	55	
pH_5	6.61	6.64	0.190
SEM	0.02	0.02	
pH_u	5.68	5.77	0.000
SEM	0.02	0.02	
Total glucose (µmol/g)	6.45	3.77	0.000
SEM	0.50	0.43	
Lactate (µmol/g)	105.08	97.32	0.007
SEM	1.75	2.23	
Glycolytic potential (µmol/g)	117.98	105.07	0.000
SEM	2.15	2.55	

pH_5: pH 5 minutes after slaughter, pH_u; ultimate pH next day, SEM; standard error of mean.

4.1.4 Age

All of the studied parameters showed significant differences between ages ($p < 0.007$) (Table 13).

Table 13. pH values and glycolytic potential between different ages.

	20 days old	30 days old	41 days old	Significance
N	40	38	36	
pH_5	6.65^a	6.55^b	6.69^a	0.000
SEM	0.02	0.02	0.02	
pH_u	5.62^a	5.74^b	5.82^c	0.000
SEM	0.02	0.01	0.02	
Total glucose (µmol/g)	6.65^a	5.87^a	2.74^b	0.000
SEM	0.50	0.66	0.49	
Lactate (µmol/g)	105.72^a	102.70^{ab}	95.01^b	0.007
SEM	1.77	3.08	2.27	
Glycolytic potential (µmol/g)	119.03^a	114.46^a	100.81^b	0.000
SEM	2.15	3.48	2.66	

^{a,b,c} Means having the same letter do not differ from each other ($p > 0.05$). pH_5: pH 5 minutes after slaughter, pH_u; ultimate pH next day, SEM; standard error of mean.

The pH_5 of 20 days old broilers (6.65 ± 0.02) did not differ from the pH_5 of 41 days old broilers (6.69 ± 0.02), but pH_5 of 30 days old group (6.55 ± 0.02) differed from the other age groups ($p < 0.001$), but the difference cannot be considered reliable. The pH_u 5.82 ± 0.02 value was the highest in the oldest broilers (41 days old), and lowest in youngest broilers (20 days old) 5.62 ± 0.02. In the oldest broilers (41 days old), the total glucose content was the lowest (2.74 ± 0.49 µmol/g; $p < 0.05$). The youngest group (20 days old) and 30 days old group did not differ from each other ($p > 0.05$).

Lactate content was the lowest for broilers of the oldest age group (95.01 ± 2.27 µmol/g), which differed from the youngest age group with lactate content of 105.72 ± 1.77 µmol/g ($p < 0.05$). The lactate content of 30 days old broilers did not differ from the youngest (20 days old) or from the oldest group (41 days old) ($p > 0.05$).

Glycolytic potential was the lowest in the group of 41 days old broilers (100 ± 2.66 µmol/g) which differed from the group of 20 days old broilers (119.03 ± 2.15 µmol/g) and from the group of 30 days old broilers (114.46 ± 3.48 µmol/g) ($p < 0.05$). Glycolytic potential of the 20 days old group and 30 days old group did not differ from each other ($p > 0.05$). The results show that the total glucose content, lactate content as well as the glycolytic potential had a tendency to decrease with age, while pH_5 and pH_u values had a tendency to increase with age.

4.2 Discussion

The aim of the main project was to study the effects of long-term exercise (wing flapping) on the prevalence of WB myopathy in *MPM* muscle of broiler chickens. The aim of the current thesis was to study whether exercise, WB status or age have effects on ultimate pH and glycolytic potential (sum of total glucose and lactic acid content, given as lactate) in *MPM* muscle of modern broilers chickens.

There is a significant dependence between WB scores and the variables (Table 10), which justified experimental design of the study. However, there were no interactions due to age and group (Appendices 15 & 16).

Effect of exercise

In the current study, exercise had no effect on pH_u nor glycolytic potential (Table 11). However, exercise has been found to improve the cardiac output, increase the size and number of mitochondria and mitochondrial enzyme activities in the skeletal muscles that are involved in the exercise, as well as to increase the GLUT4 glucose transporter activity (Ingjer, 1979; Carter et al., 2000; Holloszy, 2008). According to Nakamura (1970), exercise just before the death rises pH_u and decreases glycolytic potential of *MPM* muscle. However, it must be taken into a consideration that in the current study, the exercised group had to exercise every day from the beginning of the growth period, in contrast to the study by Nakamura (1970), where the exhaustive exercise was applied to the birds only just before slaughter.

Normal breast muscle and WB breast muscle

Between normal and WB breast muscle, no differences were found in pH_5 (5 minutes *post mortem*), however, differences were detected in pH_u (24 hours *post mortem*). The phenomenon is similar to that in Baldi et al. (2020b) study. In the current study, pH_u was 0.1 units higher in WB-affected breast muscle compared to normal unaffected breast muscle (Table 12). As expected, the results of current study are similar to those that other researchers have found, but however, even higher pH_u values in normal and WB breasts than in the current study have been found (Table 6), possible due to the low pretermination stress in the current study. In the current study, glycolytic potential was lower in WB-affected breast muscle compared to that in normal breast muscle (Table 12). However, even larger differences in glycolytic potential between

normal cases compared to WB cases have been found in the study of Baldi et al. 2020b. Differences may be due to the facts, that broilers in that study had experienced withdrawal before slaughter, the broilers were older than in the current study, and the samples were collected from commercial broiler processing plant and the broilers had experienced transport stress when transported from farms to the slaughter plant. In addition, in the current study, broilers had access to feed until they were collected, weighed, and terminated. In the current study, WB-affected breast muscles showed lower lactate content than normal breast muscles (Table 12). A similar phenomenon was found by Malila et al. (2019) and Baldi et al. (2020b) 24 hours *post mortem*, which may be explained by the decreased lactate dehydrogenase activity in WB muscles (Malila et al., 2019; Zhao et al., 2020). Total glucose content was also significantly decreased in WB-affected breast muscles compared to normal breast muscles (Table 12). The total glucose content was about half in the WB-affected breast muscles compared to that in normal breast muscles. This could be a result of a phenomenon found by Berri et al. (2007) that when the muscle fiber size increases, the muscle glycogen content decreases. The total glucose content for WB breast muscle in the current study is similar to found by Baldi et al. (2020b), but however, the results for normal breast muscle is slightly higher than in the current study. The results are always affected by from which part of the muscle the sample is taken, how the sample is taken and what is the severity of WB myopathy in the breast muscle. The disadvantage for comparison in the current study was that there were birds of different ages in the groups of normal and WB. In addition, the sample was taken from the middle part of the muscle (Figure 13), and in focal WB cases, the middle part/deep layer was usually not WB-affected.

Age

Termination age showed significant differences in all the parameters studied (Table 13). In the current study, there was no differences in the pH_5 values (5 minutes *post mortem*) between non-exercised and exercised group nor between WB and normal, but however, the pH_5 value of 30 days old broilers was lower than the pH_5 of other two the ages (20 days old and 41 days old) (Table 13), but the difference cannot be considered reliable. The lowest glycolytic potential between different ages was observed in broilers of 41 days old (100.81 ± 2.66 µmol lactate equivalents/g) (Table 13). Likewise, the highest pH_u value between different ages was observed in broilers of 41 days old (Table 13). In the study of Baldi et al. (2020b), the glycolytic potential of 48 days old broilers was lower (approximately 95 µmol lactate equivalents/g) than in the

study of Ylä-Ajos et al. (2007) (116.6 ± 2.5 µmol lactate equivalents/g) where the broilers were 35 days old, which corresponds to the phenomena of the current the study that glycolytic potential decreases with age. The decreased glycolytic potential and the higher pH_u may be due to the decrease of the glycogen content in breast muscle with age, when the muscle fiber size increases (Berri et al., 2007). Differences in the levels of the actual parameters between the current study and literature may be also due to, again, that broilers in the current study did not experience the transport stress as they experience when transported from the farm to the slaughterhouse. Again, broilers were fed until they were collected (without feed withdrawal time), weighted, and then terminated. In addition, in the current study, the sample for pH_5 analyses was taken 5 minutes after the bird has been taken from the pen and terminated. Also, slow carcass chilling in the current study may have resulted low total glucose contents. In the industry, carcass chilling is faster, while in the current study chilling was done by putting the carcasses, packaged in plastic bags with feathers and skin on, in ice. Slow chilling may have allowed glycolysis to be completed because the GDE enzyme is temperature dependent and its activity is strongly decreased at the temperatures below 15°C (Ylä-Ajos et al., 2007).

pH_u and buffering capacity

The post-rigor overall average pH_u value in the current study for *MPM* muscle was 5.72, while in the literature, the overall pH_u value has been reported to be mostly within the range 5.80 to 5.95 (Table 6). The average pH_u value of breast muscle in this study was lower than reported in the literature, which may be due to that stress was minimized before the termination as the broilers were taken from pens less than one minute before termination, the broilers had access to feed and water until they were terminated and the broilers did not experience the transport stress, like they experience when transported from the farms to slaughter plant.

In the current study, the average lactic acid content of post-rigor broiler breast muscles was 101.33 µmol/g which is similar to that Puolanne and Kivikari (2000) found from post-rigor broiler breast muscles, approximately 100 µmol/g. In the current study, the lactic acid content was different than in the study of Baldi et al. (2020b), which may be due to the fact that in their study broilers were older than in the current study and were selected from the production the line of commercial processing plant 15 minutes after slaughter, and that before slaughter, broilers had experienced transport stress and feed withdrawal. Lactic acid content of the breast

muscle measured in the study of Papinaho et al. (1996) was lower (46.9 ± 2.2 µmol/g) than in the current study. The difference is due to that lactic acid content was determined in that study at 7 to 10 minutes *post mortem* which is not comparable to the content determined in the current study. In the study of Nakamura (1970), lactic acid content (2.3 ± 1 µmol/g) was determined from the blood which is not comparable to the lactic acid content determined in the current study.

The amount of lactic acid needed to decrease pH from 7.2 to 5.72 can be calculated from Kivikari´s (1996) buffering capacity curve for broiler breast muscle (Figure 14). Degrease from pH 7.2 to 6.9 needs approximately 25.5 mmol H^+/kg and the decrease from pH 6.9 to 5.72 needs approximately 76.11 mmol H^+/kg, respectively. Consequently, based on this calculation, the total amount of lactic acid needed for pH fall from pH 7.2 to 5.72 is 101.61 mmol H^+/kg. In the current study, the value was, the average lactic acid content of post-rigor broiler breast muscle was 101.33 µmol/g which matches well with the approximation calculated from Kivikari´s (1996) buffering capacity curve for broilers breast muscle (Figure 14).

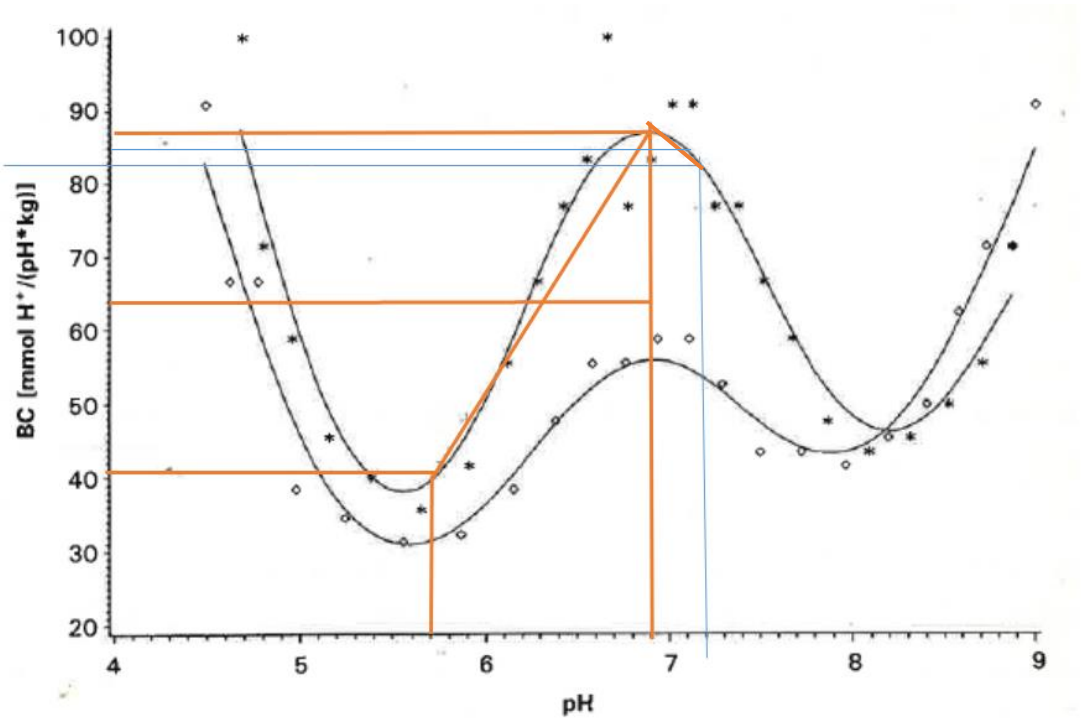

Figure 14. Buffering capacity curves of broiler breast (*) and thigh-muscles (▫) (Kivikari, 1996). Colored lines added to ease the calculations/approximations of the amount of lactic acid needed to decrease pH from 7.2 to 5.72. Degrease from pH 7.2 to 6.9 needs ca 25.5 mmol H^+/kg and the decrease from pH 6.9 to 5.72 needs ca 76.11 mmol H^+/kg. The total amount of lactic acid needed for pH fall from pH 7.2 to 5.72 is 101.61 mmol H^+/kg.

5. CONCLUSIONS

The aim of the thesis was to study whether exercise, WB status or age have effects on ultimate pH and glycolytic potential (sum of total glucose and lactic acid content, given as lactate) in *MPM* muscle of modern broilers chickens.

The results show that:

- ✓ Exercise does not affect pH_u nor glycolytic potential.
- ✓ WB has effect on pH_u and glycolytic potential. In WB-affected *MPM* muscle, pH_u is higher compared to that in normal unaffected muscle. Glycolytic potential in WB cases is lower than in normal cases.
- ✓ Age has effect on pH_u and glycolytic potential. The pH_u increases and glycolytic potential decreases with age. The decreased glycolytic potential and the higher pH_u seems to be due to the decrease of the glycogen content in breast muscle with age, when the muscle fiber size increases as well.

The results of the current study show that exercise does not have an effect on the levels of carbohydrates in *M. pectoralis major* muscle of modern broiler chickens, but WB status and increasing slaughter age result a decrease in glycolytic potential and increase in pH_u.

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APPENDICES

Appendix 1. Means report according WB score.

Report

Score		pH5	pHu	Glucose	Lactate	GP
,0	Mean	6,5770	5,6695	6,9871	106,6248	120,6116
	N	56	56	56	56	56
	Std. Deviation	,13358	,12531	3,94639	12,27461	15,13678
	Std. Error of Mean	,01785	,01675	,52736	1,64026	2,02274
,5	Mean	6,5923	5,7054	6,0108	103,9604	115,9935
	N	26	26	26	26	26
	Std. Deviation	,15637	,10148	3,84364	14,89567	17,57116
	Std. Error of Mean	,03067	,01990	,75380	2,92128	3,44599
1,0	Mean	6,6220	5,7016	5,2900	98,1648	108,7548
	N	25	25	25	25	25
	Std. Deviation	,11493	,13813	4,03379	14,38066	15,46488
	Std. Error of Mean	,02299	,02763	,80676	2,87613	3,09298
1,5	Mean	6,6273	5,7909	4,5191	97,8136	106,8600
	N	11	11	11	11	11
	Std. Deviation	,12378	,07327	3,39992	7,50313	9,79686
	Std. Error of Mean	,03732	,02209	1,02512	2,26228	2,95386
2,0	Mean	6,6445	5,8009	3,8736	96,1423	103,8964
	N	22	22	22	22	22
	Std. Deviation	,11160	,11501	3,27324	15,70597	19,65218
	Std. Error of Mean	,02379	,02452	,69786	3,34852	4,18986
2,5	Mean	6,6820	5,8020	1,3260	95,8740	98,6240
	N	5	5	5	5	5
	Std. Deviation	,08556	,08672	1,47817	17,61952	20,42960
	Std. Error of Mean	,03826	,03878	,66106	7,87969	9,13639
3,0	Mean	6,6622	5,8611	1,4556	90,2822	94,4656
	N	9	9	9	9	9
	Std. Deviation	,11627	,08283	1,01580	21,97536	22,22200
	Std. Error of Mean	,03876	,02761	,33860	7,32512	7,40733
Total	Mean	6,6085	5,7237	5,4186	101,3706	112,2951
	N	154	154	154	154	154
	Std. Deviation	,13072	,12942	3,96704	14,82374	18,25114
	Std. Error of Mean	,01053	,01043	,31967	1,19453	1,47072

Appendix 2. Pearson´s Correlations

Correlations

		Score	pH5	pHu	Glucose	Lactate	GP
Score	Pearson Correlation	1	,236**	,456**	-,418**	-,333**	-,443**
	Sig. (2-tailed)		,003	,000	,000	,000	,000
	N	154	154	154	154	154	154
pH5	Pearson Correlation	,236**	1	,211**	-,386**	-,157	-,293**
	Sig. (2-tailed)	,003		,009	,000	,052	,000
	N	154	154	154	154	154	154
pHu	Pearson Correlation	,456**	,211**	1	-,557**	-,352**	-,521**
	Sig. (2-tailed)	,000	,009		,000	,000	,000
	N	154	154	154	154	154	154
Glucose	Pearson Correlation	-,418**	-,386**	-,557**	1	,233**	,619**
	Sig. (2-tailed)	,000	,000	,000		,004	,000
	N	154	154	154	154	154	154
Lactate	Pearson Correlation	-,333**	-,157	-,352**	,233**	1	,907**
	Sig. (2-tailed)	,000	,052	,000	,004		,000
	N	154	154	154	154	154	154
GP	Pearson Correlation	-,443**	-,293**	-,521**	,619**	,907**	1
	Sig. (2-tailed)	,000	,000	,000	,000	,000	
	N	154	154	154	154	154	154

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 3. Means Table of the groups A (no exercise) and group C (exercise)

Report

Group		pH5	pHu	glucose	lactate	GP
A, no excercise	Mean	6,6329	5,7356	5,4113	99,7571	110,7282
	N	59	59	59	59	59
	Std. Deviation	,12824	,13040	4,25636	15,12226	19,97290
	Std. Error of Mean	,01670	,01698	,55413	1,96875	2,60025
C, excercise	Mean	6,6229	5,7060	4,8790	103,0265	112,8513
	N	55	55	55	55	55
	Std. Deviation	,10979	,13527	3,20731	15,77543	17,50915
	Std. Error of Mean	,01480	,01824	,43247	2,12716	2,36093
Total	Mean	6,6281	5,7213	5,1545	101,3344	111,7525
	N	114	114	114	114	114
	Std. Deviation	,11927	,13301	3,77968	15,45949	18,77210
	Std. Error of Mean	,01117	,01246	,35400	1,44791	1,75817

Appendix 4. Anova Table of the group A and C.

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
pH5 * Group	Between Groups	(Combined)	,003	1	,003	,198	,658
	Within Groups		1,605	112	,014		
	Total		1,608	113			
pHu * Group	Between Groups	(Combined)	,025	1	,025	1,414	,237
	Within Groups		1,974	112	,018		
	Total		1,999	113			
glucose * Group	Between Groups	(Combined)	8,065	1	8,065	,562	,455
	Within Groups		1606,253	112	14,342		
	Total		1614,318	113			
lactate * Group	Between Groups	(Combined)	304,271	1	304,271	1,276	,261
	Within Groups		26702,275	112	238,413		
	Total		27006,546	113			
GP * Group	Between Groups	(Combined)	128,308	1	128,308	,362	,549
	Within Groups		39691,965	112	354,393		
	Total		39820,273	113			

Appendix 5. Means Table of normal and WB.

Report

WB		pH5	pHu	glucose	lactate	GP
Normal	Mean	6,6139	5,6759	6,4476	105,0771	117,9838
	N	59	59	59	59	59
	Std. Deviation	,12548	,12298	3,82571	13,46928	16,51306
	Std. Error of Mean	,01634	,01601	,49806	1,75355	2,14982
WB	Mean	6,6433	5,7700	3,7673	97,3195	105,0681
	N	55	55	55	55	55
	Std. Deviation	,11137	,12694	3,22298	16,53900	18,88385
	Std. Error of Mean	,01502	,01712	,43459	2,23012	2,54630
Total	Mean	6,6281	5,7213	5,1545	101,3344	111,7525
	N	114	114	114	114	114
	Std. Deviation	,11927	,13301	3,77968	15,45949	18,77210
	Std. Error of Mean	,01117	,01246	,35400	1,44791	1,75817

Appendix 6. Anova Table of normal and WB.

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
pH5 * WB	Between Groups	(Combined)	,025	1	,025	1,738	,190
	Within Groups		1,583	112	,014		
	Total		1,608	113			
pHu * WB	Between Groups	(Combined)	,252	1	,252	16,144	,000
	Within Groups		1,747	112	,016		
	Total		1,999	113			
glucose * WB	Between Groups	(Combined)	204,498	1	204,498	16,246	,000
	Within Groups		1409,819	112	12,588		
	Total		1614,318	113			
lactate * WB	Between Groups	(Combined)	1713,022	1	1713,022	7,585	,007
	Within Groups		25293,525	112	225,835		
	Total		27006,546	113			
GP * WB	Between Groups	(Combined)	4748,387	1	4748,387	15,164	,000
	Within Groups		35071,886	112	313,142		
	Total		39820,273	113			

Appendix 7. Means Table of ages.

Report

Age		pH5	pHu	glucose	lactate	GP
20 days old	Mean	6,6513	5,6150	6,6465	105,7246	119,0294
	N	40	40	40	40	40
	Std. Deviation	,11402	,11638	3,15926	11,17958	13,58929
	Std. Error of Mean	,01803	,01840	,49952	1,76765	2,14865
30 days old	Mean	6,5484	5,7389	5,8737	102,7011	114,4590
	N	38	38	38	38	38
	Std. Deviation	,10676	,08825	4,05681	19,00767	21,43959
	Std. Error of Mean	,01732	,01432	,65810	3,08345	3,47796
41 days old	Mean	6,6864	5,8208	2,7374	95,0139	100,8102
	N	36	36	36	36	36
	Std. Deviation	,09163	,10177	2,91348	13,61125	15,94990
	Std. Error of Mean	,01527	,01696	,48558	2,26854	2,65832
Total	Mean	6,6281	5,7213	5,1545	101,3344	111,7525
	N	114	114	114	114	114
	Std. Deviation	,11927	,13301	3,77968	15,45949	18,77210
	Std. Error of Mean	,01117	,01246	,35400	1,44791	1,75817

Appendix 8. Anova Table of ages.

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
pH5 * Age	Between Groups	(Combined)	,385	2	,193	17,478	,000
	Within Groups		1,223	111	,011		
	Total		1,608	113			
pHu * Age	Between Groups	(Combined)	,820	2	,410	38,628	,000
	Within Groups		1,179	111	,011		
	Total		1,999	113			
glucose * Age	Between Groups	(Combined)	319,034	2	159,517	13,670	,000
	Within Groups		1295,284	111	11,669		
	Total		1614,318	113			
lactate * Age	Between Groups	(Combined)	2280,109	2	1140,054	5,118	,007
	Within Groups		24726,438	111	222,761		
	Total		27006,546	113			
GP * Age	Between Groups	(Combined)	6706,947	2	3353,474	11,241	,000
	Within Groups		33113,326	111	298,318		
	Total		39820,273	113			

Appendix 9. T-Test of group A (no exercise) and group C (exercise).

T-Test

Group Statistics

	Group	N	Mean	Std. Deviation	Std. Error Mean
pH5	A, no excercise	59	6,6329	,12824	,01670
	C, excercise	55	6,6229	,10979	,01480
pHu	A, no excercise	59	5,7356	,13040	,01698
	C, excercise	55	5,7060	,13527	,01824
glucose	A, no excercise	59	5,4113	4,25636	,55413
	C, excercise	55	4,8790	3,20731	,43247
lactate	A, no excercise	59	99,7571	15,12226	1,96875
	C, excercise	55	103,0265	15,77543	2,12716
GP	A, no excercise	59	110,7282	19,97290	2,60025
	C, excercise	55	112,8513	17,50915	2,36093

Appendix 10. T-Test of group A (no exercise) and group C (exercise).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH5	Equal variances assumed	1,156	,285	,444	112	,658	,00997	,02244	-,03448	,05443
	Equal variances not assumed			,447	111,216	,656	,00997	,02231	-,03424	,05419
pHu	Equal variances assumed	,085	,771	1,189	112	,237	,02959	,02489	-,01971	,07890
	Equal variances not assumed			1,188	110,722	,238	,02959	,02492	-,01978	,07897
glucose	Equal variances assumed	7,469	,007	,750	112	,455	,53227	,70981	-,87413	1,93868
	Equal variances not assumed			,757	107,383	,451	,53227	,70292	-,86112	1,92567
lactate	Equal variances assumed	,355	,553	-1,130	112	,261	-3,26946	2,89408	-9,00370	2,46479
	Equal variances not assumed			-1,128	110,587	,262	-3,26946	2,89841	-9,01309	2,47418
GP	Equal variances assumed	,704	,403	-,602	112	,549	-2,12310	3,52848	-9,11433	4,86812
	Equal variances not assumed			-,604	111,591	,547	-2,12310	3,51217	-9,08229	4,83608

Appendix 11. T-Test of normal and WB.

T-Test

Group Statistics

	WB	N	Mean	Std. Deviation	Std. Error Mean
pH5	Normal	59	6,6139	,12548	,01634
	WB	55	6,6433	,11137	,01502
pHu	Normal	59	5,6759	,12298	,01601
	WB	55	5,7700	,12694	,01712
glucose	Normal	59	6,4476	3,82571	,49806
	WB	55	3,7673	3,22298	,43459
lactate	Normal	59	105,0771	13,46928	1,75355
	WB	55	97,3195	16,53900	2,23012
GP	Normal	59	117,9838	16,51306	2,14982
	WB	55	105,0681	18,88385	2,54630

Appendix 12. T-Test of normal and WB.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
pH5	Equal variances assumed	,985	,323	-1,318	112	,190	-,02937	,02228	-,07353	,01478
	Equal variances not assumed			-1,324	111,740	,188	-,02937	,02219	-,07334	,01459
pHu	Equal variances assumed	,000	,999	-4,018	112	,000	-,09407	,02341	-,14046	-,04768
	Equal variances not assumed			-4,013	110,836	,000	-,09407	,02344	-,14051	-,04762
glucose	Equal variances assumed	1,918	,169	4,031	112	,000	2,68034	,66499	1,36274	3,99794
	Equal variances not assumed			4,055	110,894	,000	2,68034	,66101	1,37049	3,99019
lactate	Equal variances assumed	3,536	,063	2,754	112	,007	7,75758	2,81670	2,17665	13,33852
	Equal variances not assumed			2,734	104,297	,007	7,75758	2,83696	2,13197	13,38320
GP	Equal variances assumed	2,720	,102	3,894	112	,000	12,91571	3,31677	6,34395	19,48746
	Equal variances not assumed			3,876	107,546	,000	12,91571	3,33247	6,30986	19,52155

Appendix 13. Tukey test of ages, descriptives.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
pH5	20 days old	40	6,6512	,11402	,01803	6,6148	6,6877	6,33	6,84
	30 days old	38	6,5484	,10676	,01732	6,5133	6,5835	6,29	6,82
	41 days old	36	6,6864	,09163	,01527	6,6554	6,7174	6,36	6,87
	Total	114	6,6281	,11927	,01117	6,6059	6,6502	6,29	6,87
pHu	20 days old	40	5,6150	,11638	,01840	5,5778	5,6522	5,42	5,88
	30 days old	38	5,7389	,08825	,01432	5,7099	5,7680	5,57	5,94
	41 days old	36	5,8208	,10177	,01696	5,7864	5,8553	5,49	6,01
	Total	114	5,7213	,13301	,01246	5,6966	5,7460	5,42	6,01
glucose	20 days old	40	6,6465	3,15926	,49952	5,6361	7,6569	,00	13,77
	30 days old	38	5,8737	4,05681	,65810	4,5403	7,2072	,07	13,89
	41 days old	36	2,7374	2,91348	,48558	1,7516	3,7231	,00	11,87
	Total	114	5,1545	3,77968	,35400	4,4531	5,8558	,00	13,89
lactate	20 days old	40	105,7246	11,17958	1,76765	102,1492	109,3000	68,61	123,78
	30 days old	38	102,7011	19,00767	3,08345	96,4534	108,9488	66,38	150,04
	41 days old	36	95,0139	13,61125	2,26854	90,4085	99,6192	65,69	125,60
	Total	114	101,3344	15,45949	1,44791	98,4659	104,2030	65,69	150,04
GP	20 days old	40	119,0294	13,58929	2,14865	114,6834	123,3755	75,01	144,20
	30 days old	38	114,4590	21,43959	3,47796	107,4120	121,5061	71,28	177,84
	41 days old	36	100,8102	15,94990	2,65832	95,4135	106,2069	72,53	135,34
	Total	114	111,7525	18,77210	1,75817	108,2693	115,2358	71,28	177,84

Appendix 14. Anova table of Tukey test (ages).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
pH5	Between Groups	,385	2	,193	17,478	,000
	Within Groups	1,223	111	,011		
	Total	1,608	113			
pHu	Between Groups	,820	2	,410	38,628	,000
	Within Groups	1,179	111	,011		
	Total	1,999	113			
glucose	Between Groups	319,034	2	159,517	13,670	,000
	Within Groups	1295,284	111	11,669		
	Total	1614,318	113			
lactate	Between Groups	2280,109	2	1140,054	5,118	,007
	Within Groups	24726,438	111	222,761		
	Total	27006,546	113			
GP	Between Groups	6706,947	2	3353,474	11,241	,000
	Within Groups	33113,326	111	298,318		
	Total	39820,273	113			

Appendix 15. Interactions and covarianses of age, group and score.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	pH5	1,321[a]	41	,032	2,791	,000
	pHu	1,388[b]	41	,034	3,226	,000
	Glucose	963,069[c]	41	23,489	1,821	,007
	Lactate	11516,085[d]	41	280,880	1,423	,075
	GP	22140,383[e]	41	540,009	2,098	,001
Intercept	pH5	3165,473	1	3165,473	274178,298	,000
	pHu	2378,632	1	2378,632	226718,108	,000
	Glucose	1442,450	1	1442,450	111,821	,000
	Lactate	685064,779	1	685064,779	3471,091	,000
	GP	818571,785	1	818571,785	3180,626	,000
Age	pH5	,433	2	,216	18,747	,000
	pHu	,334	2	,167	15,896	,000
	Glucose	146,954	2	73,477	5,696	,004
	Lactate	153,842	2	76,921	,390	,678
	GP	894,814	2	447,407	1,738	,181
Group	pH5	,023	2	,011	,984	,377
	pHu	,014	2	,007	,682	,508
	Glucose	10,692	2	5,346	,414	,662
	Lactate	64,503	2	32,252	,163	,849
	GP	18,461	2	9,231	,036	,965
Score	pH5	,057	6	,010	,830	,549
	pHu	,119	6	,020	1,884	,090
	Glucose	184,045	6	30,674	2,378	,034
	Lactate	2866,049	6	477,675	2,420	,031
	GP	5883,600	6	980,600	3,810	,002
Age * Group	pH5	,005	3	,002	,139	,936
	pHu	,020	3	,007	,647	,587
	Glucose	,920	3	,307	,024	,995
	Lactate	686,526	3	228,842	1,159	,329
	GP	782,279	3	260,760	1,013	,390
Age * Score	pH5	,103	8	,013	1,120	,355
	pHu	,045	8	,006	,542	,823
	Glucose	108,968	8	13,621	1,056	,399

	Lactate	1973,948	8	246,744	1,250	,277
	GP	3304,263	8	413,033	1,605	,131
Group * Score	pH5	,080	11	,007	,631	,799
	pHu	,069	11	,006	,597	,828
	Glucose	93,805	11	8,528	,661	,772
	Lactate	1355,464	11	123,224	,624	,805
	GP	2465,113	11	224,101	,871	,571
Age * Group * Score	pH5	,174	9	,019	1,674	,103
	pHu	,145	9	,016	1,532	,145
	Glucose	106,806	9	11,867	,920	,511
	Lactate	2469,048	9	274,339	1,390	,201
	GP	3602,691	9	400,299	1,555	,137
Error	pH5	1,293	112	,012		
	pHu	1,175	112	,010		
	Glucose	1444,759	112	12,900		
	Lactate	22104,652	112	197,363		
	GP	28824,531	112	257,362		
Total	pH5	6728,158	154			
	pHu	5047,719	154			
	Glucose	6929,518	154			
	Lactate	1616124,026	154			
	GP	1992932,882	154			
Corrected Total	pH5	2,614	153			
	pHu	2,563	153			
	Glucose	2407,828	153			
	Lactate	33620,736	153			
	GP	50964,914	153			

a. R Squared = ,505 (Adjusted R Squared = ,324)

b. R Squared = ,541 (Adjusted R Squared = ,374)

c. R Squared = ,400 (Adjusted R Squared = ,180)

d. R Squared = ,343 (Adjusted R Squared = ,102)

e. R Squared = ,434 (Adjusted R Squared = ,227)

Appendix 16. Interactions and covarianses of group and age.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	pH5	,892[a]	7	,127	10,797	,000
	pHu	,951[b]	7	,136	12,301	,000
	Glucose	504,350[c]	7	72,050	5,526	,000
	Lactate	3361,972[d]	7	480,282	2,317	,029
	GP	8595,746[e]	7	1227,964	4,231	,000
	Score	38,505[f]	7	5,501	8,514	,000
Intercept	pH5	6538,667	1	6538,667	554177,266	,000
	pHu	4891,784	1	4891,784	443068,038	,000
	Glucose	4640,547	1	4640,547	355,938	,000
	Lactate	1551680,743	1	1551680,743	7486,934	,000
	GP	1912202,544	1	1912202,544	6589,263	,000
	Score	104,700	1	104,700	162,046	,000
Group	pH5	,098	2	,049	4,173	,017
	pHu	,078	2	,039	3,510	,032
	Glucose	101,509	2	50,755	3,893	,023
	Lactate	398,278	2	199,139	,961	,385
	GP	1006,927	2	503,464	1,735	,180
	Score	11,872	2	5,936	9,187	,000
Age	pH5	,682	2	,341	28,903	,000
	pHu	,907	2	,453	41,069	,000
	Glucose	442,492	2	221,246	16,970	,000
	Lactate	2025,409	2	1012,705	4,886	,009
	GP	7240,845	2	3620,422	12,476	,000
	Score	34,521	2	17,260	26,714	,000
Group * Age	pH5	,037	3	,012	1,056	,370
	pHu	,010	3	,003	,310	,818
	Glucose	12,611	3	4,204	,322	,809
	Lactate	947,156	3	315,719	1,523	,211
	GP	856,055	3	285,352	,983	,402
	Score	,405	3	,135	,209	,890
Error	pH5	1,723	146	,012		
	pHu	1,612	146	,011		
	Glucose	1903,478	146	13,038		

	Lactate	30258,765	146	207,252		
	GP	42369,168	146	290,200		
	Score	94,332	146	,646		
Total	pH5	6728,158	154			
	pHu	5047,719	154			
	Glucose	6929,518	154			
	Lactate	1616124,026	154			
	GP	1992932,882	154			
	Score	256,500	154			
Corrected Total	pH5	2,614	153			
	pHu	2,563	153			
	Glucose	2407,828	153			
	Lactate	33620,736	153			
	GP	50964,914	153			
	Score	132,838	153			

a. R Squared = ,341 (Adjusted R Squared = ,309)

b. R Squared = ,371 (Adjusted R Squared = ,341)

c. R Squared = ,209 (Adjusted R Squared = ,172)

d. R Squared = ,100 (Adjusted R Squared = ,057)

e. R Squared = ,169 (Adjusted R Squared = ,129)

f. R Squared = ,290 (Adjusted R Squared = ,256)