# GROWTH ASSOCIATED AND STRESS-INDUCED MYOPATHIES IN THE DOMESTIC CHICKEN (*Gallus domesticus*)

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PhD

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2004

**Abstract**

Genetic selection of broiler chickens for growth related production traits may have led to an increased susceptibility to both growth and stress induced myopathies. A comparison of a broiler (B); broiler great-grandparent (GGP) and layer (L) line revealed that by 25 weeks of age, the mean body weights reached by the B, GGP and L lines were 5.1 kg, 5.2 kg and 1.9 kg. Increased *Pectoralis major* (*Pm*) breast muscle yield through increased fibres sizes may contribute to the greater B and GGP body weights. At 25 weeks, the mean *Pm* breast muscle fibre size reached by the B and GGP lines (65.9 μm and 59.8 μm respectively) were 1.5 times greater than that of the L line (38.1 μm). Furthermore, there was divergence in muscle fibre growth between the mean *Pm* and *Biceps femoris* (*Bf*) leg muscle fibres of the B and GGP lines, but not the L line during growth. Cores, rims and split fibres observed in the leg muscles from older B and GGP birds may result from metabolic stress associated with larger fibre sizes and inadequate capillary support. Enzyme markers of muscle damage were indicative of a greater growth associated myopathy in the B and GGP lines compared to the L line. Histopathological assessments also revealed muscle damage. The type and incidence of structural changes were related to bird line (B>GGP>L), age (prominent at 5 and 18-23 weeks), muscle (*Ps*: necrotic and basophilic fibres; *Bf*: hyaline and basophilic fibres), and circulating steroid levels (regenerative rather than degenerative processes associated with estrogen secretion). A reduction in enzyme markers of muscle damage preceded egg yolk precursor production and increased calcium ($Ca^{2+}$) uptake for egg-shell synthesis. Estrogen may induce increased satellite cell activity and fibre regeneration, protecting muscle from the potential threat of $Ca^{2+}$-induced muscle damage due the increased $Ca^{2+}$ uptake. The demonstration of the alpha and beta estrogen receptor mRNA in chicken skeletal muscle indicates that the myoprotective effect of estrogen may involve receptor mediated gene regulation.

The profile of muscle damage during the 48 hour period following exposure to acute heat stress was determined in birds from the B line. All or a combination of catching, handling sampling and crating procedures induced a hypocapnic alkalosis in the blood, which was associated with subsequent muscle damage. The heat stress

was not severe enough to exacerbate this response, with the body temperature of the birds rising to just 42.5°C during heat stress exposure. Enzyme markers of muscle damage peaked between 12 and 48 hours following exposure to heat stress/control conditions. Hyaline fibres were observed in *Ps* and *Bf* muscles of some birds sampled after 12, 24 and 48 hours following exposure to heat stress/control conditions. However, inflammatory responses associated with a bacterial infection in many muscle sections limit interpretation. Commercial handling procedures are far rougher than those used in this study, and may therefore induce greater levels of muscle damage.

A comparison of the breast muscles of three GGP lines 48 hours following exposure to acute heat stress revealed a slight increase in the incidence of structural muscle damage in two of the lines. No heat-stress induced alterations in meat quality attributes were observed. The line with the smallest body weight and breast yield produced breast fillets with the least haemorrhaging and water loss. However, fillet texture, the most important consumer attribute, did not differ between the lines. This project has advanced our knowledge of the associations between genetic selection of broiler chickens, growth and stress-induced myopathies and alterations in meat quality.

## Declaration

I declare that this thesis is my own composition and that the work presented in it is my own and has not been submitted for any other degree or professional qualification. All assistance received has been acknowledged.

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Edinburgh
January 2004

**Acknowledgements**

I would like to thank my supervisors Dr Malcolm Mitchell, Mr Mike Mahon and Professor John Russell, and also Dr Dale Sandercock, Miss Sally Gilpin, Mr Richard Hunter, Miss Ailsa Carlisle, Mr Bob Flemming and Mr Nick Gonet for all of their help, advice and support during the experiments of this project. I would also like to thank Mr Richard Talbot and Dr Tim Boswell for their help and advice on molecular biology procedures, and for designing the primers and performing the sequencing. The work was funded by Cobb-Vantress Incorporated, and I am grateful for their financial support, interest and encouragement. I would like to give a special thanks to Gordon, and to my family and friends for their support throughout this project.

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**Contents**

## 1.1 Introduction

Poultry meat is now consumed in substantial quantities throughout the world (Roenigk, 1999). The rapid rise in poultry meat consumption is driven by many social and economic factors, including competitive pricing and freedom from religious taboos (Etches, 1996). In 2002, over 73 million tonnes of poultry meat was produced (FAOSTAT database). Over 63 million tonnes of this was chicken meat, derived from the slaughter of over 45,000 million birds (FAOSTAT database).

Consideration of poultry as a source of meat rather than just for egg production, started in the early 1900's. Founded in 1916, Cobb-Vantress Incorporated, the industrial sponsor of this study, is today one of the world's largest broiler (meat chicken) breeding companies (Cobb-Vantress website). In developing and maintaining a commercial strain of broilers, industry geneticists must consider a balance of features related to growth and reproduction which can be influenced by the genetic make-up of the bird (Leeson and Summers, 2000). The growth related characteristics include growth rate; weight-for age; meat yield; body conformation; skeletal integrity; feathering cover, rate and colour; feed efficiency and livability. Reproduction related characteristics include egg number and size; fertility; libido; egg hatchability; livability and aggressiveness (Leeson and Summers, 2000).

Commercial broiler chickens are typically derived from four different, highly selected, 'pedigree' great-grandparent lines, through a four-way cross breeding system (Figure 1.1). Male great-grandparent birds are mated with female great-grandparent birds to produce the grandparent generation. Only the highest performing birds are selected from the grandparent generation to continue in the breeding programme. The next stage involves the mating of males from one of the male lines (AA) with females from the second male line (BB) to produce parent males (AB). At the same time, males from one of the female lines (CC) are mated to females from the second female line (DD) to produce parent females (CD). In the

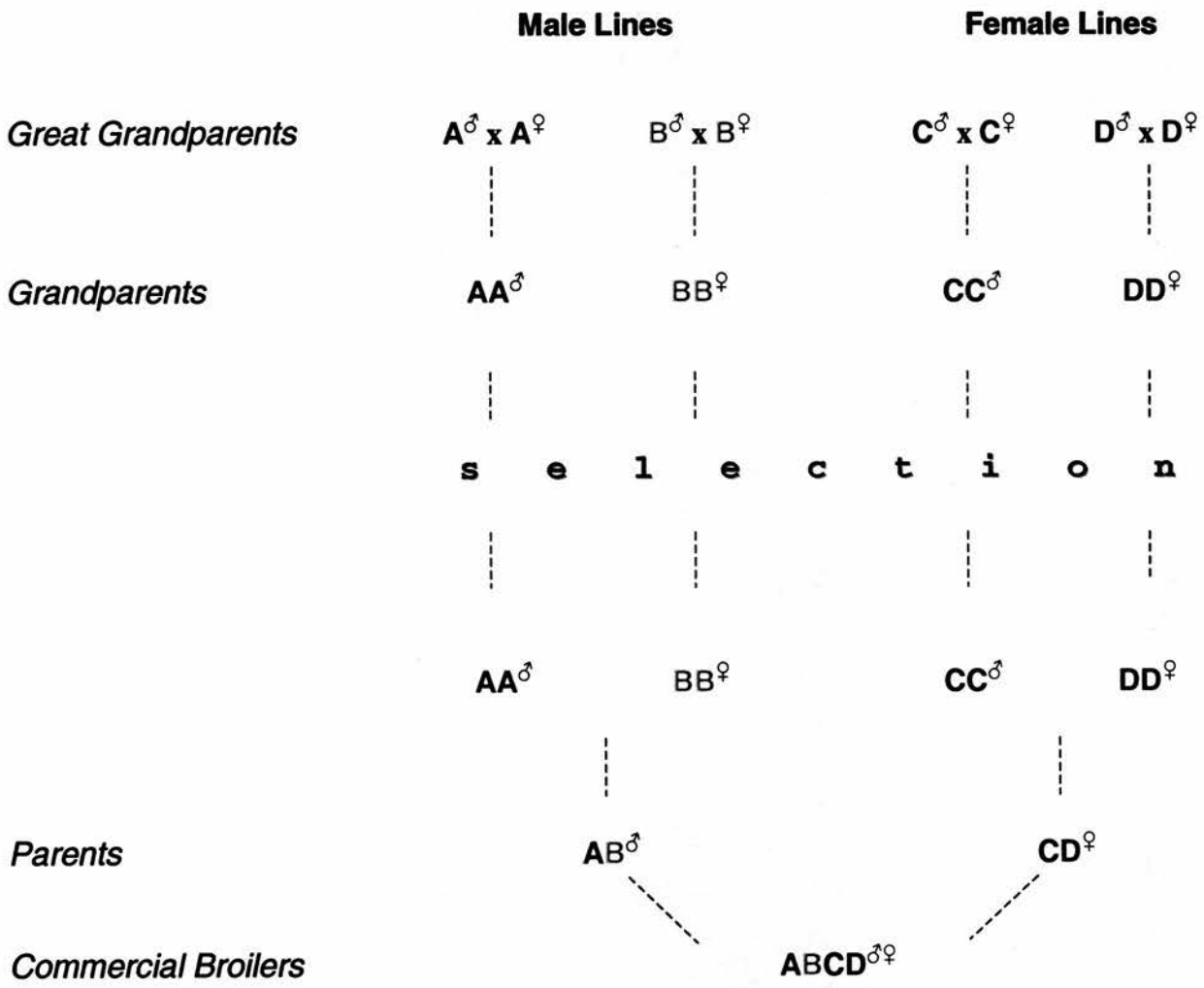



Figure 1.1. Four-way cross commercial breeding programme for broiler chickens (adapted from Leeson and Summers, 2000).

succeeding generation, parent males (AB) are mated with parent females (CD) to produce commercial broiler chickens (ABCD).

Genetic selection for desirable production traits has been extremely successful in broiler chickens. However, the traditional selection for these traits may have been almost fully exploited; at 42 days of age an average broiler chicken may reach a body weight of 2.2 kg having consumed only 1.82 g of food per 1.0 g of body weight gain (Mitchell, 1998). This genetic selection, which has led to a gross over-development of the broiler breast muscle *Pectoralis major*, may have resulted in increased susceptibility to spontaneous and stress-induced myopathy (muscle damage) (Mitchell, 1999a). This may compromise bird welfare and induce changes in poultry meat quality (Sandercock, Hunter, Nute, Hocking and Mitchell, 2001). The experiments detailed in this thesis will focus on characterising the idiopathic myopathy associated with broiler growth and development; characterising the myopathy induced by heat stress, a stressor commonly experienced by broiler chickens in the commercial environment; and investigating associations between myopathy and meat quality.

## 1.1 Skeletal muscle structure and function

### 1.2.1 Muscle structure and contraction

Both mammalian and avian skeletal muscle is a syncytium that is formed by single nucleated mesodermal cells that undergo terminal differentiation to form myoblasts, which then fuse to form multinucleated muscle fibres (Goldspink and Yang, 1999). The fibres are arranged longitudinally in parallel fasicles surrounded by perimysial connective tissue. Additionally, each individual muscle fibre is surrounded by endomysial connective tissue, through which it exchanges metabolites with blood capillaries. In poultry, myonuclei may be located in internal sarcoplasmic positions, as well as at the periphery of the muscle fibre (Figure 1.2). In contrast, all myonuclei in mammalian fibres are sarcolemmal (Carpenter, 2001). The muscle fibres contain rod-like contractile myofibrils, made up of protein filaments, which

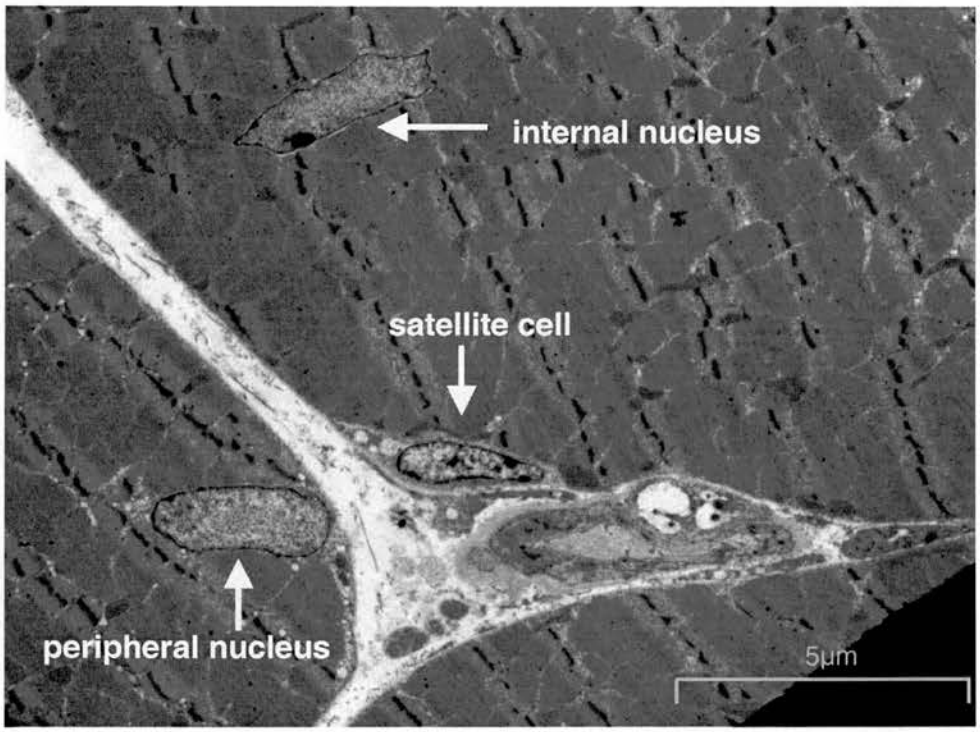



Figure 1.2. Electronmicrograph showing an internal nucleus, a peripheral nucleus and a satellite cell in neighbouring muscle fibres. Section is from the *Gastrocnemius* muscle of a 6 week old broiler chicken.

are arranged in units called sarcomeres. Each sarcomere consists of 1 set of thick (myosin) filaments and 2 sets of thin (actin) filaments. Actin and myosin filaments are arranged in an interdigited pattern, and contain the regulatory proteins troponins I, C and T, tropomyosin and actin. During contraction actin binds with myosin to form acto-myosin cross bridges. The actin filaments are pulled in over the myosin filaments so that each sarcomere shortens and generates force (Figure 1.3).

A motor nerve axon originating from the spinal cord makes contact with a muscle fibre through contact with the fibre's motor endplate (Figure 1.4). Individual axons branch in the muscle, and therefore each nerve controls many muscle fibres. The resulting functional grouping of a nerve and its associated muscle fibres is called a motor unit. When an action potential (nerve impulse) is elicited in a motor nerve, acetylcholine is released, which binds to the motor endplate, resulting in the production of a temporary electrical depolarisation. The depolarisation travels rapidly along the surface membrane of the muscle fibres in the motor unit. As depolarisation spreads along the transverse tubule system (Figure 1.4), it results in the release of calcium contained within vesicles of the sarcoplasmic reticulum (Figure 1.4), into the sarcoplasm. Contraction is initiated when calcium ions bind to the regulatory protein troponin, which is bound to tropomyosin in the actin filament. This binding induces a conformational change, freeing the binding the sites for acto-myosin cross bridge formation (Harvey and Marshall, 1986; Goldspink and Yang, 1999; Mills, 1998 and Murphy, 1996)

### 1.2.2 Fibre types

Chicken skeletal muscle fibres can be divided into one type of tonic and three types of twitch muscle fibres, the proportions of which vary between muscles (Barnard, Lyles and Pizzey, 1982). The fibre types differ in their speed of contraction due to fast, intermediate and slow myosin ATPase isoforms, which affect the rate of myosin cross-bridge cycling (Goldspink, 1996).

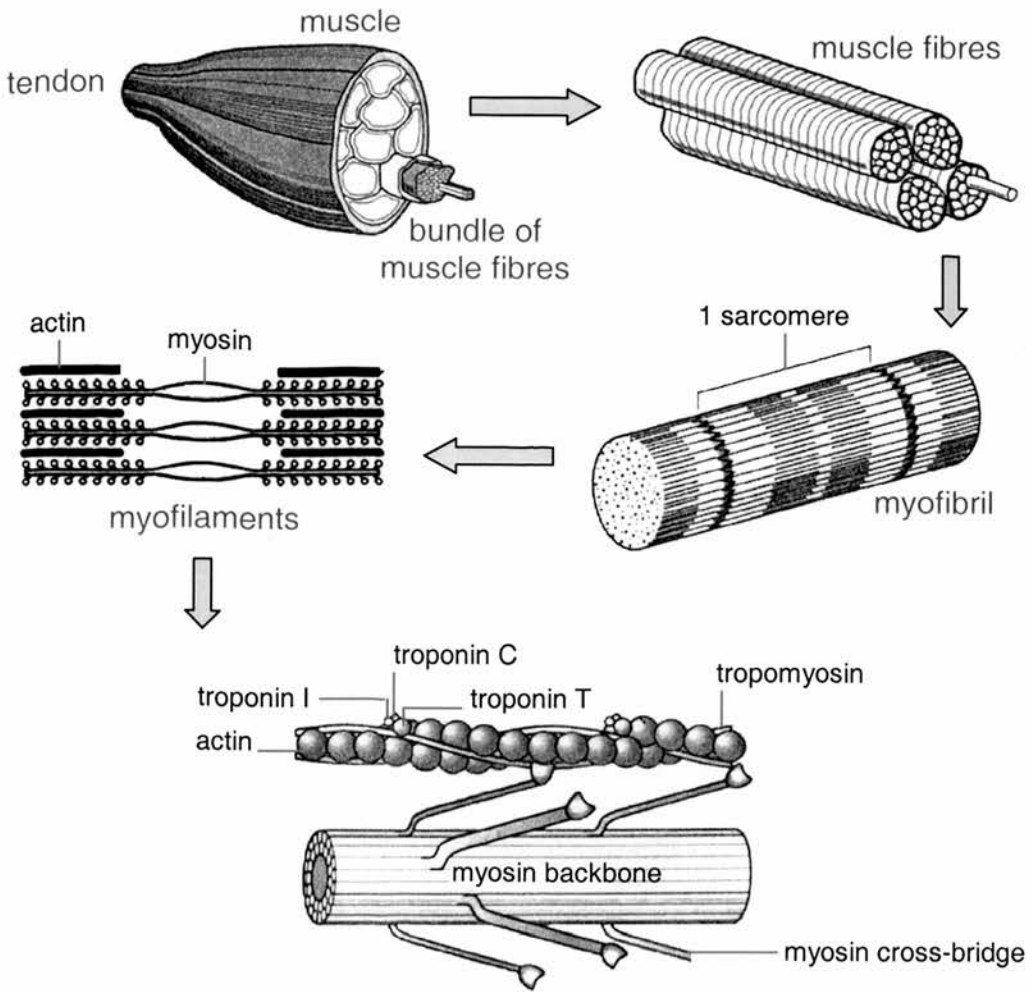



Figure 1.3 Macro, micro and molecular organisation of skeletal muscle (adapted from Goldspink, 1992).

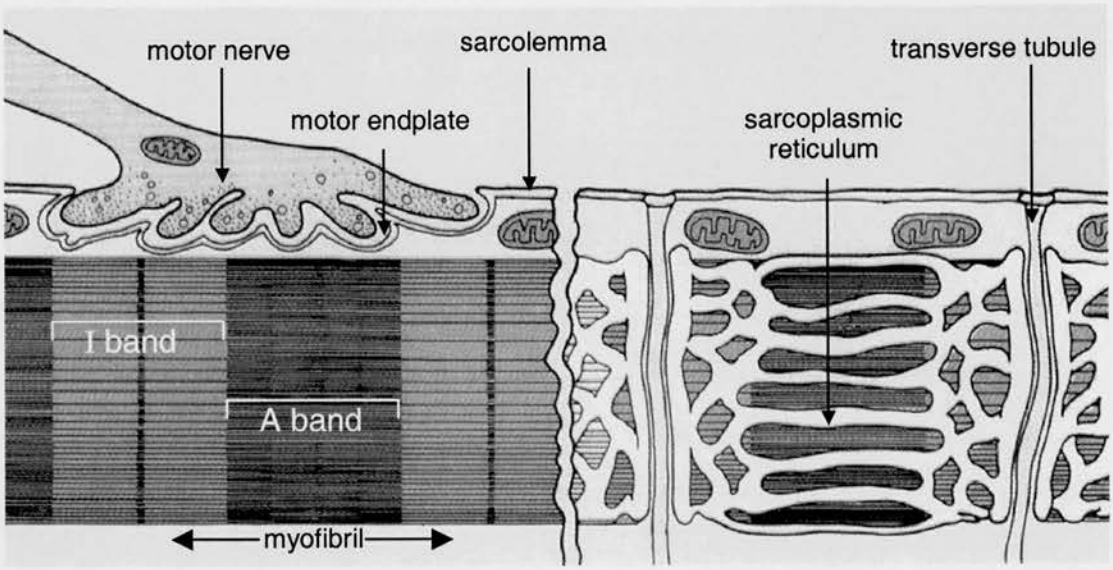



Figure 1.4. The arrangement of the membranes of a myofibril (adapted from Murphy, 1996). It should be noted that in avian muscle, the transverse tubules encircle the fibril at the two A-I band boundaries (Harvey and Marshall, 1986).

Slow tonic fibres are found in muscles such as the *Anterior latissimus dorsi*, which holds the wings back against the body. The muscle is contracted most of the time, but does so with very little ATP expenditure (Goldspink and Yang, 1999).

Slow, oxidative Type I twitch fibres are fatigue resistant and produce slow repetitive movements and sustain isometric force and are associated with postural muscles, such as the *Soleus* leg muscle, that are activated virtually all the time during standing, walking and running (Goldspink and Yang, 1999).

Type II twitch fibres develop force rapidly. Fast, glycolytic Type IIb fibres are adapted for a high power output over a short period and are ideal for functions such as an escape response (Rosser and George, 1986). These fibres fatigue quickly due to the build up of lactate during anaerobic glycolysis. The pectoral muscles of poultry are almost entirely made up of Type IIb fibres (Mills, 2001; Remignon, Gardahaut, Marche and Ricard, 1995). Fast, glycolytic and oxidative Type IIa fibres are adapted for a high-power output over longer periods of time. They have more mitochondria, and therefore a more oxidative metabolism than Type IIb fibres (Goldspink and Yang, 1999).

### 1.2.3 Avian muscle development and growth

During embryonic development, precursor cells of limb muscle enter the limb bud and then undergo terminal differentiation. The myoblasts then fuse together forming myotubes, which develop into muscle fibres (Stockdale, 1992). It has been proposed that during the postnatal period, avian muscle growth occurs only by hypertrophy, and not by hyperplasia, with fibre number becoming fixed before or shortly after hatch (Goldspink and Yang, 1999). However, genetic selection for increased growth of broiler muscles, in particular the *Pectoralis major* breast muscle, appears to have resulted in a larger muscle fibre number set and greater post-hatch growth potential (Remignon *et al*, 1995).

Increases in muscle fibre size can occur by (i) increase in length by the serial addition of sarcomeres to the ends of myofibrils (ii) increase in circumference by (a) the addition of new myofilaments to the periphery of the fibre and (b) myofibrils above a critical size may split longitudinally to form two or more daughter myofibrils within a fibre (Goldspink and Yang, 1999).

There is a considerable increase in the nuclei number during chicken muscle fibre growth (Moss, 1968). These extra nuclei are provided by satellite cells (Figure 1.2) in skeletal muscle (Moss and Leblond, 1971). Satellite cells are undifferentiated precursor muscle cells located between the membrane and basal lamina of the fibre (Bischoff, 1997). They are quiescent in mature muscle, but re-enter the cell cycle after (i) increased functional demand induces a hypertrophic response through satellite cell fusion and (ii) muscle injury generates a population of active satellite cells, which may migrate and fuse together to form new myofibres (Goldspink and Yang, 1999). Oxidative muscles generally have a higher density of satellite cells than glycolytic muscles. The reason for the difference in distribution among fibres is not known, but could be related to the innervation or recruitment pattern of individual fibre types (Schultz and McCormick, 1994).

## 1.3 Mechanisms of muscle damage

Muscle injury and disrupted cell membrane integrity may be induced by metabolic events or by mechanical factors such as high specific tensions produced in muscle fibres during eccentric contractions (Armstrong, 1990). High specific tensions could mechanically disrupt sarcolemma, sarcoplasmic reticulum or myofilaments. They could also permit the lipase phospholipaseA2 to physically come into contact with, and lyse the phospholipid substrates in the cell membrane, releasing arachidonic acid, which serves as a precursor to various eicosanoids involved in the inflammation cascade associated with muscle damage (Lehninger, Nelson and Cox, 1993). Metabolic events that could induce muscle damage include high temperature, insufficient mitochondrial respiration, free oxygen radical production and lowered pH (Armstrong, 1990). Whilst cells subjected to damage pass through

a number of different stages, damage ultimately occurs through one of a relatively few common final pathways. Of these, the primary pathways are loss of calcium homeostasis, loss of energy supply to the cell and over-activity of oxidising free radical-mediated reactions (McArdle and Jackson, 1997).

### 1.3.1 Calcium homeostasis disruption

Calcium is a highly versatile intracellular signal that operates over a wide temporal range to regulate many different cellular processes (reviewed in Berridge, Bootman and Roderick, 2003). Therefore the precise spatial and temporal regulation of calcium release and cytoplasmic concentration in the muscle fibre is essential, in order to avoid any potentially toxic effects of disrupted calcium homeostasis (Mitchell, 1999a). Therefore at rest, cells maintain a cytosolic free calcium concentration which is $10^4$ fold less than extracellular calcium concentration by having a very low plasma membrane permeability to calcium; controlling extrusion, release and uptake; buffering by proteins and calcium storage in membrane-bound compartments (Ashley, 1995). Voltage-operated, receptor-operated and store-operated channels in the plasma membrane control the influx of calcium required to fill the sarcoplasmic reticulum (Berridge, 1997). Muscular contraction increases the cytoplasmic calcium concentration, which is reduced by calcium-ATPases and sodium-calcium exchangers in the plasma membrane (Carafoli, 1987). Both the sarcoplasmic and the endoplasmic reticulum possess calcium pumps that can transport calcium into the respective organelle against a high concentration gradient (Carafoli, 1987).

Intracellular calcium accumulation induces damage to normal mammalian skeletal muscle by several different processes. These include the stimulation of calcium-activated proteases (e.g. calpain type I and calpain type II) and lipases (e.g. phospholipases which utilise cell membrane phospholipids as substrates) and mitochondrial calcium overload (McArdle and Jackson, 1997). Increased mitochondrial calcium content inhibits mitochondrial respiration, resulting in the mitochondria trying to maintain calcium homeostasis at the expense of energy

production. When no longer able to cope with the levels of calcium, the mitochondria become overloaded and release their calcium stores into the cytoplasm (McArdle and Jackson, 1997). Elevated intracellular calcium can also lead to excessive hypercontraction of some sarcomeres, producing hypercontracted (hyaline) fibres (Hoffman, 2001).

The effect of calcium homeostasis disruption on broiler skeletal muscle has been studied using in vitro muscle preparations (Sandercock, 1997). Elevated intracellular calcium, by either increased entry of external calcium (by calcium specific ionophores) or release from sarcoplasmic stores leads to disrupted cell membrane integrity (Sandercock and Mitchell, 1996). Membrane damage of chicken skeletal muscles also appears to involve activation of phospholipase A2, as a direct consequence of raised intracellular calcium (Sandercock and Mitchell, 2003). Broiler skeletal muscle treated with monensin (Sandercock and Mitchell, 1996), resulted in increased entry of sodium into cells by sodium-proton exchange (Hoya and Venosa, 1992). This causes increased calcium entry by the sodium-calcium exchange mechanisms in the sarcolemma; calcium release into the myoplasm via the ryanodine receptor and disrupted cell membrane integrity (Sandercock and Mitchell, 1996).

### 1.3.2 Loss of energy supply

Sarcoplasmic calcium regulation by mitochondrial calcium-ATPase pumps occurs at the expense of mitochondrial ATP production (Duchen, 1999). Under extreme conditions this may lead to ATP exhaustion (Wrogemann and Pena, 1976), as may a mitochrondrial defect, which would lead to sustained calcium accumulation in the sarcoplasm. A low energy supply would reduce the function of the ATP dependant calcium pumps of the sarcoplasmic reticulum and plasma membrane, leading to a gradual accumulation of cytosolic calcium (McArdle and Jackson, 1997). A lack of ATP could also lead to an influx of external calcium driven by the large extracellular:intracellular electrochemical calcium gradient (McArdle and Jackson, 1997). Therefore the muscle fibre enters a vicious cycle of ATP exhaustion and

increasing intracellular calcium concentrations with associated activation of degradative processes (Mills, 2001).

### 1.3.3 Free radical-mediated damage

There are a number of potential sites for the production of free radicals within muscle, including the mitochondrial electron system, membrane-bound oxidases and infiltrating phagocytic cells (McArdle and Jackson, 1997). Oxidative phosphorylation of ATP increases in response to energy demand, with a concomitant increase in free radicals. Free radicals will attack cellular components, especially lipids. The attack on lipids initiates a chain reaction called lipid peroxidation, which leads to generation of more free radicals and reactive oxygen species (Urso and Clarkson, 2003). Free radicals can also cause oxidative damage to DNA and to SR and plasma membrane proteins (McArdle and Jackson, 1997). Consequently, free-radical activity may lead to disruption of membrane pumps, damage to internal organelles and disrupted cell membrane integrity and ultimately disrupted calcium homeostasis (Mills, 2001).

### 1.3.4 Inter-relationships between mechanisms

The mechanisms described above (calcium overload, decreased energy supply and free radical formation) can interact as well as operate in isolation. Overt damage to muscle may lead to a loss of cell viability with a consequent failure of cells to maintain trans-membrane gradients of ions such as calcium, a loss of cellular energy supply, and stimulation of free radical-mediated degenerative processes, such as lipid peroxidation (McArdle and Jackson, 1997).

One or a combination of calcium overload, decreased energy supply and free radical formation eventually lead to muscle fibre degradation and necrosis (Armstrong, 1990). Damaged muscle fibres release factors including cytokines, which stimulate an inflammatory response, characterised by the movement of fluid, plasma proteins and leucocytes (primarily neutrophils and macrophages) into the tissue in response

to injury (Kendall and Eston, 2002). Leucocytes attack, remove and breakdown cell debris. The invasion by macrophages may stimulate satellite cell activity, leading to fibre repair and regeneration (Kendall and Eston, 2002).

## 1.4 Assessment of myopathy

### 1.4.1 Intracellular markers

Intracellular markers are released into the circulation in response to changes in muscle cell membrane integrity, providing a powerful diagnostic tool in human and veterinary clinical medicine (Hamburg, Friedman and Perryman, 1991).

The intracellular enzyme creatine (phospho)kinase (CK) is the most commonly employed indicator of muscle pathology, due to it's high activity in muscle tissue. CK is involved in the reversible transphosphorylation of adenosine diphosphate (ADP) and creatine, and is central to intracellular energy maintenance by the spatio-temporal buffering of ATP concentrations (Lehninger *et al*, 1993). Transport of broilers from farms to processing plants has been shown to result in a large elevation in plasma CK activity (Mitchell, Kettlewell and Maxwell, 1992). 97% of circulating CK in modern broiler chickens originates from skeletal muscle and it is specifically this isoform that is increased during transportation (Mitchell and Sandercock, 1995a). Therefore, measurements of plasma CK activities in poultry are a very useful index of the extent and nature of muscle cell damage induced by a range of treatments, environments and challenges (Mitchell, 1999a).

Plasma lactate dehydrogenase (LDH) activity is another commonly used measure of muscle pathology. However, LDH is also found in cardiac muscle, liver, kidney bone and erythrocytes, as well as skeletal muscle. Therefore elevations can be observed with either haemolysis or the disruption of any of these tissues, and therefore are extremely non-specific (Harris, 2002).

Elevations of plasma aspartate aminotransferase (AST) activity are frequently associated with muscle damage or liver disease. Elevated plasma AST activity without a concurrent elevation in plasma CK activity is highly suggestive of hepatocellular disruption (Harris, 2002). Conversely, elevated activities of both plasma AST and CK activities are suggestive of disrupted muscle cell membrane integrity and therefore muscle damage.

### 1.4.2 Muscle biopsies

Histological verification has been termed the 'gold standard' in assessing muscle damage. It is the only method that truly justifies the term 'damage', as damage to the structure of skeletal muscle can be visualised and quantified (Bar, Reijneveld, Wokke, Jaobs and Bootsma, 1997). However samples of muscle tissue only represent a 'snapshot in time and space' of a continuous process of adaptation and pathology. Additionally the end stages of most myopathologies usually look very similar, therefore care should be taken when interpreting the underlying cause (Mahon, 1999).

## 1.5 Poulty myopathies

### 1.5.1 Deep pectoral myopathy

Deep Pectoral Myopathy (DPM) is a polygenic recessive trait (Harper, Bernier and Thompson-Cowley, 1983). It is a degenerative myopathy of the deep *Coracobrachialis* breast muscle in broilers and turkeys (Siller, 1985). Genetic selection for desirable production traits such as increased growth rate and altered body conformation appear to be associated with this condition (Siller, 1985). DPM is recognisable by a green discolouration of the muscle and oedema. Histopathological features include fibre atrophy, hyalinisation and necrosis (Harper, Bernier, Helfer and Schmitz, 1975), accompanied by elevations in plasma creatine kinase activity (Hollands, Gruner, Williams and Gavora, 1980).

### 1.5.2 Genetic muscular dystrophy

This inherited muscular dystrophy is characterised by muscle weakness and disability, with muscle degeneration and fibrosis. Fast twitch, glycolytic fibres are mostly affected (Wilson, 1990).

### 1.5.3 Focal myopathy and growth associated myopathy

Focal myopathy is a term used to describe a muscle abnormality that occurs in young, rapidly growing turkeys. Pathological features include hypercontraction of fibres, scattered focal necrosis, fatty tissue replacement, fibrosis and infiltration of mononuclear cells (Sosnicki, Cassens, Vimini and Greaser 1991; Wilson, Nieberg and Buhr 1990). Despite these characteristics, and elevated plasma creatine kinase activities (Wilson, 1990), affected birds show no signs of disability. A muscle abnormality observed in broiler breeders termed 'generalised myopathy' (Randall, 1981) may be similar to focal myopathy in turkeys. A comparative study of meat-type and layer-type chickens of the same age revealed a higher prevalence of degenerative changes such as cross striation and necrosis in the meat-type, primarily in the breast rather than leg muscles (Soike and Bergmann, 1998). However, in contrast to the comparable turkey studies (Sosnicki *et al*, 1991, Wilson *et al*, 1990), infiltration by mononuclear cells, fibrosis or fatty replacement occurred only very rarely.

Using plasma creatine kinase activity as an indicator of myopathy, studies have shown that muscle damage increases with age in commercial broilers (Mitchell and Sandercock, 1994) and turkeys (Hocking, Mitchell, Bernard and Sandercock, 1998; Mills, Mahon and Mitchell, 1998). In both species there was significantly greater muscle damage in the commercial lines compared to their genetic predecessors or more traditional lines. A histological comparison of three commercial turkey lines has shown an increased incidence of muscle abnormalities coincident with the rapid growth phase in both breast and leg muscles (Mahon, Gilpin, Nixey and French, 1995). This was also true of the commercial turkey line in a comparison of a

commercial and an unselected traditional turkey line (Mills *et al*, 1998), with the unselected line showing very few structural abnormalities. In this study a large increase in plasma creatine kinase occurred in the commercial turkeys at 15 weeks of age onwards, coincident with a greatly increased incidence of muscle pathology. Histopathological features seen in turkey muscle include hyaline fibres, loose packing and oedematous connective tissue, necrosis, phagocytosis, inflammation, enzyme changes, increased esterase activity, regeneration and extensive basophilia and multinucleation. Additionally, the number of internalised myonuclei per muscle fibre cross-section increased with age in both leg and breast muscles (Mahon *et al*, 1995). A more recent histological turkey study compared a random-bred control line, a subline from the random-bred line selected for long-term for increased 16 week body weight and a commercial sire line (Velleman, Anderson, Coy and Nestor, 2003). Degenerative muscle fibre hypercontraction was predominant in the selected subline, suggesting that growth selection for body weight may be associated with post hatch muscle damage.

### 1.5.4 Capture myopathy

The trapping and transportation of birds can cause an acute degeneration of breast, wing and leg muscles due to intense muscular exertion and trauma, and may occasionally lead to paralysis and death (Spraker, Adrian and Lance, 1987; Dabbert and Powell, 1993). Trapping of wild turkeys appears to result in lesions characterised by pale, white streaked areas in the muscle, basophilia, hyalinisation, myogibrillar disruption, necrosis and oedema. This pathology may be linked to cellular hypoxia and lactic acidosis (Spraker *et al*, 1987). Relative humidity, ambient temperature and plasma creatine kinase activity at time of capture have been suggested as the best predictors of wild turkey mortality within 14 days post-capture (Nicholson, Lochmiller, Stewart, Masters and Leslie, 2000). During the capture of wild ducks, the time of struggling positively correlates to plasma creatine kinase activities (Dabbert and Powell, 1993). In a review of postmortem examinations of captive and imported Houbara bustards (Bailey, Nicholls, Samour, Naldo, Wernery and Howlett, 1996), capture myopathy is cited as a common cause

of death in adult birds. Plasma creatine kinase and lactate dehydrogenase activities are markedly elevated in Houbara bustards following capture, handling and translocation, and peak 24 hours after capture (Bailey, Wernery, Naldo and Samour, 1997).

### 1.5.5 Sudden death syndrome

The sudden death of apparently healthy broilers may be induced by reduced sarcoplasmic calcium regulation in skeletal muscle (Reiner, Hartmann and Dzapo, 1995). Sarcoplasmic regulation of calcium in broiler chickens shows lower calcium transport rates and transport efficiencies compared to layer chickens. This may cause greater myofibre calcium concentrations and muscle hypersensitivity, followed by lactic acid accumulation and cardiovascular failure (Reiner *et al*, 1995).

### 1.5.6 Toxicity

The most frequently described agent responsible for toxic myopathies in poultry is the anticoccidial agent monensin (Dowling, 1992). This myopathy is characterised by weakness and paralysis, with histopathological features including hyalinisation and necrosis (Julian, 1991). Elevated plasma creatine kinase and aspartate aminotransferase activites are indicative of ionophore toxicity (Dowling, 1992). It has been proposed that monensin, a sodium ionophore (Hoya and Venosa, 1992), disrupts sodium-potassium equilibrium across the sarcolemma, resulting in increased intracellular calcium, which would result in cellular damage (Trump, Berezesky, Smith, Phelps and Elliget, 1989).

The plant *Senna occidentalis* is a common agricultural contaminant. The seeds produce a toxic mitochondrial myopathy in poultry, with histopathological features including sarcolemmal nuclei proliferation and necrosis (Cavaliere, Calore, Haraguchi, Gorniak, Dagli, Raspantini, Calore and Weg, 1997). Histological studies have revealed an intense fibre atrophy in both type I and type II muscle fibres in chickens fed the external tegument of the seed compared to birds fed the

whole seed or other parts of the seed (Haraguchi, Calore, Dagli, Cavaliere, Calore, Weg, Raspantini and Gorniak, 1998).

### 1.5.7 Dietary deficiencies

Deficiencies of vitamin E and selenium are associated with myopathy in poultry. Symptoms include haemorrhaging, calcium deposits and vascular lesions (Hassan, Hakkarainen, Jonsonn and Tyopponen, 1990). Vitamin E appears to exert a myo-protective effect by preventing the damage of cell membranes by oxidative free radicals (Formigli, Manneschi, Tani, Gandini, Adembri, Pratesi, Novelli and Orlandini, 1997).

### 1.5.8 Heat stress-induced myopathy

Heat stress is commonly experienced by broiler chickens in the commercial environment (Mitchell and Kettlewell, 1998). Plasma creatine kinase activity following acute exposure to heat stress is greater in selected broilers compared to a control line (Mitchell and Sandercock, 1995a). This is associated with a larger heat stress induced increase in metabolic heat production in the broilers (Mitchell and Sandercock, 1995b). However, in a series of controlled environmental studies on turkeys, there was no increase in muscle damage that could be attributable to heat stress (Mills, 2001). Avian thermoregulation and the pattern of elevated plasma enzyme activities in response to muscle trauma are reviewed in the introduction of Chapter 5. Associations between acute pre-slaughter stress and alterations in meat quality attributes are described in the introduction of Chapter 6.

## 1.6 Myo-protective effect of estrogen[1]

Estrogen has been reported to reduce the incidence of myopathy induced by exercise in both rats and man (reviewed in Amelink, Koot, Erich, Van Gijn and Bar, 1990).

[1] American spelling used as this corresponds to the standard abbreviation for estrogen receptor (ER), which is used frequently in the thesis, and as many of the relevant references use this spelling.

Also, the spontaneous myopathy observed in broilers (Mitchell and Sandercock, 1994) reduces both at the onset of ovarian estradiol secretion in female birds (Mitchell and Sandercock, 1996) and following estrogen administration to immature broilers (Carlisle, Mitchell and Tritten, 1997). An understanding of this action may help identify the lesions mediating the predisposition of modern rapidly growing poultry to spontaneous or stress-induced muscle damage (Mitchell, 1999a).

### 1.6.1 Indices of sexual development

In female birds, plasma concentrations of estrogen rise steadily for several weeks prior to the onset of lay, with the maximum concentration of estrogen occurring 3-5 weeks before the onset of lay (Etches, 1996). Estrogen stimulates production of the yolk precursors vitellogenin and very low density lipo-protein (VLDL). These are synthesised by the liver, transported to the developing follicle via the blood, and deposited in the yolk by specific receptor-mediated mechanisms (Beekman, Wijnholds, Schippers, Pot, Gruber and Ab, 1991; McEwan, Saluz and Jost, 1991). Physiological adaptations facilitate the absorption, deposition and storage of calcium from feed, and utilise $CO_2$ dissolved in the blood to synthesise the carbonate ion in the eggshell (Etches, 1996). Therefore the measurement of calcium and the egg yolk precursors vitellogenin and VLDL in the plasma can be used to detect ovarian activity (Mitchell and Carlisle, 1991).

### 1.6.2 Muscle damage during growth and development

Plasma CK activities have been shown to increase with age in commercial broilers (Mitchell and Sandercock, 1994). However, at the onset of ovarian estradiol secretion a dramatic fall in broiler plasma CK activity occurs, coincident with significant elevations of plasma concentrations of calcium and the egg yolk precursors vitellogenin and VLDL, which reflect ovarian activity (Mitchell and Sandercock, 1996). Studies in White Italian geese have shown that the plasma activity of CK is lower in adult female compared to male geese (Czekaj, Samorek-Salamonowicz, Kozdrun and Bik, 2003).

### 1.6.3 Estradiol studies

In mammals, estradiol appears to exert a protective effect against muscle damage (Amelink *et al*, 1990; Bar and Amelink, 1997). Exercise induces greater post-exercise serum CK, AST and LDH activities in male compared to female rats (Van der Meulen, Kuipers and Drukker, 1991). A similar course of exercise-induced histopathological changes have been observed in muscles from both sexes, with females showing less marked changes compared to males (Komulainen, Koskinen, Kalliokoski, Takala and Vihko, 1999). Ovariectomised compared to control female rats also show greater serum CK activities following exercise (Amelink and Bar, 1986). The administration of estradiol to male rats has been shown to reduce both serum CK activity and histological muscle damage (Reijineveld, Ferrington, Amelink and Bar, 1994). Tamoxifen, a selective estrogen receptor modulator (SERM), is as equally effective as estrogen in protecting muscle membranes when administered to rats (Koot, Amelink, Blankenstein and Bar, 1991). A direct anti-oxidative role of estrogen has been proposed, whereby estrogen reduces the damage caused by free radical generation and lipid peroxidation in the cell membrane (Bar and Amelink, 1997).

The administration of estradiol to immature female broilers causes a marked decrease in plasma creatine kinase activities (Mitchell and Sandercock, 1996). The administration of tamoxifen alone has no effect, and the myoprotective action of estradiol can be blocked by the simultaneous administration of tamoxifen (Carlisle, *et al*, 1997). Tamoxifen also inhibits the estrogen dependent synthesis of egg yolk precursors. These same effects of tamoxifen administration have also been shown in sexually mature white leghorn laying hens (Carlisle, Mitchell and Hunter, 1999). As VLDL and vitellogenin synthesis are mediated through estrogen receptor occupation in the liver (Beekman *et al*, 1991; McEwan *et al*, 1991), the myoprotective effect of estradiol in avian skeletal muscle may also be mediated by estrogen receptor occupation, in contrast to reports in mammals. The literature associated with mammalian and estrogen receptors is reviewed in detail in the introduction of Chapter 4.

## 1.6 Experimental aims

(i) To establish the time course of idiopathic myopathy development in chicken lines contrasting markedly in growth rate (Chapter 3).

(ii) To study the mechanisms that may mediate the apparent myo-protective effects of estrogen observed at the onset of ovarian steroid secretion in female chickens (Chapter 4).

(iii) To induce a stress-associated myopathy in broiler chickens and to determine the subsequent myopathy profile (Chapter 5).

(iv) To study the associations between myopathy, stress, and meat quality attributes (Chapter 6).

## 2.1 Chicken lines

### 2.1.1 Commercial broiler

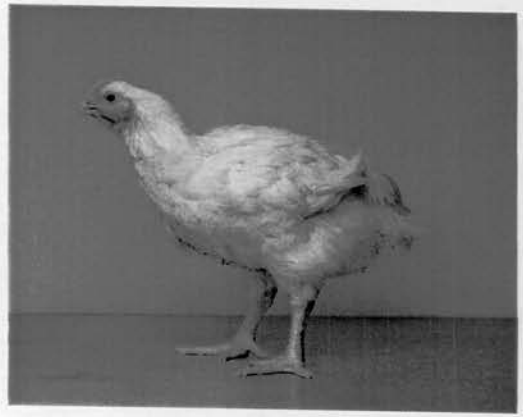
The commercial broiler used for all of the experiments was the Cobb 500 (Figure 2.1a) (Cobb Vantress website), which is phenotypically selected for traits including growth rate, body conformation and meat yield. Female birds were obtained as day old chicks. The broilers used in the study described in Chapter 5 were sourced from Grampian Hatchery, Newbridge, Scotland, UK). The broilers used in the study described in Chapter 3 were sourced from Cobb UK, Chelmsford, UK as other lines were required from this source for the study. There was no difference in growth performance between the broilers obtained from the two locations.

### 2.1.2 Commercial broiler great-grandparents

These "pedigree" birds were hatched on a speciality farm (Cobb UK, Chelmsford, UK) and were from flocks that have been selected primarily for growth related characteristics, but will also have undergone selection for reproductive characteristics such as egg productivity and fertility. Female birds from the three great-grandparent lines used commercially (Lines A, B and C) (Figure 2.1c-e) were obtained as day old chicks (Cobb UK, Chelmsford, UK).

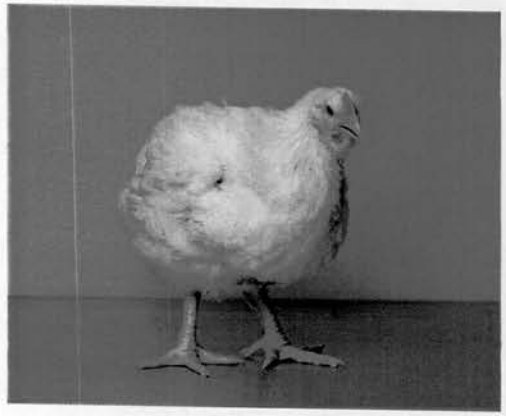
### 2.1.3 Commercial layers

The Brown Lohman (Lohmann Tierzucht website) (Figure 2.1b) and ISA Brown (Hubbard ISA website) commercial laying lines were used. The ISA Brown line has a slightly better performance specification in terms of egg productivity. These lines are selected for reproductive traits including fertility, egg number, size and hatchability and not growth related traits.

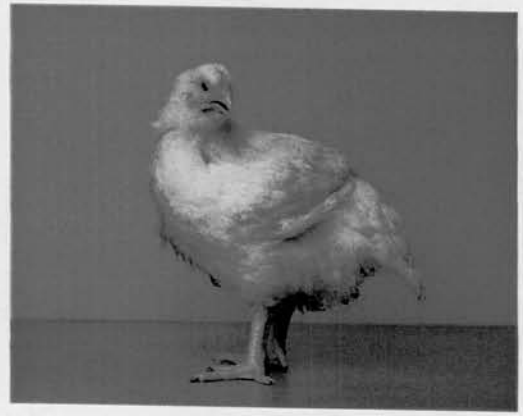


(a) Cobb 500 commercial broiler

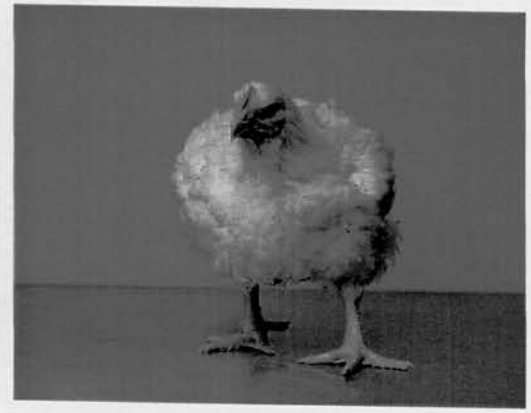
(b) Lohman (Brown) commercial layer



(c) Cobb Line A broiler great-grandparent



(d) Cobb Line B broiler great-grandparent



(e) Cobb Line C broiler great-grandparent

Figure 2.1 (a) Cobb 500 commercial broiler; (b) Lohman (Brown) commercial layer, and (c-e) Cobb broiler great-grandparents from Lines A, B and C respectively (all birds at 6 weeks of age).

## 2.2 Husbandry

The different lines were reared separately in 3.6m$^2$ pens littered with wood shavings on a light regime of 14 hours light:10 hours dark, at 21°C and 55% relative humidity. During the first 3 weeks, brooders were placed in the pens, in order to provide a spot temperature of 38°C and ambient temperature of 21-25°C. Food and water were available ad libitum. The birds were fed a series of commercial diets (Roslin Nutrition, Midlothian, UK). The layer starter diet was fed to the birds from 0-8 weeks of age and contained 26.5% soya (protein) and 68.1% wheat (carbohydrate). This was followed by a grower diet that was fed to the birds from 8-16 weeks of age and contained 15.2% soya and 80.1% carbohydrate. From 16 weeks onwards, the birds were fed a diet appropriate for sexual development and egg production, which contained 16.5% soya, 70.1% wheat and 8.2% limestone (as a source of calcium). The broiler starter diet was fed to the broiler and broiler great-grandparent chickens from 0-6 weeks of age and contained 29.8% soya and 62.2% wheat. From 6 weeks onwards the broilers and broiler great-grandparents were fed a finisher diet that contained 20.4% soya and 71.0% wheat.

## 2.3 Ethical approval

All experiments involving live birds had received ethical approval from the Roslin Institute Ethical Review Process and were undertaken under an appropriate home office licence.

***Procedures undertaken on live birds***

## 2.4 Blood sampling and euthanising

Blood samples (2ml) were obtained by venepuncture of the brachial vein using a 2.0ml syringe, fitted with a 25 gauge, 5/8 inch needle. Each blood sample was transferred to a 5ml blood collection tube (Teklab, UK) containing 50 units Li-heparin anti-coagulant and placed on ice. The samples were centrifuged at 1500g

(MSE-Mistral 2000R) for 5 minutes. The plasma supernatant was pipetted into plasma tubes and immediately frozen at -20°C, pending analysis. Birds were euthanised using an intravenous injection of sodium pentobarbitone. The birds were deemed dead when there was cessation of visible respiratory movements; a loss of withdrawl reflexes; a fixed dilated pupil and when the third eyelid ceased to move in response to corneal stimulation.

## 2.5 Body temperature

A thermistor probe (R.S. Components Ltd, UK) was inserted 5cm into the rectum and maintained in position until the digital readout displayed a constant value.

## 2.6 Continuous recording of deep body temperature

This technique was developed for transport and thermal stress studies in broiler chickens. Tinytalk II data loggers (RS Component Ltd, Northants, UK) (Figure 2.2) were placed in a waterproof latex cover, sterilised in an iodine containing solution and implanted in the peritoneal cavity of the three lines of broiler great-grandparents. The data loggers continuously recorded the deep body temperature of the birds during control, heat stress and recovery periods. Dr M. A. Mitchell performed the implantations of the broiler great-grandparent chickens with data loggers.

### 2.6.1 Anaesthesia

A beak tube was used to deliver the volatile anaesthetic. Induction of deep, surgical anaesthesia was achieved in less than three minutes using 4% halothane in pure oxygen at an oxygen flow rate of 2.0-2.5 $lmin^{-1}$. Subsequently, the appropriate plane of anaesthesia was maintained with 1.0-3.0% halothane at 1.0-1.5 $lmin^{-1}$.

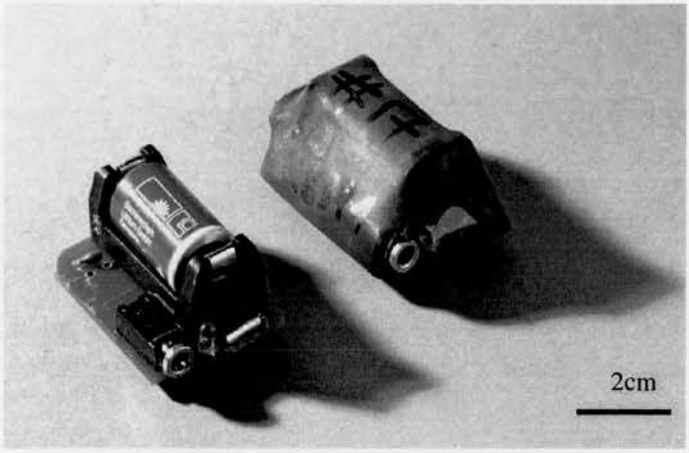



Figure 2.2 The Tinytalk II datalogger

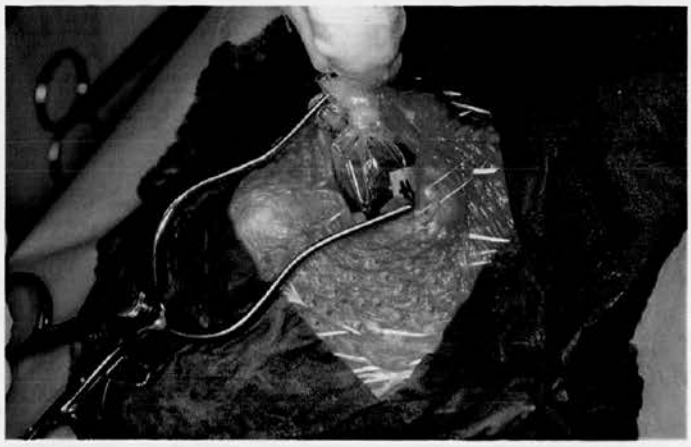


Figure 2.3 Insertion of the datalogger into the peritoneal cavity

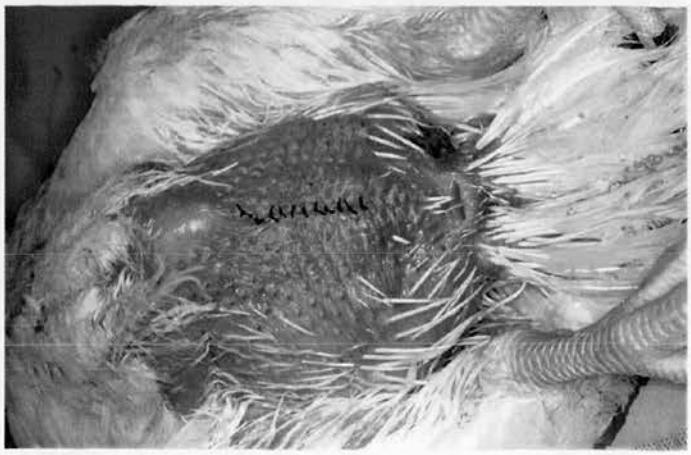


Figure 2.4 Following implantation of the datalogger, the skin was sutured and cleansed.

### 2.6.2 Surgery

Feathers in the region of the operative field were plucked and the follicles and skin were cleaned thoroughly with pevidine and alcohol. The operative field was then prepared with sterile drapes. The primary incision was 1-2 cm below the keel arch, in the anterior mid-line. This incision was of the skin only, and approximately 4 cm in length. The secondary incision was through the body wall. The muscle was clamped on either side with artery forceps. Finally, the abdominal air sac was cut, and the data logger was inserted into the intraperitoneal cavity (Figure 2.3). The body wall and skin were sutured separately, and the wound area was cleaned thoroughly (Figure 2.4).

### 2.6.3 Recovery

Following the surgery, the birds were placed in a closed poultry box, in a quiet, warm room. When each bird had fully regained consciousness, it was placed in a 'recovery' pen and allowed at least 5 days of recovery before being placed in the experiment.

***Procedures undertaken on chicken muscle samples***

## 2.7 Muscle sampling for histocytochemical studies

Standard histological procedures involving tissue fixation and paraffin embedding were not used for the muscle tissue samples as they do not allow muscle fibre typing and also may cause fibre shrinkage and a reduction in enzyme activity (Cumming, Fulthorpe, Hudgson and Mahon, 1994). Therefore the tissue was frozen for cryostat sectioning to ensure optimum tissue preservation.

### 2.7.1 Muscles selected for sampling

The large number of birds, and the time taken to process each sample restricted the number of muscles sampled from each bird. The *Pectoralis major* (Figures 2.5 and 2.6), *Coracobrachialis* (Figures 2.7 and 2.8), *Biceps femoris* (Figures 2.9 and 2.10) *Peroneus longus* (Figures 2.9 and 2.11) and *Gastrocnemius* (lateral head) (Figures 2.9 and 2.12) muscles were sampled, as these muscles provided contrasting morphological and physiological characteristics (Mahon *et al*, 1995). The origin, insertion and action of the muscles are described in Table 2.1.

### 2.7.2 Muscle dissection and freezing

Approximately $2cm^3$ samples originating from standardised regions of the *Pectoralis major* (Figure 2.6), *Coracobrachialis* (Figures 2.8), *Biceps femoris* (Figure 2.10) *Peroneus longus* (Figure 2.11) and *Gastrocnemius* (Figure 2.12) muscles were orientated for transverse fibre sectioning and mounted on labelled cork discs using the viscous embedding compound OCT Tissue-Tek (Sakura, Netherlands). The samples were snap frozen by immersion in liquid nitrogen – cooled Isceon [45] (Hotfrost, UK) (Figures 2.13 and 2.14), and stored at -70°C until sectioned.

### 2.7.3 Sectioning frozen muscle samples

10μm serial transverse sections were cut from each muscle sample using a cryostat (Bright Instruments, UK) at –16 to -24°C. The first section of each block was stained with Toluidine Blue (1% toluidine blue in 1% sodium tetraborate). This stained tissue components blue and enabled the confirmation of the orientation and preservation of each block. The slides were frozen at -70°C pending staining.

### 2.7.4 Staining procedures

Histological procedures were used to study muscle architecture and muscle fibre morphometry by dye staining. Histochemical procedures were used to reveal

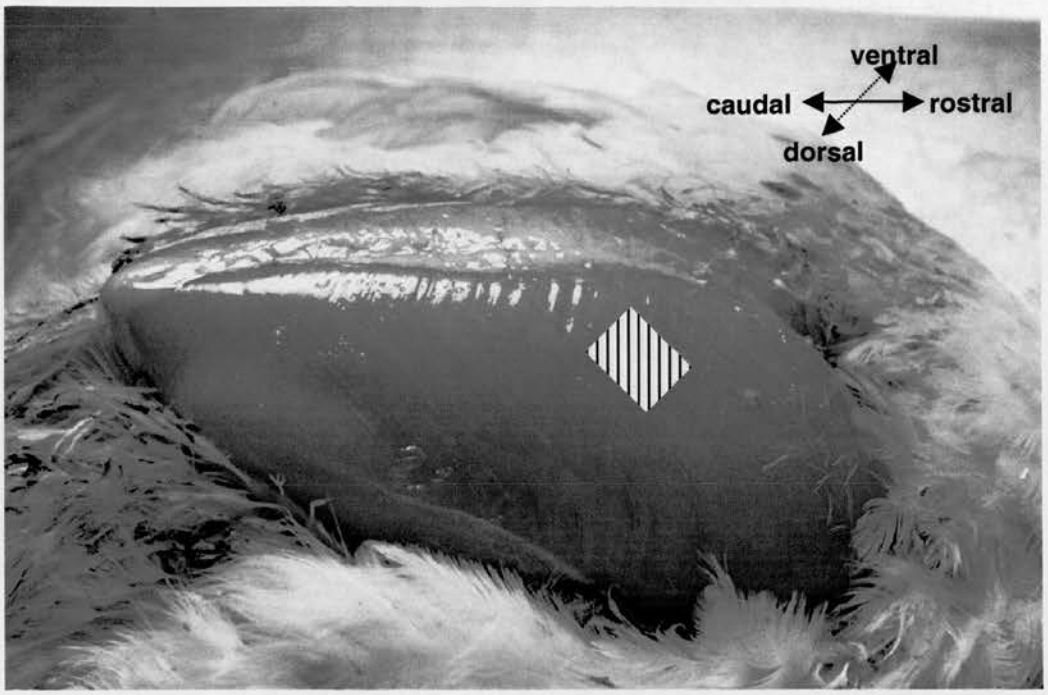



Figure 2.5 Ventral view of the left *Pectoralis major* breast muscle.

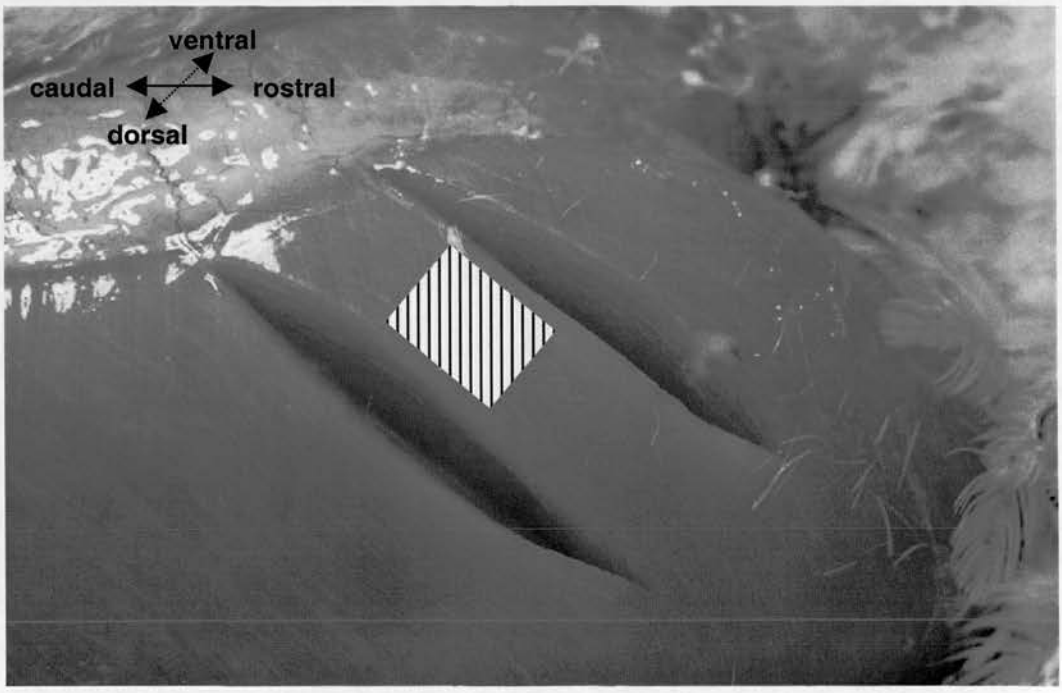



Figure 2.6 Ventral view of the region from which the left *Pectoralis major* breast muscle was sampled.

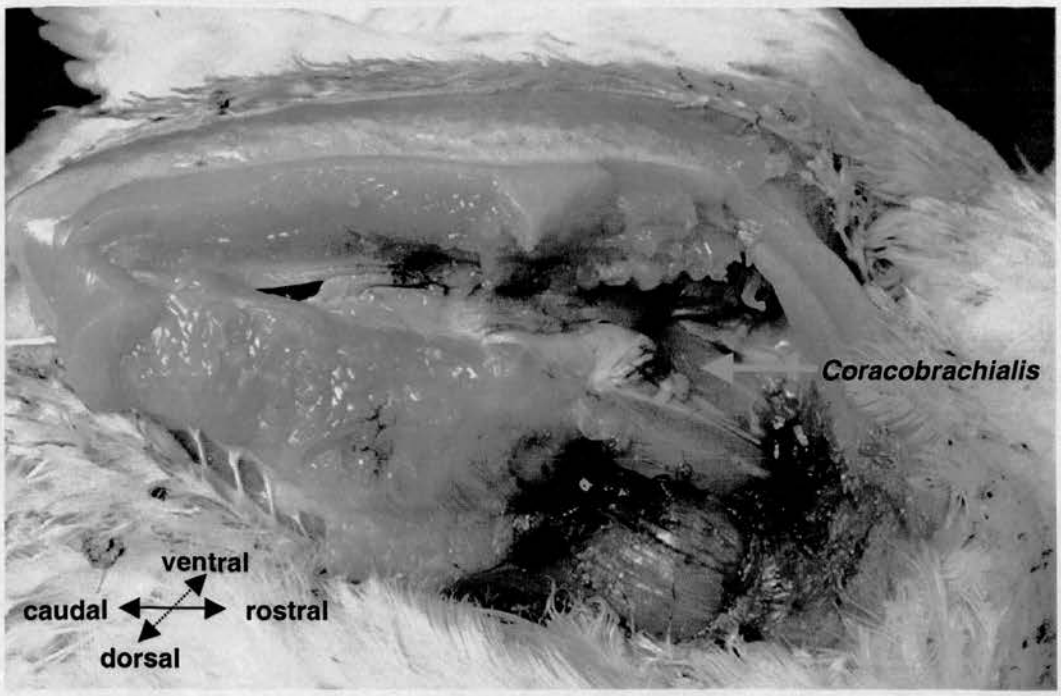



Figure 2.7 Ventral view of the left *Coracobrachialis* breast muscle.

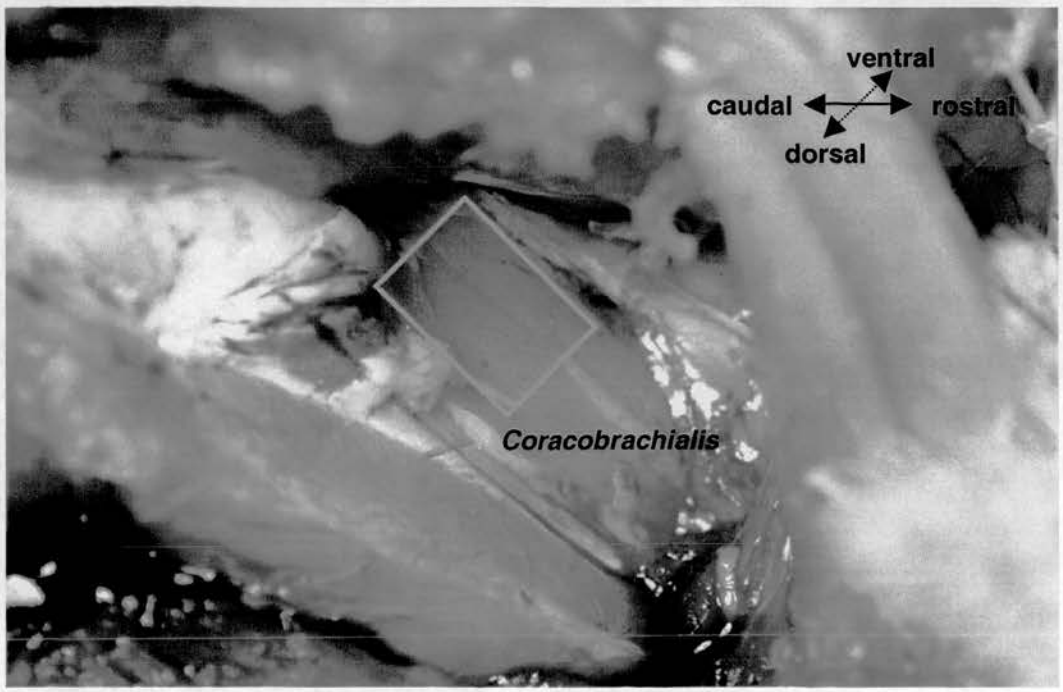



Figure 2.8 Ventral view of the region from which the left *Coracobrachialis* breast muscle was sampled.

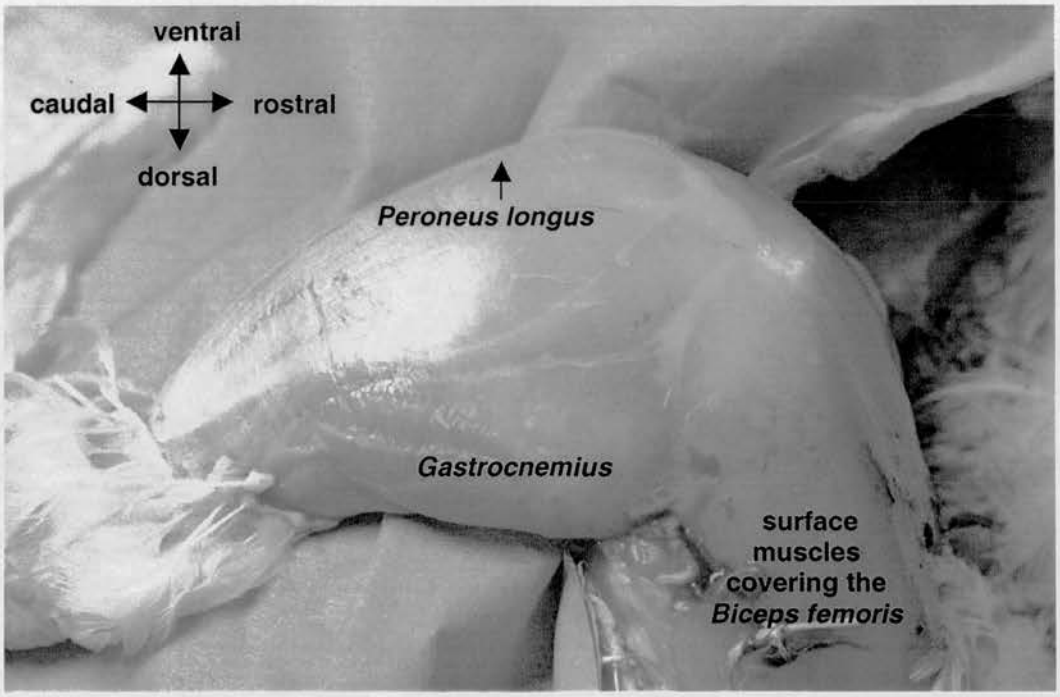



Figure 2.9 Left lateral view of the left *Peroneus longus,* and left *Gastrocnemius* muscles and of the surface muscles covering the left *Biceps femoris* muscle.

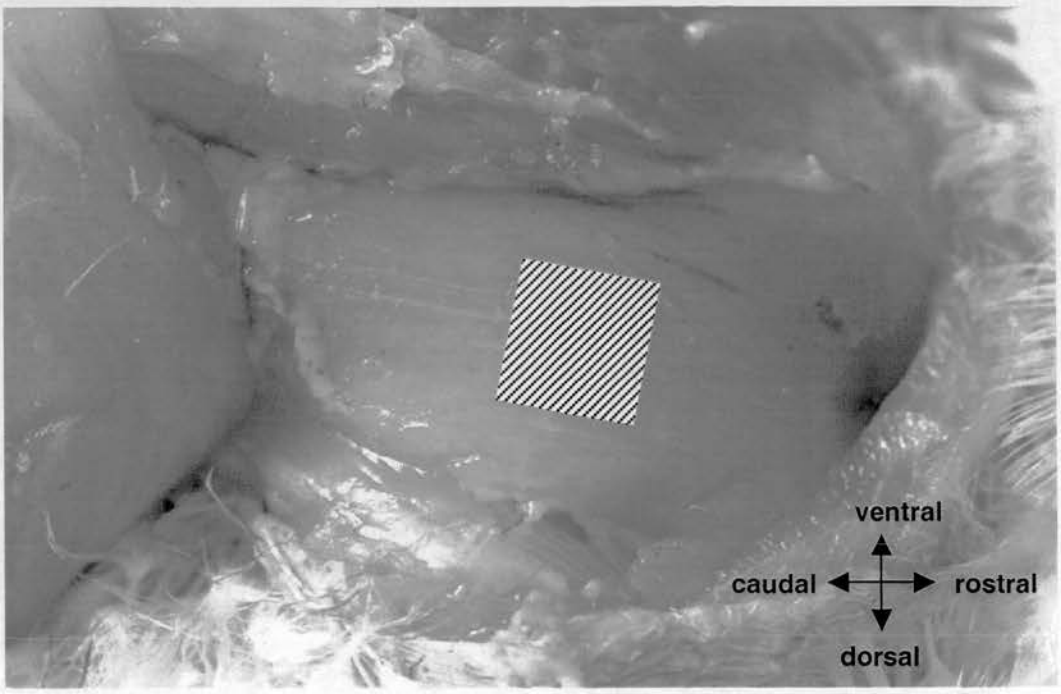



Figure 2.10 Left lateral view of the region from which the left *Biceps femoris* leg muscle was sampled.

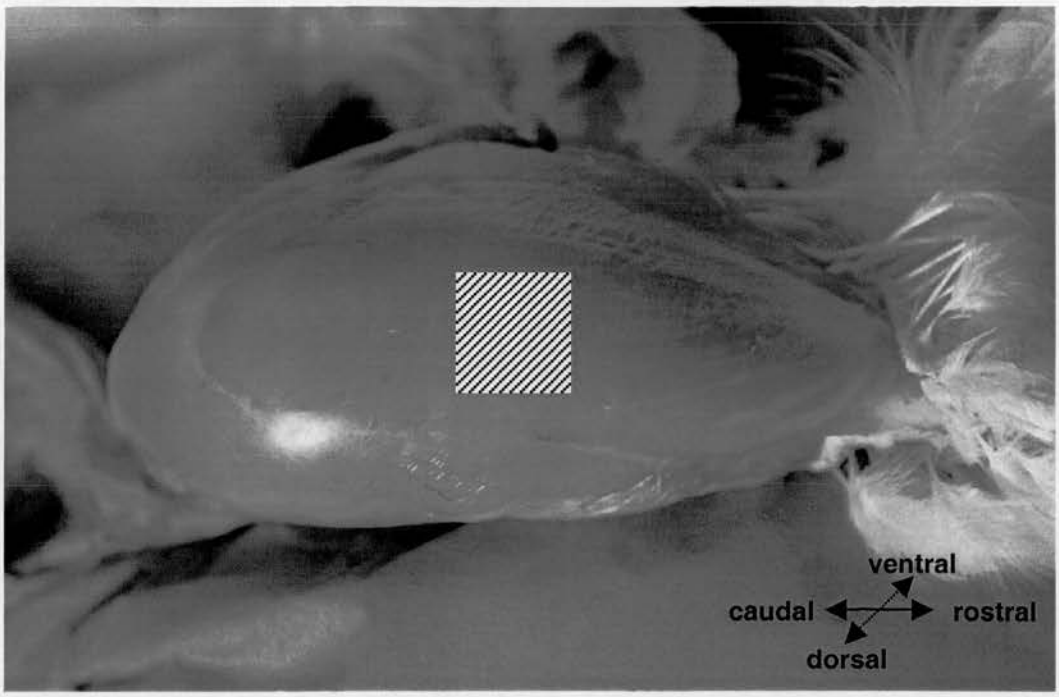



Figure 2.11 Ventral view of the region from which the left *Peroneus Longus* leg muscle was sampled.

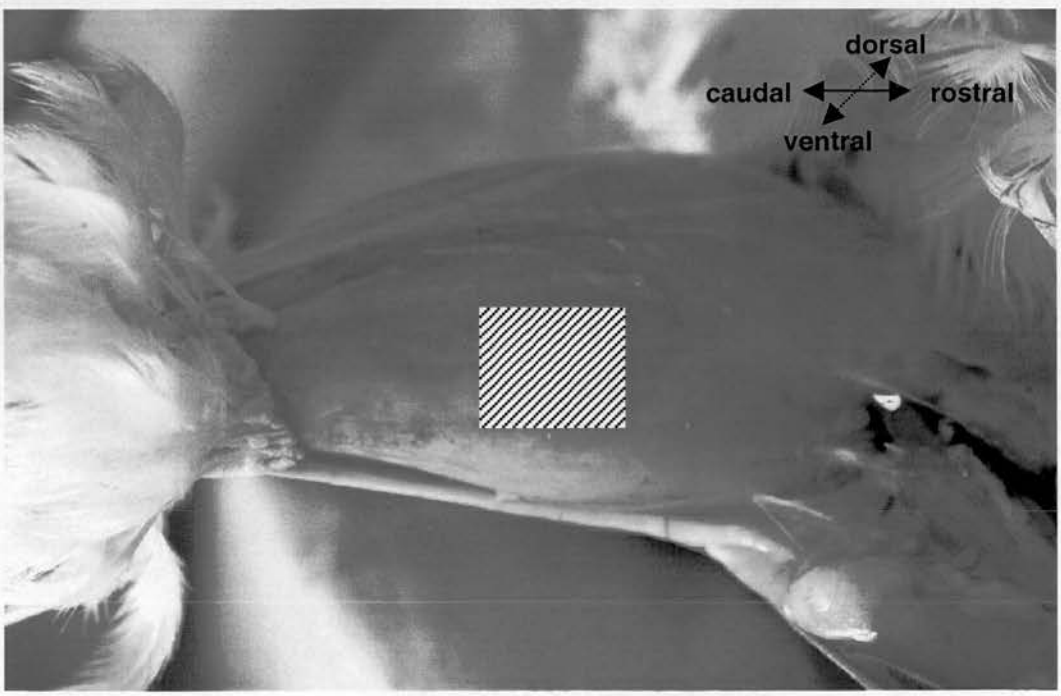



Figure 2.12 Dorsal view of the region from which the left *Gastrocnemius* leg muscle was sampled.

| Muscle | Origin | Insertion | Action |
|---|---|---|---|
| *Pectoralis major* | Clavicle, keel of sternum and the sternal ribs | Greater tuberosity of humerus | Depresses wing |
| *Coracobrachialis* | Anterolateral surface of coracoid and sternum | Proximal surface of humerus | Depresses wing |
| *Biceps femoris* | Posterior surface of ilium | Fibula, through a fibrous loop | Extends hip and flexes knee |
| *Peroneus longus* | Proximal tibiotarsus and patella | Tendon of digital flexors | Flexes digits |
| *Gastrocnemius* | 1. proximal tibiotarsus<br>2. laterodistal femur<br>3. lateral ilium | Tarsometatarsus | Flexes tarsometatarsus |

Table 2.1 The origin, insertion and action of the *Pectoralis major, Coracobrachialis, Biceps femoris*, *Peroneus longus* and *Gastrocnemius* muscles (Nickel, Schummer and Seiferle, 1977; Robinson, 1970).

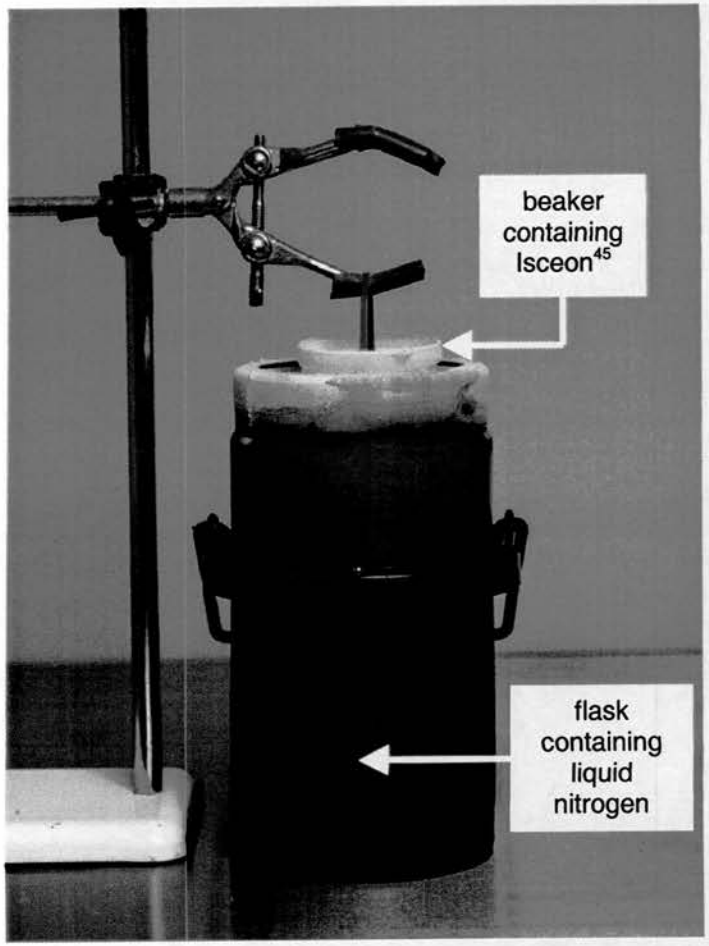



Figure 2.13 Apparatus for freezing muscle samples.

Figure 2.14 A muscle block being snap frozen by immersion in liquid nitrogen – cooled Isceon [45].

differences in metabolic properties between muscle fibres using chemical reactions. The protocols for the staining procedures outlined (2.7.4.1. and 2.7.4.2.) are detailed in Cumming *et al.* (1994).

### 2.7.4.1 Histological procedures

#### 2.7.4.1.1 Haematoxylin and Eosin (H&E)

Haematoxylin is a basic dye, and therefore has an affinity for the nucleic acids of the cell nucleus. Nuclei and basophilic material stain dark blue. Eosin is an acidic dye with an affinity for cytoplasmic components of the cell, and stains the cytoplasm dark pink. This stain was used to assess muscle fibre architecture; fibre splitting; position of nuclei; hyaline fibres (deep pink and rounded), regenerating fibres (basophilic fibres stained blue due to high RNA content); necrotic fibres (disrupted cytoplasm) and mononuclear infiltration.

#### 2.7.4.1.2 Masson's Trichrome

Generally, when the protein component of a tissue is exposed to a fixative agent, a three-dimensional, insoluble, protein 'network' is formed. Muscle cells form an open structure with large pores. However in comparison, collagen shows a less dense network and therefore the greater porosity. During this staining procedure, haematoxylin stained the nuclei blue/black. Ponceau de xylidine, (medium sized dye molecules of molecular weight 494) penetrated and stained the muscle fibres and collagen red/brown. Phosphotungstic acid was used as a differentiator and mordant. Fast green, (larger dye sized molecules of molecular weight 809) only penetrated and stained the collagen green. The Masson's trichrome stain demonstrated muscle fibre architecture and connective tissue amount.

#### 2.7.4.1.3 Acridine orange

This fluorescent stain auto-fluoresces orange in association with RNA and yellow-green with DNA. The yellow-green autofluorescence is a result of the acridine orange binding to an RNA histone protein.

### 2.7.4.2 Histochemical staining techniques

#### 2.7.4.2.1 Nicotinamide adenine dinucleotide tetrazolium reductase (NADH-Tr)

Oxidative enzymes were demonstrated by a simultaneous coupling method that involved the oxidation of NADH, and the consequent reduction of nitro blue tetrazolium, which produced a blue formazan deposit at the site of enzyme activity (mitochondria). This stain was also used for fibre typing; Type I fibres stained dark blue, Type IIa intermediate and Type IIb pale blue. NADH-Tr has been abbreviated to NADH in the remainder of the thesis.

#### 2.7.4.2.2 Myofibrillar adenosine triphosphatase (ATPase)

Myofibrillar ATPase activity was demonstrated by a simultaneous coupling reaction, in which ATPase produced phosphate from the substrate ATP. The released phosphate ions combined with calcium ions to produce a precipitate of calcium phosphate, which in turn was converted to a colourless precipitate of cobalt phosphate by treatment with cobalt nitrate. Further treatment with ammonium sulphide produced a visible black precipitate of cobalt sulphide. This was seen as a dark brown precipitate at the site of enzyme activity. Serial sections were pre-incubated at pH 4.35, 4.6 and 10.2, prior to incubation at pH 9.5 and development. This allowed the differentiation and identification of muscle fibre types as described in Cummings *et al*, 1994 (e.g. Figure 3.7).

#### 2.7.4.2.3 Non-Specific Esterase

Esterases hydrolyse carboxylic acid esters. The majority of esterase enzymes are called 'non-specific' esterases, and are able to hydrolyse α-napthyl acetate as a

substrate. In this technique, the non-specific esterases hydrolysed α-napthyl acetate, releasing α-napthol. The α-napthol was then coupled with hexazonium pararosanilin, which produced an insoluble dark brown dye at the site of enzyme activity, allowing the identification of sites of lysosomal activity.

### 2.7.5 Image capture

#### 2.7.5.1 Light microscopy

Digital images were captured from stained sections viewed on a Vickers M17 light microscope using a digital camera in conjunction with SPOT computer software for Windows.

#### 2.7.5.2 Fluorescence microscopy

Sections stained with acridine orange were viewed on an Olympus BHS reflected light fluorescence microscope with an excitation wavelength of 490nm (blue) with a blue dichroic mirror and a barrier filter of 515nm (the peak emission of acridine orange is at 530nm). S Plan objectives of x10 and x20 magnifications were used. Photographs of the sections were taken using an Olympus OM2N camera, with Kodak Ektachrome 160T film.

### 2.7.6 Analysis of muscle sections

#### 2.7.6.1 Muscle pathology assessment

##### 2.7.6.1.1 Detailed evaluation

Damaged fibres, structural changes, and abnormal distributions of enzymes were identified and described during assessments of histological and histochemical sections, using adapted human muscle biopsy evaluation forms.

#### 2.7.6.1.2. Approximate estimations of percentage damaged fibres

H&E sections were viewed using a x10 objective. The number of complete hyaline, basophilic and necrotic fibres and the total number of complete fibres present within the grid of an eye piece graticule (100 X 1 $mm^2$ divisions) were recorded. This was repeated over representative fields of view until approximately 1500 fibres had been counted. The percentage of fibres that were hyaline, basophilic and necrotic was then calculated.

### 2.7.6.2 Morphometric assessment

The morphometric assessment of the sections involved the estimation of muscle fibre size and connective tissue content. Direct morphometry was used to evaluate muscle fibre sizes, by direct measurement of the transverse profiles of the fibres using image analysis software (Scion Image for Windows). Stereology, the measurement of form in two dimension sections/images to obtain information of specimens in three dimensions (Gunderson, 1986; Williams, 1977), was used to measure connective tissue content.

#### 2.7.6.2.1 Muscle fibre size

The minimum fibre diameter is commonly employed to provide an estimation of muscle fibre size, as it is least prone to the influences of section obliquity and muscle fibre kinking (Dubowitz and Brooke 1973). Minimum fibre diameter is defined as the distance across the narrowest part of the fibre profile passing through the centroid (Dubowitz and Brooke 1973; Cumming *et al*, 1994).

In normal human muscle, the measurement of 100-200 fibres per section provides a precise mean (Dubowitz and Brooke, 1973; Cumming *et al*, 1994). The progressive mean (Williams, 1977) of the minimum fibre diameters of 500 *Pectoralis major* muscle fibres of a five-week old commercial broiler was calculated and plotted

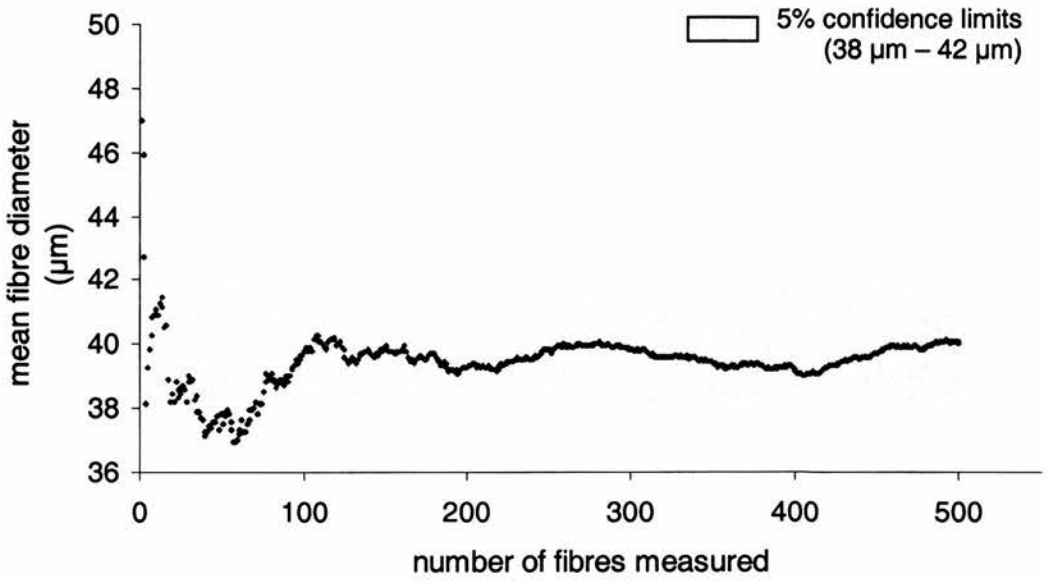



Figure 2.15 Progressive means of 500 fibre diameter measurements. The shaded region represents the 5% confidence limits (final mean +/- 5%) and indicates that the mean is stable by n=100.

(Figure 2.15). As the number of measured fibres increased, the mean value became more reliable. However, above 73 measured fibres, the mean remained stable (within +/- 5% confidence limits). It was concluded that 100 fibres per muscle section should be measured to obtain a reliable mean, in order to allow for possible variation between individual birds, lines, ages and muscles.

#### 2.7.6.2.2 Detailed evaluation of muscle fibre size variation

The minimum diameters of 500 fibres were measured in order to obtain a detailed evaluation of alterations in muscle fibre size variation.

#### 2.7.6.2.3 Connective tissue content

Connective tissue content is defined as the proportion of tissue cross-sectional area occupied by non-muscle fibre components (Cummings *et al*, 1994). The connective tissue content of sections stained with H&E was estimated by point-counting stereology. A preliminary study was undertaken to determine the number of points that should be counted to reduce the statistical error of measurement to an insignificant amount, by calculating the 'relative standard error' (RSE). To be statistically precise the RSE should be less than or equal to 0.05 (Williams, 1977).

*Calculation of the RSE*

The percentage of connective tissue was studied in sections from the *Pectoralis major* and *Biceps femoris* muscles of 24 birds of different lines and ages. A 100-square eyepiece graticule was used to count 100 points intersecting the measured component. The number of points that intersected connective tissue were recorded. This was repeated over 5 random fields. RSE was calculated using the following equation.

$$RSE = \frac{\sqrt{(1\text{-volume fraction})}}{(\sqrt{n})}$$

*n = total number of points applied (100, 200, 300, 400 and 500)*

$$\textit{volume fraction} = \frac{\textit{number of points intersecting the connective tissue}}{\textit{total number of points applied}}$$

When the total number of points counted were 500, the RSE ranged from 0.038 to 0.043. It was concluded that 500 points provided a reliable estimate of the percentage of connective tissue in muscle.

### 2.7.7 Validation of measurements

Operator error and reproducibility of minimum fibre diameter measurements and connective tissue point counts were assessed on selected muscle sections over time. Fibre diameter measurements of 10 muscle fibres were repeated 10 times on 10 different days, revealing an operator error of less than 2%. Repeated fibre diameter measurements of 10 muscle sections (100 fibres per section) were not significantly different from the original measurements. Repeated connective tissue point counts of 10 muscle sections were not significantly different either.

## 2.8 Molecular biology techniques

### 2.8.1 Primer design

Primer are short, single-stranded oligonucleotides, which, when attached by base pairing to a single-stranded template molecule, acts as the start point for complementary strand synthesis directed by a DNA polymerase enzyme (Brown, 1998). Oligonucleotide primer pairs (MWG Biotech Inc, US) were designed from complementary DNA (cDNA) sequences of the chicken estrogen receptor alpha (ERα) (Genbank/Embl accession number XO3805, forward primer position 1736-1755, reverse primer position 2017-1997) and estrogen receptor beta (ERβ) (accession number AB036414, forward primer position 1907-1927, reverse primer position 2256-2236) across exon/intron/exon boundaries. The primers were

designed to amplify a target region unique to the appropriate estrogen receptor subtype. The primers were designed by Mr R Talbot and Dr T Boswell.

### 2.8.2 Muscle dissection and freezing

Surfaces and equipment were sprayed with a solution free of ribonuclease (RNAase) activity called RNAzol B (Whitney, UK). 100mg *Pectoralis major* breast muscle and *Iliotibialis lateralis* leg muscle samples were removed, placed in sterile tubes, frozen in liquid nitrogen and stored at -70°C.

### 2.8.3 Isolation of total RNA

Each muscle sample was homogenised in RNAzol B solution. The addition of chloroform caused the precipitation of the total RNA as a pellet. The optical density of an aliquot of the re-suspended pellet was determined by UV spectrophotometry at 260 nm and 280 nm. Total RNA content was assessed by absorbance at 260nm and purity by A260/A280 ratios. Each sample was then diluted with distilled water to a pre-determined RNA concentration and stored at -70°C.

### 2.8.4 Reverse transcription

The isolated RNA was reverse transcribed to complementary DNA (cDNA) at 37°C, using a first strand synthesis kit (Amersham Pharmacia Biotech Ltd, UK), which contained the enzyme reverse transcriptase.

### 2.8.5 Polymerase chain reaction (PCR)

PCR involved the selective amplification (by the thermostable *Taq* DNA polymerase enzyme), of the region of the cDNA molecule delimited by the primers (Brown, 1998). The PCR was set up using reactants supplied as a kit (Amersham Pharmacia Biotech Ltd, UK). Reactions were performed in microcentrifuge tubes with PCR buffer, nucleotides and *Taq* polymerase present in the ratios 20:2:1, and final primer

concentrations of 1 $\mu moll^{-1}$. The tubes were placed in the hot block of a PCR machine (Hybaid, UK). The PCR machine was pre-programmed with the number of cycles (40), and the desired melting (94°C), annealing (57°C for the ERα reaction; 60°C for the ERβ reaction) and extending temperatures (74°C). The melting temperature induces newly synthesised DNA strands to detach from the DNA template; the annealing temperature enables more primers to hybridise at their respective positions and the extending temperature induces the synthesis of new complementary DNA strands (Brown, 1998).

### 2.8.6 Analysis of PCR products

PCR products were visualised by running them on a 3% agarose gel containing ethidium bromide. Amplified fragments were ligated into pBluescript II SK+ plasmids (Stratagene Ltd, Cambridge, UK.). A plasmid is a usually circular piece of DNA, primarily independent of the host chromosome, and often found in bacterial cells. Certain plasmids will readily incorporate 'foreign DNA' into their DNA (Brown, 1998). The plasmids were transformed into E. coli XL1-Blue strain bacteria (Stratagene Ltd, Cambridge, UK.), and sequenced with a Thermo Sequenase cycle-sequencing kit (Amersham Pharmacia Biotech, UK). The sequences were analysed using the GCG Wisconsin package by Mr R Talbot and Dr T Boswell.

## 2.9 Indices of meat quality

### 2.9.1 Thaw loss

Birds were killed and the carcasses chilled for 24 hours at 4°C. The right breast muscle was removed from the carcass, weighed, placed in a self-seal food bag of known weight and freely suspended on a rack (via a cable tie inserted through both the bag and muscle). The rack was then placed in a chilling room maintained at 4°C for a further 24 hours. The amount of water and exudates collected in the bottom of each bag was weighed, and thaw loss was calculated.

### 2.9.2 Colour

Birds were killed and the carcasses chilled for 24 hours at 4°C. The right breast muscle was removed from the carcass and evaluated for colour (lightness [L*], redness [a*] and yellowness [b*]) using reflectance colorimetry (Minolta CR-300, CIELab). Three colour readings were taken along the length of the ventral side of the breast muscle.

### 2.9.3 Haemorrhages

Birds were killed and the carcasses chilled for 24 hours at 4°C. The right breast muscle was removed from the carcass and evaluated for haemorrhaging using a visual scoring system (ID-DLO, Lelystad, The Netherlands). Haemorrhages were scored using a threshold model consisting of a discontinuous 5 point scale with 4 cut off points, which were determined by photographs of breast muscles showing a particular severity of haemorrhage (1 = none to 5 = extensive).

### 2.9.4 Texture analysis

The breast fillets were cooked for 30 minutes at 90°C and left to cool for 5 hours. Assessment of meat texture (Warner Bratzler shear testing apparatus with the V configuration blade, Lloyds Instruments LRX5) was performed on standard sections of cooked meat. Indices measured were hardness (N), work done during the first compression of a simulated bite (Nmm), springiness index (distance sample springs back after the first compression/maximum distance sample deformed), cohesiveness (work done during the first compression / work done during the second compression) and adhesive force (N) (peak force required to pull the probe away from the sample after applying a compressive force).

*Procedures undertaken on chicken blood samples*

### 2.10.1 Blood carbon dioxide ($CO_2$) and pH

A 238 pH/Blood Gas auto-analyser (Ciba-Corning Diagnostics Ltd, UK), which contained electrodes sensitive to protons and gaseous $CO_2$, was used to measure blood pH and partial pressure of carbon dioxide ($pCO_2$). The measurements were corrected for body temperature by the analysers. Blood analysis was either undertaken immediately after the sample had been removed, or the sample was placed on ice in sealed heparin coated tubes and analysed as soon as possible. This was to minimise the blood gases coming out of solution due to atmospheric exposure (Mills, 2001).

### 2.10.2 Sodium ($Na^+$) and potassium ($K^+$) ions

Whole blood concentrations of $Na^+$ and $K^+$ were measured using a clinical electrolyte auto-analyser (Ciba-Corning Diagnostics Ltd, UK). The electrodes were sensitive to the ions, and detected concentrations in the appropriate ranges for measurement in poultry (80 -200 $mmoll^{-1}$ and 0.5 - 9.99 $mmoll^{-1}$ for $Na^+$ and $K^+$ respectively).

*Procedures undertaken on chicken plasma samples*

## 2.11 Biochemical assays

Plasma indices were measured using commercially available kits that were modified for use in a spectrophotometer (ELX808IU ultra microplate reader, Fisher Scientific, UK) that read 96 well micro-plates (Alpha laboratories, UK).

### 2.11.1 Creatine Kinase (CK) (Randox, Ireland).

Procedure reference cited by kit datasheet: German Society for Clinical Chemistry, 1977. Reference for use with avian plasma: Mitchell and Sandercock, 1995a.

This was a kinetic assay. The contents of the reagent included creatine phosphate, adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP), nicotinamide-adenine dinucleotide phosphate (NADP+), reduced nicotinamide-adenine dinucleotide phosphate (NADPH), hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH). The assay involved the following reactions.

$$\text{Creatine phosphate} + \text{ADP} \overset{\text{CK}}{\leftrightarrow} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \overset{\text{HK}}{\leftrightarrow} \text{ADP} + \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + \text{NADP}^{+} \overset{\text{G-6-PDH}}{\leftrightarrow} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

The NADPH produced in the final reaction was proportional to the creatine produced in the initial reaction, and therefore proportional to the CK activity. The increase in NADPH was determined by measuring the change in absorbance per minute over the linear part of the reaction during a 30 minute period, at 340nm.

**2.11.2 Lactate Dehydrogenase (LDH)** (Sigma Aldrich, UK).
Procedure reference cited by kit datasheet: German Society for Clinical Chemistry, 1970.

This assay was also kinetic. Lactate dehydrogenase catalysed the interconversion of lactate and pyruvate. The determination of LDH activity was based on the reduction of pyruvate to lactate by the following reaction.

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \overset{\text{LDH}}{\leftrightarrow} \text{Lactate} + \text{NAD}^{+}$$

During the reduction of pyruvate an equimolar amount of NADH was oxidised to $NAD^{+}$. The oxidation of NADH resulted in a decrease directly proportional to LDH activity. The decrease in NADH was determined by measuring the change in

absorbance per minute over the linear part of the reaction during a 20 minute period, at 340nm.

**2.11.3 Aspartate Aminotransaminase (AST)** (Thermotrace, Australia).
Procedure reference cited by kit datasheet: IFCC, 1986.

This was a kinetic assay. AST catalysed the transfer of the amino group from L-aspartate to 2-oxoglutarate, forming oxalacetate and L-glatamate. Oxalacetate in the presence of NADH and malate dehydrogenase (MDH) was reduced to L-malate. The reaction was monitored by measuring the rate of decrease in absorbance due to the oxidation of NADH to NAD over the linear part of the reaction during a 20 minute period at 340nm. The lactate dehydrogenase present in the reagent reduced any endogenous sample pyruvate so that it did not interfere with assay.

$$\text{L-Aspartate} + \text{2-oxoglutarate} \overset{\text{AST}}{\leftrightarrow} \text{oxalacetate} + \text{L-glatamate}$$

$$\text{oxalacetate} + \text{NADH} \overset{\text{MDH}}{\leftrightarrow} \text{malate} + \text{NAD}$$

**2.11.4 Triglyceride** (Wako Chemicals GmbH, Germany)
Procedure reference cited by kit datasheet: McGowan, Artiss, Strandbergh and Zak, 1983. Reference for use with avian plasma: Carlisle *et al*, 1991.

Plasma triglycerides were hydrolysed to glycerol and free fatty acids by a reaction catalysed by lipoprotein lipase. Glycerol was converted to glycerol-3-phosphate by glycerokinase in the presence of ATP. The glycerol-3-phosphate was then oxidised by glycerol-3-phosphate oxidase in a reaction that produced hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and 4-Chlorophenol to produce yielded a red coloured dye. The amount of triglyceride in the sample was determined by measuring the absorption of the developed colour at 595nm.

**2.11.5 Zinc** (Wako Chemicals GmbH, Germany)

Procedure reference cited by kit datasheet: Makino, Saito, Horiguchi and Kina, 1982. Reference for use with avian plasma: Carlisle *et al*, 1991.

A solution of trichloroacetic acid was added to the plasma samples to precipitate proteins, leaving zinc in solution. After proteins were removed, a reagent mixture that contained 2-(5-Bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino-)-phenol (5-Br-PAPS) was added to an aliquot of the supernatant fluid. Zinc bound to the sodium salt of (5-Br-PAPS), forming a reddish-violet chelate. The absorbance of this chelate was measured at 560nm, and was directly proportional to the concentration of zinc in the specimen.

**2.4.6 Calcium** (Wako Chemicals GmbH, Germany).

Procedure reference cited by kit data sheet: Michaylova and Ilkova, 1972.
Reference for use with avian plasma: Carlisle *et al*, 1991.

The supplied reagent mixture contained Arsenazo III (1,8-dihydroxynaphthalene-3 6-disulphonic acid-2, 7-bis [(azo-2)-phenylarsonic acid]) and imidazole buffer (pH 6.5). The assay was based on a dye-binding method. Arsenazo III formed a 1:1 violet complex with calcium, which had the maximum absorbance at 650nm. The change in absorbance at 650nm was proportional to the calcium concentration in the sample.

## *Statistical analysis*

### 2.12 Statistical analysis

Statistical analyses were performed using Minitab Data Analysis Software (Minitab 12 for Windows) and Genstat (Release 6.1, 6$^{th}$ edition). Probability was assumed significant when less than 0.05.

Exploratory data analysis incorporating the Anderson-Darling Normality Test was performed on all raw data to assess the normality of data distributions. If necessary, transformation of the raw data was undertaken. Parametric tests used to compare means included analysis of variance (ANOVA) and the student's T-test. Non-parametric tests used were the Kruskal-Wallis test for independent samples (equivalent to a 1 way ANOVA) and the Mann Whitney U test. Regression analyses were undertaken to determine relationships between parameters.

## 3.1 Introduction

It has been proposed that genetic selection of commercial broiler chickens and turkeys for improved production traits, such as increased growth rate and greater muscularity, may have resulted in an increased susceptibility to growth-associated myopathy (Mitchell, 1999a). Many of the poultry muscle myopathies described in the literature appear to be growth related, suggesting that some aspects of muscle growth have not kept pace with bird growth, and muscle degeneration has occurred (Mahon, 1999). These myopathies include deep pectoral myopathy (Siller, 1985), focal myopathy (Wilson *et al*, 1990), capture myopathy (Spraker *et al*, 1987) and heat stress-induced myopathy (Mitchell and Sandercock, 1995a). The pathological features that describe these myopathies are similar, and appear to be linked for selection for increased musculature. However, it would be unwise to clump together differing pathologies until a proper basis for their aetiology is established (Mahon, 1999).

Extensive histological studies in male commercial turkeys have shown that plasma activities of the intracellular enzyme marker creatine kinase and the incidence of structural muscle abnormalities increase with age (Mahon *et al*, 1995; Mills *et al*, 1998). In commercial turkeys, a large increase in plasma creatine kinase activity occurs at body weights of ~10 kg (equivalent to approximately 15 weeks of age), which corresponds to mean minimum fibre diameters greater than 70 μm and an increased incidence of structural muscle abnormalities (Mills *et al*, 1998).

Plasma creatine kinase activities have been shown to increase with age in chickens, with commercial broilers showing greater activity values than their genetic predecessors (Mitchell and Sandercock, 1994). The only histological study in the literature that has compared broiler and layer muscle pathology demonstrated a more frequent occurrence of structural muscle abnormalities in muscles of broilers compared to layers, but no alteration in incidence with age (Soike and Bergmann,

1998). At the onset of sexual maturity, a dramatic fall in plasma creatine kinase activity occurs in female broilers (Mitchell and Sandercock, 1994a). However, it is not known whether this is accompanied by a reduction in the incidence of myopathology.

## 3.2 Experimental aims

(i) To determine the effect of genetic selection for desirable production traits on muscle fibre growth and the incidence of muscle damage in broiler chickens during development.

(ii) To establish whether the reduction in muscle damage associated with increased ovarian estrogen secretion in female birds, indicated by reduced plasma activity of intracellular enzyme markers of myopathy, is accompanied by a reduced incidence of structural abnormalities.

To assess the effects of genetic selection on broiler muscle growth and development, it was necessary to compare the broiler with a chicken line that has not been selected for high growth rate and increased muscularity. The layer line was chosen as it is selected for reproductive in contrast to growth related traits. A female broiler great-grandparent line was also included in the study, to examine whether genetic selection for both growth and reproductive related traits effects the degree of muscle damage reduction during increased ovarian estrogen secretion, compared with selection for solely growth (broiler) or reproduction (layer).

### Study outline

*Part I: Reproductive development*

To determine when increased egg yolk precursor production and calcium uptake occurs in female birds from the three chicken lines.

*Part II: Muscle growth*

To characterise and compare the growth of a representative breast and leg muscle in female birds from the three chicken lines.

*Part III: Muscle damage*

To determine the incidence of skeletal muscle damage in a representative breast and leg muscle during growth using histocytochemical techniques, and to measure the plasma activity of intracellular enzyme markers of muscle damage during growth in female birds from the three chicken lines.

*Part IV: Assessment of further broiler muscles*

To characterise the muscle growth and the incidence of damage in additional breast and leg muscles of the broiler line at age intervals when structural damage is observed in the representative muscles examined in Part III.

## 3.3 Methods

### 3.3.1 Animals and Husbandry

The three lines of chickens used in this study were a layer line (Brown Lohmann) (L), a commercial broiler line (Cobb 500) (B), and a female great-grandparent line (Cobb) (GGP). All birds were reared in floor pens under standard conditions and fed commercial broiler or layer rations as appropriate from 4-26 weeks of age (Chapter 2). From 8 weeks of age, the B and GGP lines were allowed 65% of calculated ad libitum intake to optimise welfare whilst maintaining a high growth rate. The restricted intake was calculated based on commercial guidelines (Leeson and Summers, 2000) and assuming commercial restriction was an average of 40% of ad libitum intake (Hocking, Maxwell and Mitchell, 1996).

### 3.3.2 Samples

Four different females from each line were weighed, blood sampled and killed at 5, 9, 13, 18, 20, 23 and 25 weeks of age. Samples of the *Pectoralis major* and *Coracobrachialis* breast muscles, and the *Peroneus longus*, *Gastrocnemius* and *Biceps femoris* leg muscles were removed from standardised regions of the left hand side of the bird. The muscle samples were frozen at –70°C pending histocytochemical assessment. Plasma samples were prepared from the blood samples and stored at –20°C pending analysis.

### 3.3.3 Measurements

#### 3.3.3.1 Plasma samples

The plasma concentrations of the egg yolk precursors vitellogenin and Very Low Density Lipoprotein (VLDL) were measured to detect increased ovarian estrogen secretion. VLDL concentration was indirectly determined by measurement of plasma triglyceride levels, and vitellogenin concentration by estimation of plasma

zinc (Mitchell and Carlisle, 1991). Plasma levels of calcium were measured directly, as estrogen stimulates increased calcium uptake for egg-shell synthesis.

Plasma activities of three commonly employed intracellular enzyme markers of muscle damage were determined: creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransaminase (AST).

### 3.3.3.2 Muscle samples

The frozen muscle samples were sectioned and stained for Haematoxylin and Eosin (H&E), Masson's Trichrome, NADH, ATPase and non-specific esterase.

The minimum muscle fibre diameter of 100 fibres, connective tissue content and the percentage of fibres with ≥1 internal nucleus were estimated from *Pectoralis major* and *Biceps femoris* muscle sections from each bird at each age group. The minimum fibre diameter of 500 fibres was estimated from *Pectoralis major* and *Biceps femoris* muscle sections from one randomly selected broiler, great-grandparent and layer at 5 and 25 weeks of age.

A qualitative, descriptive evaluation of muscle pathology was performed on *Pectoralis major* and *Biceps femoris* muscle sections from each bird at each age group, using adapted human biopsy evaluation forms. The percentage of hyaline, basophilic and necrotic fibres were estimated from *Pectoralis major* and *Biceps femoris* muscle sections from each layer and broiler at each age. 15 serial sections were cut (one 10 μm section every 50 μm) from a five week broiler *Pectoralis major* muscle sample and a twenty week broiler *Biceps femoris* muscle sample and abnormal fibres were 'tracked' along the fibres. The percentage of abnormal fibres in the same fascicle of the *Biceps femoris* muscle sample was determined in each serial section.

The minimum fibre diameter of 100 fibres, connective tissue content and the percentage of fibres with ≥1 internal nucleus were estimated from the

*Coracobrachialis* muscle sections from each broiler at 5 weeks of age, and from the *Peroneus longus* and *Gastrocnemius* muscle sections from each broiler at 5, 16, 18, 20 and 23 weeks of age. A qualitative evaluation of muscle pathology was performed on the sections, and the percentage of hyaline, basophilic and necrotic fibres present were estimated. The birds from which additional muscles were assessed were assigned individual bird numbers. The four birds sampled at 5, 16, 18, 20 and 23 weeks of age were numbered birds 1-4, 5-8, 9-12, 13-16 and 17-20 respectively.

A summary of the data recorded from the muscle samples according to line, age and muscle is given in Table 3.1 (fibre sizes) and Table 3.2 (pathology).

### 3.3.4 Statistics

Exploratory data analysis incorporating the Anderson-Darling Normality Test was performed to assess the normality of data distributions, and if necessary data transformation was undertaken. The natural log (ln) transformation was applied to the plasma CK, LDH and AST activities data. $Log_{10}$ transformation was applied to the fibre diameter measurements. The arcsin transformation for proportions was applied to the percentage connective tissue data and the percentage of fibres with $\geq 1$ internal nucleus data due to the constraints of a fixed scale (0-100%).

#### 3.3.4.1 Plasma data analysis

Two-way analysis of variance (ANOVA) was performed to determine the effect of age and line. General Linear Model analysis incorporating pairwise comparisons using Tukey's Test was undertaken to compare groups within the ANOVA model.

#### 3.3.4.2 Fibre diameter data analysis

Split-plot ANOVA was undertaken on the fibre diameter, connective tissue content and nuclei data of each bird, to allow for the comparison of the different muscles

| | | muscle | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Pectoralis major* | | *Coracobrachialis* | | *Biceps femoris* | | *Peroneus longus* | | *Gastrocnemius* | |
| age | line | n=100 | n=500 | n=100 | n=500 | n=100 | n=500 | n=100 | n=500 | n=100 | n=500 |
| 5 | B | 4 | 1 | 4 | | 4 | 1 | 4 | | 4 | |
| | GGP | 4 | 1 | | | 4 | 1 | | | | |
| | L | 4 | 1 | | | 4 | 1 | | | | |
| 9 | B | 4 | | | | 4 | | | | | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 13 | B | 4 | | | | 4 | | | | | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 16 | B | 4 | | | | 4 | | 4 | | 4 | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 18 | B | 4 | | | | 4 | | 4 | | 4 | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 20 | B | 4 | | | | 4 | | 4 | | 4 | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 23 | B | 4 | | | | 4 | | 4 | | 4 | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | | | | 4 | | | | | |
| 25 | B | 4 | 1 | | | 4 | 1 | | | | |
| | GGP | 4 | 1 | | | 4 | 1 | | | | |
| | L | 4 | 1 | | | 4 | 1 | | | | |

Table 3.1 Summary of the number of samples according to line, age and muscle on which 100 (n=100) and 500 (n=500) minimum fibre diameter measurements were made.

| age | line | muscle | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Pectoralis major* | | *Coracobrachialis* | | *Biceps femoris* | | *Peroneus longus* | | *Gastrocnemius* | |
| | | evaluation | damaged fibres (%) | evaluation | damaged fibres (%) | evaluation | damaged fibres (%) | evaluation | damaged fibres (%) | evaluation | damaged fibres (%) |
| 5 | B | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 9 | B | 4 | 4 | | | 4 | 4 | | | | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 13 | B | 4 | 4 | | | 4 | 4 | | | | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 16 | B | 4 | 4 | | | 4 | 4 | 4 | 4 | 4 | 4 |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 18 | B | 4 | 4 | | | 4 | 4 | 4 | 4 | 4 | 4 |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 20 | B | 4 | 4 | | | 4 | 4 | 4 | 4 | 4 | 4 |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 23 | B | 4 | 4 | | | 4 | 4 | 4 | 4 | 4 | 4 |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |
| 25 | B | 4 | 4 | | | 4 | 4 | | | | |
| | GGP | 4 | | | | 4 | | | | | |
| | L | 4 | 4 | | | 4 | 4 | | | | |

Table 3.2 Summary of the number of samples according to line, age and muscle on which pathology was evaluated and percentage (%) damaged fibres estimated from sections.

(individual bird = plot, muscle = split-plot), as well as determining the effect of age and line.

Correlations between (i) $\log_{10}$ body weight and $\log_{10}$ fibre diameter and (ii) $\log_{10}$ percentage of fibres with $\geq 1$ internal nucleus and $\log_{10}$ fibre diameter within each line and muscle where determined using the Pearson correlation coefficient. Multiple regression analyses were performed to obtain the equations of the best fitting lines and the R-squared values.

#### 3.3.4.3 Muscle pathology data

The verdict of 'pathology' is a result of a descriptive evaluation of the whole muscle section rather than due to significant increases or decreases in a certain feature. The percentage of hyaline, basophilic and necrotic fibres data was collected to visualise any extreme changes in the frequencies of occurrence of the fibres, rather than to undergo statistical analysis.

## 3.4 Results I: Reproductive development

### 3.4.1 Plasma zinc concentration

There was a significant effect of age ($P<0.05$) and line ($P<0.05$) on plasma zinc concentrations ($P<0.05$) (Table 3.3 and Figure 3.1). There was a significant increase in the plasma zinc concentrations of both the broiler and great-grandparent lines between 23 and 25 weeks of age, from 1.0 $\mu gml^{-1}$ to 2.4 $\mu gml^{-1}$ ($P<0.05$) and from 0.8 $\mu gml^{-1}$ to 2.3 $\mu gml^{-1}$ ($P<0.05$) respectively. Plasma zinc concentrations significantly increased in the layer line between 16 and 18 weeks of age from 1.1 $\mu gml^{-1}$ to 2.5 $\mu gml^{-1}$ ($P<0.05$).

### 3.4.2 Plasma triglyceride concentration

Plasma triglyceride concentrations were significantly affected by age ($P<0.05$) and line ($P<0.05$) (Table 3.3 and Figure 3.2). In the layer line, plasma triglyceride concentrations significantly increased between 16 and 18 weeks of age from 1.0 $mgml^{-1}$ to 5.8 $mgml^{-1}$ ($P<0.05$). In the broiler line and great-grandparent line there was a significant increase between 23 and 25 weeks of age from 0.5 $mgml^{-1}$ to 7.2 $mgml^{-1}$ ($P<0.05$) and from 0.5 $mgml^{-1}$ to 8.5 $mgml^{-1}$ ($P<0.05$) respectively.

### 3.4.3 Plasma calcium concentration

There was a significant effect of age ($P<0.05$) and line ($P<0.05$) on plasma calcium concentrations (Table 3.3 and Figure 3.3). In the layer line, the increase in plasma calcium concentrations from 16 to 18 weeks (3.0 $mmoll^{-1}$ to 4.5 $mmoll^{-1}$) was short of significance, but increased significantly between 16 and 20 weeks of age (3.0 $mmoll^{-1}$ to 5.7 $mmoll^{-1}$) ($P<0.05$). In the broiler line there was a significant increase between 23 and 25 weeks of age from 2.3 $mmoll^{-1}$ to 4.9 $mmoll^{-1}$ ($P<0.05$). In the great-grandparent line there was also a significant increase between 23 and 25 weeks of age, from 2.3 $mmoll^{-1}$ to 5.0 $mmoll^{-1}$ ($P<0.05$).

| age (weeks) | line | plasma [trigyceride] ($mgml^{-1}$) | plasma [zinc] ($\mu gml^{-1}$) | plasma [calcium] ($mmoll^{-1}$) |
|---|---|---|---|---|
| | B | 0.5 (0.4) | 1.2 (0.4) | 3.1 (0.6) |
| 5 | GGP | 0.4 (0.2) | 0.9 (0.3) | 2.9 (0.9) |
| | L | 0.6 (0.2) | 1.4 (0.1) | 3.4 (0.6) |
| | B | 0.8 (0.4) | 1.1 (0.4) | 3.1 (0.4) |
| 9 | GGP | 0.5 (0.1) | 1.1 (0.3) | 3.0 (0.1) |
| | L | 0.4 (0.1) | 1.7 (0.2) | 3.0 (0.4) |
| | B | 0.9 (0.4) | 0.8 (0.1) | 2.3 (0.3) |
| 13 | GGP | 0.5 (0.2) | 0.8 (0.1) | 2.0 (0.2) |
| | L | 0.6 (0.2) | 1.5 (0.1) | 2.3 (0.4) |
| | B | 1.0 (0.7) | 1.0 (0.1) | 2.7 (0.4) |
| 16 | GGP | 1.1 (0.6) | 0.8 (0.1) | 2.6 (0.3) |
| | L | 1.0 (0.2) | 1.1 (0.2) | 3.0 (0.2) |
| | B | 0.8 (0.2) | 0.8 (0.2) | 2.5 (0.2) |
| 18 | GGP | 0.6 (0.2) | 0.8 (0.1) | 2.6 (0.3) |
| | L | 5.8 (4.6) | 2.5 (1.1) | 4.5 (1.9) |
| | B | 0.5 (0.5) | 1.0 (0.2) | 2.3 (0.1) |
| 20 | GGP | 1.9 (3.5) | 1.6 (1.6) | 3.3 (2.1) |
| | L | 5.6 (2.1) | 3.5 (0.5) | 5.7 (1.0) |
| | B | 0.5 (0.2) | 1.0 (0.1) | 2.3 (0.1) |
| 23 | GGP | 0.5 (0.1) | 0.8 (0.2) | 2.3 (0.1) |
| | L | 8.5 (0.6) | 2.9 (0.5) | 5.7 (0.3) |
| | B | 7.2 (4.0) | 2.4 (1.0) | 4.9 (1.7) |
| 25 | GGP | 8.5 (2.1) | 2.3 (0.4) | 5.0 (1.0) |
| | L | 7.6 (2.5) | 2.7 (0.7) | 5.6 (1.4) |

Table 3.3 Mean plasma concentrations of triglyceride, zinc and calcium of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

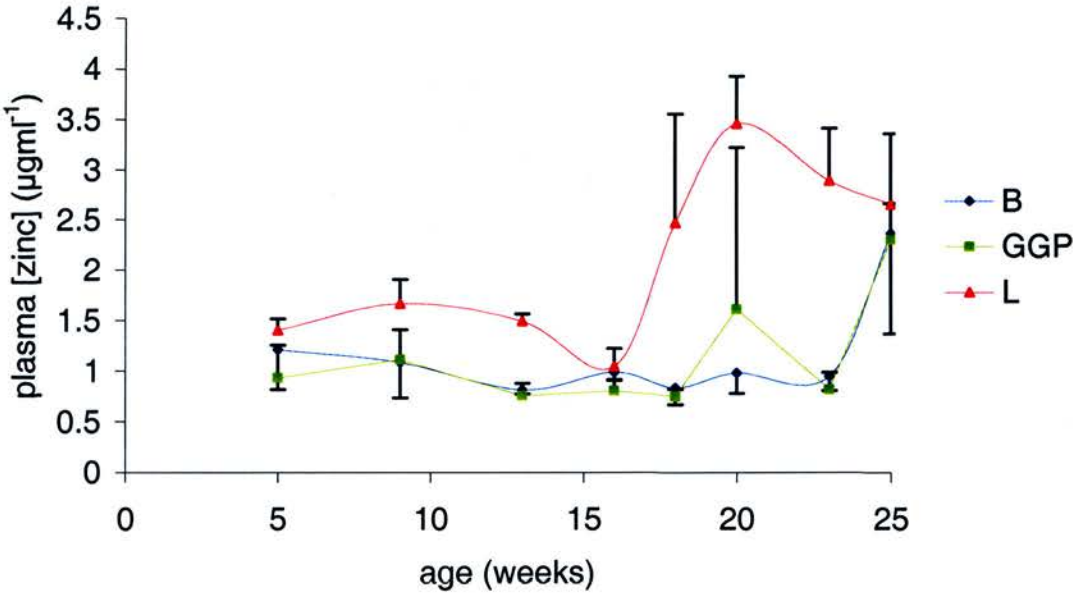



Figure 3.1 Mean plasma zinc concentrations ($\mu gml^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

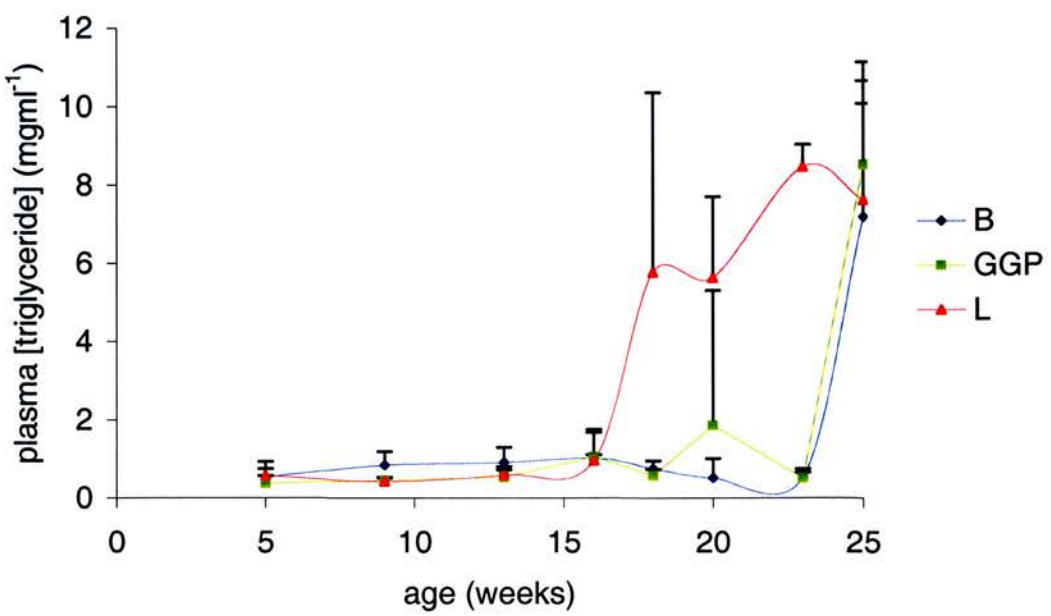



Figure 3.2 Mean plasma triglyceride concentrations ($mgml^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

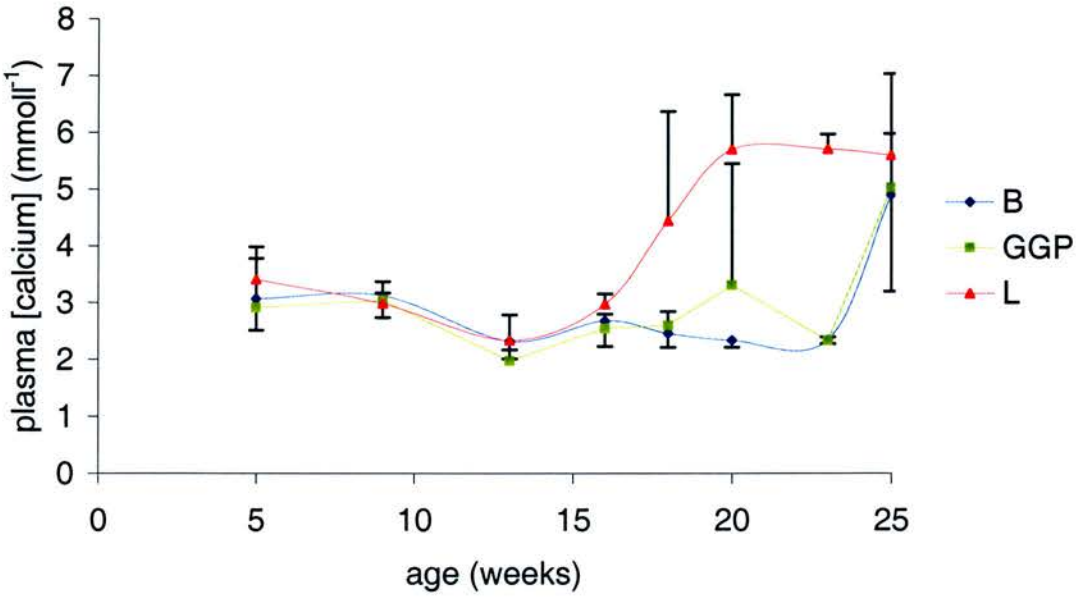



Figure 3.3 Mean plasma calcium concentrations ($mmoll^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

## 3.5 Results II: Muscle growth

### 3.5.1 Body weight

Mean body weight increased significantly with age ($P<0.001$) in the broiler, great-grandparent and layer lines (Table 3.4 and Figure 3.4). Both the broiler and great-grandparent lines had significantly greater mean body weights than the layer line at all ages ($P<0.001$). There was no significant difference between broiler and great-grandparent mean body weight at any age. There was a significant interaction between age and line ($P<0.05$), suggesting that the mean body weights of the broiler and great-grandparent lines diverged from those of the layer line with age. Peak mean body weights were achieved at 25 weeks of age in the broiler (5.1 kg) and the great-grandparent (5.2 kg), and at 20 weeks in the layer line (2.1 kg).

### 3.5.2 Muscle fibre typing

Myofibrillar ATPase and NADH staining revealed that the *Pectoralis major* and *Coracobrachialis* breast muscles were almost entirely made up of Type IIb, fast twitch fibres (Figure 3.5 and Figure 3.6). In contrast, the fibre type distribution of the *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles were more heterogeneous, and contained slow twitch, oxidative type I fibres as well as the type II muscle fibres (Figure 3.7 and 3.8).

### 3.5.3 Muscle fibre growth

#### 3.5.3.1 Estimation of mean fibre size

*Pectoralis major*

The mean Minimum Fibre Diameter (MFD) (n=100) of the breast muscle *Pectoralis major* increased significantly with age ($P<0.001$) in all three chicken lines (Table 3.5 and Figure 3.9). With the exception of the group aged 16 weeks, the broiler line had

| age (weeks) | weight (kg) | | |
|---|---|---|---|
| | B | GGP | L |
| 5 | 1.8 (0.1) | 1.6 (0.2) | 0.5 (0.1) |
| 9 | 3.4 (0.1) | 3.1 (0.3) | 0.9 (0.1) |
| 13 | 4.2 (0.1) | 3.8 (0.6) | 1.3 (0.1) |
| 16 | 3.7 0.4) | 4.3 (0.3) | 1.8 (0.1) |
| 18 | 4.5 (0.4) | 4.2 (0.5) | 1.9 (0.2) |
| 20 | 4.2 (0.1) | 3.9 (0.8) | 2.1 (0.3) |
| 23 | 3.4 (0.3) | 3.9 (0.4) | 1.9 (0.1) |
| 25 | 5.1 (0.3) | 5.2 (0.3) | 1.9 (0.1) |

Table 3.4 Mean body weight (kg) (1 standard deviation in parentheses) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

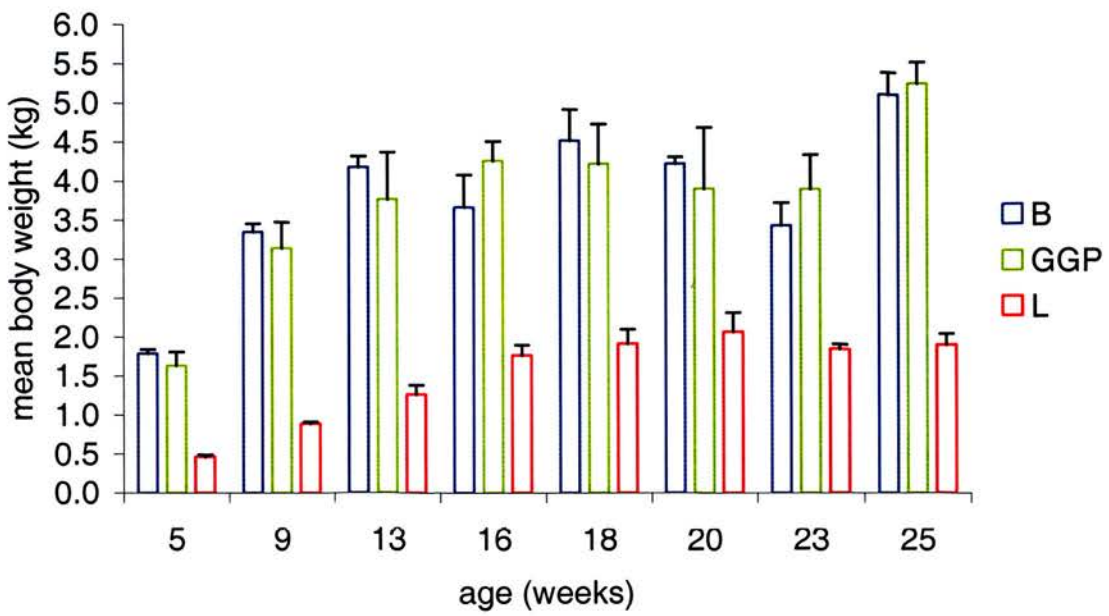



Figure 3.4 Mean body weight (kg) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

Figure 3.5. Typical homogenous distribution of fibre types in the *Pectoralis major* and *Coracobrachialis* breast muscles of all three chicken lines. The fibres are Type IIb. The section is from a 5 week old broiler *Pectoralis major* muscle sample (ATPase stain, preincubation pH of 4.35, magnification x10).

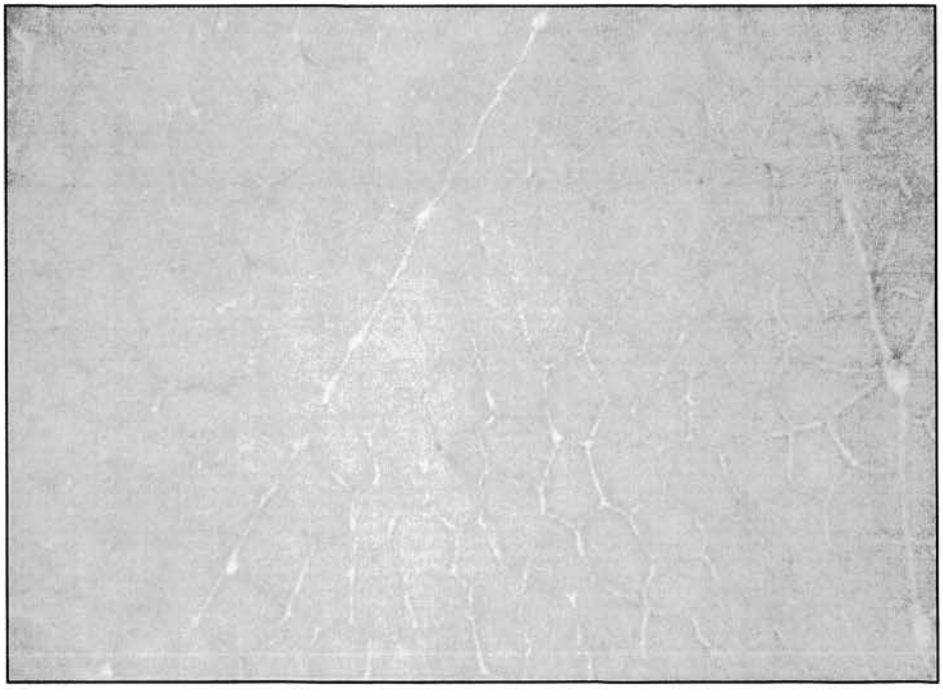


Figure 3.6 Typical homogeneous distribution of fibre types in the *Pectoralis major* and *Coracobrachialis* breast of all three chicken lines. The pale blue fibres are Type IIb. The section is from a 20 week old broiler *Pectoralis* muscle sample (NADH stain, magnification x10).

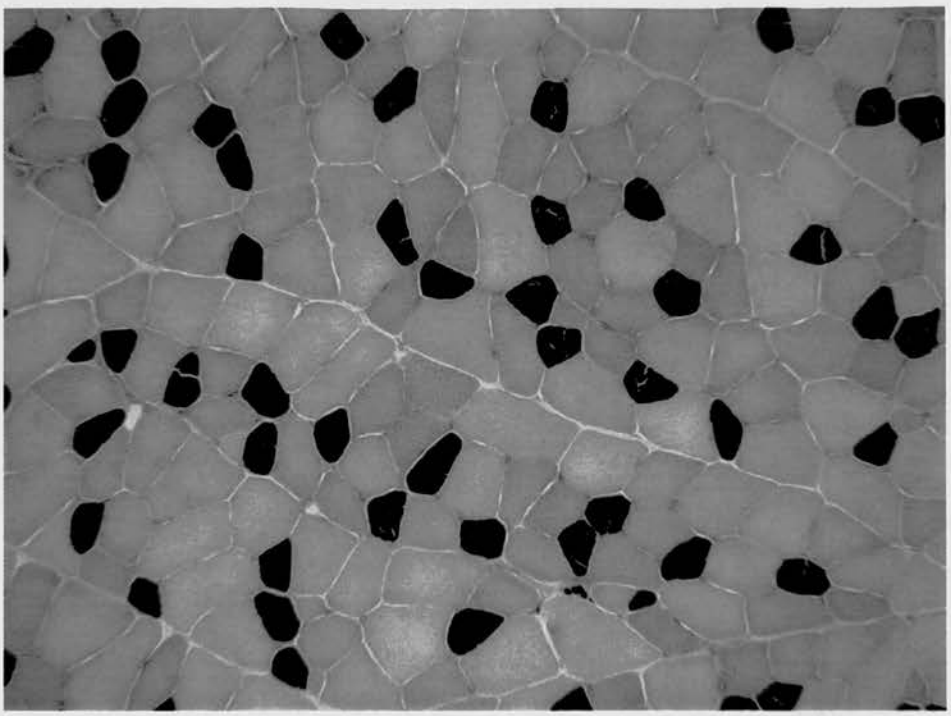


Figure 3.7 Typical heterogeneous distribution of fibre types in the *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles of all three chicken lines. Black fibres are Type I, white fibres are Type IIb and intermediate fibres are Type IIa. The section is from a 5 week old broiler *Gastrocnemius* muscle sample (ATPase stain, preincubation pH of 4.35, magnification x10).

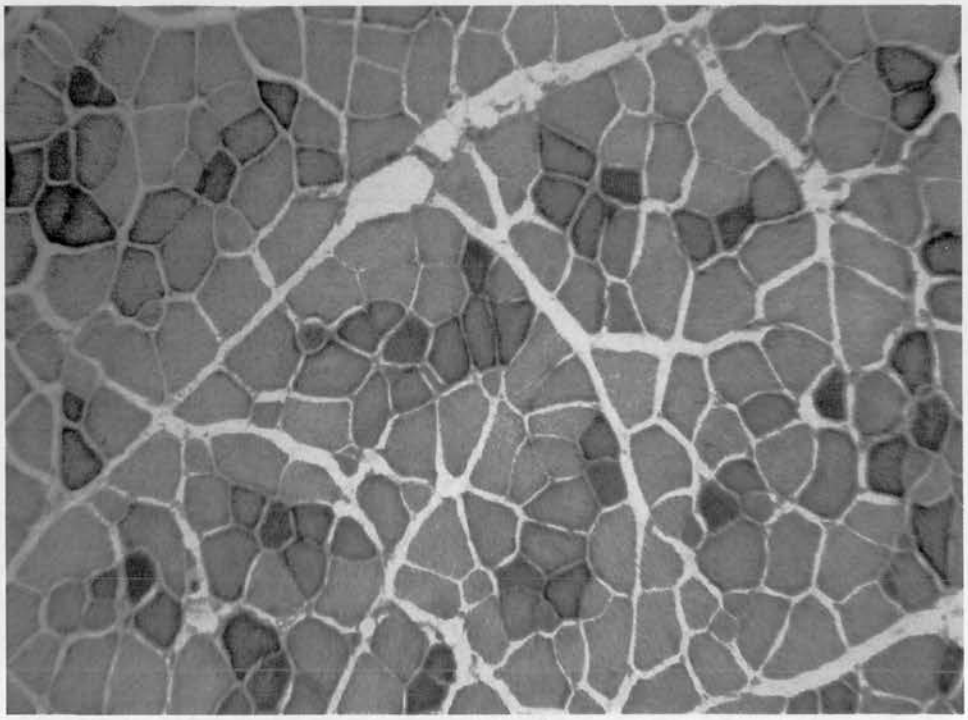


Figure 3.8 Typical heterogeneous distribution of fibre types in the *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles of all lines. Dark blue fibres are Type I, and pale blue fibres are Type II. The section is from a 16 week old great-grandparent *Biceps femoris* muscle sample (NADH stain, magnification x10).

| age (weeks) | line | connective tissue content (%) | Minimum Fibre Diameter (MFD) (μm) | | | | mean coefficient of variation (%) |
|---|---|---|---|---|---|---|---|
| | | | mean | minimum | maximum | range | |
| | B | 10.6 (0.8) | 45.9 (0.9) | 5.5 | 76.2 | 70.7 | 33.2 |
| 5 | GGP | 15.3 (0.8) | 39.2 (1.2) | 7.7 | 66.5 | 58.8 | 30.7 |
| | L | 13.1 (3.5) | 21.5 (0.6) | 4.3 | 43.2 | 38.9 | 30.0 |
| | B | 15.4 (2.2) | 58.5 (7.7) | 4.4 | 105.4 | 100.9 | 35.4 |
| 9 | GGP | 12.5 (1.2) | 45.4 (8.5) | 3.9 | 99.7 | 95.8 | 38.6 |
| | L | 14.5 (4.9) | 24.7 (2.5) | 2.5 | 46.8 | 44.3 | 33.4 |
| | B | 16.7 (3.4) | 56.2 (4.9) | 4.4 | 101.6 | 97.2 | 34.5 |
| 13 | GGP | 13.1 (1.7) | 56.6 (10.8) | 5.6 | 103.8 | 98.2 | 33.9 |
| | L | 18.2 (5.8) | 31.2 (3.3) | 5.1 | 60.1 | 55.0 | 30.7 |
| | B | 15.1 (1.1) | 48.8 (5.6) | 10.5 | 96.8 | 86.3 | 31.0 |
| 16 | GGP | 13.9 (1.6) | 60.5 (7.1) | 5.6 | 115.9 | 110.3 | 32.6 |
| | L | 19.3 (0.2) | 39.3 (7.8) | 4.7 | 70.0 | 65.3 | 29.1 |
| | B | 15.7 (0.9) | 61.9 (4.8) | 5.3 | 115.7 | 110.4 | 34.3 |
| 18 | GGP | 17.4 (1.3) | 59.9 (5.6) | 3.8 | 102.7 | 98.9 | 34.9 |
| | L | 19.1 (0.9) | 41.1 (2.2) | 8.0 | 73.8 | 65.8 | 33.3 |
| | B | 14.9 (1.8) | 57.8 (8.5) | 5.1 | 128.7 | 123.6 | 36.8 |
| 20 | GGP | 17.2 (0.4) | 60.2 (2.6) | 6.5 | 116.3 | 109.8 | 34.7 |
| | L | 22.5 (3.0) | 39.9 (1.7) | 5.3 | 71.7 | 66.4 | 34.3 |
| | B | 17.1 (0.4) | 55.3 (6.4) | 6.7 | 127.5 | 120.8 | 41.7 |
| 23 | GGP | 16.6 (1.1) | 60.8 (6.4) | 9.8 | 122.5 | 112.7 | 34.2 |
| | L | 17.0 (1.3) | 38.5 (1.2) | 5.6 | 70.0 | 64.4 | 32.8 |
| | B | 12.5 (1.5) | 65.9 (10.8) | 10.6 | 128.2 | 117.6 | 42.2 |
| 25 | GGP | 14.6 (0.8) | 59.8 (2.3) | 5.2 | 121.6 | 116.4 | 40.6 |
| | L | 16.9 (0.8) | 38.1 (2.4) | 5.6 | 68.4 | 62.8 | 35.1 |

Table 3.5 Mean connective tissue content (%) (n=4), mean minimum fibre diameter (MFD) (μm) (standard deviations in parentheses) (n=400 per muscle), minimum MFD (μm), maximum MFD (μm), MFD range (μm) and mean coefficient of variation (%) (n=4) of the *Pectoralis major* muscle fibres of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

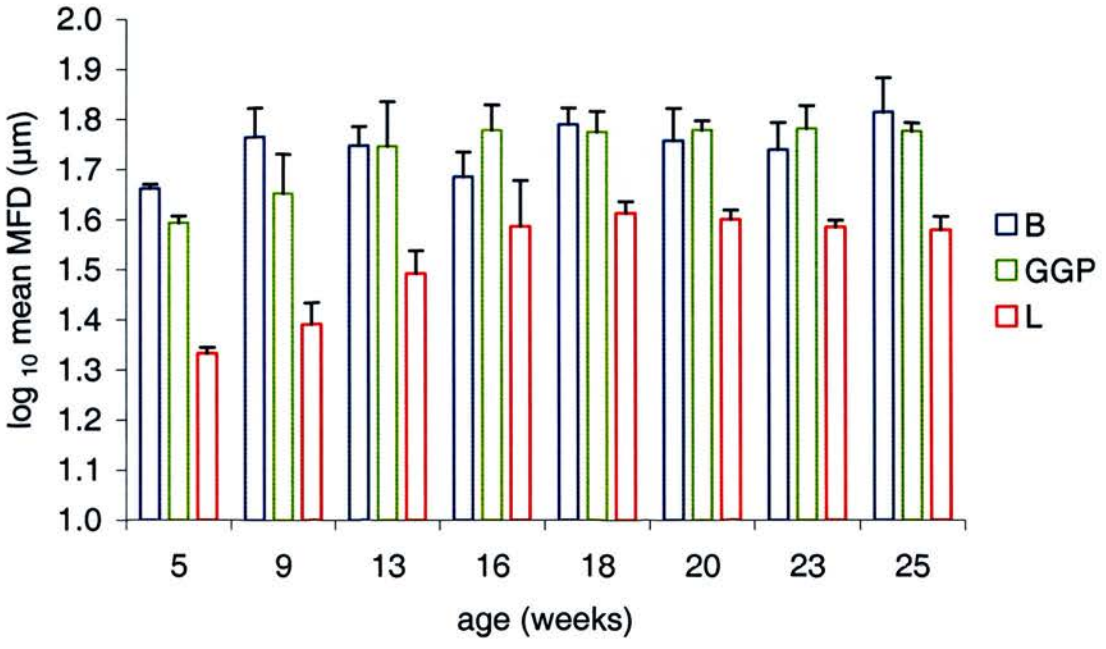



Figure 3.9 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Pectoralis major* muscle fibres of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

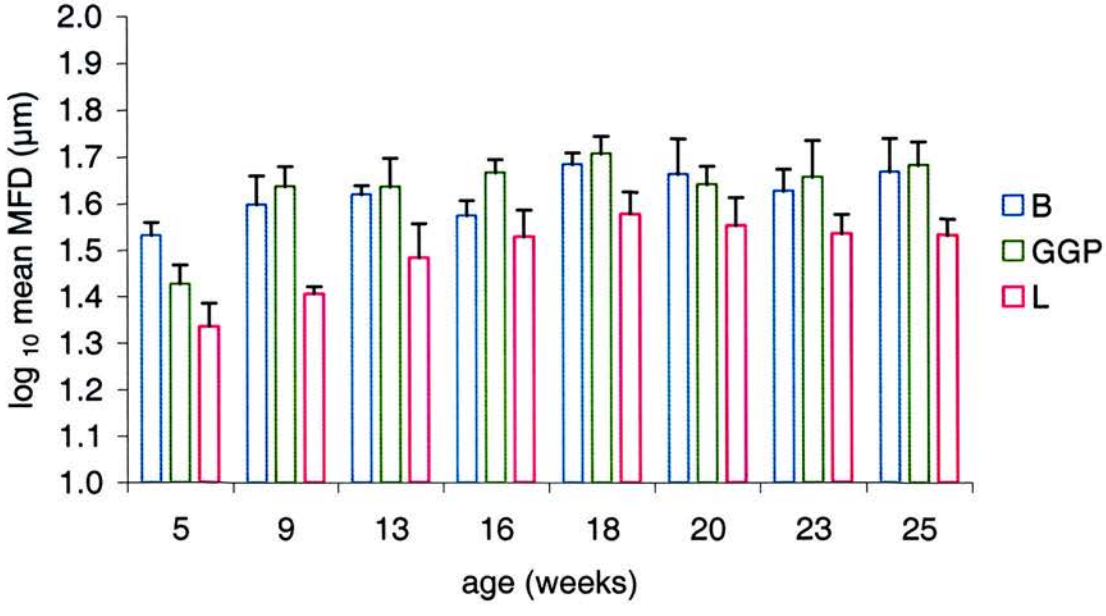



Figure 3.10 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Biceps femoris* muscle fibres of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

significantly larger mean MFD values than the layer line at all ages ($P<0.05$). The great-grandparent line had significantly larger mean MFD values than the layer line at all ages ($P<0.05$). The mean MFD values did not differ significantly between the broiler and great-grandparent lines at any age. There was a significant interaction between age and line ($P<0.001$), suggesting that the mean MFD values of the broiler and great-grandparent lines diverged from the mean MFD values of the layer line with age.

Peak mean MFD values were achieved at 25 weeks in the broiler (65.9 μm), at 23 weeks in the great-grandparent line (60.8 μm), and at 18 weeks in the layer line (41.1 μm). The smallest MFD value recorded in the broiler line was at both 9 and 13 weeks (4.4 μm), at 18 weeks in the great-grandparent line (3.8 μm), and at 9 weeks in the layer line (2.5 μm). The largest MFD value was measured in the broiler line at 20 weeks of age (128.7 μm), at 23 weeks in the great-grandparent line (122.5 μm), and at 18 weeks in the layer line (73.8 μm).

The mean MFD values appeared to reach an asymptote at 16 weeks of age in the layer and great-grandparent lines, 'plateau-ing' at approximately 60 μm and 39 μm respectively. The mean MFD values were quite variable over the corresponding age intervals of the broiler line, with no clear plateau.

*Biceps femoris*

In the broiler, great-grandparent and layer lines, the mean Minimum Fibre Diameter (MFD) (n=100) of the leg muscle *Biceps femoris* increased significantly with age ($P<0.001$) (Table 3.6 and Figure 3.10). The broiler line had significantly larger mean MFD values than the layer line at 5, 9, 13 and 25 weeks of age ($P<0.05$). The great-grandparent line had significantly larger mean MFD values than the layer line at 9, 13 and 16 weeks of age ($P<0.05$). The mean MFD values did not differ significantly between the broiler and great-grandparent lines at any age. There was a significant interaction between age and line ($P<0.001$), suggesting that the mean MFD values of

| age (weeks) | line | connective tissue content (%) | Minimum Fibre Diameter (MFD) (μm) | | | | mean coefficient of variation (%) |
|---|---|---|---|---|---|---|---|
| | | | mean | minimum | maximum | range | |
| | B | 17.9 (3.0) | 34.1 (2.1) | 8.7 | 55.9 | 47.2 | 26.8 |
| 5 | GGP | 15.4 (1.0) | 26.9 (2.5) | 4.1 | 65.8 | 61.7 | 37.6 |
| | L | 14.4 (2.7) | 21.8 (2.4) | 5.8 | 39.9 | 34.1 | 25.7 |
| | B | 19.8 (1.7) | 39.9 (5.5) | 6.1 | 81.2 | 75.1 | 33.3 |
| 9 | GGP | 13.8 (1.3) | 43.6 (4.2) | 3.9 | 91.2 | 87.3 | 34.5 |
| | L | 17.7 (3.9) | 25.5 (0.9) | 6.9 | 43.9 | 37.0 | 28.8 |
| | B | 21.2 (1.3) | 41.7 (1.9) | 8.8 | 76.7 | 67.9 | 29.7 |
| 13 | GGP | 18.1 (2.8) | 43.6 (6.3) | 8.5 | 100.2 | 91.7 | 33.6 |
| | L | 17.6 (4.2) | 30.8 (5.4) | 4.7 | 47.2 | 42.5 | 30.9 |
| | B | 19.6 (2.6) | 37.7 (2.8) | 7.2 | 70.5 | 63.3 | 28.0 |
| 16 | GGP | 17.2 (3.2) | 46.5 (3.0) | 2.7 | 96.7 | 94.0 | 32.4 |
| | L | 21.0 (1.8) | 34.1 (4.4) | 6.0 | 63.4 | 57.4 | 26.7 |
| | B | 23.3 (2.2) | 48.3 (2.8) | 6.5 | 107.9 | 101.4 | 33.6 |
| 18 | GGP | 21.8 (3.4) | 51.0 (4.3) | 8.1 | 94.2 | 86.1 | 32.7 |
| | L | 23.8 (4.4) | 38.0 (4.1) | 5.3 | 75.8 | 70.5 | 34.9 |
| | B | 26.1 (6.3) | 46.6 (7.7) | 10.7 | 94.8 | 84.1 | 29.6 |
| 20 | GGP | 25.4 (5.6) | 43.9 (3.8) | 5.6 | 110.4 | 104.8 | 34.5 |
| | L | 25.5 (3.8) | 36.0 (5.1) | 7.4 | 67.2 | 59.8 | 29.9 |
| | B | 21.4 (2.7) | 42.6 (4.4) | 4.7 | 88.9 | 84.2 | 32.9 |
| 23 | GGP | 20.9 (1.4) | 45.9 (7.6) | 7.3 | 88.6 | 81.3 | 35.3 |
| | L | 22.3 (1.3) | 34.4 (3.3) | 2.1 | 64.2 | 62.1 | 35.8 |
| | B | 20.1 (1.6) | 46.9 (7.4) | 6.7 | 91.5 | 84.8 | 31.6 |
| 25 | GGP | 20.7 (2.1) | 48.3 (5.4) | 3.3 | 97.7 | 94.4 | 40.3 |
| | L | 25.8 (3.2) | 34.1 (2.6) | 6.7 | 60.0 | 53.3 | 32.6 |

Table 3.6 Mean connective tissue content (%) (n=4), mean minimum fibre diameter (MFD) (μm) (standard deviations in parentheses) (n=400 per muscle), minimum MFD (μm), maximum MFD (μm), MFD range (μm) and mean coefficient of variation (%) (n=4) of the *Biceps femoris* muscle fibres of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

the broiler and great-grandparent lines and the mean MFD values of the layer line converged with age.

Peak mean MFD values were achieved at 18 weeks in the broiler, great-grandparent and layer line (48.3 μm, 51.0 μm and 38.0 μm respectively). The smallest MFD value recorded in the broiler and layer lines was at 23 weeks (4.7 μm and 2.1 μm respectively) and at 16 weeks in the great-grandparent line (2.7 μm). The largest MFD value was measured at 18 weeks of age in the broiler and layer lines (107.9 μm and 75.8 μm respectively) and at 20 weeks in the great-grandparent line (110.4 μm).

The mean MFD values seemed to reach an asymptote at 16 weeks of age in the layer line, 'plateau-ing' at approximately 35 μm. The mean MFD values of the broiler and great-grandparent lines were variable over the later age intervals (16 weeks of age onwards), with no clear plateau.

*Comparing Pectoralis major and Biceps femoris*

There was a significant effect of muscle type on mean MFD ($P<0.001$) and a significant interaction between muscle type and line ($P<0.001$). The mean MFD of the *Pectoralis major* muscle of the broiler line was significantly greater than the mean MFD of the *Biceps femoris* muscle at all ages ($P<0.05$), with the exception of group aged 20 weeks ($P = 0.06$) (Figure 3.11). The mean MFD of the *Pectoralis major* muscle was significantly greater than the mean MFD of the *Biceps femoris* muscle at 5, 16, 20 and 25 weeks of age in the great-grandparent line ($P<0.05$) (Figure 3.12). There was no significant difference between the mean MFD of the two muscles at any age interval in the layer line (Figure 3.13). The mean *Pectoralis major* MFD: *Biceps femoris* MFD ratios over the eight age intervals were 1.33, 1.27 and 1.06 for the broiler, great-grandparent and layer lines respectively.

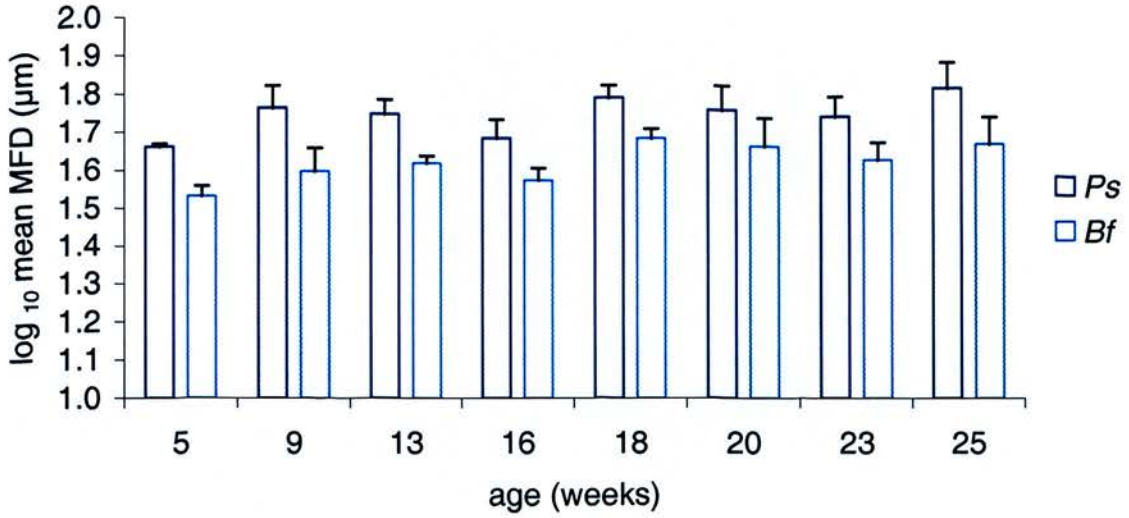



Figure 3.11 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Pectoralis major* (*Ps*) and *Biceps femoris* (*Bf*) muscle fibres of the broiler line at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

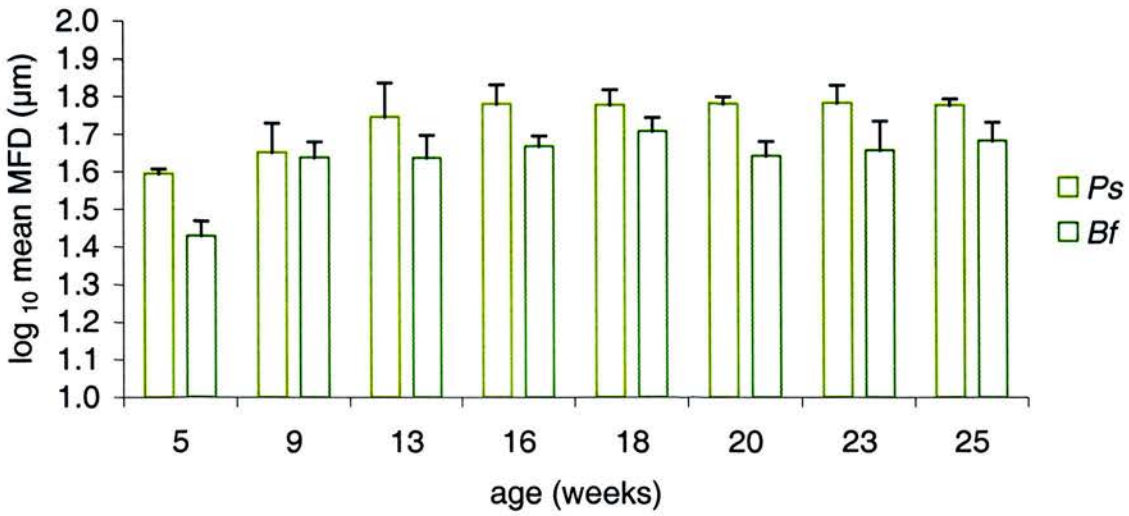



Figure 3.12 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Pectoralis major* (*Ps*) and *Biceps femoris* (*Bf*) muscle fibres of the great-grandparent line at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

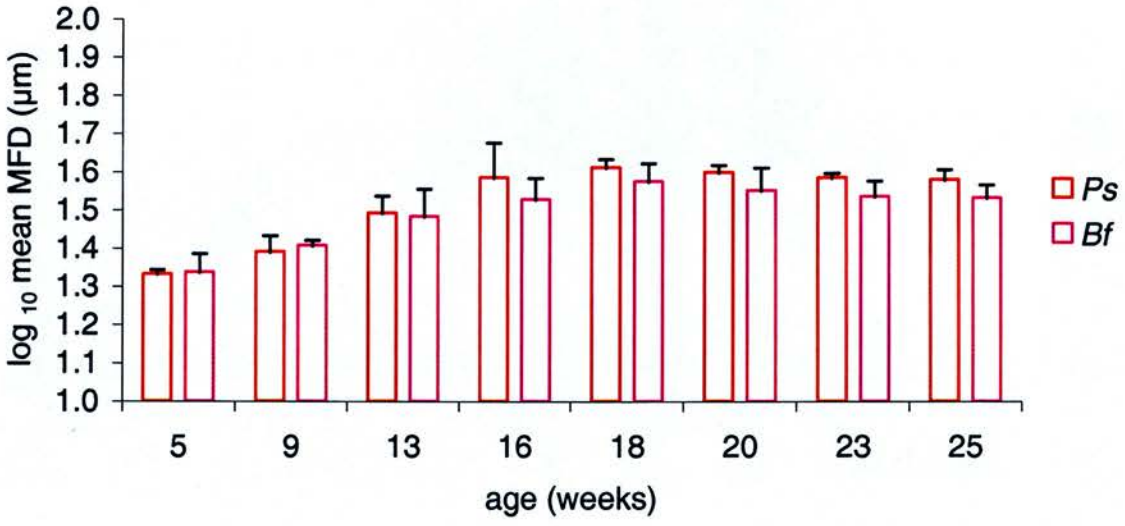



Figure 3.13 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Pectoralis major* (*Ps*) and *Biceps femoris* (*Bf*) muscle fibres of the layer line at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of (n=400 per muscle).

*Fibre size and body weight*

Regression analysis revealed significant linear relationships ($P<0.05$) between $\log_{10}$ mean MFD of the *Pectoralis major* and the corresponding $\log_{10}$ $3\sqrt{}$body weight for the broiler ($y = 0.92x + 1.57$, $R^2 = 0.40$), great grandparent ($y = 1.31x + 1.50$; $R^2 = 0.62$) and layer ($y = 1.38x + 1.46$; $R^2 = 0.85$) lines (Figure 3.14). Regression analysis revealed significant linear relationships ($P<0.05$) between mean $\log_{10}$ MFD of the *Biceps femoris* and the corresponding $\log_{10}$ $3\sqrt{}$body weight for the broiler ($y = 0.95x + 1.44$, $R^2 = 0.43$), great grandparent ($y = 1.55x + 1.35$; $R^2 = 0.69$) and layer ($y = 1.05x + 1.45$; $R^2 = 0.71$) lines (Figure 3.15).

### 3.5.3.2 Connective tissue content

*Pectoralis major*

There was a significant effect of age ($P<0.001$) and line ($P<0.001$) on the connective tissue content of the *Pectoralis major*, and a significant interaction between age and line ($P<0.001$) (Table 3.5). Pairwise comparisons did not reveal any significant differences between the connective tissue content the broiler, great-grandparent and layer lines at any age, with the exception of significantly greater mean connective tissue content of the layer line compared to the broiler line at 20 weeks of age ($P<0.05$).

*Biceps femoris*

There was a significant effect of age ($P<0.001$) and line ($P<0.001$) on the connective tissue content of the *Biceps femoris*, and an insignificant interaction (Table 3.6). Pairwise comparisons did not reveal any significant differences between the connective tissue content the broiler, great-grandparent and layer lines at any age.

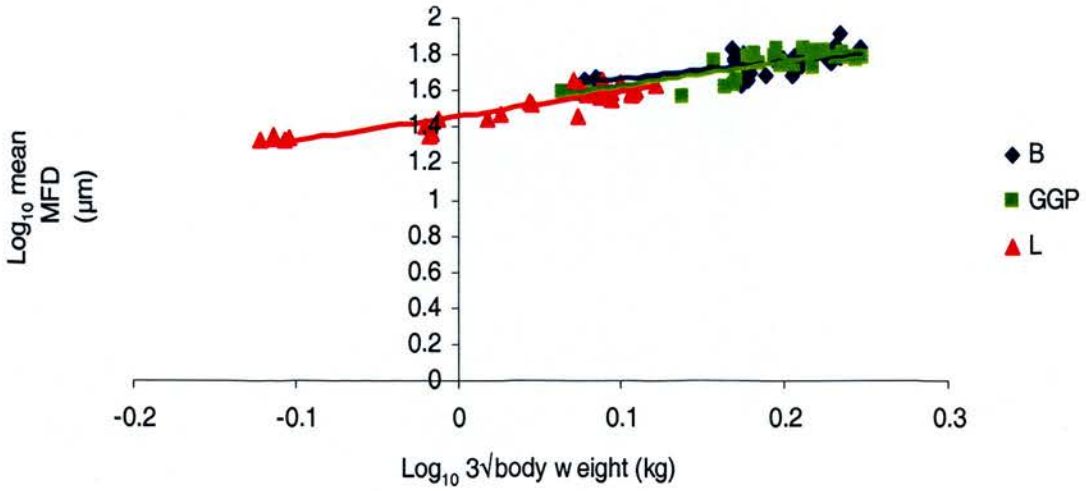



Figure 3.14 The linear relationship between $\log_{10}$ mean minimum fibre diameter (MFD) (µm) of the *Pectoralis major* muscle fibres and the corresponding $\log_{10}$ 3√body weight (kg) of each broiler (B) (y = 0.92x + 1.57, $R^2$ = 0.40), great grandparent (GGP) (y = 1.31x + 1.50; $R^2$ = 0.62) and layer (L) (y = 1.38x + 1.46; $R^2$ = 0.85) (n=32).

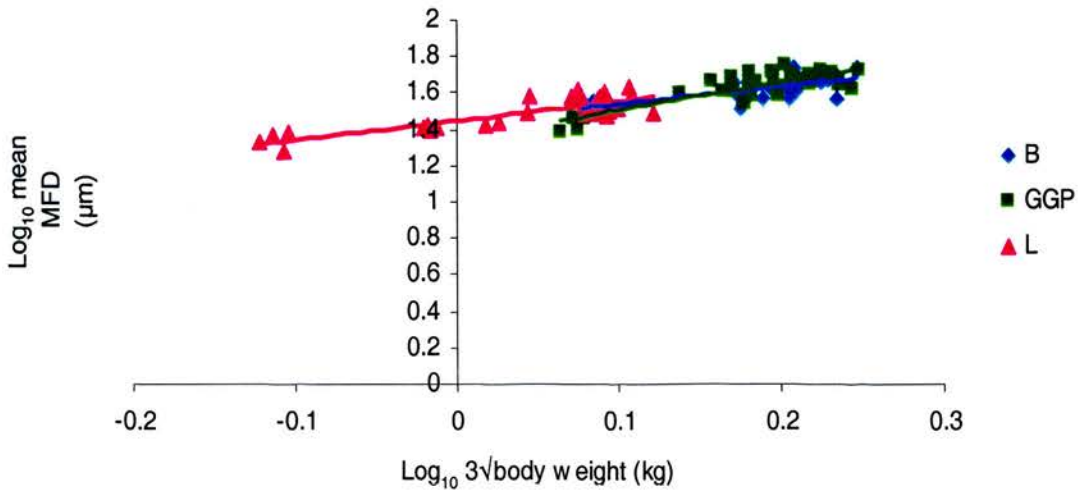



Figure 3.15 The linear relationship between $\log_{10}$ mean minimum fibre diameter (MFD) (µm) of the *Biceps femoris* muscle fibres and the corresponding $\log_{10}$ 3√body weight (kg) of each broiler (B) (y = 0.95x + 1.44, $R^2$ = 0.43), great grandparent (GGP) (y = 1.55x + 1.35; $R^2$ = 0.69) and layer (L) (y = 1.05x + 1.45; $R^2$ = 0.71) chicken (n=32).

*Comparing Pectoralis major and Biceps femoris*

There was a significant effect of muscle type on the connective tissue content and significant interactions between muscle type and line ($P<0.05$) and muscle type and age ($P<0.001$) (Table 3.5 and Table 3.6). In the broiler line, the mean connective tissue content of the *Pectoralis major* was significantly lower ($P<0.05$) than the *Biceps femoris* at all age intervals, with the exception of the muscles from the 13 week age group, where there was no significant difference. There were only significant differences in mean connective tissue content between the *Pectoralis major* the *Biceps femoris* at 23 and 25 weeks of age in the great-grandparent line ($P<0.05$), and at 20, 23 and 25 weeks in the layer line ($P<0.05$).

### 3.5.3.3 Nuclei distribution

*Pectoralis major*

There was a significant effect of both line ($P<0.05$) and age ($P<0.001$) on the percentage of fibres of the breast muscle *Pectoralis major* with ≥1 internal nucleus (fibres $_{\geq 1\ I\ N}$) (Table 3.7 and Figure 3.16). There was no interaction between line and age. The mean percentage of fibres $_{\geq 1\ I\ N}$ was between 90 and 100% in both the broiler and great-grandparent lines at all age intervals. In the layer line, the mean percentage of fibres $_{\geq 1\ I\ N}$ increased from 89% at 5 weeks, to 97% by 25 weeks of age. Pairwise comparisons did not reveal significant differences between the mean percentage of fibres $_{\geq 1\ I\ N}$ of the broiler, great-grandparent and layer lines at any age.

*Biceps femoris*

There was a significant effect of both line ($P<0.001$) and age ($P<0.05$) on the percentage of fibres of the leg muscle *Biceps femoris* with ≥1 internal nucleus (fibres $_{\geq 1\ I\ N}$) (Table 3.7 and Figure 3.17). There was a significant interaction between line and age ($P<0.001$). The mean percentage of fibres $_{\geq 1\ I\ N}$ varied between 41-88% over the age intervals studied in the broiler line. In the great-grandparent line, the

| age (weeks) | muscle | % fibres with ≥1 internal nucleus | | |
|---|---|---|---|---|
| | | B | GGP | L |
| 5 | *Ps* | 97 | 97 | 89 |
| | *Bf* | 88 | 24 | 7 |
| 9 | *Ps* | 99 | 96 | 94 |
| | *Bf* | 61 | 59 | 9 |
| 13 | *Ps* | 97 | 98 | 99 |
| | *Bf* | 67 | 63 | 43 |
| 16 | *Ps* | 100 | 98 | 97 |
| | *Bf* | 78 | 69 | 54 |
| 18 | *Ps* | 97 | 96 | 93 |
| | *Bf* | 41 | 68 | 70 |
| 20 | *Ps* | 96 | 97 | 94 |
| | *Bf* | 50 | 71 | 36 |
| 23 | *Ps* | 99 | 99 | 94 |
| | *Bf* | 48 | 71 | 22 |
| 25 | *Ps* | 98 | 99 | 97 |
| | *Bf* | 77 | 77 | 45 |

Table 3.7 Percentage (%) fibres with ≥1 internal nucleus in the *Pectoralis major* (*Ps*) and *Biceps femoris* (*Bf*) muscles of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

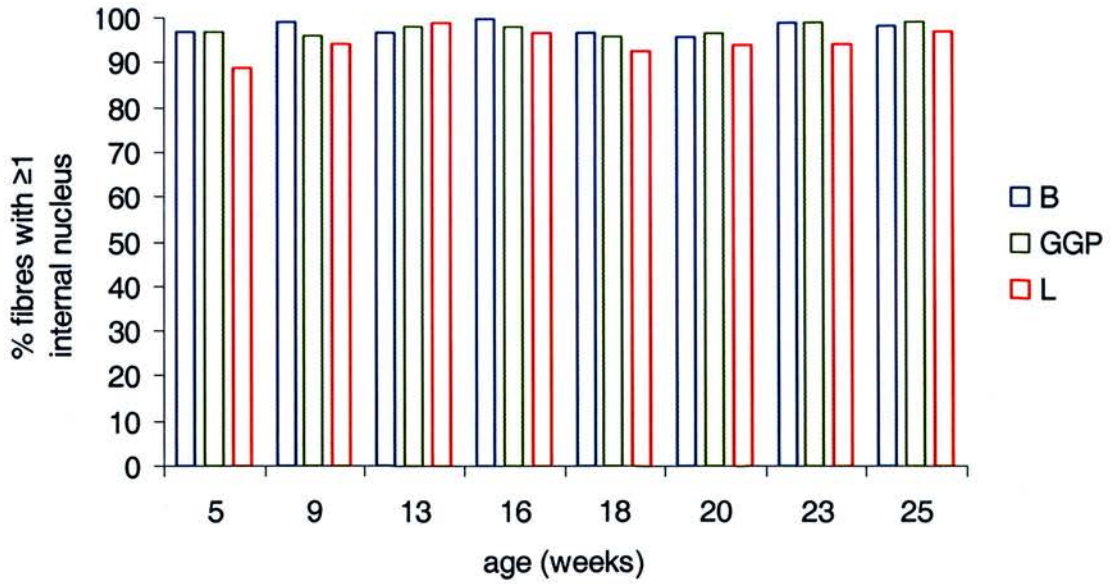



Figure 3.16 Percentage (%) fibres with ≥1 internal nucleus in the *Pectoralis major* muscle of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

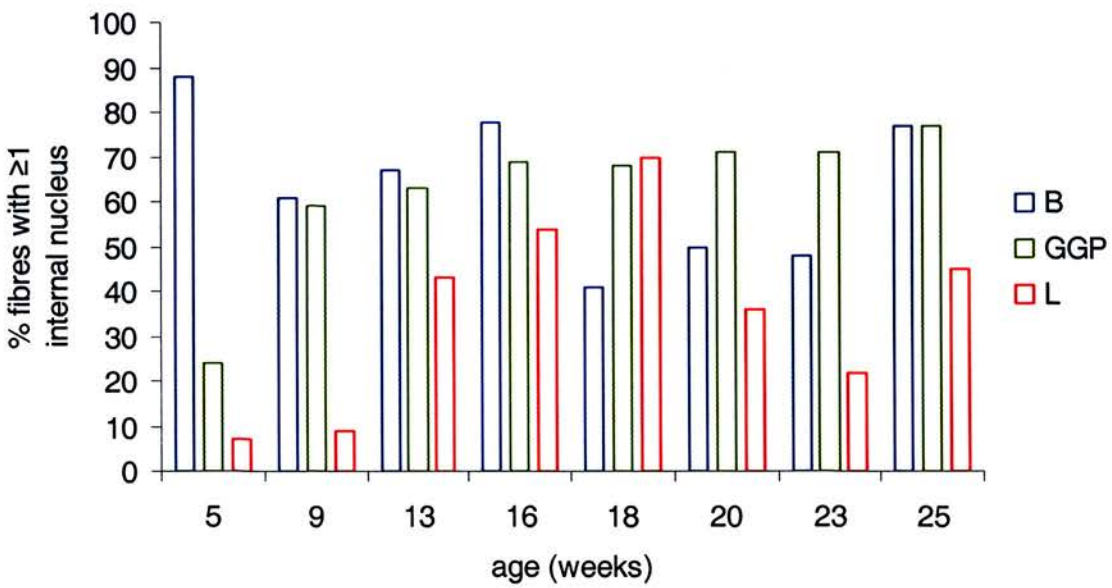



Figure 3.17 Percentage (%) fibres with ≥1 internal nucleus in the *Biceps femoris* muscle of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=400 per muscle).

mean percentage of fibres $_{\geq 1\ I\ N}$ increased with age from 24% at 5 weeks of age, peaking at 77% by 25 weeks. In the layer line, the mean percentage of fibres $_{\geq 1\ I\ N}$ increased from 7% at 5 weeks of age, peaking at 70% by 18 weeks. At 25 weeks of age the mean percentage of fibres $_{\geq 1\ I\ N}$ increased was 45%. There were no significant pairwise differences between the mean percentage of fibres $_{\geq 1\ I\ N}$ of the broiler, great-grandparent and layer lines at all ages with the exception of the 5 and 23 week intervals. The mean percentage of fibres $_{\geq 1\ I\ N}$ of the *Biceps femoris* of the broiler line was significantly greater ($P<0.05$) than that of the great-grandparent and layer lines at 5 weeks of age. The mean percentage of fibres $_{\geq 1\ I\ N}$ of the *Biceps femoris* of the great-grandparent line was significantly greater ($P<0.05$) than that of the layer line at 23 weeks of age.

*Comparing Pectoralis major and Biceps femoris*

There was a significant effect of muscle type on the percentage of fibres $_{\geq 1\ I\ N}$ ($P<0.001$) and significant interactions between (i) muscle type and line ($P<0.001$), (ii) muscle type and age ($P<0.05$) and (iii) muscle type and line and age ($P<0.001$). The mean percentage of fibres of the *Pectoralis major* with ≥1 internal nucleus (fibres $_{\geq 1\ I\ N}$) was significantly greater ($P<0.05$) than the *Biceps femoris* at all age intervals, with the exception of the muscles from the 25 week old great-grandparent line (Table 3.7).

*Fibre size and nuclei distribution*

Regression analysis did not show any significant linear relationships between the $\log_{10}$ arcsin percentage of fibres $_{\geq 1\ I\ N}$ and the corresponding $\log_{10}$ mean MFD of the *Pectoralis major* for any of the three lines. However, regression analysis revealed significant linear relationships ($P<0.05$) between $\log_{10}$ arcsin percentage of fibres $_{\geq 1\ I\ N}$ and the corresponding $\log_{10}$ mean MFD of the *Biceps femoris* for the great-grandparent ($y = 1.76x - 3.13$, $R^2 = 0.46$) and layer ($y = 3.65x - 6.07$, $R^2 = 0.50$) lines (Figure 3.18).

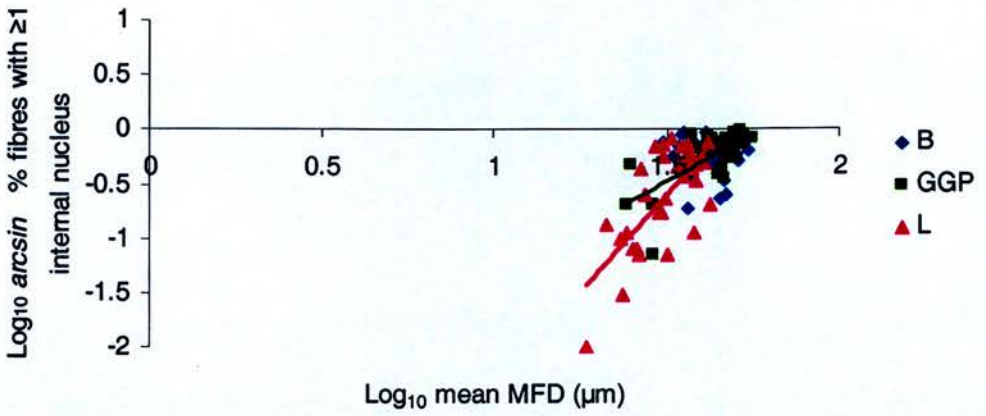



Figure 3.18 The relationship between $\log_{10}$ arcsin percentage (%) fibres with ≥1 internal nucleus in the *Biceps femoris* muscle fibres and the corresponding $\log_{10}$ mean minimum fibre diameter (MFD) of each broiler (B) (no linear relationship), great-grandparent (GGP) ($y = 1.76x - 3.13$; $R^2 = 0.46$) and layer (L) ($y = 3.65x - 4.10$; $R^2 = 0.50$) (n=32).

## 3.6 Results III: Muscle damage

### 3.6.1 Enzyme markers of myopathy

#### 3.6.1.1 Plasma creatine kinase (CK) activity

Plasma CK activity differed significantly with age ($P<0.05$) and between line ($P<0.05$) (Table 3.8 and Figure 3.19). The plasma CK activity of the layer line increased significantly from 5 to 16 weeks of age (244 $IUl^{-1}$ to 489 $IUl^{-1}$) ($P<0.05$). By 25 weeks, the plasma CK activity of the layer line had fallen significantly to 143 $IUl^{-1}$ ($P<0.05$). In the broiler line plasma CK activity increased significantly from 5 to 18 weeks of age (955 $IUl^{-1}$ to 1894 $IUl^{-1}$) ($P<0.05$). By 25 weeks, the plasma CK activity of the broiler line had fallen significantly to 723 $IUl^{-1}$ ($P<0.05$). The plasma CK activity of the great-grandparent line also increased significantly from 5 to 18 weeks of age (728 $IUl^{-1}$ to 1801 $IUl^{-1}$) ($P<0.05$). The plasma CK activity of the great-grandparent line had fallen significantly to 621 $IUl^{-1}$ by 25 weeks of age ($P<0.05$). The peak of plasma CK activity in the broiler and great-grandparent lines were significantly greater compared to that of the layer line ($P<0.05$).

#### 3.6.1.2 Plasma lactate dehydrogenase (LDH) activity

Mean plasma LDH activity differed significantly with age ($P<0.001$) and between line ($P<0.001$), with no significant interaction between age and line (Table 3.8 and Figure 3.20). The peak mean plasma LDH activity of the layer was recorded at 5 and 16 weeks of age (184 $IUl^{-1}$), with little change at 9 and 13 weeks of age. By 25 weeks, the mean plasma LDH activity of the layer line had fallen to 104 $IUl^{-1}$. In the broiler line mean plasma LDH activity peaked at 315 $Ul^{-1}$ at 9 weeks of age, with a smaller peak at 18 weeks at 268 $IUl^{-1}$. By 25 weeks, the mean plasma LDH activity of the broiler line had fallen to 165 $IUl^{-1}$. The mean plasma LDH activity of the great-grandparent line also peaked at 9 weeks of age, at 264 $Ul^{-1}$, with a smaller peak at 18 weeks at 215 $IUl^{-1}$. The mean plasma LDH activity of the great-grandparent line had fallen to 109 $IUl^{-1}$ by 25 weeks of age.

| age (weeks) | line | plasma CK activity ($IUl^{-1}$) | plasma LDH activity ($IUl^{-1}$) | plasma AST activity ($IUl^{-1}$) |
|---|---|---|---|---|
| 5 | B | 955 (397) | 258 (20) | 35.1 (8.3) |
| | GGP | 728 (479) | 170 (41) | 46.0 (6.8) |
| | L | 244 (87) | 184 (21) | 28.6 (1.2) |
| 9 | B | 1088 (430) | 315 (36) | 41.1 (6.9) |
| | GGP | 1385 (460) | 264 (29) | 61.1 (7.0) |
| | L | 238 (98) | 173 (28) | 28.9 (1.3) |
| 13 | B | 1035 (302) | 248 (64) | 44.2 (11.9) |
| | GGP | 1677 (1175) | 169 (79) | 64.4 (12.8) |
| | L | 199 (65) | 159 (34) | 32.1 (3.5) |
| 16 | B | 808 (364) | 201 (51) | 40.2 (9.2) |
| | GGP | 1426 (614) | 190 (40) | 61.4 (12.0) |
| | L | 489 (40) | 184 (37) | 33.1 (3.2) |
| 18 | B | 1894 (609) | 268 (54) | 68.0 (14.5) |
| | GGP | 1801 (846) | 215 (67) | 62.8 (15.6) |
| | L | 348 (85) | 178 (23) | 49.1 (20.5) |
| 20 | B | 1199 (371) | 221 (21) | 48.7 (6.9) |
| | GGP | 1296 (260) | 213 (61) | 60.6 (9.2) |
| | L | 229 (120) | 121 (13) | 36.4 (13.4) |
| 23 | B | 703 (266) | 173 (63) | 39.9 (2.7) |
| | GGP | 1107 (802) | 156 (73) | 50.5 (10.8) |
| | L | 143 (36) | 106 (14) | 44.9 (9.7) |
| 25 | B | 723 (305) | 165 (41) | 49.5 (18.8) |
| | GGP | 621 (201) | 109 (44) | 53.8 (7.5) |
| | L | 143 (71) | 104 ( 7) | 48.6 (31.3) |

Table 3.8 Mean plasma activities of creatine kinase (CK) ($IUl^{-1}$), lactate dehydrogenase (LDH) ($IUl^{-1}$) and aspartate aminotransferase (AST) ($IUl^{-1}$) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

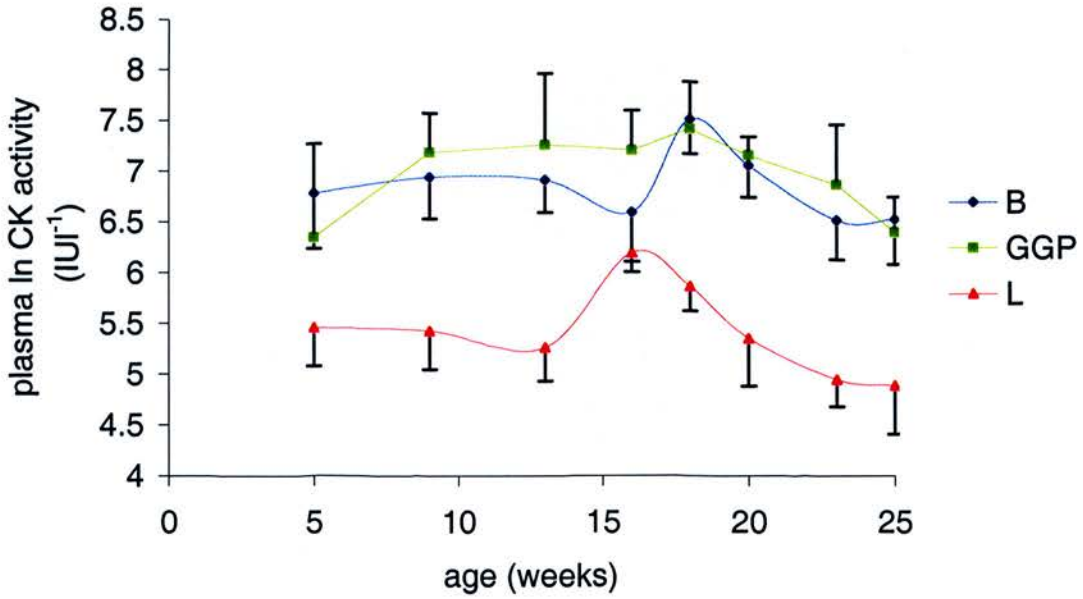



Figure 3.19 Mean plasma creatine kinase (CK) activity ($IUl^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

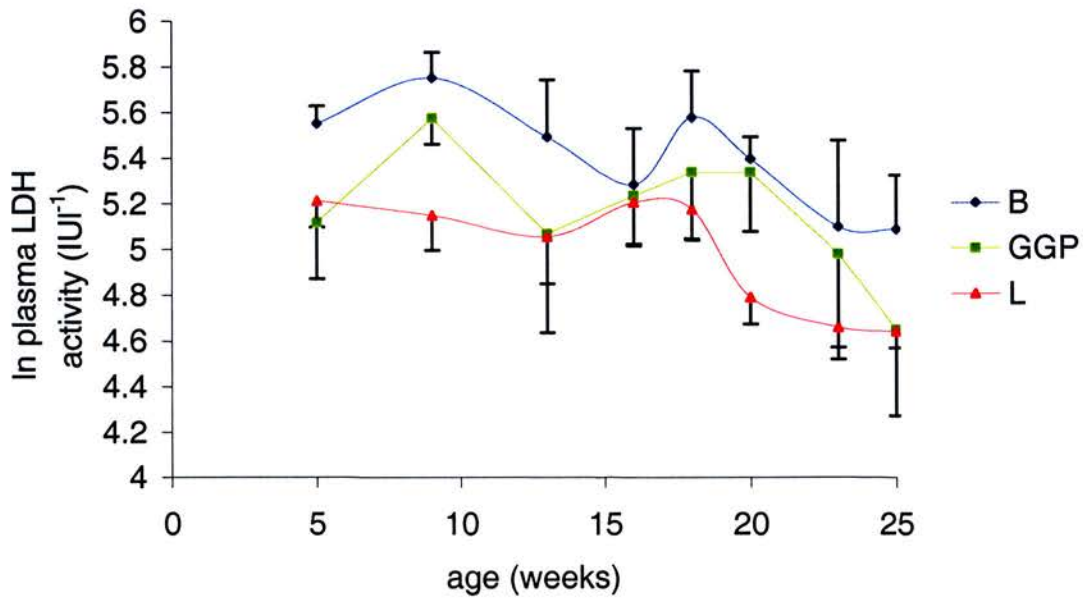



Figure 3.20 Mean plasma lactate dehydrogenase (LDH) activity ($IUl^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

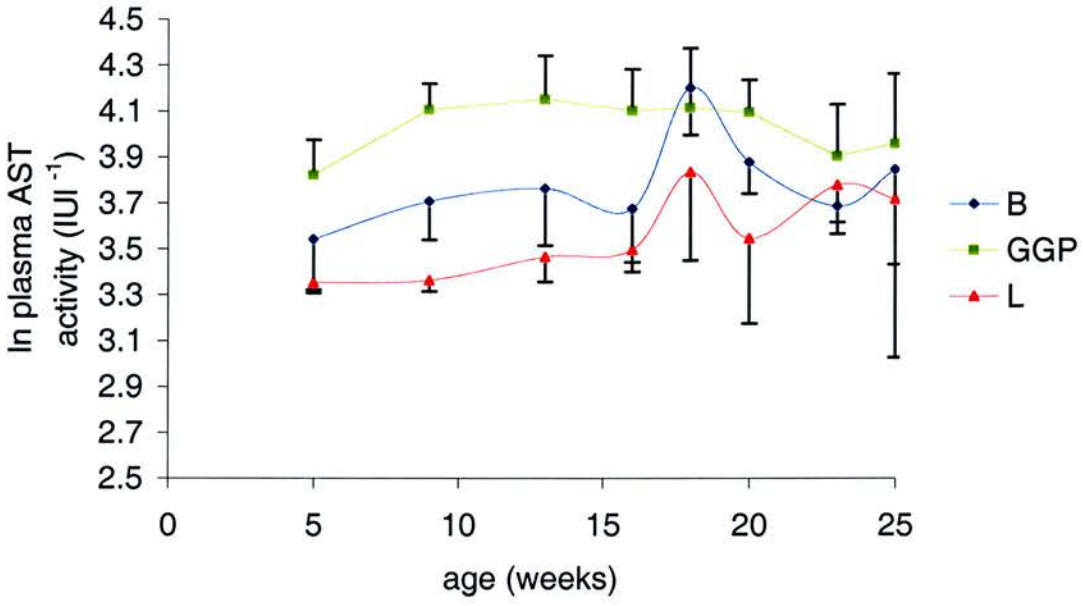



Figure 3.21 Mean ln plasma aspartate aminotransaminase (AST) activity ($IUl^{-1}$) (error bars represent 1 standard deviation) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age (n=4).

### 3.6.1.3 Plasma aspartate aminotransaminase (AST) activity

Plasma AST activity differed significantly with age ($P<0.05$) and between line ($P<0.001$) (Table 3.8 and Figure 3.21), with no significant interaction between age and line. No pair-wise comparisons between appropriate line and age groups were significant. Plasma AST activity increased numerically from 5 to 18 weeks of age in the layer (28.6 $IUl^{-1}$ to 49.1 $IUl^{-1}$), broiler (35.1 $IUl^{-1}$ to 68.0 $IUl^{-1}$) and great-grandparent line (46.0 $IUl^{-1}$ to 62.8 $IUl^{-1}$) lines. The peak plasma AST activity was numerically larger in the broiler and great-grandparent lines (68.0 $IUl^{-1}$ and 62.8 $IUl^{-1}$ respectively) compared to that of the layer line (49.1 $IUl^{-1}$). At 25 weeks of age, the plasma AST activities were still elevated compared to the values at 5 weeks, and were 48.6 $IUl^{-1}$, 49.5 $IUl^{-1}$ and 53.8 $IUl^{-1}$ in the layer, broiler and great-grandparent lines respectively.

## 3.6.2 Muscle pathology assessment

### 3.6.2.1 Detailed evaluation

Abnormal structural features generally occurred at a greater frequency (i) in the broiler *Pectoralis major* and *Biceps femoris* muscles, compared to the great-grandparent *Pectoralis major* and *Biceps femoris* muscles, and (ii) in the great-grandparent muscles compared to the layer muscles. The type of abnormal feature observed appeared to be related to both the type of muscle and the age of the bird. Features that were observed in most sections included 'tiny' fibres (<10 μm), fibre size variation and fibre splitting (Figure 3.22 and Figure 3.23). The detailed pathology evaluations undertaken for each individual bird are summarised below according to line and muscle type. The abnormal features described include fibres that were necrotic (Figure 3.24 and Figure 3.25), basophilic (Figure 3.26) and hyaline (Figure 3.27), and fibres with NADH negative cores or NADH rich rims (Figure 3.28).

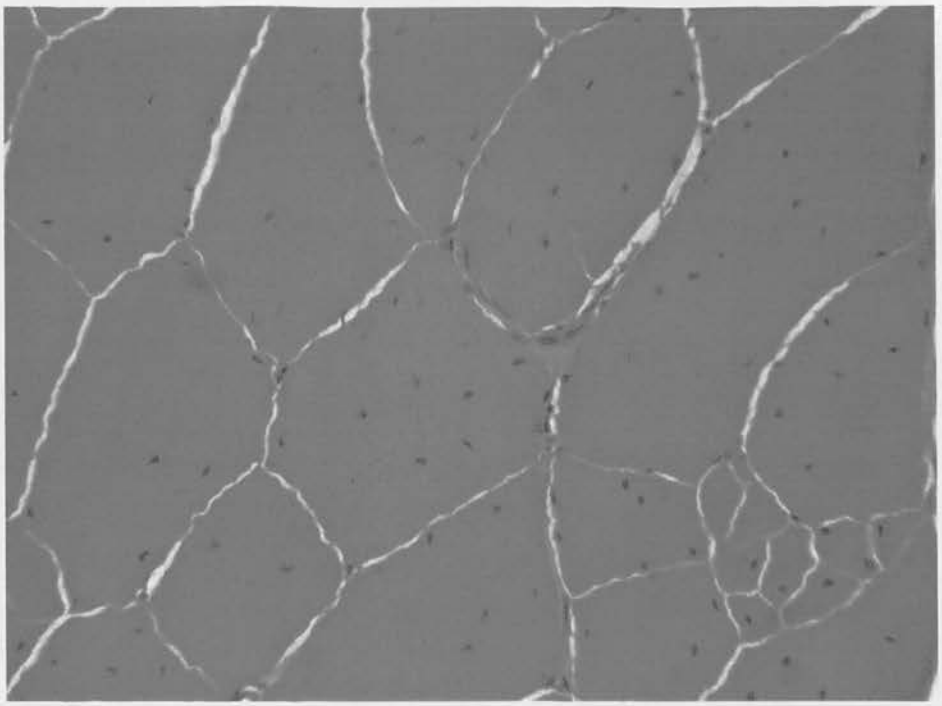


Figure 3.22 Fibre 'splitting' in a section from an 18 week old broiler *Pectoralis major* muscle sample (H&E stain, magnification x40)

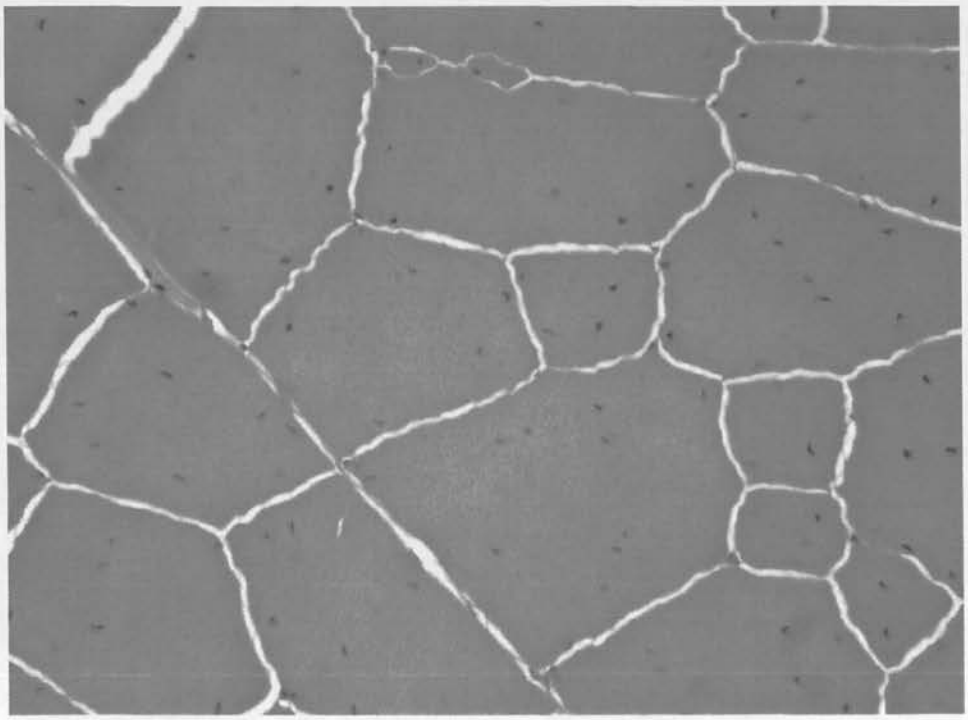


Figure 3.23 Fibre size variation in a section from a 18 week old broiler *Pectoralis major* muscle sample (H&E stain, magnification x40).

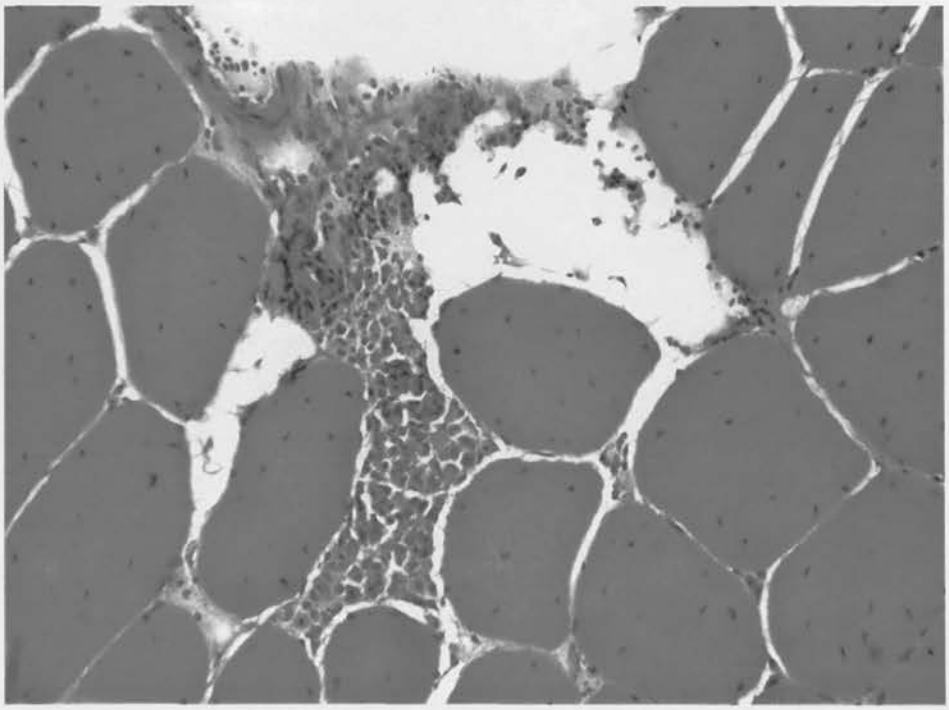


Figure 3.24 Necrotic fibres with fatty tissue replacement in a section from a 20 week old broiler *Pectoralis major* muscle sample (H&E stain, magnification x25).

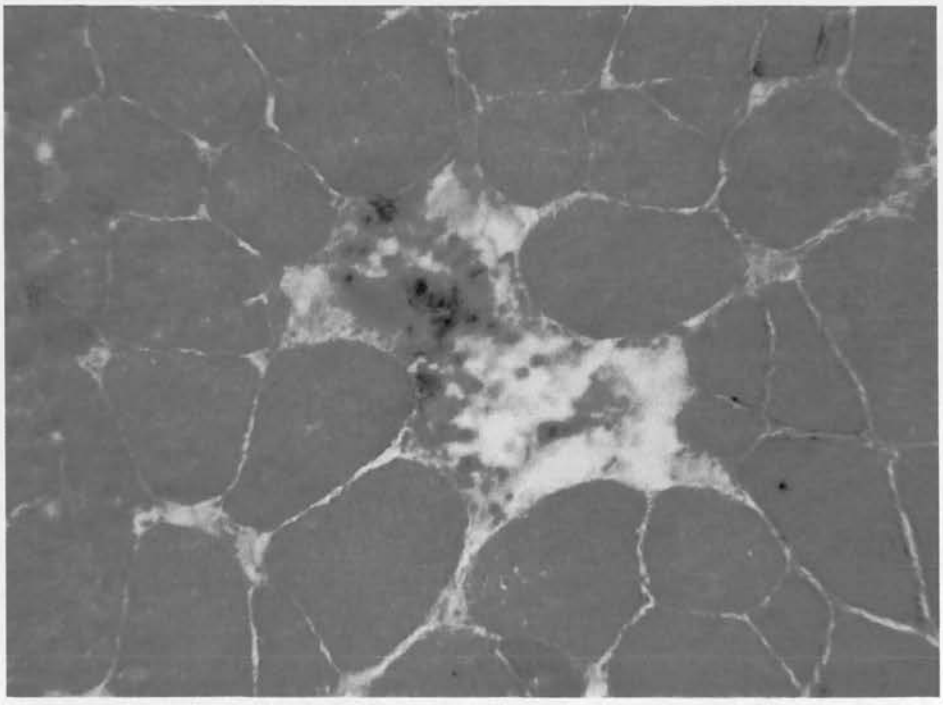


Figure 3.25 Sites of lysosomal activity in two necrotic fibres in a section from a 5 week old broiler *Pectoralis major* muscle sample (esterase stain, magnification x25).

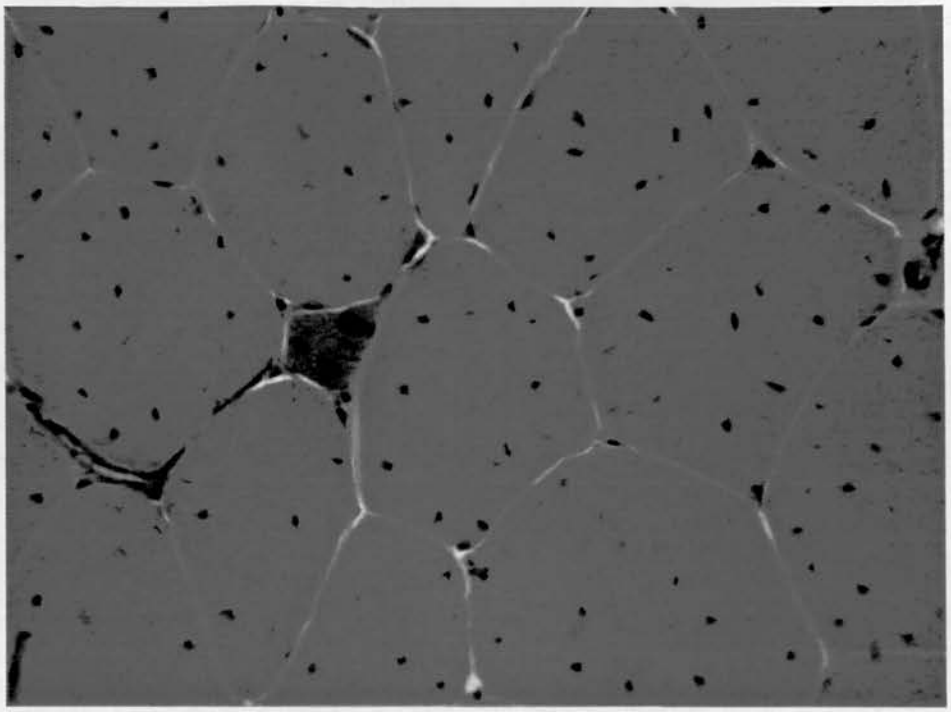


Figure 3.26 A basophilic fibre in a section from a 20 week old broiler *Pectoralis major* muscle sample (H&E stain, magnification x40).

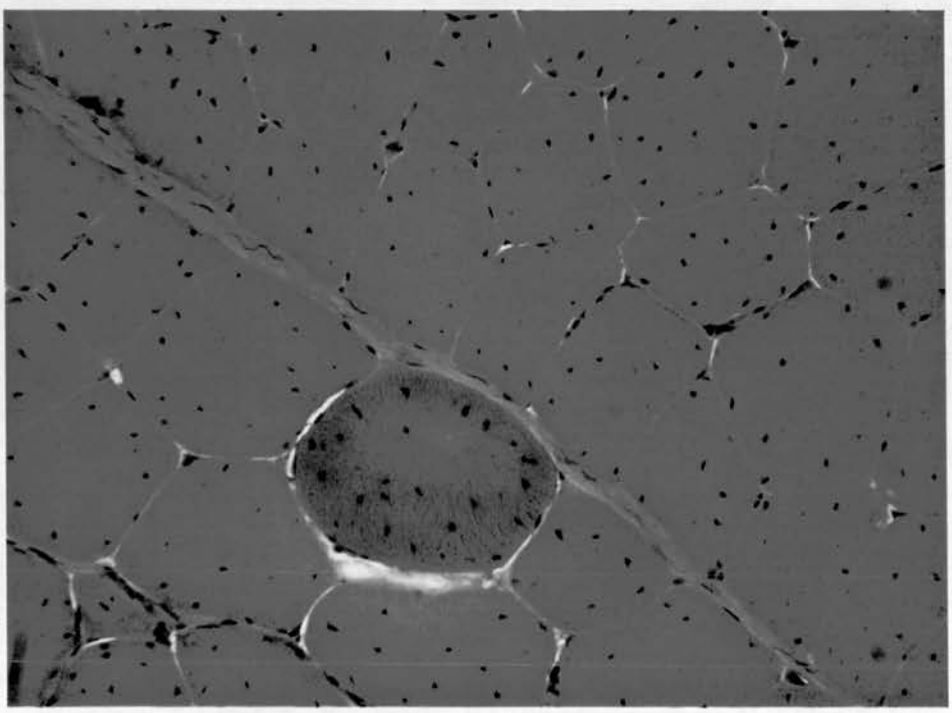


Figure 3.27 A hyaline fibre in a section from a 20 week old broiler *Biceps femoris* muscle sample (H&E stain, magnification x25).

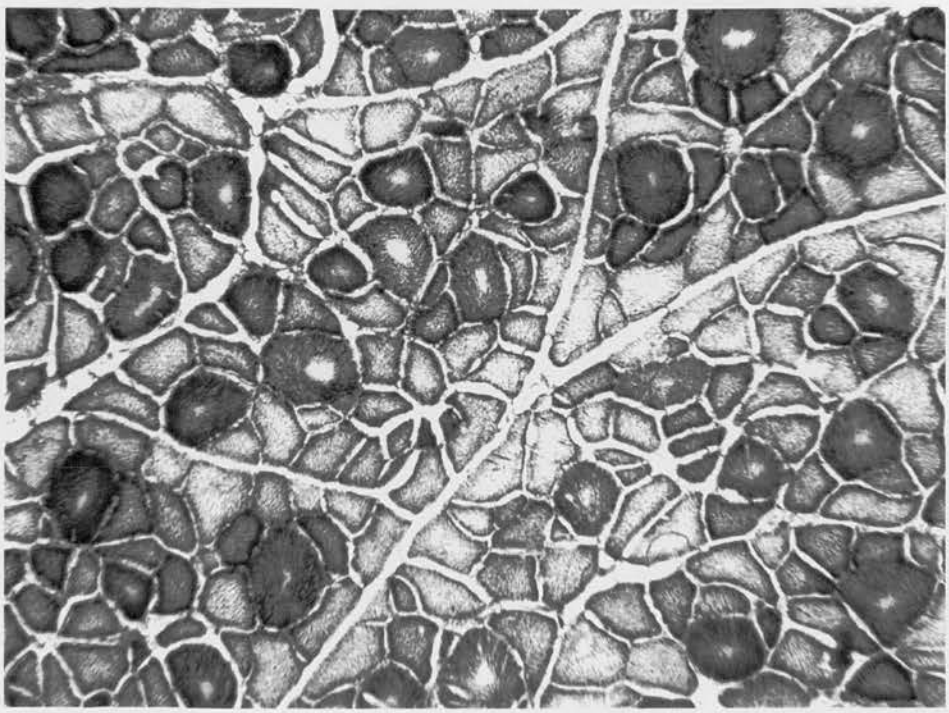


Figure 3.28 Rims and cores in a section from a 20 week old broiler *Biceps femoris* muscle sample (NADH stain, magnification x10).

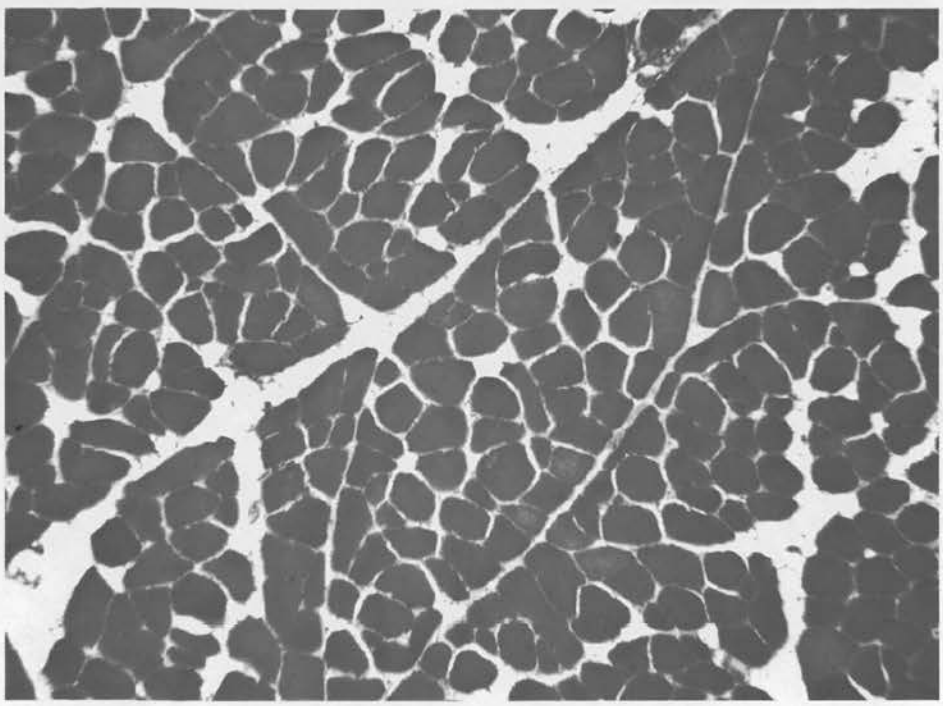


Figure 3.29 Fatty tissue replacing fibres within fascicles and merging with fat between fasicles, in a section from an 18 week old broiler *Biceps femoris* muscle sample (Trichrome stain, magnification x10).

*Broiler Pectoralis major*

There was a high incidence of basophilic fibres in two of the four sections of *Pectoralis major* muscle samples taken at 5, 9, 18, and 20 weeks of age, and one section at 13 and 23 weeks of age. Necrotic fibres were prominent in two sections of muscle samples removed at 5 weeks and one section at 20 weeks of age. A very low/no incidence of abnormal fibres was observed in the sections of muscle samples collected at 25 weeks of age.

*Broiler Biceps femoris*

A few necrotic and basophilic fibres were present in two sections of *Biceps femoris* muscle samples removed at 5 weeks of age. There was a low incidence of basophilic fibres in one of the four sections of muscle samples taken at 18, 20 and 23 weeks of age. Hyaline fibres were prominent in most sections from muscle samples removed at 18-20 weeks of age. There was a very low/no incidence of abnormal fibres in the sections of muscle samples collected at 25 weeks. Fat was present both within and between fascicles of most sections of muscle samples taken from 18 weeks of age onwards (Figure 3.29). NADH rich rims were observed in most of the *Biceps femoris* sections sampled during the study. NADH negative cores were also present in 1-2 of the four sections from muscle samples taken at 18, 20, 23 and 25 weeks of age.

*Great-grandparent Pectoralis major*

1-2 of the four sections of muscle samples removed at 5, 9 and 16 weeks of age contained a few necrotic fibres. Basophilic fibres appeared at a low frequency in most sections of *Pectoralis major* muscle samples taken at 5, 9 and 13 weeks, and in 1-2 of the four sections at 16, 18, 20, 23 and 25 weeks of age.

*Great-grandparent Biceps femoris*

The sections of *Biceps femoris* muscle samples collected at 5 and 9 weeks of age showed a very low/no incidence of abnormal fibres. Most sections from muscle samples removed at 13 weeks of age contained a few hyaline fibres. Large numbers of hyaline fibres were observed in two of the four sections from muscle samples taken at 16, 18, 20, 23 and 25 weeks of age. Most of the sections of muscle samples taken from 18 weeks of age onwards showed the presence of fat both within and between fascicles. NADH rich rims were present in 2-3 sections from muscle samples taken at each age interval between 9 and 25 weeks of age. NADH negative cores were also present in 1 of the four sections from muscle samples taken at 18, 23 and 25 weeks of age.

*Layer Pectoralis major*

There was a very low/no incidence of abnormal fibres in the sections of *Pectoralis major* muscle samples removed at 5, 9, 13, 16, 18 and 25 weeks of age. A few basophilic fibres were present in one section of muscle sample taken at 20 weeks and 23 weeks of age.

*Layer Biceps femoris*

The sections of *Biceps femoris* muscle samples taken at 5, 9, 13 and 18 weeks of age contained a very low/no incidence of abnormal fibres. Hyaline fibres were observed at a low frequency in two of the four sections from muscle samples removed at 16, 20, 23 and 25 weeks of age. A low incidence ($\leq$3 fibres/section) of NADH rich rims was seen in 2-4 of the *Biceps femoris* sections sampled from 9 weeks of age onwards. NADH negative cores were not observed in any of the sections.

### 3.6.2.2 Estimation of the percentage of hyaline, basophilic and necrotic fibres

*Broiler Pectoralis major*

The main type of structural abnormality seen in the sections of the broiler *Pectoralis major* muscle samples was basophilia (Figure 3.26 and Figure 3.30). The largest estimates of basophilic fibres recorded were 26.8% in a section from a muscle sample removed at 23 weeks of age, and 10.1% in a section from a muscle sample removed at 18 weeks of age. All sections from muscle samples removed at 5 and 9 weeks of age contained basophilic fibres (estimated percentages ranged from 0.2% – 6.3%). Basophilic fibres were observed in most sections from muscle samples removed at 18 and 20 weeks of age (estimated percentages ranged from 0.1% – 10.1%). One or two sections from muscle samples removed at 23 and 25 weeks of age contained basophilic fibres, (estimated percentages ranged from 2.1% – 26.8%). A low incidence of necrotic fibres (estimated percentages ranged from 0.3% – 1.1%) was observed in most sections from muscle samples removed at 5 and 9 weeks of age.

*Broiler Biceps femoris*

In the sections of the broiler *Biceps femoris* muscle samples, hyaline fibres were the main type of abnormality observed (Figure 3.27 and Figure 3.31). Most sections from muscle samples removed at 16-23 weeks of age contained hyaline fibres (estimated percentages ranged from 0.3% – 5.4%). Necrotic fibres were recorded in most sections from muscle samples removed at 5 weeks of age (estimated percentages ranged from 0.4% -0.5%). A few sections from muscle samples removed between 5 and 18 weeks of age contained a low number of basophilic fibres (estimated percentages ranged from 0.1% – 0.4%). Basophilic fibres were observed in a section from a muscle sample removed at 20 and 23 weeks (estimated percentages of 1.5% and 1.2% respectively).

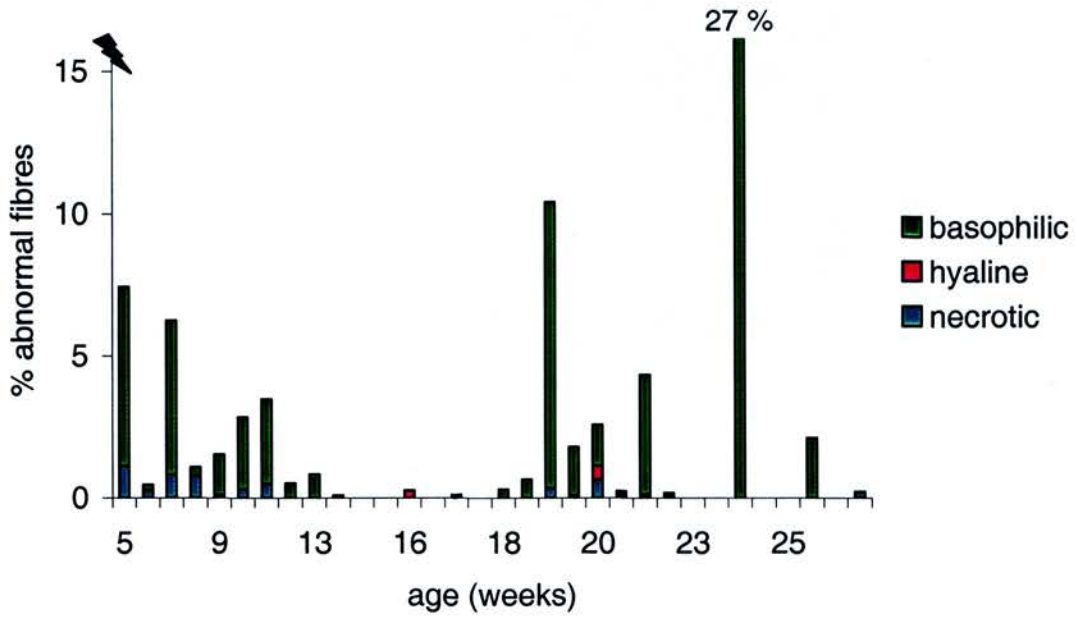



Figure 3.30 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Pectoralis major* muscle sections at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

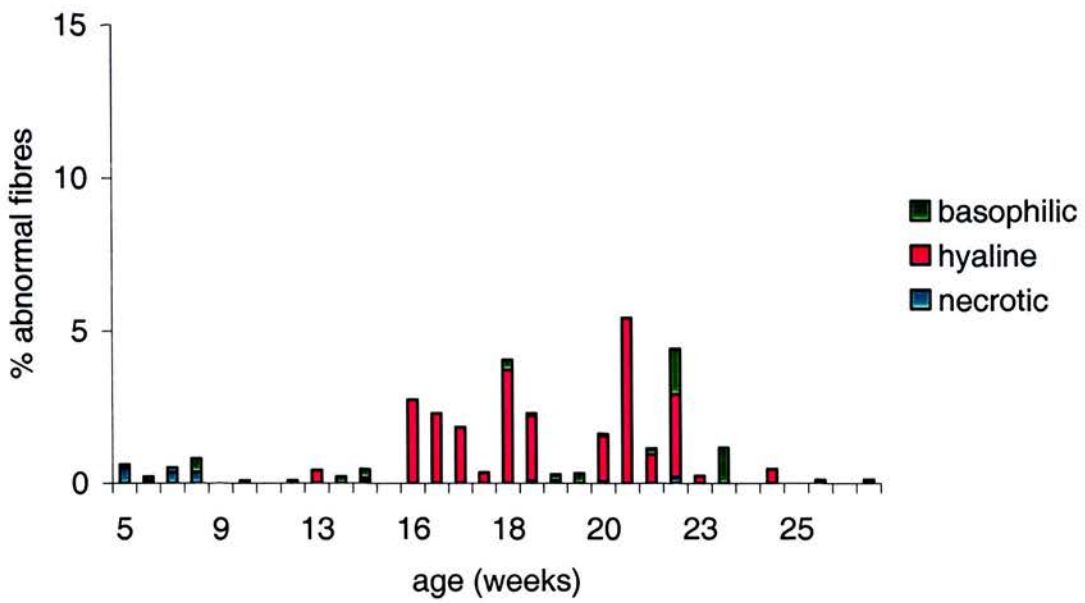



Figure 3.31 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Biceps femoris* sections at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

*Great-grandparent Pectoralis major*

Basophilic fibres were the principal type of structural abnormality seen in the sections of the great-grandparent *Pectoralis major* muscle samples (Figure 3.26 and Figure 3.32). One or two sections from muscle samples removed at 9, 13, 16, 18, 20, 23 and 25 weeks of age contained a small number of basophilic fibres, (estimated percentages ranged from 0.3% – 1.1%).

*Great-grandparent Biceps femoris*

In the sections of the great-grandparent *Biceps femoris* muscle samples, hyaline fibres were the main type of abnormality observed (Figure 3.27 and Figure 3.33). Generally, 2-3 of the four sections from muscle samples removed at 13, 16, 18, 20, 23 and 25 weeks of age contained hyaline fibres (estimated percentages ranged from 0.2% – 8.4%).

*Layer Pectoralis major*

The only type of structural abnormality seen in the sections of the layer *Pectoralis major* muscle samples was basophilic fibres (Figure 3.26 and Figure 3.34). One or two sections from muscle samples removed at 13, 18 and 20 weeks of age contained a small number of basophilic fibres, (estimated percentages were 0.1% for all samples).

*Layer Biceps femoris*

In the sections of the layer *Biceps femoris* muscle samples, hyaline fibres were the main type of abnormality observed (Figure 3.27 and Figure 3.35). The largest estimates of hyaline fibres recorded was 2.2% in a section from a muscle sample removed at 20 weeks of age. Three sections from muscle samples removed at 20 weeks of age contained hyaline fibres (estimated percentages ranged from 0.3% – 2.2%). Hyaline fibres were observed in two sections from muscle samples removed

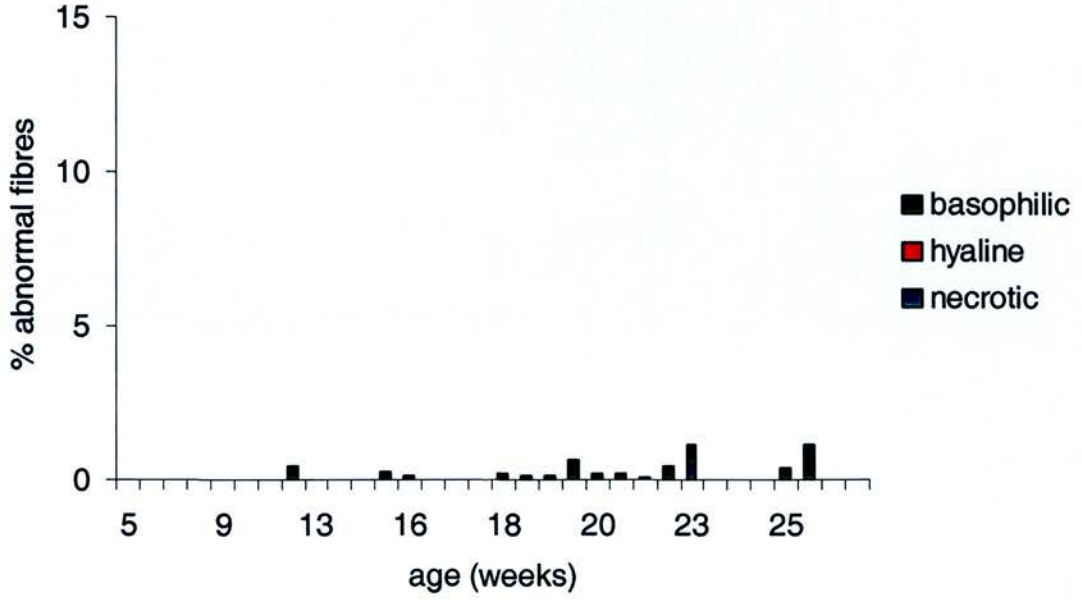



Figure 3.32 Percentage (%) basophilic, hyaline and necrotic fibres recorded in great-grandparent *Pectoralis major* sections of at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

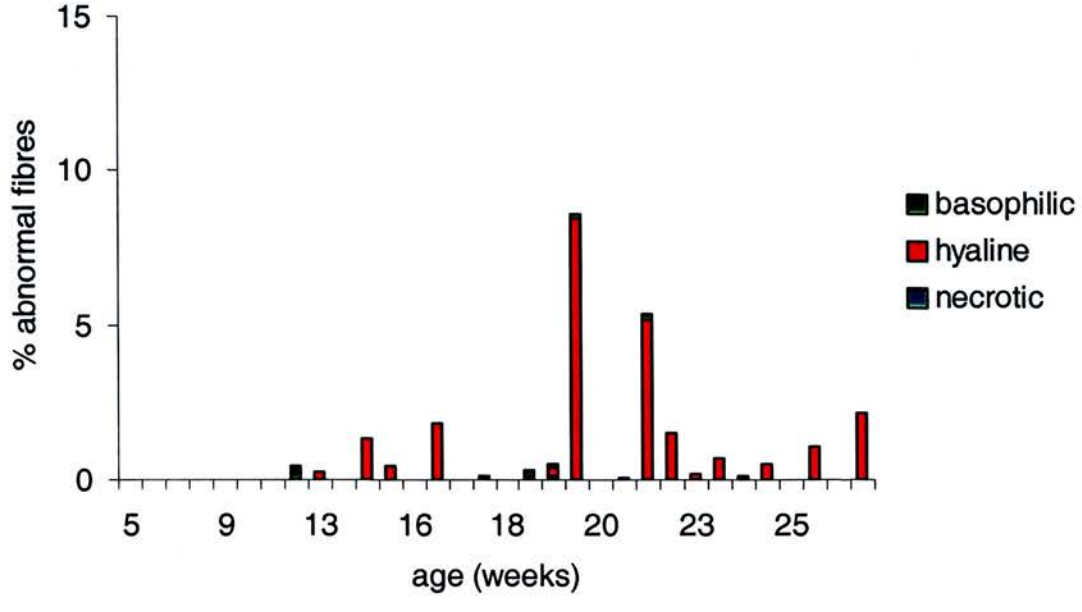



Figure 3.33 Percentage (%) basophilic, hyaline and necrotic fibres recorded in great-grandparent *Biceps femoris* sections at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

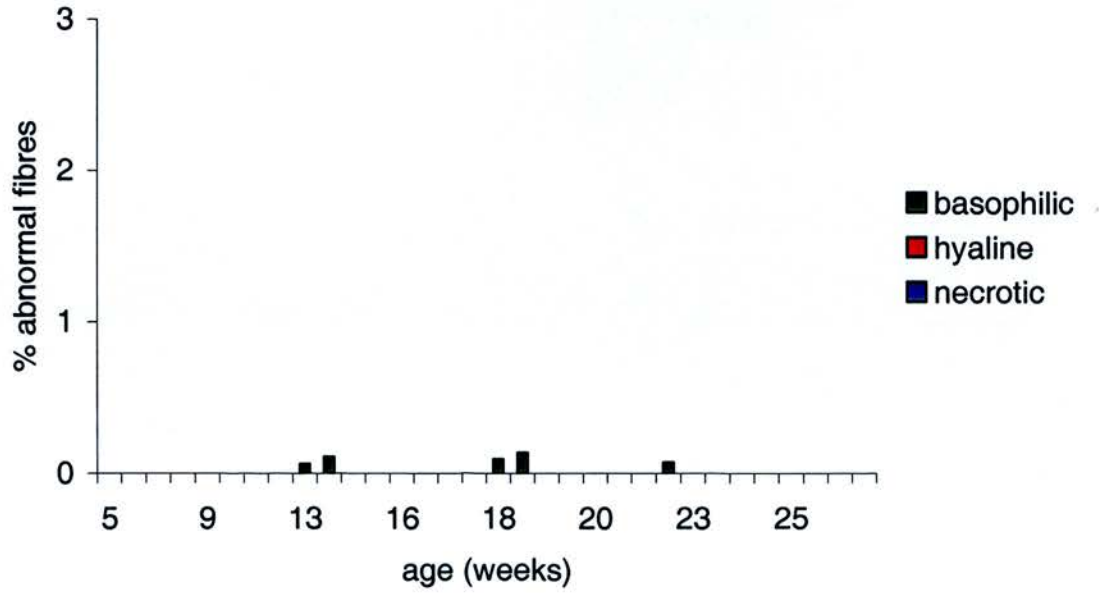



Figure 3.34 Percentage (%) basophilic, hyaline and necrotic fibres recorded in layer *Pectoralis major* sections of at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

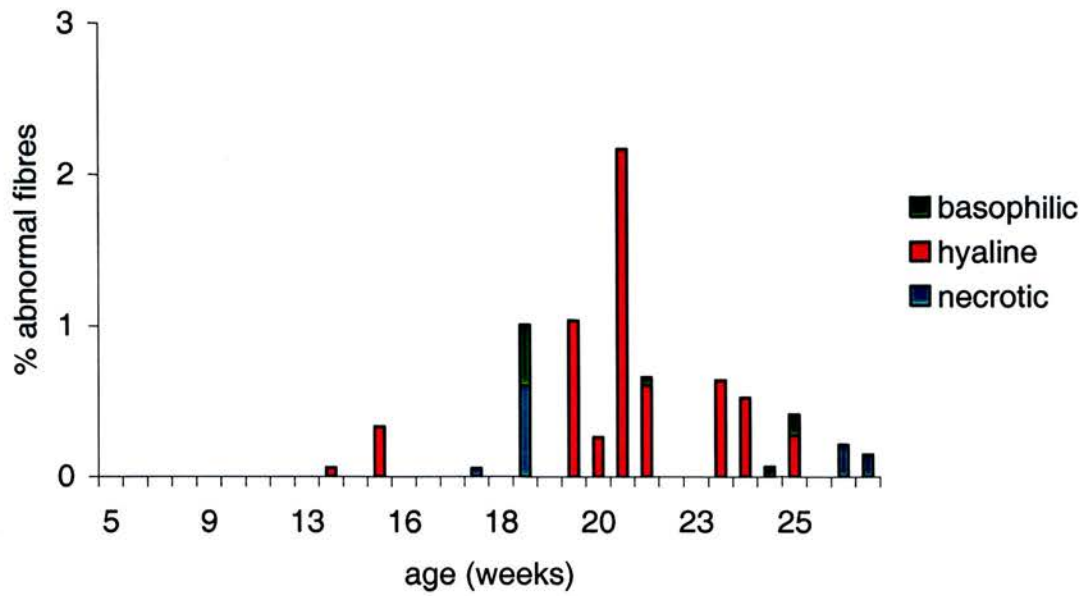



Figure 3.35 Percentage (%) basophilic, hyaline and necrotic fibres recorded in layer *Biceps femoris* sections at 5, 9, 13, 16, 18, 20, 23 and 25 weeks of age.

at 16 weeks (estimated percentages were 0.1% and 0.3%) and 23 weeks (estimated percentages were 0.5% and 0.6%) of age. One section from a muscle sample removed at 18 and 25 weeks of age contained hyaline fibres (estimated percentages were 1.0% and 0.3% respectively). Necrotic fibres were recorded in one or two sections from muscle samples removed at 16, 18 and 25 weeks of age (estimated percentages ranged from 0.1% - 0.6%). One of the four sections from a muscle sample removed at 18, 20, 23 and 25 weeks of age contained a low number of basophilic fibres (estimated percentages ranged from 0.1% – 0.4%).

### 3.6.3 Frequency distribution of fibre size

*Broiler Pectoralis major*

At 5 weeks of age, the frequency distribution of the 500 fibre diameter values of the broiler *Pectoralis major* muscle was slightly positively skewed (Table 3.9 and Figure 3.36). The frequency distribution peak corresponded to the diameters of 128 fibres measuring between 41-50 μm. This interval also contained the mean fibre size (48.3 +/- 4.6 μm). The distribution of fibre diameters ranged from 1-10 μm (1 fibre) to 81-90 μm (5 fibres). The frequency distribution of the 500 *Pectoralis major* fibre diameter values of the muscle was more positively skewed by 25 weeks of age (Table 3.9 and Figure 3.37). The peak of the frequency distribution corresponded to 89 fibres diameters with values between 61-70 μm. The mean fibre size value (67.5 +/-8.2 μm) fell in this interval. The fibre diameter values ranged from 1-10 μm (5 fibres) to 121-130 μm (1 fibre). The number of fibres with very small diameters (≤10 μm) did not dramatically alter between 5 and 25 weeks. At 5 weeks, there were no fibres with relatively large fibre diameters (≥90 μm). By 25 weeks of age, 77 fibres had diameters ≥90 μm. The frequency distributions of the 500 broiler *Pectoralis major* muscle fibre diameter values at 5 and 25 weeks of age were unimodal.

| | | | frequency of minimum fibre diameters (μm) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| age | muscle | line | 1-10 | 11-20 | 21-30 | 31-40 | 41-50 | 51-60 | 61-70 | 71-80 | 81-90 | 91-100 | 101-110 | 111-120 | 121-130 | 131-140 |
| | | B | 1 | 13 | 53 | 125 | 128 | 95 | 62 | 18 | 5 | 0 | 0 | 0 | 0 | 0 |
| 5 | | GGP | 8 | 52 | 63 | 124 | 166 | 75 | 11 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | L | 30 | 182 | 201 | 84 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Ps* | | | | | | | | | | | | | | | |
| | | B | 5 | 12 | 17 | 34 | 43 | 62 | 89 | 83 | 78 | 43 | 31 | 2 | 1 | 0 |
| 25 | | GGP | 9 | 12 | 16 | 38 | 58 | 81 | 130 | 93 | 37 | 20 | 4 | 2 | 0 | 0 |
| | | L | 4 | 48 | 44 | 79 | 133 | 137 | 52 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | B | 0 | 5 | 79 | 198 | 150 | 53 | 13 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | | GGP | 11 | 80 | 154 | 160 | 81 | 12 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | L | 95 | 308 | 90 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Bf* | | | | | | | | | | | | | | | |
| | | B | 5 | 16 | 49 | 56 | 112 | 126 | 100 | 31 | 4 | 0 | 0 | 1 | 0 | 0 |
| 25 | | GGP | 9 | 23 | 42 | 62 | 112 | 111 | 89 | 45 | 5 | 2 | 0 | 0 | 0 | 0 |
| | | L | 5 | 50 | 87 | 159 | 141 | 51 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 3.9 Frequencies of the *Pectoralis major* and *Biceps femoris* muscle fibre minimum diameters (μm) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5 and 25 weeks of age (n=500).

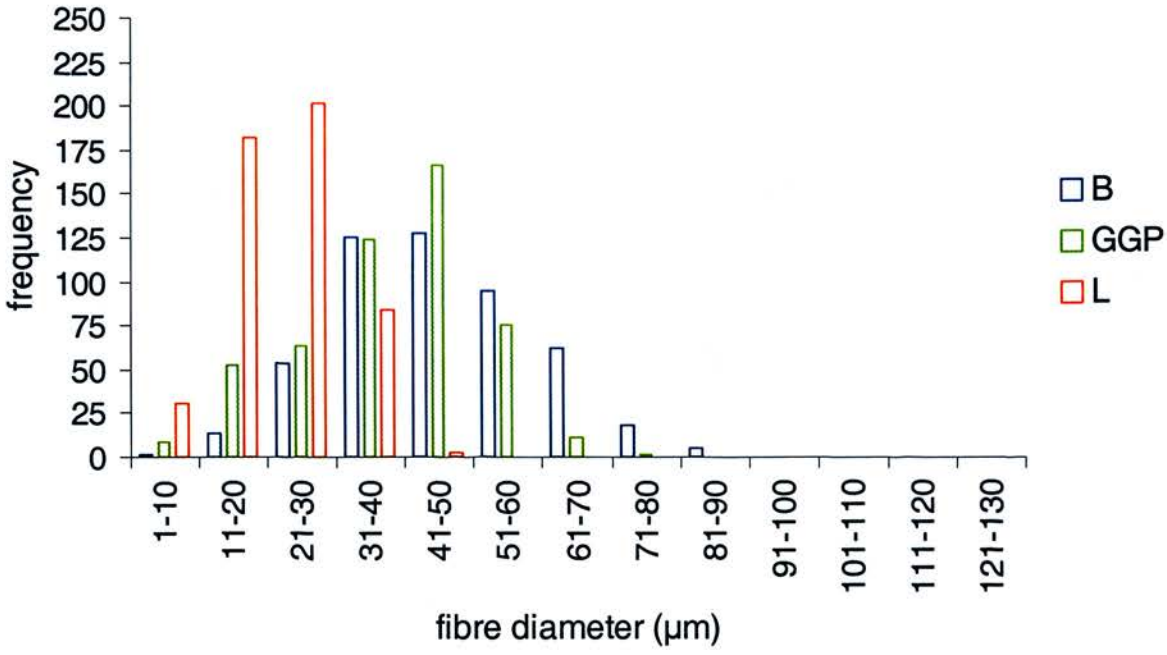



Figure 3.36 Frequencies of the *Pectoralis major* muscle fibre minimum diameters (µm) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5 weeks of age (n=500).

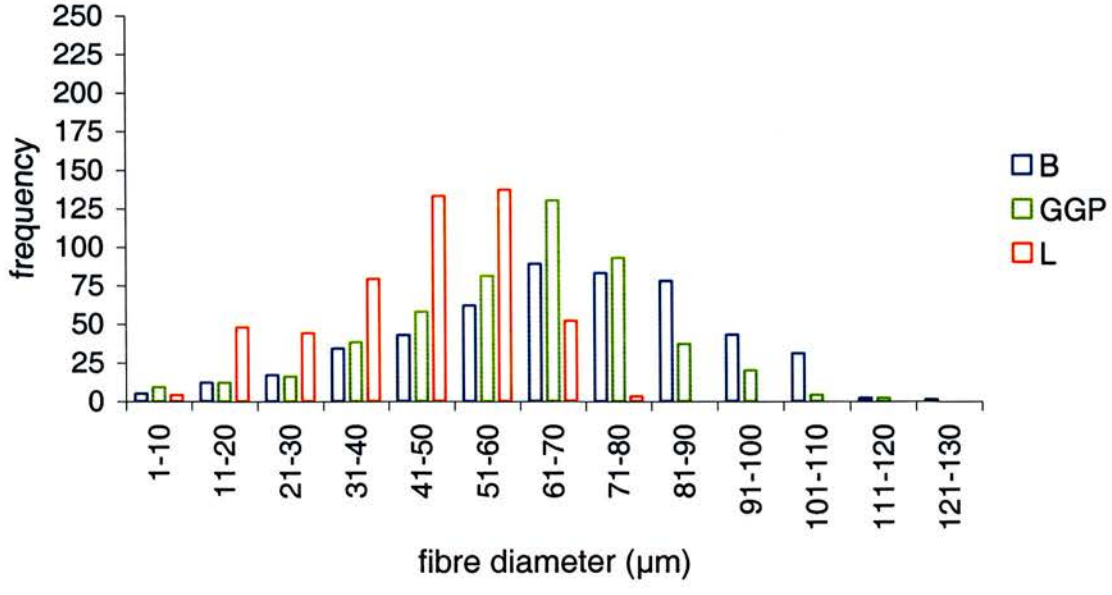



Figure 3.37 Frequencies of the *Pectoralis major* muscle fibre minimum diameters (µm) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 25 weeks of age (n=500).

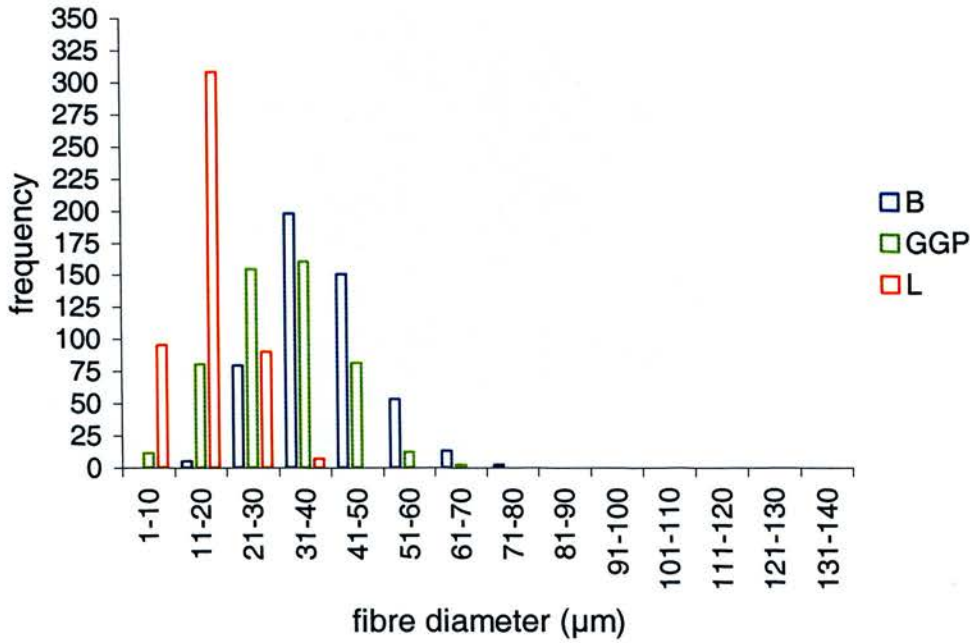



Figure 3.38 Frequencies of the *Biceps femoris* muscle fibre minimum diameters (µm) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 5 weeks of age (n=500).

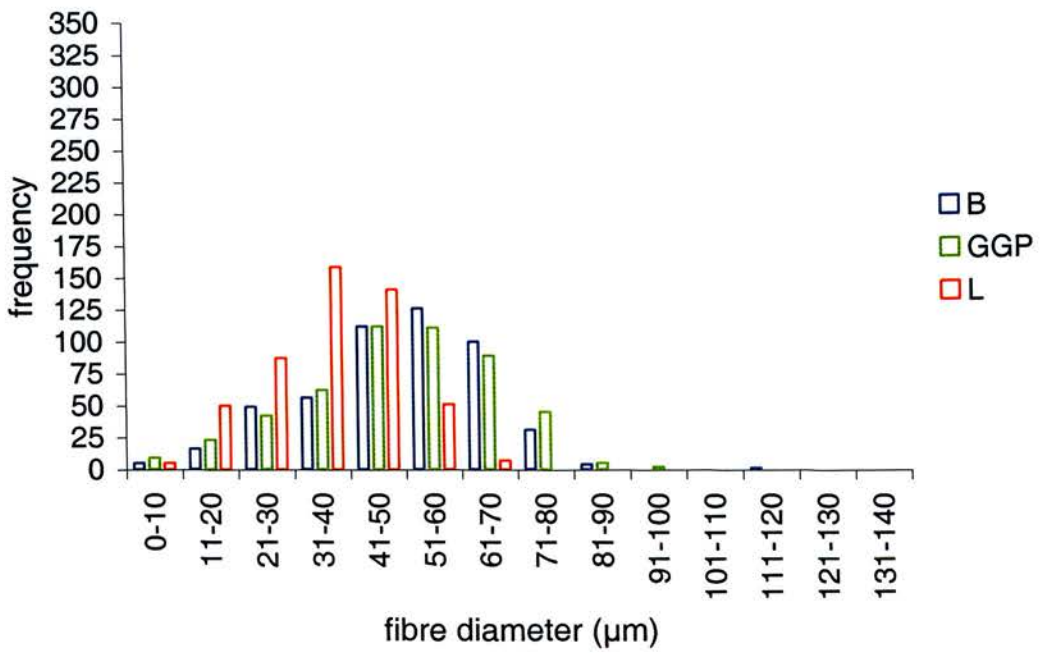



Figure 3.39 Frequencies of the *Biceps femoris* muscle fibre minimum diameters (µm) of the broiler (B), great-grandparent (GGP) and layer (L) lines at 25 weeks of age (n=500).

*Great-grandparent Pectoralis major*

At 5 weeks of age, the frequency distribution peak corresponded to the diameters of 166 fibres measuring between 41-50 μm (Table 3.9 and Figure 3.36). The preceding interval contained the mean fibre size value (38.8 +/- 5.3 μm) due to the positive skew of the frequency distribution. The measurements of the fibre diameters ranged from 1-10 μm (8 fibres) to 71-80 μm (1 fibre). By 25 weeks of age, the frequency distribution of the 500 fibre diameter values was more positively skewed (Table 3.9 and Figure 3.37). The frequency distribution peak corresponded to the diameters of 130 fibres measuring between 61-70 μm. This interval also contained the mean fibre size (60.8 +/- 4.7 μm). The fibre diameter values ranged from 1-10 μm (9 fibres) to 111-120 μm (2 fibres). There was no noticeable change in the number of fibres with very small diameters (≤10 μm) from 5 to 25 weeks. At 5 weeks, there were no fibres with relatively large fibre diameters (≥90 μm). By 25 weeks of age, 26 fibres had diameters ≥90 μm. The frequency distributions of the 500 muscle fibre diameter values at 5 and 25 weeks of age were unimodal.

*Layer Pectoralis major*

The frequency distribution of the 500 muscle fibre diameter values of the 5 week old layer was normal (Table 3.9 and Figure 3.36). The peak of the frequency distribution corresponded to the diameters of 201 fibres measuring between 21-30 μm. The mean fibre size (22.5 +/- 3.5 μm) fell in this interval. Fibre diameters ranged from 1-10 μm (30 fibres) to 41-50 μm (3 fibres). By 25 weeks of age, the frequency distribution of the 500 fibre diameter values of the layer *Pectoralis major* muscle was positively skewed (Table 3.9 and Figure 3.37). The frequency distribution peak corresponded to the diameters of 133 fibres measuring between 41-50 μm. This interval also contained the mean fibre size (43.8 +/- 6.5 μm). The distribution of fibre diameters ranged from 1-10 μm (4 fibres) to 61-70 μm (3 fibres). The number of fibres with very small diameters (≤10 μm) decreased from 5 to 25 weeks (30 to 4 fibres). The frequency distributions of the 500 layer *Pectoralis major* muscle fibre diameter values were unimodal at 5 and 25 weeks of age.

*Broiler Biceps femoris*

At 5 weeks of age, the frequency distribution peak corresponded to the diameters of 198 fibres measuring between 31-40 μm (Table 3.9 and Figure 3.38). The preceding interval contained the mean fibre size value (37.2 +/- 3.8 μm), due to the positive skew of the frequency distribution. The distribution of fibre diameters ranged from 11-20 μm (5 fibres) to 71-80 μm (2 fibres). The frequency distribution of the fibre diameter values was more positively skewed at 25 weeks of age (Table 3.9 and Figure 3.39). The frequency distribution peak corresponded to the diameters of 126 fibres measuring between 51-60 μm. The interval also contained the mean fibre size (49.8 +/- 2.1 μm). The distribution of fibre diameters ranged from 1-10 μm (5 fibres) to 81-90 μm (4 fibres). Between 5 and 25 weeks the number of fibres with the smallest diameters (≤10 μm) did not change. The frequency distributions of the fibre diameter values at 5 and 25 weeks of age were unimodal.

*Great-grandparent Biceps femoris*

At 5 weeks of age, the frequency distribution of the 500 fibre diameter values was normal (Table 3.9 and Figure 3.38). The frequency distribution peak corresponded to the diameters of 160 fibres measuring between 31-40 μm. This interval contained the mean fibre size (27.8 +/- 3.3 μm). The distribution of fibre diameters ranged from 1-10 μm (11 fibres) to 61-70 μm (2 fibres). By 25 weeks of age, the frequency distribution of the 500 fibre diameter values of the great-grandparent *Biceps femoris* muscle was more positively skewed (Table 3.9 and Figure 3.39). The frequency distribution peak corresponded to the diameters of 112 fibres measuring between 41-50 μm. This interval also contained the mean fibre size (49.4 +/- 6.5 μm). The distribution of fibre diameters ranged from 1-10 μm (9 fibres) to 91-100 μm (2 fibres). The number of fibres with very small diameters (≤10 μm) did not noticeably change between 5 and 25 weeks. The frequency distributions of the 500 great-grandparent *Biceps femoris* muscle fibre diameter values at 5 and 25 weeks of age were unimodal.

*Layer Biceps femoris*

At 5 weeks of age, the frequency distribution of the 500 fibre diameter values of the layer *Biceps femoris* muscle was slightly negatively skewed (Table 3.9 and Figure 3.38). The frequency distribution peak corresponded to the diameters of 308 fibres measuring between 11-20 μm. This interval contained the mean fibre size value (22.4 +/- 4.6 μm). The distribution of fibre diameters ranged from 1-10 μm (95 fibres) to 31-40 μm (7 fibres). By 25 weeks of age, the frequency distribution of the 500 fibre diameter values was slightly positively skewed (Table 3.9 and Figure 3.39). The frequency distribution peak corresponded to the diameters of 159 fibres measuring between 31-40 μm. This interval also contained the mean fibre size (36.7 +/- 6.7 μm). The distribution of fibre diameters ranged from 1-10 μm (5 fibres) to 61-70 μm (7 fibres). The number of fibres with very small diameters (≤10) decreased from 5 to 25 weeks (95 to 5 fibres). The frequency distributions of the 500 diameter values were unimodal at 5 and 25 weeks of age.

*Comparing broiler Pectoralis major and Biceps femoris at 25 weeks of age*

The frequency distributions of the broiler 500 fibre diameter values were unimodal at 25 weeks of age for both the *Pectoralis major* (composed of predominantly Type IIb fibres) and *Biceps femoris* (composed of Type I, Type 11a and Type IIb fibres). 6.8% of the *Pectoralis major* fibre diameters were greater than 100 μm. In contrast, only 0.2% of the *Biceps femoris* fibre diameters were greater than 100 μm. This comparison confirms that the divergence in size between the *Pectoralis major and Biceps femoris* described earlier in the thesis (Chapter 3, Results II) is not simply due to the *Pectoralis major* consisting of predominantly Type IIb fibres (typically larger than Type I and Type IIa). The lack of *Biceps femoris* Type IIb fibres with diameters comparable to those observed in the *Pectoralis major* suggests that the larger *Pectoralis major* fibre diameters in the broiler are due to genetic selection for increased breast yield.

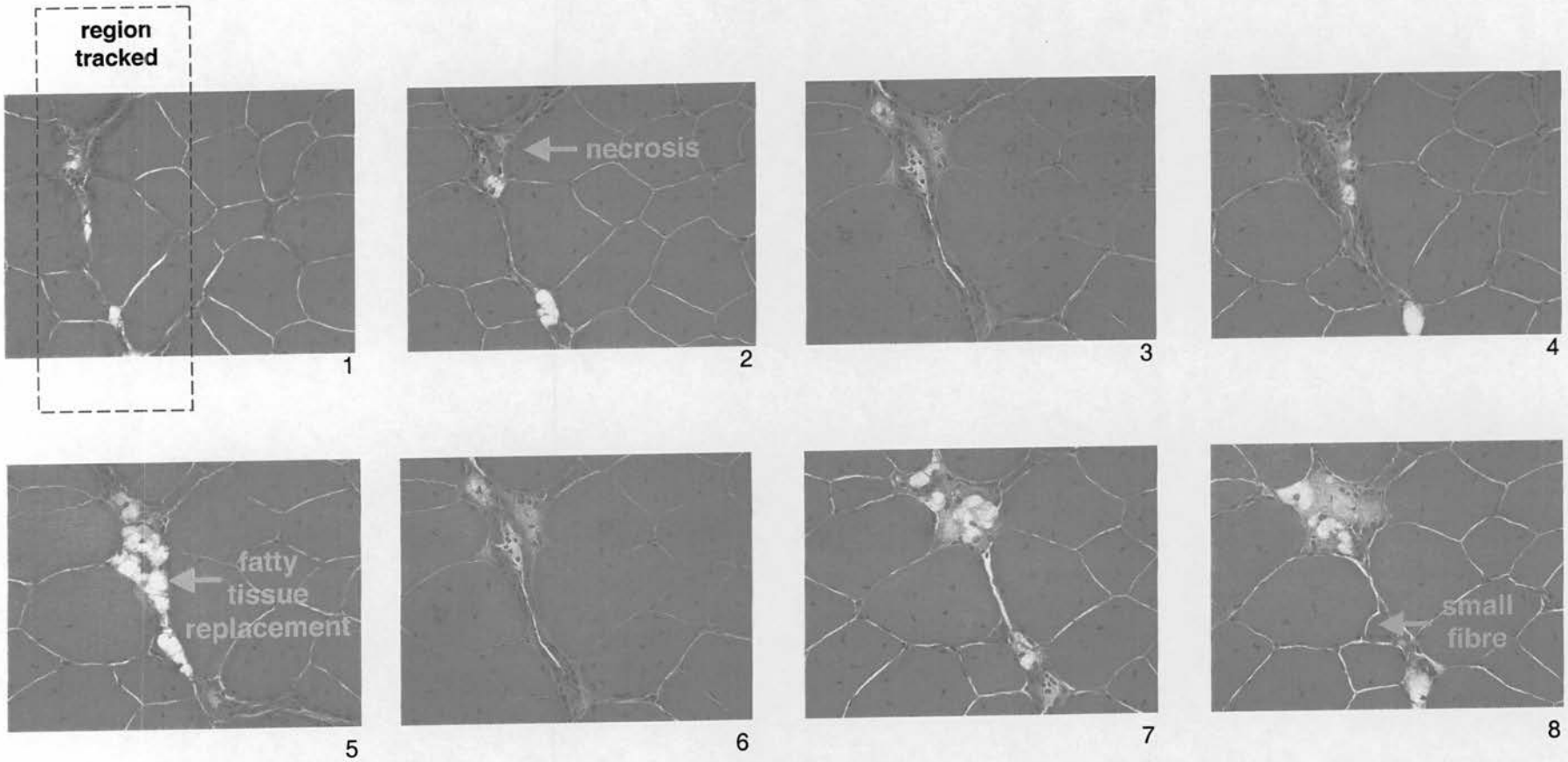



Figure 3.40 Serial sections 1-8 (taken every 50 μm along the fibre) of a five week old broiler *Pectoralis major* muscle sample.

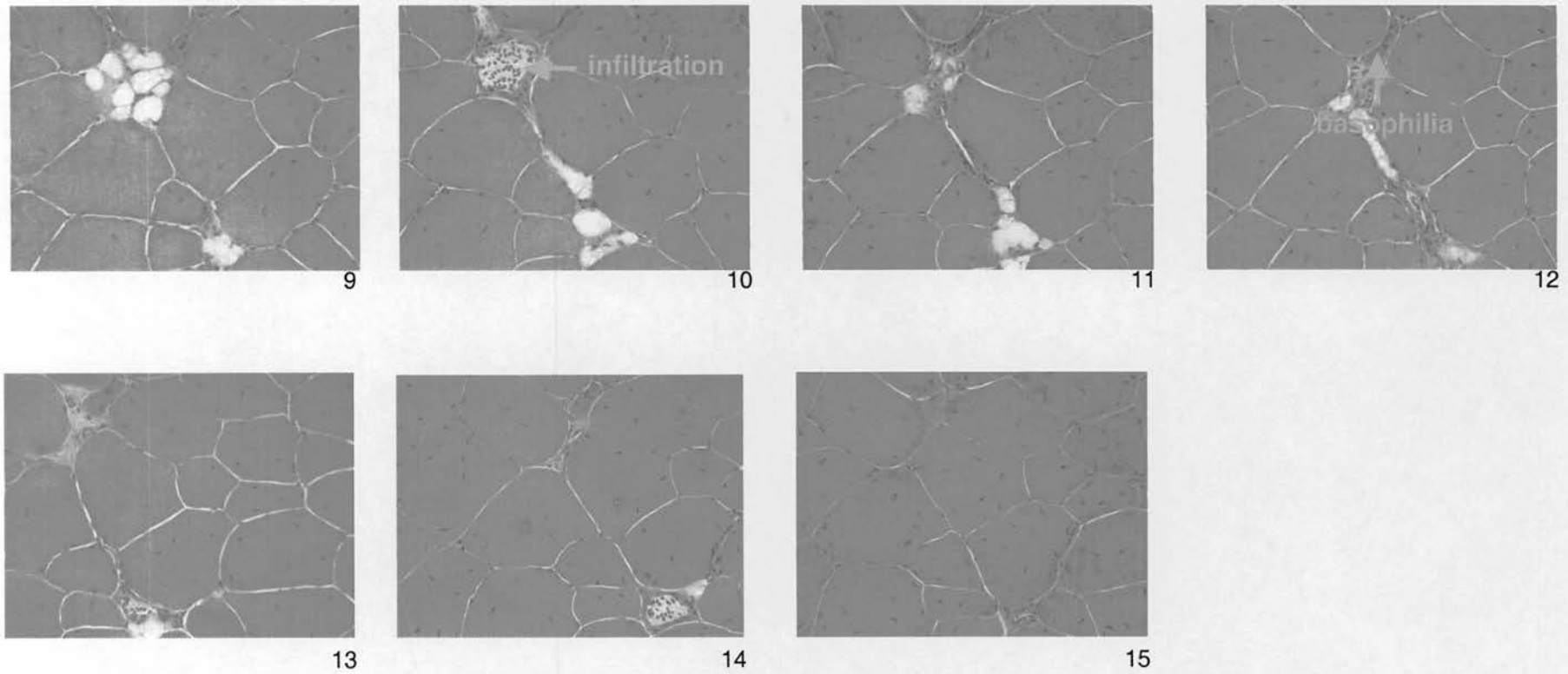



Figure 3.40 (continued) Serial sections 9-15 (taken every 50 µm along the fibre) of a five week old broiler *Pectoralis major* muscle sample.

### 3.6.4 Serial section analysis

#### 3.6.4.1 Fibre tracking

Assessment of 15 serial sections cut at 50μm intervals from a five week old broiler *Pectoralis major* sample (Figure 3.40) revealed that the characteristics of a muscle fibre cross section changed along the 800μm length. Some sections displaying overt structural changes (e.g. section 10 of Figure 3.40), whilst others looking relatively normal in appearance (e.g. section 15 of Figure 3.40). Histopathological features observed in the sections include basophilia (e.g. section 12 of Figure 3.40), mononuclear infiltration (e.g. section 10 of Figure 3.40), fatty tissue replacement (e.g. section 5 of Figure 3.40), necrosis (e.g. section 2 of Figure 3.40) and small fibres (e.g. section 8 of Figure 3.40). However, all of these features were often not present in all of the sections.

#### 3.6.4.2 Changes in damage along a fascicle

Assessment of 15 serial sections of a fascicle cut at 50 μm intervals from a twenty week old broiler *Biceps femoris* sample (Figure 3.41), revealed that the percentage of damaged fibres observed varied from 2.6% to 12.3% along an 800 μm length of a fascicle.

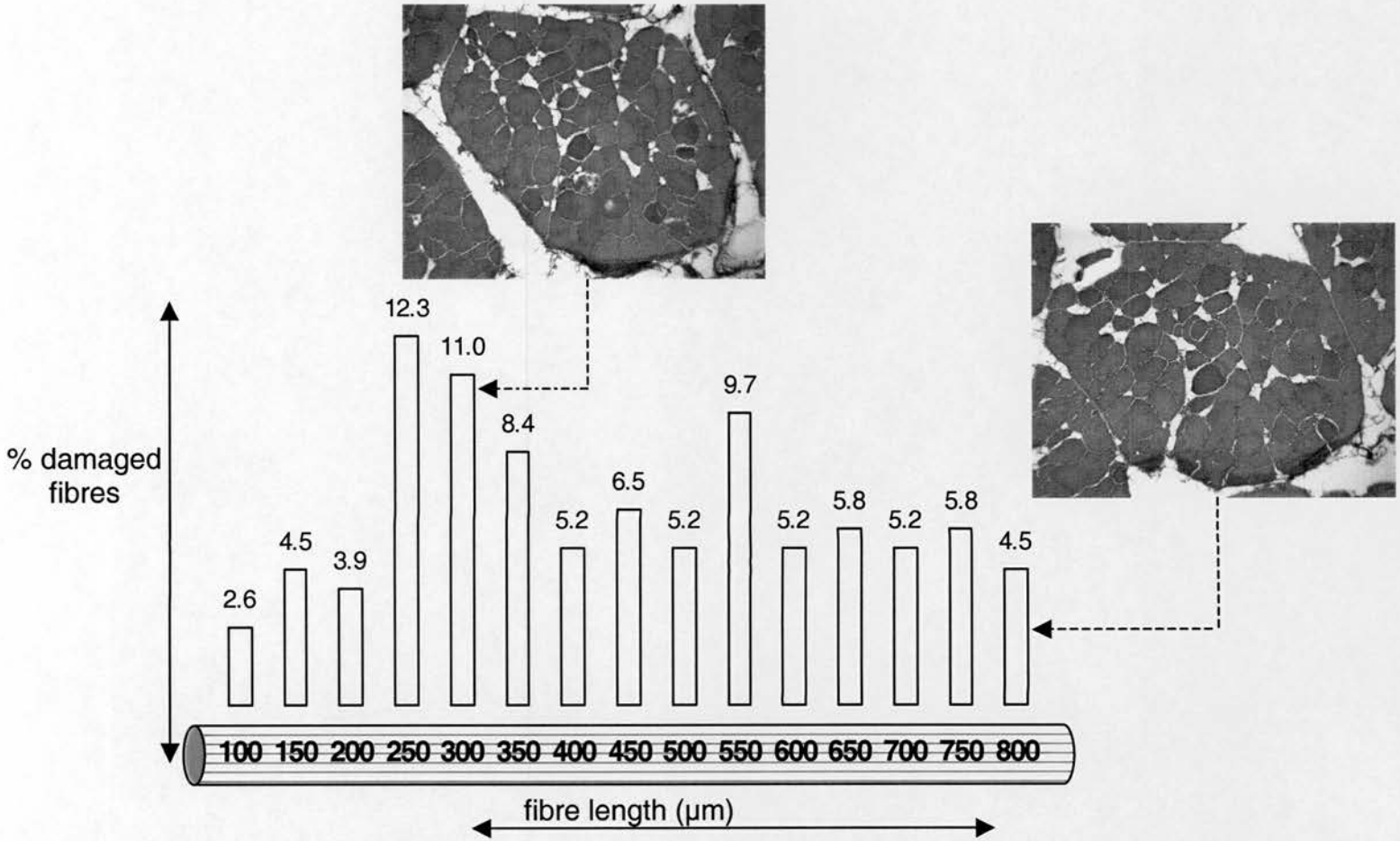



Figure 3.41 Percentage of damaged fibres (%) in serial sections (taken every 50 µm along the fibre) of a twenty week old broiler *Biceps femoris* muscle fascicle.

## 3.7 Results IV: Assessment of further broiler muscles

At the age intervals where prominent structural damage was observed in the broiler *Pectoralis major* (5 weeks of age) and *Biceps femoris* (5 weeks and 16-23 weeks of age), the corresponding breast (*Coracobrachialis*) or leg (*Peroneus longus* and *Gastrocnemius*) muscle blocks were sectioned, stained and assessed.

### 3.7.1 Estimation of mean fibre size

At 5 weeks of age, there was no significant difference between the mean minimum fibre diameter (MFD) values of the three broiler leg muscles (Table 3.10 and Figure 3.42). The smallest MFD values recorded in the *Biceps femoris*, *Gastrocnemius* and *Peroneus longus* were 8.7 μm, 7.2 μm and 4.4 μm respectively. The corresponding maximum MFD values recorded were 55.9 μm, 77.9 μm and 76.0 μm respectively. The mean MFD value of the broiler *Pectoralis major* muscle was significantly greater than that of the *Coracobrachialis* muscle ($P<0.05$) at 5 weeks of age. The smallest MFD values recorded in *Pectoralis major* and *Coracobrachialis* at this age interval were 5.5 μm and 6.5 μm respectively. The largest MFD value recorded was 76.2 μm in the *Pectoralis major* and 76.3 μm in the *Coracobrachialis*. Comparison of all five muscles of the broiler line at 5 weeks of age revealed a significant effect of muscle type on mean MFD ($P<0.05$), with the mean MFD values of both the *Coracobrachialis* and *Biceps femoris* muscles being significantly smaller than the mean MFD value of the *Pectoralis major* ($P<0.05$).

*Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles of the broiler line were compared at 16, 18, 20 and 23 weeks of age (Table 3.11 and Figure 3.43). There was a significant effect of age ($P<0.05$) and muscle type ($P<0.001$) on mean MFD and a significant interaction between muscle type and age ($P<0.05$). The mean MFD of the *Biceps femoris* muscle was significantly smaller than that of both the *Peroneus longus* and *Gastrocnemius* muscles at all age intervals ($P<0.001$). There was no significant difference between the mean MFD of the *Peroneus longus* and *Gastrocnemius* muscles at any age interval. The peak mean MFD value of the

| muscle | connective tissue content (%) | Minimum Fibre Diameter (MFD) (µm) | | | | mean coefficient of variation (%) |
|---|---|---|---|---|---|---|
| | | mean | minimum value | maximum value | range | |
| *Bf* | 17.9 (3.0) | 34.1 (2.1) | 8.7 | 55.9 | 47.2 | 26.8 |
| *Pl* | 20.0 (1.4) | 39.6 (5.0) | 4.4 | 76.0 | 71.6 | 30.7 |
| *Ga* | 16.4 (2.4) | 38.8 (5.5) | 7.2 | 77.9 | 70.7 | 28.0 |
| *Ps* | 10.6 (0.8) | 45.9 (0.9) | 5.5 | 76.2 | 70.7 | 33.2 |
| *Co* | 17.8 (0.4) | 38.9 (2.4) | 6.5 | 76.3 | 69.8 | 33.9 |

Table 3.10 Mean connective tissue content (%) (n=4), mean minimum fibre diameter (MFD) (µm) (standard deviations in parentheses) (n=400 per muscle), and mean coefficient of variation (%) of the *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*), *Gastrocnemius* (*Ga*), *Pectoralis major* (*Ps*) and *Coracobrachialis* muscle fibres of the broiler line at 5 weeks of age.

| age | muscle | connective tissue content (%) | Minimum Fibre Diameter (MFD) (μm) | | | | mean coefficient of variation (%) |
|---|---|---|---|---|---|---|---|
| | | | mean | minimum value | maximum value | range | |
| 16 | *Bf* | 19.6 (2.6) | 37.7 (2.8) | 7.2 | 70.5 | 63.3 | 28.0 |
| | *Pl* | 14.3 (2.8) | 66.4 (3.3) | 19.4 | 108.4 | 89.0 | 24.4 |
| | *Ga* | 13.1 (1.9) | 53.0 (4.5) | 5.1 | 93.1 | 88.0 | 21.9 |
| 18 | *Bf* | 23.3 (2.2) | 48.3 (2.8) | 6.5 | 107.9 | 101.4 | 33.6 |
| | *Pl* | 15.7 (2.8) | 65.3 (5.6) | 9.2 | 115.7 | 106.5 | 26.5 |
| | *Ga* | 11.7 (1.8) | 63.2 (7.2) | 9.9 | 115.7 | 105.8 | 33.9 |
| 20 | *Bf* | 26.1 (6.3) | 46.6 (7.7) | 10.7 | 94.8 | 84.1 | 29.6 |
| | *Pl* | 14.6 (2.0) | 62.3 (9.9) | 8.4 | 107.9 | 99.5 | 22.7 |
| | *Ga* | 12.9 (1.3) | 56.6 (1.2) | 3.5 | 101.7 | 98.2 | 30.5 |
| 23 | *Bf* | 21.4 (2.7) | 42.6 (4.4) | 4.7 | 88.9 | 84.2 | 32.9 |
| | *Pl* | 17.9 (2.9) | 60.7 (4.5) | 15.7 | 109.3 | 93.6 | 22.3 |
| | *Ga* | 19.4 (1.7) | 52.4 (12.5) | 9.2 | 123.6 | 114.4 | 27.2 |

Table 3.11 Mean connective tissue content (%) (n=4), mean minimum fibre diameter (MFD) (μm) (standard deviations in parentheses) (n=400 per muscle), minimum MFD (μm), maximum MFD (μm), MFD range (μm) and mean coefficient of variation (%) of the *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*) and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 16, 18, 20 and 23 weeks of age.

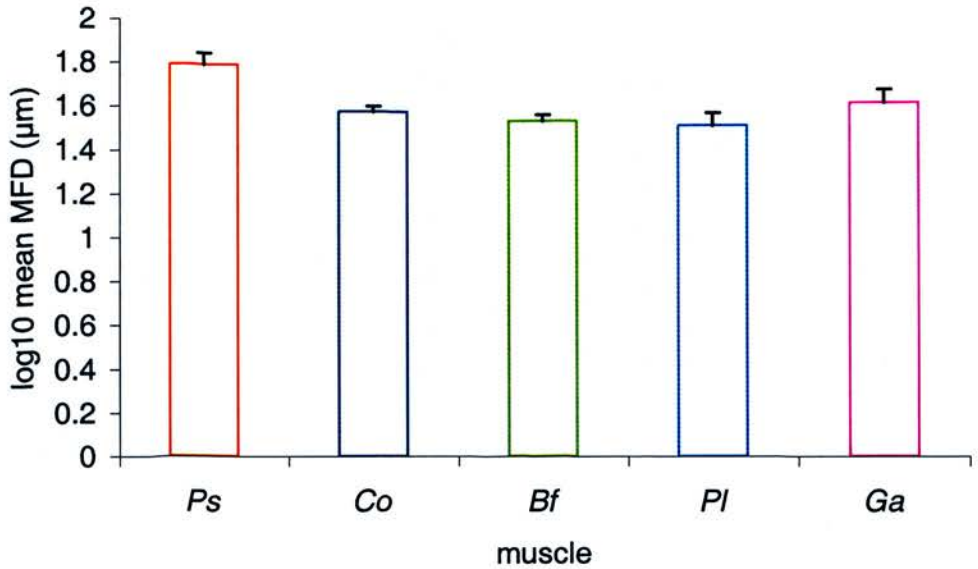



Figure 3.42 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Pectoralis major* (*Ps*), *Coracobrachialis* (*Co*) *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*), and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 5 weeks of (n=400 per muscle).

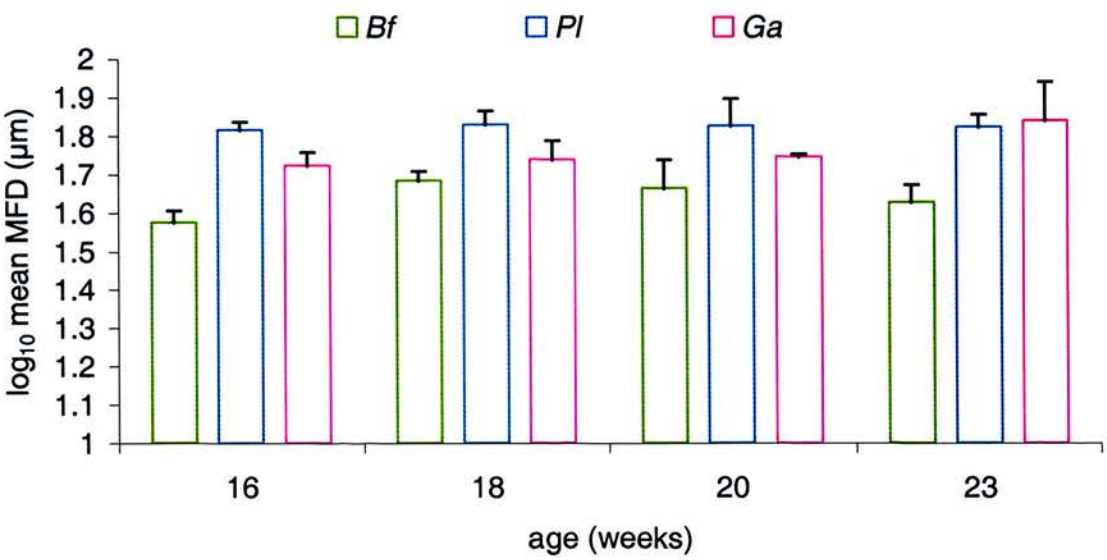



Figure 3.43 $Log_{10}$ mean minimum fibre diameter (MFD) (µm) (error bars represent 1 standard deviation) of the *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*) and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 16, 18, 20 and 23 weeks of age (n=400 per muscle).

*Biceps femoris* and *Gastrocnemius* muscles were recorded at 18 weeks of age (48.3 μm and 63.2 μm respectively). The peak mean MFD value of the *Peroneus longus* muscle was observed at 16 weeks of age (66.4 μm). The smallest MFD value was recorded at 23 weeks of age in the *Biceps femoris* (4.7 μm), at 20 weeks of age in the *Gastrocnemius* (3.5μm) and at 20 weeks of age in the *Peroneus longus*. 8.4 μm). The largest MFD value was recorded at 18 weeks of age in the *Biceps femoris* and *Peroneus longus* (107.9 μm and 115.7 μm respectively), and at 23 weeks of age in the *Gastrocnemius* (123.6 μm).

### 3.7.2 Connective tissue content

At 5 weeks of age there was no significant difference between the mean connective tissue content of the three leg muscles of the broiler line (Table 3.10). Comparison of the two breast muscles demonstrated that the mean connective tissue content of the *Pectoralis major* muscle was significantly smaller than that of the *Coracobrachialis* muscle ($P<0.001$) (Table 3.10). When all five muscles of the broiler line were compared, there was a significant effect of muscle type on mean connective tissue content ($P<0.001$). The mean connective tissue content of the *Pectoralis major* muscle was significantly smaller than that of the *Coracobrachialis*, *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* muscles ($P<0.05$).

Statistical analysis of the leg muscles of the broiler line at 16-23 weeks of age revealed a significant effect of muscle type ($P<0.001$) on mean connective tissue content and a significant interaction between muscle type and age ($P<0.05$) (Table 3.11). The effect of age on mean connective tissue content was just short of significance ($P=0.05$). The mean connective tissue content of the *Biceps femoris* muscle was significantly greater than that of both the *Peroneus longus* and *Gastrocnemius* muscles at 18 and 20 weeks of age ($P<0.001$). There was no significant difference between the mean MFD of the *Peroneus longus* and *Gastrocnemius* muscles at any age interval.

### 3.7.3 Nuclei distribution

The mean percentage of broiler *Pectoralis major* muscle fibres with ≥1 internal nucleus (fibres $_{\geq 1\ I\ N}$) (97%) was significantly smaller than that of the *Coracobrachialis* muscle (91%) ($P<0.05$) at 5 weeks of age (Table 3.12 and figure 3.44). Comparison of all five muscles of the broiler line at 5 weeks of age revealed an overall significant effect of muscle type on the mean percentage of muscle fibres $_{\geq 1\ I\ N}$ ($P<0.05$). However, pair-wise comparisons using Tukey's Test did not reveal any significant differences between groups. The mean percentage of *Peroneus longus* and *Gastrocnemius* muscle fibres $_{\geq 1\ I\ N}$ (both 59%) were numerically lower than that of the *Biceps femoris*, *Pectoralis major* and *Coracobrachialis* (88%, 97% and 91% respectively).

There was a significant effect of muscle type ($P<0.001$) on the percentage of leg muscle fibres $_{\geq 1\ I\ N}$ in the broiler line at 16-23 weeks of age (Table 3.13 and figure 3.45). There was no significant effect of age on the mean percentage of fibres $_{\geq 1\ I\ N}$, and the interaction between muscle and age was just short of significance ($P=0.07$). The mean percentage of *Peroneus longus* muscle fibres $_{\geq 1\ I\ N}$ (98%) were significantly greater than that of both the *Biceps femoris* (50%) and *Gastrocnemius* (40%) muscles at 20 weeks of age. The mean percentage of *Peroneus longus* muscle fibres $_{\geq 1\ I\ N}$ were numerically greater than that of both the *Biceps femoris* and *Gastrocnemius* muscles at 18 weeks of age (89% compared to 41% and 57%) and 23 weeks of age (92% compared to 48% and 66%).

### 3.7.4 Muscle pathology assessment

#### 3.7.4.1 Detailed evaluation

Detailed pathology evaluations of the *Coracobrachialis* breast muscle, and the *Peroneus longus* and *Gastrocnemius* leg muscles of the broiler line were undertaken at 5 weeks of age. This allowed pathology comparisons with the corresponding *Pectoralis major* breast muscle and *Biceps femoris* leg muscles described in the previous results section (3.6).

| | % fibres with ≥1 internal nucleus | | | | |
|---|---|---|---|---|---|
| age (weeks) | *Ps* | *Co* | *Bf* | *Pl* | *Ga* |
| 5 | 97 | 91 | 88 | 59 | 59 |

Table 3.12 Percentage (%) fibres with ≥1 internal nucleus in the *Pectoralis major* (*Ps*), *Coracobrachialis* (*Co*) *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*), and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 5 weeks of age (n=400 per muscle).

| | % fibres with ≥1 internal nucleus | | |
|---|---|---|---|
| age (weeks) | *Bf* | *Pl* | *Ga* |
| 16 | 78 | 86 | 87 |
| 18 | 41 | 89 | 57 |
| 20 | 50 | 98 | 40 |
| 23 | 48 | 92 | 66 |

Table 3.13 Percentage (%) fibres with ≥1 internal nucleus in the *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*) and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 16, 18, 20 and 23 weeks of age (n=400 per muscle).

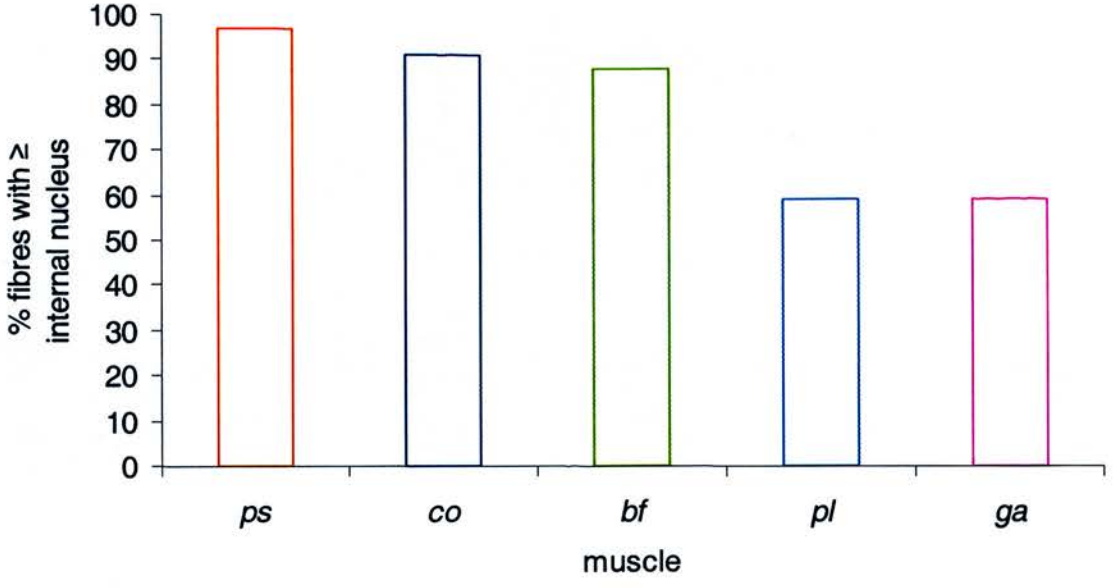



Figure 3.44 Percentage (%) fibres with ≥1 internal nucleus in the *Pectoralis major* (*Ps*), *Coracobrachialis* (*Co*) *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*), and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 5 weeks of age (n=400 per muscle).

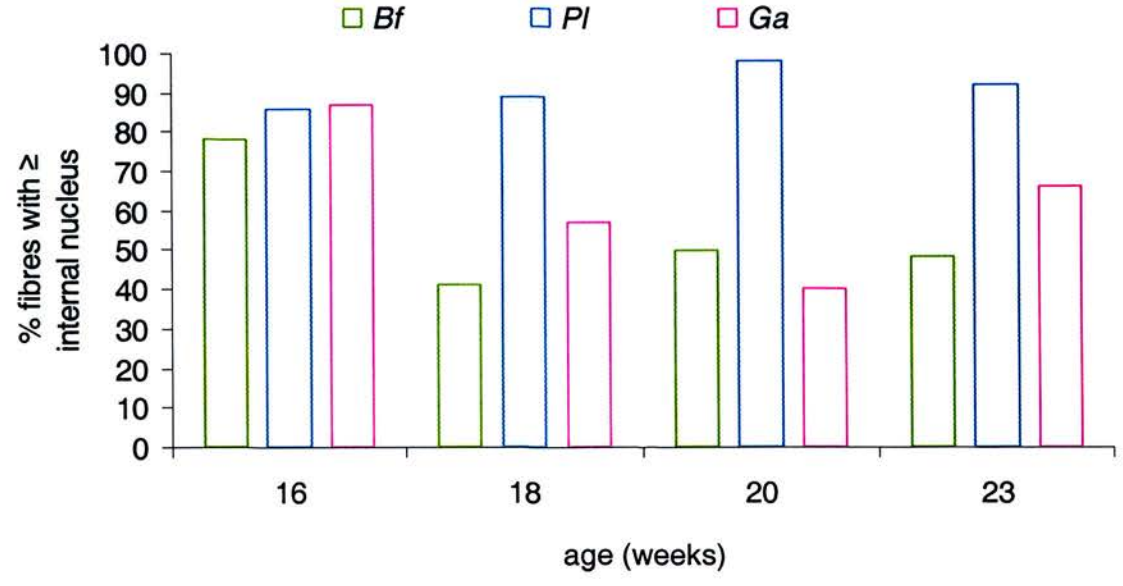



Figure 3.45 Percentage (%) fibres with ≥1 internal nucleus in the *Biceps femoris* (*Bf*), *Peroneus longus* (*Pl*) and *Gastrocnemius* (*Ga*) muscle fibres of the broiler line at 16, 18, 20 and 23 weeks of age (n=400 per muscle).

At 5 weeks of age, prominent necrotic (>15) and basophilic (>15) fibres were observed in the sections of two broiler *Pectoralis major* sections (bird 1 and bird 3). There was a low incidence of necrotic fibres (<5) (Figure 3.24) in one of the broiler *Coracobrachialis* sections (bird 1). A low incidence of necrosis (<10) and basophilia (<10) was observed in the sections of two broiler *Biceps femoris* sections (bird 1 and bird 4). There was a high incidence of necrotic fibres (>15) throughout the sections of two broiler *Gastrocnemius* samples (bird 1 and bird 3). In one of these samples there was also a low incidence of basophilic fibres (<5) (Figure 3.26) throughout the section (bird 1). There was a high incidence of basophilic (>30) and necrotic (>20) fibres throughout the section of one broiler *Peroneus longus* sample (bird 1) and a low incidence of basophilic fibres throughout the section of another *Peroneus longus* sample (bird 4). Fibre splitting, fibre size variation and 'tiny' fibres (Figure 3.22 and 3.23) were observed in most *Gastrocnemius* and *Peroneus longus* muscle sections. NADH staining revealed that cores were present in the section from one *Peroneus longus* muscle sample (bird 1), and rims were present in one *Gastrocnemius* muscle sample (bird 2). All of the muscles of bird 1 contained some degree of pathology. The sections from bird 3 showed signs of pathology in the *Gastrocnemius* but not the *Peroneus longus*, whereas the opposite was true for bird 4.

Detailed pathology evaluations of the *Peroneus longus* and *Gastrocnemius* leg muscles of the broiler line were undertaken at 16, 18, 20 and 23 weeks of age. This allowed pathology comparisons with the corresponding *Biceps femoris* muscles.

Fibre splitting, fibre size variation and 'tiny' fibres were observed in most sections, although the mean coefficient of variation values were generally lower for the *Peroneus longus* compared to the *Biceps femoris* and *Gastrocnemius* fibre sizes (Table 3.11). Fat was noted within and between some fascicles of most sections (Figure 3.29), but was more prevalent in sections from the *Biceps femoris* compared to the *Peroneus longus* and *Gastrocnemius* muscle samples. ≥5 basophilic (Figure 3.26) fibres were only observed in sections from the *Peroneus longus* muscle samples (e.g. birds 7-9) and there was a low/no incidence of hyaline fibres in many

sections from *Peroneus longus* muscle samples (e.g. birds 13-15 and birds 17-20). ≥5 hyaline (Figure 3.27) fibres per field were only observed in sections from the *Biceps femoris* and *Gastrocnemius* muscle samples. In many birds, hyaline fibres were present in most fields of the sections from both the *Biceps femoris* and corresponding *Gastrocnemius* muscle samples (e.g. birds 5-10 and birds 13-17). However, the incidence was generally much greater in a *Biceps femoris* section compared to the corresponding *Gastrocnemius* of an individual bird (e.g. bird 14). Prominent necrosis was not observed in any of the sections.

Rims were observed in most of the broiler *Biceps femoris* sections sampled at 16-23 week of age. Cores were also present in 1-2 sections from *Biceps femoris* muscle samples taken at 18, 20, 23 and 25 weeks. Rims were observed in 2-3 broiler *Gastrocnemius* sections sampled at 16-23 week of age, and cores were observed infrequently. In contrast, cores were observed in 2-4 broiler *Peroneus longus* sections sampled at 16-23 weeks of age, with rims occurring in only 1 section at each age interval.

### 3.7.4.2 Estimation of the percentage of hyaline, basophilic and necrotic fibres

At 5 weeks of age, necrotic and basophilic fibres were recorded in all four of the broiler *Pectoralis major* breast muscle sections (Figure 3.46). The estimates of basophilic fibres ranged from 0.2% (bird 2) to 6.3% (bird 1), and the estimates of necrotic fibres ranged from 0.2% (bird 2) to 1.1% (bird 1). Basophilic fibres were only observed in the *Coracobrachialis* breast muscle section of bird 1 (Figure 3.46), at an estimated percentage of 0.1%. No necrotic fibres were observed in the *Coracobrachialis* sections.

Basophilic fibres were recorded in all four of the broiler *Biceps femoris* leg muscle sections at 5 weeks of age (Figure 3.46) (estimated percentages ranged from 0.1% (birds 1, 2 and 3) – 0.4% (bird 4)). Necrotic fibres were also observed in all four sections (estimated percentages ranged from 0.1 % (bird 2) and 0.5 % (bird 1)). Basophilic fibres were observed in two of the broiler *Peroneus longus* leg muscle

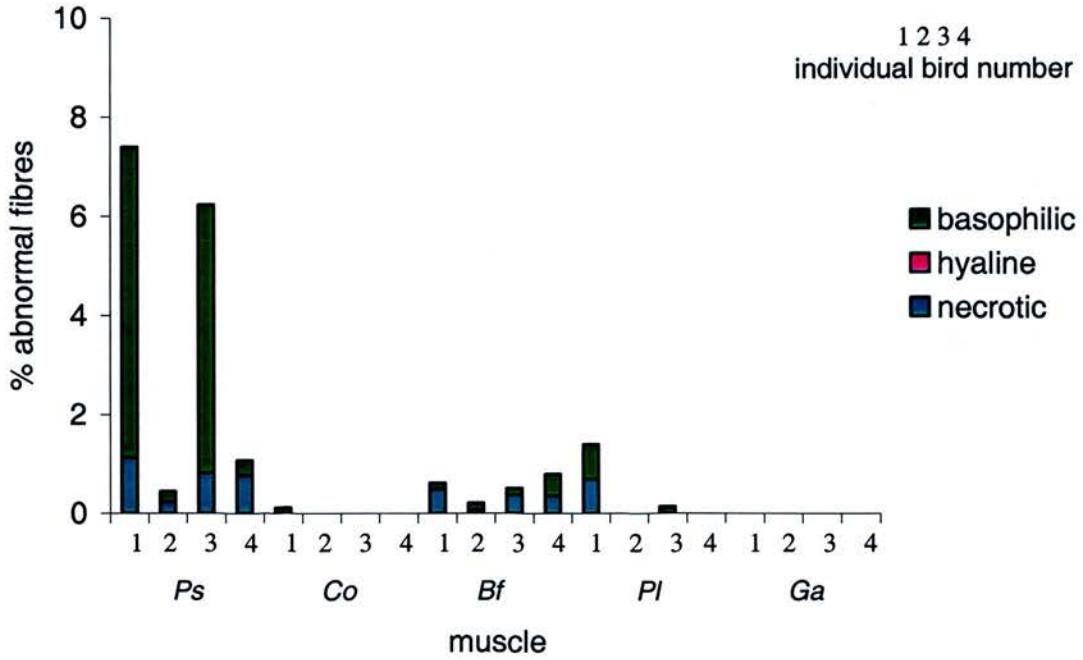



Figure 3.46 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Pectoralis major (Ps)*, *Coracobrachialis (Co)*, *Biceps femoris (Bf)*, *Peroneus longus (Pl)* and *Gastrconemius (Ga)* sections of at 5 weeks of age.

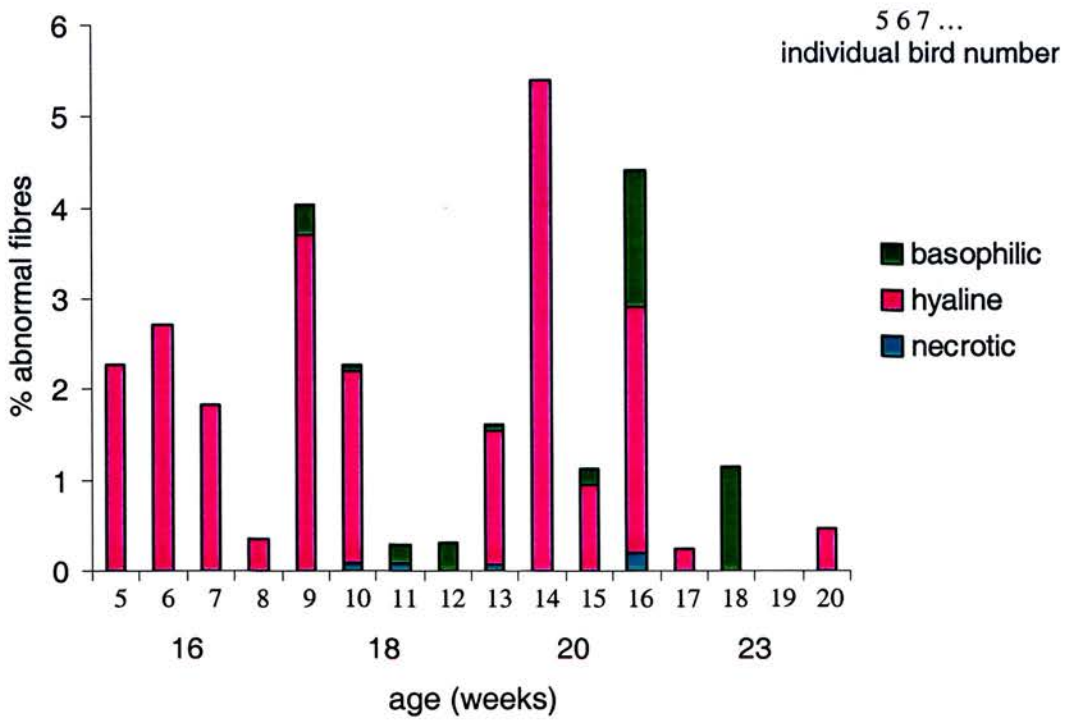



Figure 3.47 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Biceps femoris* sections at 16, 18, 20 and 23 weeks of age.

sections at 5 weeks of age (Figure 3.46) (estimated percentages were from 0.1% (bird 3) and 0.7% (bird 1)). The *Peroneus longus* muscle section from bird 1 also contained necrotic fibres (0.7%). No structural abnormalities were observed in the broiler *Gastrocnemius* sections at 5 weeks of age (Figure 3.46).

Many of the broiler *Biceps femoris* sections from muscle samples removed at 16-23 weeks of age contained hyaline and basophilic fibres (Figure 3.47). At 16 weeks of age, all four sections contained hyaline fibres (estimated percentages ranged from 0.3% (bird 8) to 2.7% (bird 5). At 18 weeks of age, two sections contained hyaline fibres (estimated percentages were 2.1% (bird 10) and 3.7% (bird 9)). Basophilic fibres were also observed in the section from bird 9 (estimated percentage was 0.3%). The *Biceps femoris* sections from bird 11 and bird 12 both contained a low incidence of basophilic fibres (estimated percentages were 0.2% and 0.3% respectively). At 20 weeks of age, the sections from all four birds contained hyaline fibres (estimated percentages ranged from 0.9% (bird 15) and 5.4% (bird 14). The sections from bird 15 and bird 16 also contained basophilic fibres (estimated percentages were 0.2% and 1.5% respectively). At 23 weeks of age, sections from bird 17 and bird 20 contained hyaline fibres (estimated percentages were 0.3% and 0.5% respectively). The section from bird 18 contained basophilic fibres (estimated percentage was 1.2%).

Hyaline and basophilic fibres were the main type of structural abnormality observed in the broiler *Peroneus longus* sections (Figure 3.48), primarily at 16 and 18 weeks of age. At 16 weeks of age, the sections from bird 5, bird 6 and bird 7 contained hyaline fibres (estimated percentages were 0.3% (bird 5 and bird 7) and 1.5% (bird 6)). Basophilic fibres were observed in the section from bird 7 and bird 8 (estimated percentages were 1.3% and 0.6% respectively). At 18 weeks of age, the sections from bird 9, bird 11 and bird 12 contained basophilic fibres (estimated percentages were 0.4% (bird 12) and 1.2% (bird 9 and bird 11)). Hyaline fibres were observed in the section from bird 10 and bird 12 (estimated percentages were 0.3% and 0.2% respectively). At 20 weeks of age, the section from bird 13 contained both hyaline and necrotic fibres (estimated percentages were 0.2% and 0.4% respectively). The

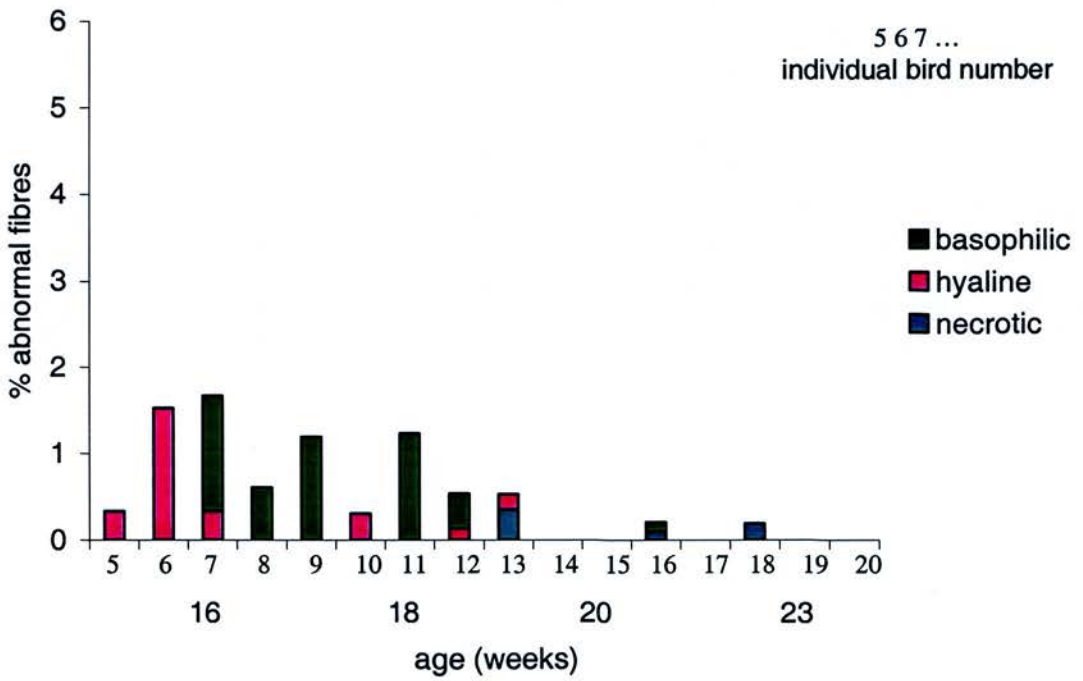



Figure 3.48 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Peroneus longus* sections at 16, 18, 20 and 23 weeks of age.

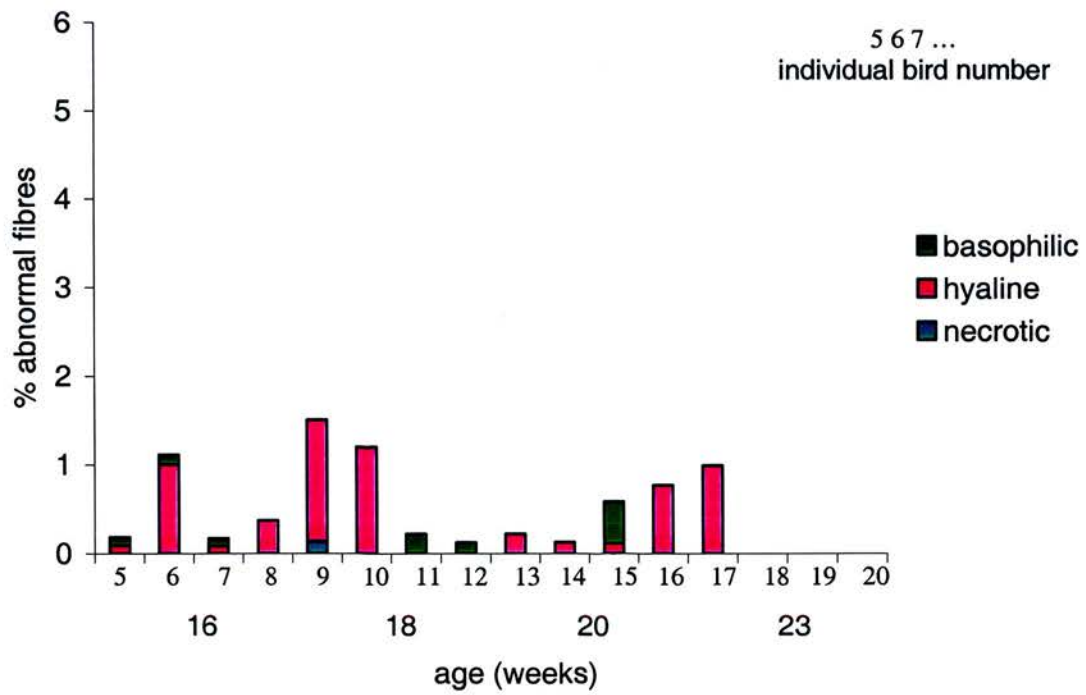



Figure 3.49 Percentage (%) basophilic, hyaline and necrotic fibres recorded in broiler *Gastrocnemius* sections at 16, 18, 20 and 23 weeks of age.

section from bird 16 contained both basophilic and necrotic fibres (estimated percentages were both 0.1%). The section from bird 12 also contained necrotic fibres (estimated percentage was 0.4%). At 23 weeks of age, the section from bird 18 contained necrotic fibres (estimated percentages were 0.2%).

Between 16 and 23 weeks of age, hyaline fibres were the main type of structural abnormality observed in the broiler *Gastrocnemius* sections (Figure 3.49). At 16 weeks of age, hyaline fibres were present in the sections from all four birds (estimated percentages ranged from 0.1% (bird 1 and bird 3) to 1.4% (bird 6)). The sections from bird 5, bird 6 and bird 7 also contained basophilic fibres (estimated percentages were 0.1% for all 3 birds). At 18 weeks of age, hyaline fibres were present in the sections from bird 9 and bird 10 (estimated percentages were 1.4% and 1.2% respectively). The section from bird 9 also contained necrotic fibres (estimated percentage was 0.1%). Basophilic fibres were present in the sections from bird 11 and bird 12 (estimated percentages were 0.2% and 0.1% respectively). At 20 weeks of age, hyaline fibres were present in the sections from all four birds (estimated percentages ranged from 0.1% (bird 14 and bird 15) to 0.7% (bird 6)). The section from bird 15 also contained basophilic fibres (estimated percentage was 0.5%). Hyaline fibres were present in the section from bird 17 at 23 weeks of age (estimated percentage was 1.0%).

## 3.8 Discussion

### 3.8.1 Reproductive status

Large elevations of plasma concentrations of the egg yolk precursors vitellogenin (estimated by plasma zinc concentrations) (Table 3.3 and Figure 3.1) and VLDL (measured indirectly as plasma triglyceride concentrations) (Table 3.3 and Figure 3.2) and calcium (Table 3.3 and Figure 3.3) are indicative of increased ovarian estrogen secretion in female birds.

In both the broiler and great-grandparent lines, all three indices significantly increased between 23 and 25 weeks of age, demonstrating that increased egg yolk precursor production and calcium uptake had occurred by 25 weeks (Table 3.14). Whilst the elevation in mean plasma calcium concentrations from 16 to 18 weeks was numerically large in the layer line, it was not statistically significant. However, taken in conjunction with the significant increases in the plasma concentrations of both of the egg yolk precursors during this time period, it can be concluded that increased egg yolk precursor production and calcium uptake had occurred by 18 weeks in the layer line.

| line | age at which egg yolk precursor production and calcium uptake increased (weeks) |
|---|---|
| B | 25 |
| GGP | 25 |
| L | 18 |

Table 3.14 Age (weeks) at which egg yolk precursor production and calcium uptake increased in the broiler (B), great-grandparent (GGP) and layer (L) lines.

Changes in egg yolk precursor production and calcium uptake in the broiler, great-grandparent and layer lines in relation to muscle damage is considered later in the discussion.

### 3.8.2 Body weight

Even in this experimental situation involving feed restriction, body weight was significantly greater in both the broiler and great-grandparent lines compared to the layer line at all age intervals (Table 3.4 and Figure 3.4). By 25 weeks of age, the mean body weight of the broiler and great-grandparent lines were more than twice the mean body weight of the layer line. These differences reflect the genetic selection of commercial broilers and broiler great-grandparents for increase growth rate and breast yield. From 9 weeks of age, both the broiler and great-grandparent lines were restricted to 65% of the calculated *ad libitum* feed intake in order to optimise welfare whilst maintaining a high growth rate. Had the birds been grown with *ad libitum* access to food, the mean body weight of the two lines would have been considerably greater by 25 weeks of age. In a previous study, broilers were grown to sexual maturity with *ad libitum* access to food, and achieved mean body weights of approximately 6.0 kg (Sandercock and Mitchell, 1996). However it was necessary to cull a large proportion of the birds during the growth period, as they suffered from cardiovascular and leg conditions.

### 3.8.3 Muscle fibre types

The *Pectoralis major* and *Coracobrachialis* breast muscles were almost entirely made up of Type IIb, fast 'glycolytic' fibres (Figure 3.5 and Figure 3.6), whereas the *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles contained slow, oxidative type I fibres as well as the type II muscle fibres (Figure 3.7 and 3.8). Previous poultry fibre typing studies have found similar fibre type distributions in breast and leg muscles (Mahon *et al*, 1995; Mills, 2001; Remignon *et al*, 1995; Suzuki, 1978). It has been proposed that the major difference between slow and fast growing birds is fibre size, rather than fibre type, and that the frequency of fibre types in chicken muscles is a conservative property depending on the function of the muscle (Horak, Sevcikova and Knizetova, 1989).

The action of the *Pectoralis major* and *Coracobrachialis* breast muscles is to depress the wing during flight. Chickens do not have the ability for long-term flight, therefore the breast muscles do not require slow, oxidative type I fibres, but rely on fast, glycolytic Type IIb fibres, which generate a high power output over a short period, for short-term intense flight or flapping activites (Suzuki, 1978). In contrast, the leg muscles are frequently in use during standing and walking activities. The *Biceps femoris* muscle extends the hip and flexes the knee, the *Peroneus longus* flexes the digits, and the *Gastrocnemius* flexes the tarsometatarsus (Nickel *et al*, 1977; Robinson, 1970). These muscles all contain the fatigue resistant slow, oxidative type I fibres, which are adapted for producing slow repetitive movements and sustaining isometric force.

### 3.8.4 Muscle fibre growth

Radial fibre size (estimated by measurements of minimum fibre diameter) of the *Pectoralis major* and *Biceps femoris* muscles increased with age, and was generally much greater in the broiler and great-grandparent lines compared to the layer line (Table 3.5; Figure 3.9 and Figure 3.10). Significant linear relationships existed between ($\log_{10}$) radial fibre size of the *Pectoralis major* and *Biceps femoris* and the corresponding ($\log_{10}$ of the cube root) body weight for all three lines (Figure 3.14 and Figure 3.15). The radial fibre sizes of the *Pectoralis major* muscles of the broiler and great-grandparent lines were generally much greater than the corresponding fibre sizes of the *Biceps femoris* muscles (Figure 3.11 and Figure 3.12). This was reflected in the mean *Pectoralis major* MFD: *Biceps femoris* MFD ratios over the eight age intervals, which were considerably greater than 1 for the broiler and great-grandparent lines (1.33 and 1.27 respectively). At 5 weeks of age, the broiler *Pectoralis major* muscles were considerably larger than the corresponding *Coracobrachialis*, *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles (Figure 3.42). However, the relative fibre sizes of the *Pectoralis major* and *Biceps femoris* muscles did not differ in the layer line (Figure 3.13). Again, this was reflected in the mean *Pectoralis major* MFD: *Biceps femoris* MFD ratios over the eight age intervals, which were close to one 1 for the layer line

(1.06). The greater growth of the *Pectoralis major* compared to the other muscles assessed in the broiler and great-grandparent lines is likely to be a consequence of genetic selection for increased breast yield, and could contribute to the heavier body weights observed in the broiler and great-grandparent lines compared to the layer line. In a study that compared eleven muscles from four commercial lines of turkeys, differential growth between breast and leg muscles was also observed (Mahon *et al*, 1995). It is possible that there is an upper size constraint on the Type I fibres of the leg muscles, as they need to employ an efficient oxidative metabolism. They therefore need relatively small diameters so that oxygen diffusion into the fibre does not become limiting and because these slow fibres need only generate low forces (Hughes and Schiaffino, 1999). Conversely, the fast, glycolytic type IIb muscle fibres of the breast muscles are used for high force movements transiently and rarely, and therefore can function metabolically with large diameters. Without the metabolic constraints on fibre size, the type IIb fibres may be more responsive to selection for increased fibre size as a result of genetic selection strategies for increased breast yield.

Fibre sizes of the broiler *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles between 16 and 23 weeks all generally increased with age (Table 3.10 and Figure 3.42). However, the *Biceps femoris* muscle fibres were smaller than both the *Peroneus longus* and *Gastrocnemius* fibres, which were similar in size. During this study, fibre type distributions were only qualitatively assessed. A more detailed, quantitative evaluation may have revealed differences in fibre type composition in the three leg muscles, which may contribute to the size differences observed. Fibre size differences may be related to the physiological and metabolic demands placed on the different muscles. Extending the hip and flexing the knee (*Biceps femoris*) may require small fibre diameters that allow a small oxygen diffusion distance and a very efficient oxidative metabolism. Flexing the digits (*Peroneus longus*) and flexing the tarsometatarsus (*Gastrocnemius*) may not require such an efficient oxidative metabolism, and so the fibre sizes may grow to larger sizes. The fibres of the broiler *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles did not differ in size at 5 weeks of age, suggesting that the divergence in size occurs after

the commercial slaughter age, and may be related to the large body weights that the muscles are supporting at the older age groups. From a commercial perspective, employing selection strategies for the divergence in leg fibre sizes at commercially relevant ages could result in increased leg muscle yield.

There are a number of factors that were not measured in the study, but could have contributed to the growth of the breast and leg muscles. These factors include increased longitudinal fibre growth, increased fibre number (hyperplasia) and a greater fibre number set in the embryo.

The degree of longitudinal compared to radial fibre growth has been shown to vary between muscles in turkeys. The *Supracorocoideus* breast muscle develops by greater radial than longitudinal growth (Swatland, 1979a), whereas longitudinal muscle growth is greater in the *Sartorius* leg muscle (Swatland, 1979b). However, in a comparison between a turkey breeding line selected mainly for egg production and a line selected for meat and egg production, the latter line had a greater pectoral growth rate and breast length, but no differences were found in muscle depth, muscle fibre number or fibre cross sectional area (Swatland, 1989).

Evidence of new fibre formation or post-hatch hyperplasia in poultry includes the appearance of small fibres expressing embryonic myosin heavy chain isoforms following experimental chronic stretch of the chicken *Anterior latissimus dorsi* wing muscle (McCormick and Schultz, 1992). Additionally, increased radial fibre growth and increased fibre number have been shown to contribute to muscle growth in a 'heavy weight' turkey line, whereas solely increased radial fibre growth contributed to muscle growth in a 'light weight' turkey line (Cherel, Hurtrel, Gardahaut, Merly, Magras-Resch, Fountaine-Perus and Wyers, 1994). The authors proposed that during the rapid growth of turkeys selected for increased body weight, disproportionate loads on the muscles induced hyperplasia by a similar mechanism to the stimulation of hyperplasia by chronic stretching. However, recent growth studies in broiler chickens have not revealed any evidence of hyperplasia (Remignon *et al*, 1995).

In the present study, small fibres were observed in all muscle samples (minimum fibre diameter data of Table 3.5; Table 3.6; Table 3.10 and Table 3.11). These fibres may have been evidence of hyperplasia, however they could also be the tapering ends of mature fibres terminating intrafascicularly (Holly, Barnett, Ashmore, Taylor and Mole, 1980; Rosser, Waldbillig, Lovo, Armstrong and Bandman, 1995), evidence of regeneration with fusion of myotubes to damaged fibres (Winchester and Gonyea, 1992), undeveloped fibres (Schmitz and Harper, 1975; Mahon, 1999) or split fibres (Figure 3.22 and Figure 3.33).

A comparison of two chicken lines (derived from a divergent selection based on growth rate) revealed that the number of fibres in the *Pectoralis major* breast muscle and the *Anterior latissimus dorsi* leg muscle at hatch were 30% and 20% greater respectively in the line selected for the greater growth rate (Remignon *et al*, 1995). In this study, fibre number remained constant during growth to adulthood. Studies in turkeys have demonstrated that at the same breast muscle weights, muscle fibres in a traditional line were larger than those of a commercial line (Mills, 2001). The author proposed that there were more fibres contributing to breast muscle mass in the commercial line, and that with a greater number of fibres that grew at a faster rate, the commercial turkeys could attain a given breast muscle weight more rapidly than the traditional turkeys, resulting in a greater growth potential.

### 3.8.5 Connective tissue content

Connective tissue content is defined as the proportion of tissue cross-sectional area occupied by non-muscle fibre components (Cumming *et al*, 1994), and includes perimysial connective tissue (surrounding each muscle fascicle), endomysial connective tissue (surrounding each muscle fibre), neurovascular bundles, capillaries and fat deposits. It has been proposed that the division of a muscle into fascicles by the perimysial connective tissue is related to the need to accommodate shear strains as muscles change shape during contraction and extension (Purslow, 2002). Connective tissue has a central role in growth, transmission of mechanical signals to

muscle cells and co-ordination of forces between fibres within a muscle (Purslow, 2002).

It has been hypothesised that in commercial turkeys, the growth of the connective tissue in muscle does not keep pace with muscle fibre radial growth, and the fibres outgrow the supporting connective tissue, leading to muscle damage (Kranen, Lambooy, Veerkamp, Van Kuppevelt and Veerkamp, 2000; Swatland, 1990). However, comparisons between traditional and commercial turkey lines have demonstrated a general reduction in connective tissue content with age, with no differences between the two lines (Mills, 2001). These results suggest that genetic selection of commercial turkeys for desirable production traits has not affected the relationship between muscle fibre and connective tissue growth.

A study of *Quadriceps femoris* muscle samples from 80 healthy humans revealed that connective tissue varied from 8-26%, with a median of 12% (Cumming *et al*, 1994). Values recorded in the present study ranged from 11-23% in the *Pectoralis major* muscle (Table 3.5) and from 14-26% in the *Biceps femoris* muscle (Table 3.6). Therefore the chicken and human muscle samples appear to contain comparable amounts of connective tissue. In the present study, there were significant effects of age, line and muscle type on connective tissue content. The connective tissue content was generally higher in the *Biceps femoris* compared to the *Pectoralis major*. Leg muscle fibres of turkeys have also been shown to have higher connective tissue contents than breast muscle fibres (Mahon *et al*, 1995). The connective tissue content was also generally higher in the *Biceps femoris* compared to the *Peroneus longus* and *Gastrocnemius* muscles from 16-23 weeks of age (Table 3.10). The differences observed in connective tissue content may have been related to changes in muscle fascicle and fibre sizes, and the forces required of the muscles during normal function (Cumming *et al*, 1994) as the birds grew and developed.

Proliferation of perimysial connective tissue is not uncommon in normal skeletal muscle. However endomysial proliferation leads to the separation of individual muscle fibres, and is commonly seen in myopathies as a secondary feature to the

basic disease process (Dubowitz and Brooke, 1973). Therefore the structural abnormalities observed in the muscle samples (considered later in the discussion) could be associated with increases in connective tissue content.

The connective tissue content of the broiler *Pectoralis major* muscle at 5 weeks of age was very low (10.6%) relative to (i) broiler *Pectoralis major* muscle samples at older age intervals and (ii) the connective tissue content of the broiler *Coracobrachialis*, *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* muscles at 5 weeks of age. These differences are probably a consequence of genetic selection for desirable meat characteristics such as lean breast meat in commercial broilers, which are usually slaughtered at 6 weeks of age.

### 3.8.6 Nuclei distribution

In the present study, most (89-100%) fibres from the broiler, great-grandparent and layer *Pectoralis major* breast muscle samples contained at least one internal nucleus. However, fewer fibres from the corresponding *Biceps femoris* muscle samples contained at least one internal nucleus (Table 3.7; Figure 3.16 and Figure 3.17). This was also true for the broiler *Peroneus longus* and *Gastrocnemius* muscle samples taken at 16-23 weeks of age. At 5 weeks of age, numerically fewer broiler *Peroneus longus* and *Gastrocnemius* muscle fibres contained at least one internal nucleus (Table 3.13 and Figure 3.45), compared to the *Pectoralis major* fibres (Table 3.12 and Figure 3.44). A study that compared the chicken *Pectoralis major* breast muscle and *Posterior latissimus dorsi* leg muscle at 7 weeks of age showed that most breast muscle fibres contained internal nuclei, whereas considerably fewer leg muscle fibres contained internal nuclei (Pizzey and Barnard, 1983). In the present study, at most age intervals there were numerically less fibres from the layer *Biceps femoris* muscle samples that contained at least one internal nucleus compared to the broiler and great-grandparent samples.

Mammalian skeletal muscle generally contains nuclei located only at the periphery of the fibre. When more than 3% of the fibres in a transverse human muscle section

contain a nucleus in the substance of the muscle fibre and not at its periphery, the biopsy is said to demonstrate internal nuclei (Greenfield, Shy, Alvord and Berg, 1957). In mammalian skeletal muscle, the presence of internal nuclei in muscle fibres is an abnormality, and a great profusion of internal nuclei is suggestive of a myopathy (Dubowitz and Brooke, 1973). The internal nuclei may be indicative of satellite cell fusion with the damaged cell during regeneration (Carpenter, 2001).

During development, myofibrils form in the centre of myotubes, but later occupy more peripheral positions in young muscle fibres. It has been proposed that this may be responsible for a corresponding movement in myonuclei to the periphery of the fibre (Goldspink, 1974). A weakened myofibrillar matrix could allow the passive migration of myonuclei to central fibre positions, resulting in the abnormal myonuclei distribution seen in myopathic human muscle fibres (Goldspink, 1974). However, observations on control and dystrophic lines of chickens have indicated that myofibrillar pressure is not responsible for myonuclei positioning within muscle fibres, as myonuclei were predominantly found in internal locations in the *Pectoralis major* muscle of the control chickens. Furthermore, severely degenerating fibres with marked myofibrillar disorganisation in both *Pectoralis major* and *Posterior latissimus dorsi* muscles of dystrophic chickens contained similar numbers of peripheral nuclei as less affected fibres (Pizzey and Barnard, 1983). To date, the functional significance of internal nuclei in poultry skeletal muscle fibres has yet to be elucidated.

It is thought that muscle fibre size is regulated by the number of nuclei incorporated into each fibre and by the volume of cytoplasm that each nucleus supports (Hughes and Schiaffino, 1999). In birds, a constant cytoplasmic volume per nucleus is maintained in muscle fibres during post-hatch development (Moss, 1968) and following chronic stretch-induced hypertrophy (Winchester and Gonyea, 1992). The cytoplasmic volume per nucleus is higher in mammalian Type II compared to Type I fibres, and greater in Type IIb compared to Type IIa fibres (Tseng, Kasper, and Edgerton, 1994). It is not clear how the addition of new nuclei is regulated during muscle growth in mammals or birds. In the present study, regression analysis

revealed significant linear relationships between ($log_{10}$ *arcsine*) percentage of fibres containing at least one internal nucleus and the corresponding ($log_{10}$) mean minimum fibre diameter of the *Biceps femoris* for the great-grandparent and layer lines. Therefore, the centralised nuclei may be associated with larger fibre sizes in certain avian muscles.

### 3.8.7 Enzyme markers of muscle damage

This is the first study of growth-associated myopathy in poultry to use lactate dehydrogenase (LDH) and aspartate aminotransaminase (AST) as enzyme markers of muscle damage in conjunction with plasma creatine kinase (CK) activity, which has been employed in previous studies (Mills, 2001, Mitchell and Sandercock, 1994). The CK, LDH and AST enzymes showed markedly different plasma activity profiles which are unlikely to be solely due to varying rates of release and clearance for the different enzymes and/or a difference in the times at which they peak, and the rates at which they return to control levels (Noakes, 1987). Plasma CK activity peaked at 18 weeks of age in the broiler and great-grandparent lines, and at 16 weeks of age in the layer line. By 25 weeks, the plasma CK had decreased to activities similar to those determined at 5 weeks of age in all three lines (Table 3.8 and Figure 3.19). Up to 18 weeks of age, AST followed a similar profile to that of CK, and peaked or was close to the peak value at this age in all three lines (Table 3.8 and Figure 3.21). However, elevated plasma AST activities were observed in some of subsequent age intervals in all three lines. AST is also a marker of liver damage (Harris, 2002), therefore these elevations may reflect disrupted liver cell function due to the large demands placed on the liver for egg yolk precursor production in the older birds (Beekman *et al*, 1991; McEwan, *et al*, 1991). The mean plasma LDH activity of the broiler and great-grandparent lines also peaked at 18 weeks of age, like the CK profile (Table 3.8 and Figure 3.20). However, an earlier, larger LDH peak was observed 9 weeks of age in both lines. In contrast, the plasma LDH activity of the layer did not notably alter between 5 and 16 weeks. The food intake of the broiler and great-grandparent birds was restricted to 65% of *ad libitum* intake from 8 weeks of age onwards, leading to a reduced growth rate. It is likely that this

restriction regime reduced the severity of the growth-associated myopathy and may also have reduced damage to other tissues that may be induced by high growth rates. These tissues may have included cardiac muscle, liver, kidney and bone, all of which contain LDH (Harris, 2002). Therefore a reduced damage in a large number of tissues following food restriction could lead to a very large subsequent decrease in LDH efflux into the plasma. Similarly, the reduction in LDH from 18-20 weeks onwards in the three lines may not only reflect a reduction in skeletal muscle damage, but also a reduction in cardiac muscle, liver, kidney and bone tissue damage.

The enzyme markers of muscle damage were generally indicative of a growth-associated myopathy in all three lines. Greater marker activities, and therefore greater disrupted plasma membrane integrity and muscle damage, were observed primarily in the broiler and great-grandparent lines, which have been subjected to selection for increased growth rate and muscle yield.

Activities of the CK and LDH enzyme markers of muscle damage started to decrease before the elevations in plasma egg yolk precursors and calcium (Table 3.14) in all three lines. By 25 weeks of age, the activities of the enzyme markers had significantly decreased to values comparable to or even less than those recorded at 5 weeks of age. The reduction in muscle damage started before estrogen initiated physiological adaptations in the intestine, which facilitate the absorption of calcium from feed for egg-shell synthesis (Etches, 1996). The increased calcium load associated with egg-shell production could constitute a threat to tissue integrity. Therefore, a protective mechanism stimulated by estrogen that maintains muscle function in the face of the demands of reproductive activity would need to be in place before the birds absorb increased levels of calcium. The mechanism by which estrogen may exert this protective effect is investigated and discussed in Chapter 4.

### 3.8.8 Muscle pathology assessment

Abnormal features were observed in sections of *Pectoralis major* and *Biceps femoris* muscle samples taken from the broiler, great-grandparent and layer lines. However the incidence of these features was generally greatest in sections from broiler muscles, and least in sections from the layer muscles. The type of abnormal feature observed appeared to be related to bird age and muscle type. Furthermore, assessment of broiler *Coracobrachialis*, *Peroneus longus* and *Gastrocnemius* muscles at the age intervals where prominent structural damage was observed in *Pectoralis major* and *Biceps femoris* sections, revealed that the presence and incidence of structural abnormalities is often very different across the different muscles of the same individual bird.

There was generally a good correspondence between the descriptive evaluations and the extreme changes in the estimated frequencies of occurrence of hyaline, basophilic and necrotic fibres in each section. However, whilst the estimation of frequencies of abnormal fibres has the advantages of highlighting severe abnormalities and of being a relatively quick procedure, abnormalities at a lower incidence or of a more focal distribution may be missed, therefore a detailed evaluation is essential for accurate biopsy assessment. The most commonly recorded abnormalities are described below (Summarised from Cumming *et al*, 1994; Dubowitz and Brooke, 1973).

*Hyalinisation* (Figure 3.27)

High intracellular calcium concentrations stimulate segmental hypercontraction of a muscle fibre during degeneration, resulting in the formation of hyaline fibres. Hyaline fibres appeared rounded and 'glassy' in sections stained with H&E.

*Necrosis* (Figure 3.24 and Figure 3.25)

Plasma membrane disruption or structural damage to a muscle fibre, may lead to degeneration and irreversible cell death (necrosis). The infiltration of a necrotic fibre by macrophages to remove cell debris, and fatty tissue replacement of the fibre were commonly observed in sections stained with H&E. Lysosomal activity in necrotic fibres undergoing macrophage infiltration stained dark brown with the non-specific esterase stain.

*Basophilia* (Figure 3.26)

Alternatively, regenerative processes may be initiated in degenerating muscle fibres, resulting in fibre repair and/or the activation of satellite cells that fuse with the damaged fibres to form new fibres. Regenerating fibres were identified as basophilic fibres – the haematoxylin in the H&E stain was a basic dye that had an affinity for the nucleic acids of a fibre. The increased expression of RNA in regenerating fibres due to the requirement for new cellular components resulted in the fibres staining blue following H&E staining. The expression of RNA in basophilic fibres was confirmed using acridine orange staining.

*Fibre size variation* (Figure 3.22 and 3.23)

When assessing muscle pathology, it is difficult to differentiate between 'normal' fibre size variation, pathological fibre size variation and variation due to technique and sampling site (Mahon *et al*, 1984; Mahon, 1999). Small fibres were observed in all muscle samples (minimum fibre diameter data of Table 3.5; Table 3.6; Table 3.10 and Table 3.11). These could have been the tapering ends of mature fibres, newly formed, undeveloped, atrophied or split fibres. The activation of satellite cells may lead to the formation of new small fibres that fuse with damaged fibres. The muscle fibres assessed may have been growing at different rates, with some fibres not growing until the birds were older in age, therefore some fibres may be small as they are undeveloped. Atrophied fibres are fibres that have shrunk from

their original size. Causes of atrophy include fibre degeneration, denervation and disuse. Extremely large fibres were also observed, primarily in the sections from the broiler and great-grandparent *Pectoralis major* muscle samples (maximum fibre diameter data, Table 3.5). These fibres could have been approaching the maximum size at which the fibres could function. It is difficult to evaluate whether these fibres should be considered abnormally enlarged (hypertrophic) or just part of the growth spectrum. However, it is highly probable that the large diffusion distances of oxygen and metabolites to the fibre core exert a metabolic stress on the fibres, which would compromise function. To reduce the diffusion distances, the large fibres may split longitudinally or myotubes derived from activated satellite cells may have fused to existing enlarged fibres. Split fibres resulting from splitting or fusion processes were identified where a small fibre appeared to share the same cell membrane as the adjacent larger fibre, where a large fibre appeared to consist of a number of closely fitting small fibres (Figure 3.22 and Figure 3.23) or where fibrous septa within the body of the fibre appeared.

The changes in fibre size distribution of 500 muscle fibres from *Pectoralis major* and *Biceps femoris* muscles from the broiler, great-grandparent and layer line at 5 and 25 weeks of age was compared (Table 3.9; Figure 3.36; Figure 3.37; Figure 3.38 and Figure 3.39). This study confirmed that greater fibre sizes were present in the broiler and great-grandparent *Pectoralis major* muscles compared to (i) the corresponding *Biceps femoris* muscle and (ii) the layer *Pectoralis major* muscle sampled at the same age.

The fibre sizes of the *Pectoralis major* or *Biceps femoris* muscles of the layer line increased between 5 and 25 weeks of age, and the frequency distribution became more positively skewed. However, the number of small fibres ($\geq$10 μm) recorded at 25 weeks was far lower than at 5 weeks of age. This suggests that many of the small fibres observed at 5 weeks of age in the layer muscles were undeveloped, with a relatively little population of fibres of a small size due to other factors (including fibre damage) at both age intervals. Although the frequency distribution became more positively skewed, and larger fibres were recorded at 25 weeks compared to 5

weeks of age, the number of small fibres (≥10 μm) did not alter in the *Pectoralis major* or *Biceps femoris* muscles of the broiler and great-grandparent lines. This may be because (i) the small fibres observed at 5 weeks of age were undeveloped, and had not developed by 25 weeks of age (ii) the small fibres could have been undeveloped at 5 weeks, developed and been 'replaced' by a similar number of new/damaged (split/atrophied) fibres by 25 weeks of age or (iii) the small fibres could have been damaged at 5 and 25 weeks of age, with a comparable rate of regeneration/splitting/atrophy at both ages.

*Cores and rims* (Figure 3.28)

Mitochondrial activity was assessed using the NADH stain. Enhanced staining in the peripheral areas of fibres produced NADH rich rims, and a complete absence of staining in the central area of the fibre produced NADH negative cores. This may be indicative of either altered mitochondrial distributions or mitochondrial dysfunction. Cores and rims were observed in the *Biceps femoris*, *Gastrocnemius* and *Peroneus longus* leg muscles, primarily of the broiler and great-grandparent lines. Greater oxygen diffusion distances from the capillaries at the fibre surface to the fibre centre will exist in bigger fibres, which may limit aerobic capacity. To compensate for the larger fibre size, mitochondria may move to the fibre periphery, resulting in cores or rims. Alternatively the increased mitochondrial density around the periphery of the fibre may be due to mitochondria increasing in number by splitting processes (Mahon, 1999).

Abnormal structural features occurred primarily in the broiler muscles, to a lesser extent in the great-grandparent muscles, and at a comparatively very low incidence in the layer muscles. The type of structural abnormality observed seemed to be related to bird age and muscle type. Necrotic and basophilic fibres were the predominant types of abnormal fibres observed in sections of *Pectoralis major* muscle samples. In contrast hyaline and basophilic fibres were the predominant types of abnormal fibres observed in sections of *Peroneus longus* (primarily basophilic), *Biceps femoris* and *Gastrocnemius* muscle samples. These findings are

different to those of the only other comparable histopathological study in the literature, in which the skeletal muscle characteristics of meat-type and layer-type chickens were examined at 7-8, 19-20 and 60-61 weeks of age (Soike and Bergmann, 1998). This previous study did not distinguish between hyalinisation and necrosis, classifying both as degenerative processes, and did not record basophilia. Whilst the authors also reported a higher prevalence of degenerative changes in the meat-type rather compared to the layer-type birds, as in the present study, these changes were observed primarily in the breast muscles (*Pectoralis major* and *Coracobrachialis*) rather than the leg muscles (*Gastrocnemius* and *Flexor cruris medialis*). Furthermore, no effect of age was observed. Theses discrepancies may be because the previous study used different layer-type and meat-type lines, examined only three age groups, and classified all pathology as 'degeneration', rather than examining the different types of abnormal structures.

The findings of the present study also differ from the comparable study on male turkey muscle growth and development (Mills, 2001), in which the histological features were very similar to descriptions of focal myopathy (Sosnicki *et al*, 1991; Wilson *et al*, 1990). Overall, much less pathology was observed in commercial chickens compared to commercial turkeys (Mills, 2001). Furthermore, the type of structural abnormality observed in turkeys was not related to bird age and muscle type – necrosis, basophilia and hyalinisation were frequently observed in the same muscle section. Therefore it is possible that the myopathy observed in commercial broilers is distinct from that observed in turkeys. Further findings of the previous turkey study include a marked increase in plasma CK activity at 15 weeks of age which corresponded to 10 kg body weight, a mean fibre diameter greater than 70 μm and a marked increase in the incidence of pathological features. Although many of the fibres of the broiler and great-grandparent muscles were greater than 70 μm, comparable changes were not observed in the present study. This may have been because the birds were female, and the myo-protective effect of estrogen may have ameliorated any fibre size/body weight associated structural damage.

Genetic selection strategies focus on optimising growth rate and breast muscle yield up to the commercial slaughter age of 6 weeks of age. The prominent occurrence of necrotic and basophilic fibres in the broiler *Pectoralis major* at 5 weeks of age may be a consequence of muscle fibre growth outstripping that of the connective tissue (Sosnicki and Wilson, 1991) and reduced capillary density (and therefore a reduced supply of oxygen), which is associated with increased breast muscle yield (Hoving-Bolink, Kranen, Klont, Gerritson and de Greef, 2000). This hypothesis is supported by the absence of structural abnormalities present in the corresponding *Coracobrachialis* muscle sections, a muscle that has not been subjected to selection for increased size. The appearance of hyaline fibres at the later ages in the broiler *Biceps femoris* and *Gastrocnemius* may be associated with the large body weights, which may have overloaded the weight bearing leg muscles. Muscle function would be further compromised if the skeletal system were undeveloped, and could not support the weight of the tissues (Lilburn, 1994).

### 3.8.9 Satellite cells and regeneration

Although large numbers of hyaline fibres were observed in the *Biceps femoris* and *Gastrocnemius* muscle sections between 16 and 20 weeks of age, large numbers of necrotic fibres were never seen. Only regenerating fibres were observed. It therefore appears that regenerative rather than degenerative processes followed fibre hypercontraction.

In mature skeletal muscles, dormant satellite cells are activated in response to growth. They are also the principal cells involved in myofibre repair and regeneration following muscle damage (reviewed in Best and Hunter, 2000; Grounds, White, Rosenthal and Bogoyevitch, 2002; Morgan and Partridge, 2003; Schultz and McCormick, 1994). Satellite cells are quiescent mononucleated muscle precursor cells (myoblasts), which when activated, proliferate, differentiate, and fuse together to form multinucleated young muscle cells called myotubes. These myotubes then undergo further differentiation and (when innervated) mature to form fully functional muscle fibres (Bishcoff, 1997; Hawke and Garry, 2001). The

satellite cells appear to be reserve muscle precursor cells. Satellite cells may contain a sub-population of cells with stem-like characteristics that serve to replenish the satellite cells' compartment. Although heterogeneity of satellite cells is well documented, it is not clear whether these differences reflect distinct populations of cells or represent stochastic differences within one population (Zammit and Beauchamp, 2001). Myotrauma initiates the release of growth factors that influence satellite cells in a cascade of regenerative events that ultimately lead to myofibre hypertrophy (Grounds *et al*, 2002). Neutrophils and macrophages are attracted to the injury site where they phagocytose cellular debris and chemotactically attract satellite cells (Hawke and Garry, 2001).

Genetic selection of broiler muscle for rapid and sustained growth has been extremely successful. Broiler muscle may also have excellent regenerative powers since satellite cells are involved in both processes. It is likely that there are differences in satellite cell proliferation capacity between broilers and layers, due to the differences in postnatal muscle growth, as is proposed in turkeys (Merley, Magras-Resch, Rouaud, Fontaine-Perus and Gardahaut, 1998). Satellite cells appear to have a greater activity in response to mitogenic stimuli in commercial compared to unselected turkey lines (McFarland, Pesall and Gilkerson, 1993) and fast growing compared to slow growing chicken lines (Duclos, Chevalier and Simon, 1996).

It is therefore possible that much of the myopathy inherent in commercial broilers is masked by successful regeneration, and that only the 'tip of the iceberg' is being observed with respect to the amount of abnormality really present (Mahon, 1999). This would account for the elevated activities of plasma enzyme markers of muscle damage in the early 5-13 week broiler growth period, that were not accompanied by high incidences of structural muscle abnormalities. All commercial broiler muscle fibres may be defective, and only reveal problems if regeneration cannot keep pace (Mahon, 1999), which in this study appeared to be at 16 –18 weeks of age in the broiler line and the broiler great grandparent (at a lower incidence).

Estrogen produces significant beneficial, protective effects on the mammalian physiology of bone (reviewed in Balasch, 2003; Rickard, Subremaniam and Spelsberg, 1999), the nervous system (reviewed in Behl, 2002; Cho, Iannucci, Fraile, Franco, Alesius and Stefano, 2003), the cardiovascular system (reviewed in Bian, Nilsson, and Gustafsson 2001; Dubey and Jackson, 2001; Mendelsohn, 2002) and skeletal muscle (reviewed in Kendall and Eston, 2002; Tiidus, 2003). Some mammalian studies have demonstrated a protective effect of estrogen on skeletal muscle by a reduction in both histological damage and enzyme markers (Reijnevald *et al*, 1994; Komulainen *et al*, 1999). Other studies have demonstrated an estrogen-induced reduction in enzyme markers, but not an accompanying reduction in histological damage (Van der Meulen *et al*, 1991). The myo-protective effect of estrogen on avian skeletal muscle has been demonstrated by employing enzyme markers as indicators of muscle damage (Carlisle *et al*, 1997; Mitchell and Sandercock, 1996). To date, no histological studies have examined the myoprotective effect of estrogen on avian muscle structure.

Estrogen may stimulate muscle fibre regeneration and recovery thus reducing the activities of the plasma markers of muscle damage in female birds. This myo-protective response may constitute an adaptation to potentially detrimental changes in calcium economy during egg production (discussion of Chapter 4). It is possible that elevated ovarian estrogen secretion may result in increased satellite cell activation. This hypothesis is supported by the observation that the implantation of steers with trenbolone acetate and estradiol results in the activation of satellite cells and the enhancement of muscle growth (Johnson, Halstead, White, Hathaway, DiCostanzo and Dayton, 1998).

This study does reveal some correlations between the profile of structural damage and the enzyme markers of damage over the 25 week experimental period. At 25 weeks of age the CK and LDH enzyme activities are greatly reduced in both the broiler and great-grandparent lines, and there is very little or no structural damage observed in the muscles (observed in both the detailed pathology evaluations and the percentage estimations of structural abnormalities). Furthermore, the plasma CK,

LDH and AST enzyme activities of the broiler and great-grandparent lines were elevated an 18 weeks of age, which coincided with prominent hyalinisation in the *Biceps femoris* and *Gastrocnemius* muscles (again observed in both the detailed pathology evaluations and the percentage estimations of structural abnormalities). However, this study also provides examples of a lack of a relationship between the degree of structural damage and the plasma activities of the enzyme markers. For example prominent necrosis was observed in the *Pectoralis major* breast muscle sections of the five week old broilers, however the enzyme marker activities were relatively low in comparison to the peak levels achieved at later ages. Also, between 5 and 9 weeks of age, the enzyme marker activities of the broiler and great-grandparent lines noticeably increased, whereas the incidence of structural abnormalities markedly decreased in both the *Pectoralis major* and *Biceps femoris* muscles. It therefore appears that plasma enzyme markers of muscle damage are only indicative of the really large changes in pathology incidence, with little or no correspondence between relatively low levels of structural abnormalities and marker enzyme activities. This apparent lack of correspondence at the 'lower end' of the damage spectrum may be due to some or all of the limitations described below, and may be improved by studying more birds, more muscles and more regions of each individual muscle.

### 3.8.10 Limitations of myopathy assessment

Histological verification is the only method for which the term muscle damage can truly be justified, as it allows the visualisation of damage to the structure of skeletal muscle (Bar *et al*, 1997). However, the assessment of the total amount of muscle damage in the birds of this study is very difficult for the following reasons.

1. The growth associated muscle damage response differed in the muscles studied.

2. Just two muscles were assessed for the primary study, and five muscles for the evaluation of further broiler muscles. Therefore, the degree of structural damage in the chicken skeletal muscles that were not sampled is unknown.

3. Structural damage was not distributed homogenously throughout a given muscle. Tracking a small number of broiler *Pectoralis major* muscle fibres revealed that the degree and type of structural damage differed along the fibre length. Assessing the same fascicle from a broiler *Biceps femoris* sample demonstrated that the percentage of damaged fibres observed varied considerably along the length of the fascicle.

4. Evaluations of muscle pathology were performed on samples removed from a relatively small area of each muscle, and may not have been representative of the whole muscle.

5. Myopathy may be masked by successful regeneration.

6. It is not possible to know which muscles (including those not assessed in the study), or which muscle fibres released the enzyme markers of muscle damage such as creatine kinase, lactate dehydrogenase and aspartate aminotransaminase.

7. Different muscles may contain different concentrations of the enzyme markers.

### 3.8.11 Summary

Through the employment of enzyme markers of muscle damage, and histocytochemical techniques, this study has demonstrated that genetic selection of broiler and broiler great-grandparent chickens is associated with a growth rate - induced myopathy. This may predispose the birds to further heat stress induced myopathy (investigated in Chapter 5) and concomitant alterations in meat quality attributes (investigated in Chapter 6). The mechanistic pathways that may be involved in the amelioration of myopathy associated with increased ovarian estradiol secretion are examined and discussed further in Chapter 4.

## 4.1 Introduction

The enzyme marker and histological data of Chapter 3 confirmed that genetic selection for improved production traits in broiler chickens has resulted in increased susceptibility to growth related myopathies, which may be associated with uncontrolled elevations of intracellular calcium (Mitchell 1999; Sandercock, 1997). However, the female birds studied showed an apparent decrease in myopathy associated with increased ovarian estrogen secretion, reflected in a reduced incidence of structural abnormalities in muscle fibres, and reduced plasma activities of enzyme markers of muscle damage. Estradiol administration to immature female chickens also reduces muscle damage, an effect blocked by the administration of tamoxifen, a selective estrogen receptor (ER) modulator (Carlisle *et al*, 1997). Tamoxifen administration also inhibits the estrogen-receptor dependent synthesis of the egg yolk precurors vitellogenin and Very Low Density Lipoprotein (Carlisle *et al*, 1997; Carlisle *et al*, 1999). Therefore, it has been suggested that the myo-protective effect of estradiol in birds may involve ER occupation.

Until recently, estrogen was assumed to exert its physiological effects through a single estrogen receptor (today classified as ERα), which was characterised in mammals in 1986 (Green, Walter, Kumar, Krust, Bornert, Argos and Chambon, 1986). However, in 1996, a novel mammalian estrogen receptor (ERβ) was discovered (Kuipier, Enmark, Pelto-Huikko, Nilsson and Gustaffson, 1996). Both estrogen receptors belong to the steroid receptor subgroup of the nuclear receptor gene family of transcription factors. These receptors rely on ligand activation for DNA binding and transcriptional activity (Petterson & Gustafsson, 2001).

ERα and ERβ regulate the expression of specific genes through binding to specific response elements on the DNA in the nucleus and inducing transcription (Craig Jordan, 1998). Also, the existence of membrane bound estrogen receptors that exert rapid, non-genomic effects is now widely accepted, involving coupling of the receptor to a G-protein which initiates intracellular signalling cascades (reviewed in Benton, Stephan,

Lieberherr and Wunderlich, 2001; Levin, 2002; Schmidt, Gerdes, Feuring, Falkenstein, Christ and Wehling, 2000).

ERα and ERβ have different affinities for various ligands and can therefore elicit different transcriptional responses for a given compound (reviewed in Katzenellenbogen, Montano, Ediger, Sun, Ekena, Lazennec, Martini, McInerney, Delage-Mourroux, Weis and Katzenellenbogen, 2000; Petterson & Gustafsson, 2001). For example tamoxifen can act as both an agonist and antagonist on ERα, but only as a pure antagonist on ERβ (Barkhem, Carlsson, Nilsson, Enmark, Gustafsson and Nilsson 1998). Therefore, tamoxifen may block the myoprotective action of estrogen in birds (Carlisle *et al*, 1997, 1999) by acting as an antagonist on ERβ. The identification of a mammalian delta receptor (ERΔ) has also been reported (Ali, El-Shayb and El Attar, 2000), on which tamoxifen acts as a partial agonist.

ERβ is distributed in several tissues, and can coexist with ERα in the same tissue. However, the two receptors often segregate into different cell types within the tissue. It is likely that ERα and ERβ have quite distinct biological properties (Gustafsson, 2000). Indeed, it has been hypothesised that the ERα is predominantly involved in reproductive functions, whereas the ERβ is more important for non- reproductive functions (Kuiper, Shugrue, Merchenthaler and Gustafsson, 1998).

The chicken ERα gene has been characterised, and is highly homologous to mammalian ERα (Krust, Green, Argos, Kumar, Walter, Bornert and Chambon, 1986). Four isoforms of ERα mRNA have been identified in the chicken, which all code for the same ERα protein (Griffin, Flouriot, Sonntag-Buck, Nestor and Gannon, 1997), although their physiological significance has yet to be elucidated. The existence of a second ERα protein that is present in chickens and other oviparous species (the frog, *Xenopus laevis* and the rainbow trout, *Oncorhynchus mykiss*), but not in mammals has been demonstrated (Griffin, Flouriot, Sonntag-Buck and Gannon, 1999). The authors proposed that this could account for the pleiotrophic effects of estradiol in a wide range of physiological processes occurring in oviparous vertebrates. The ERβ gene has only been demonstrated in three avian species: the Japanese quail (*Coturnix japonica*) (Foidart, Lakaye, Grisar, Ball and Balthazart, 1999), the European starling (*Sturnus vulgaris* (Bernard, Bentley, Balthazart, Turek and Ball, 1999) and the canary (Gahr,

2001). The ERβ gene in chickens has been cloned (Susuki, Mizuno and Nakabayashi, 2000), but this has only been published in the Genbank database, and has yet to appear in the literature. Rapid, non-genomic response 17β-estradiol have been found in chicken granulosa cells (Morley, Whitfield, Vanderhyden, Tsang and Schwartz, 1992), implying the existence of membrane bound estrogen receptors in birds. ERα has yet to be reported in avian skeletal muscle. ERβ has only been identified in the skeletal muscle of the European starling (Bernard *et al*, 1999).

A non-genomic protective role of estradiol is generally proposed in the literature associated with mammalian exercise-induced muscle damage, whereby estrogen is thought to act as an anti-oxidant and a stabiliser of muscle membranes (Bar *et al*, 1997, Kendall and Eston, 2002). However, both ERα (Couse, Lindzey, Grandien, Gustafsson and Korach, 1997; Lemoine, Granier, Tiffoche, Berthon, Rannou-Bekono, Thieulant, Carre and Delamarche, 2002a) and ERβ (Knutsson, Glenmark, Bodin, Jansson and Glenmark, 2000) mRNA transcripts have been demonstrated in mammalian skeletal muscle. Therefore estrogen may exert myo-protective effects on mammalian skeletal muscle through receptor mediated gene regulation as well as through non-genomic pathways.

## 4.2 Experimental aim

To determine whether the ERα and ERβ isoforms are expressed in chicken skeletal muscle.

## 4.3 Methods

Studies were performed on 30 week old female ISA Brown hens. This line was selected as it is a laying type of chicken, which was sexually mature and 'in lay' at 30 weeks of age. The birds had been grown under standard conditions and fed a commercial layer diet. A representative breast (*Pectoralis major*) and leg (*Iliotibialis lateralis*) muscle were selected for sampling. Avian liver tissue contains both ERα (Griffin *et al*, 1999) and ERβ (Foidart *et al*, 1991), therefore the liver was used as a control tissue. Oligonucleotide primers were designed from complementary DNA (cDNA) sequences of chicken ERα and ERβ. 100 mg of liver, and breast and leg muscle samples were homogenised. Total RNA was isolated and content was assessed. Messenger RNA was reverse transcribed to cDNA and amplified using the polymerase chain reaction (PCR) with the gene specific primers. PCR products were visualised on a 3% agarose gel containing ethidium bromide. Amplified fragments were ligated into plasmids, transformed into bacteria, and sequenced.

## 4.4 Results

The primers produced fragments of the size expected for ERα (280 bases) (Figure 4.1 a) and ERβ (350 bases) (Figure 4.2 b) in the breast muscle *Pectoralis major* and the thigh muscle *Iliotibialis lateralis*. Sequencing of PCR products indicated 100% homology with the corresponding known chicken ERα and ERβ sequence. The presence of messenger RNA for both receptor sub-types was also demonstrated in the control liver tissue (Figures 4.1 a and b).

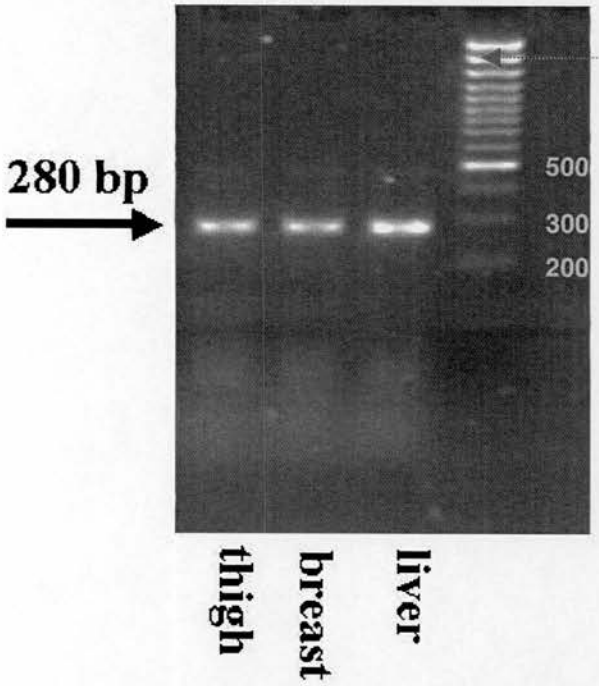



Figure 4.1 (a) Gel showing the PCR products of the ERα primers at 280 base pairs in the thigh muscle *Iliotibialis lateralis*, the breast muscle *Pectoralis major* and the liver (positive control).

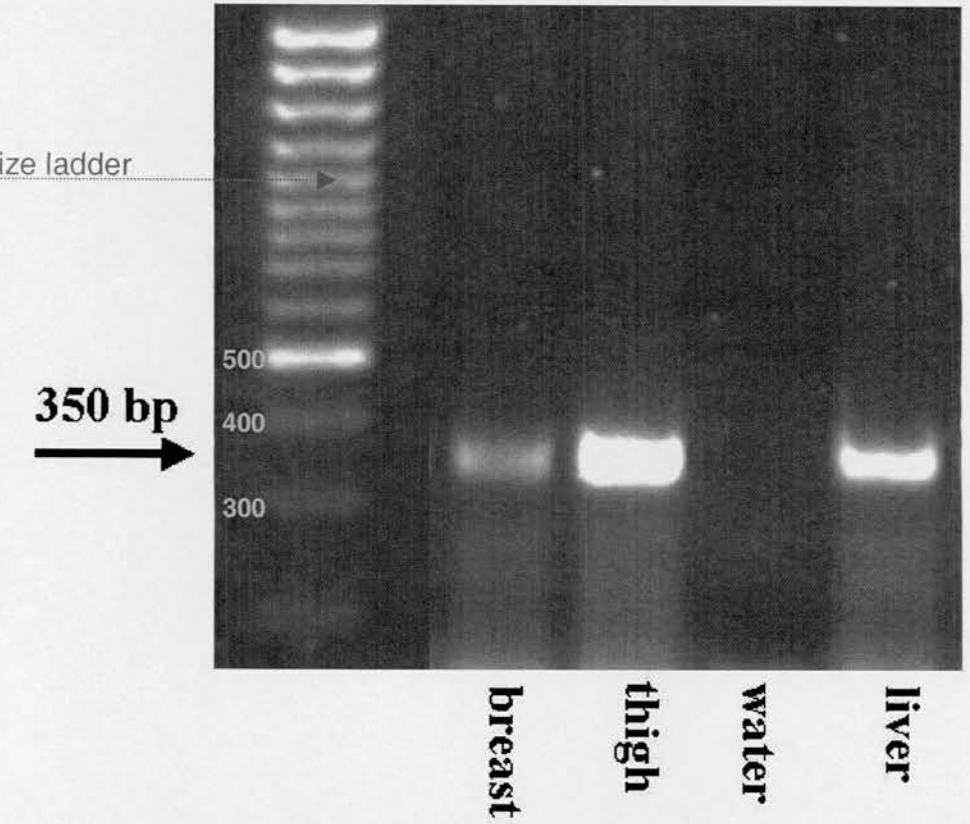



Figure 4.1 (b) Gel showing the PCR products of the ERβ primers at 350 base pairs in the thigh muscle *Iliotibialis lateralis*, the breast muscle *Pectoralis major*, the liver (positive control) and water (negative control).

## 4.5 Discussion

This is the first demonstration of ERα and ERβ mRNA expression in chicken skeletal muscle. The presence of mRNA from both estrogen receptor sub-types in both of the skeletal muscles studied shows that a receptor mediated protective effect of estradiol involving the ERα and/or ERβ may be theoretically possible in avian skeletal muscle. However, the antagonistic action of tamoxifen on the myoprotective action of estrogen in birds (Carlisle *et al*, 1997, 1999) suggests that the ERβ may be the receptor sub-type through which estrogen acts. This is because in mammals, tamoxifen can act as both an agonist and antagonist on ERα, but only as a pure antagonist on ERβ (Barkhem *et al*, 1998).

The myo-protective effect of estrogen on the cardiovascular system has been studied extensively (reviewed in Bian *et al*, 2001; Dubey and Jackson, 2001; Mendelsohn, 2002). It has been proposed that the protective effect on vascular smooth muscle cells is mediated by estrogen receptor alpha (Pare, Krust, Karas, Dupont, Aronovitz, Chambon and Mendelsohn, 2002). Estrogen has an inhibitory effect on the influx of calcium into the smooth muscle cell, and also enhances calcium efflux via a receptor-mediated mechanism (Prakesh, Togaibayeva, Kannan, Miller, Fitzpatrick and Sieck, 1999). This would reduce calcium overload, and suppress the cascade of events that lead to muscle damage. The presence of this mechanism in skeletal muscle could be an important process by which estrogen affects the inflammatory response to muscle damage (Kendall and Eston, 2002). Studies on frog skeletal twitch muscle fibres have suggested that the binding of estrogen to the receptor can affect the electrical stimulation-induced activation of calcium movement in the cytoplasm (Hatae, 2001).

Another possible mechanism through which estrogen may exert a receptor mediated protective effect may involve the transcription of calcium binding proteins. In birds, increased levels of estrogen before the onset of lay, as well as at the time of the formation of the first egg shell, are associated with increased plasma concentrations of 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$) (Nys, 1993). $1,25(OH)_2D_3$ is an

active metabolite of vitamin D, which stimulates calcium transport through a sequence of events involving the increased expression of the calcium binding protein calbindin $D_{28k}$ (Saki, Iji and Tivry, 2002; Wu, Smith, Mitchell, Peacock, Turvey and Keable, 1993). Calcium transport also appears to involve estrogen interacting with estrogen receptors as well as $1,25(OH)_2D_3$ interacting with vitamin D receptors to promote gene transcription in the intestine (Wu, Smith, Turvey, Keable and Colston, 1994).

Calbindin $D_{28k}$ binds calcium, thus avoiding enterocyte cellular damage in the face of increased calcium load. As well as posing a threat to enterocytes, the increased calcium associated with elevated plasma estrogen and eggshell production could also constitute a threat to skeletal muscle tissue integrity. Gene transcription of calcium binding proteins in the myoplasm as a result of estrogen interacting with estrogen receptors (in conjunction with $1,25(OH)_2D_3$ interacting with vitamin D receptors) would decrease intracellular free calcium concentrations thus reduce calcium-induced myopathy.

The binding of 17β-estradiol to plasma membrane estrogen receptors rapidly activates signalling systems upon ligation. One role of membrane estrogen receptors is the regulation of calcium flux in a number of mammalian cells including enterocytes, monocytes, pancreatic cells, endothelial cells and smooth muscle cells (reviewed in Kelly and Levin, 2001). Membrane vitamin D receptors are also believed to be involved in the systemic regulation of mammalian calcium homeostasis, with actions in the intestine, kidneys, bone and skeletal muscle (reviewed in Nemere and Farach-Carson, 1998). Therefore, cell surface receptors for both vitamin D metabolites and estrogen may play a role in maintaining calcium homeostasis at the onset of ovarian estrogen secretion.

It has been proposed that the myoprotective effects observed in mammals are exerted through the ant-oxidant and membrane stabilising actions of estrogen (Bar, *et al*, 1997; Kendall and Eston, 2002). An anti-oxidant is a molecule with a relatively strong reductant property to scavenge the unpaired electron from free

radical species (Kendall and Eston, 2002). The anti-oxidant properties of estrogen exert protective effects against oxidative damage to skeletal muscle (Amelink and Bar, 1997; Persky, Green, Stubley, Howell, Zaulyanov, Brazeau and Simpkins, 2000). The lipophilic properties of estrogen may allow it to intercalate into the bilayer of the cell plasma membrane, potentially altering membrane fluidity and function and increasing membrane stability as a mechanism of antioxidant action (Kendall and Eston, 2002). This may prevent calcium entry into the cell (Tiidus, Holden, Bombardier, Zajchowski, Enns and Belcastro, 2001). Increased cytosolic calcium consequent to muscle damage activates the protease calpain, which degrades specific proteins and concomitantly produces neutrophil chemoattractant peptides (Belcastro, Shewchuck and Raj, 1998). Estrogen appears to inhibit calpain activation and neutrophil infiltration, possibly by reducing calcium influx (Tiidus *et al*, 2001).

Recent developments in the mammalian exercise field suggest that the myoprotective effects of estrogen may also be mediated though estrogen receptors as well as through non-genomic actions. Both ERα (Couse *et al*, 1997; Lemoine, *et al*, 2002a) and ERβ (Knutsson *et al*, 2002) mRNA have been demonstrated in mammalian skeletal muscle. Endurance exercise training induces changes in estrogen receptor alpha mRNA levels specific to the skeletal muscle type (Lemoine, Granier, Tiffoche, Berthon, Rannou-Bekono, Thieulant, Carre and Delamarche, 2002b). Estrogen receptor alpha mRNA levels increase in the female rat *Gastrocnemius* muscle (type I and II fibres) decrease in the *Extensor digitorum longus* muscle (predominantly glycolytic type II fibres) and do not alter in the *Soleus* muscle (predominantly oxidative type I fibres). Furthermore, skeletal myoblasts contain functionally competent estrogen receptors, which may play an important role in skeletal muscle growth and reparation after injury (Kahlert, Grohe, Karas, Lobbert, Neyses and Vetter, 1997).

The protection of skeletal muscle from calcium-induced damage would be of paramount importance in female birds at the onset of ovarian activity, egg production and brooding. A protective mechanism stimulated by estrogen would

maintain muscle function in the face of the demands of reproductive activity. This would prevent the potentially increased risk of predation during egg-shell production due to calcium-induced muscle damage and therefore reduced capacity for fleeing. The estrogen receptor mediated synthesis of egg yolk precursors and increased intestinal uptake of dietary calcium may have evolved concurrently with an estrogen receptor mediated role in protection against potential calcium induced muscle damage. The positive and negative inter-relationships that may exist between estrogen, calcium and skeletal muscle are summarised in Figure 4.2.

The functional significance of ERα and ERβ in avian and mammalian muscle tissue awaits further elucidation. There is a diversity of DNA response elements mediating transcriptional regulation by estrogen receptors, which control the broad physiological effects of estrogens. Estrogen receptors can regulate gene expression by directly binding to DNA. In the absence of direct interaction with DNA, estrogen receptors can regulate gene expression via protein-protein interactions with other transcription factors or by modulating the activity of upstream signalling components (reviewed in Sanchez, Nguyen, Rocha, White and Mader, 2002). The processes involved in muscle damage are complex with many interactions between processes. Thus the way in which estrogen exerts myo-protective effects are also likely to be complex, and may involve gene regulation through nuclear and/or membrane ERα and/or ERβ, membrane stabilisation and free radical reduction (Figure 4.3) as well as other as yet unknown mechanisms and interactions between the different processes.

**Limitations of study**

Although this study demonstrated the presence of ERα and ERβ mRNA in mature chicken skeletal muscle, the presence of the respective proteins was not confirmed. Furthermore, the proteins were not localised, therefore the receptors may be distributed in the endothelial or vascular tissues, and not in the muscle tissue.

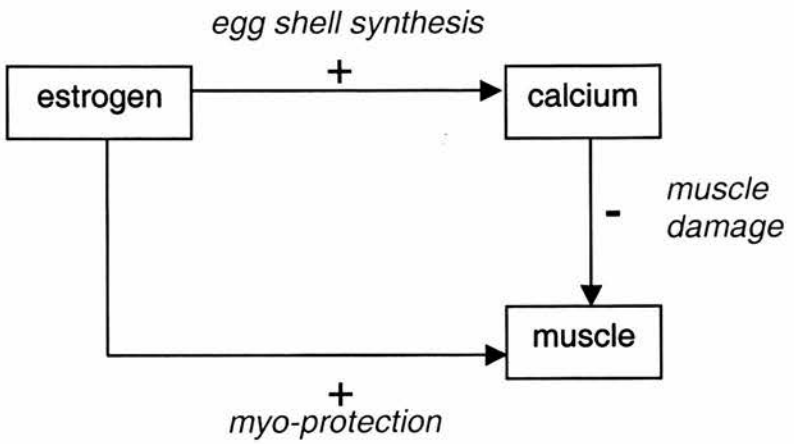



Figure 4.2 The positive and negative inter-relationships between estrogen, calcium and muscle.

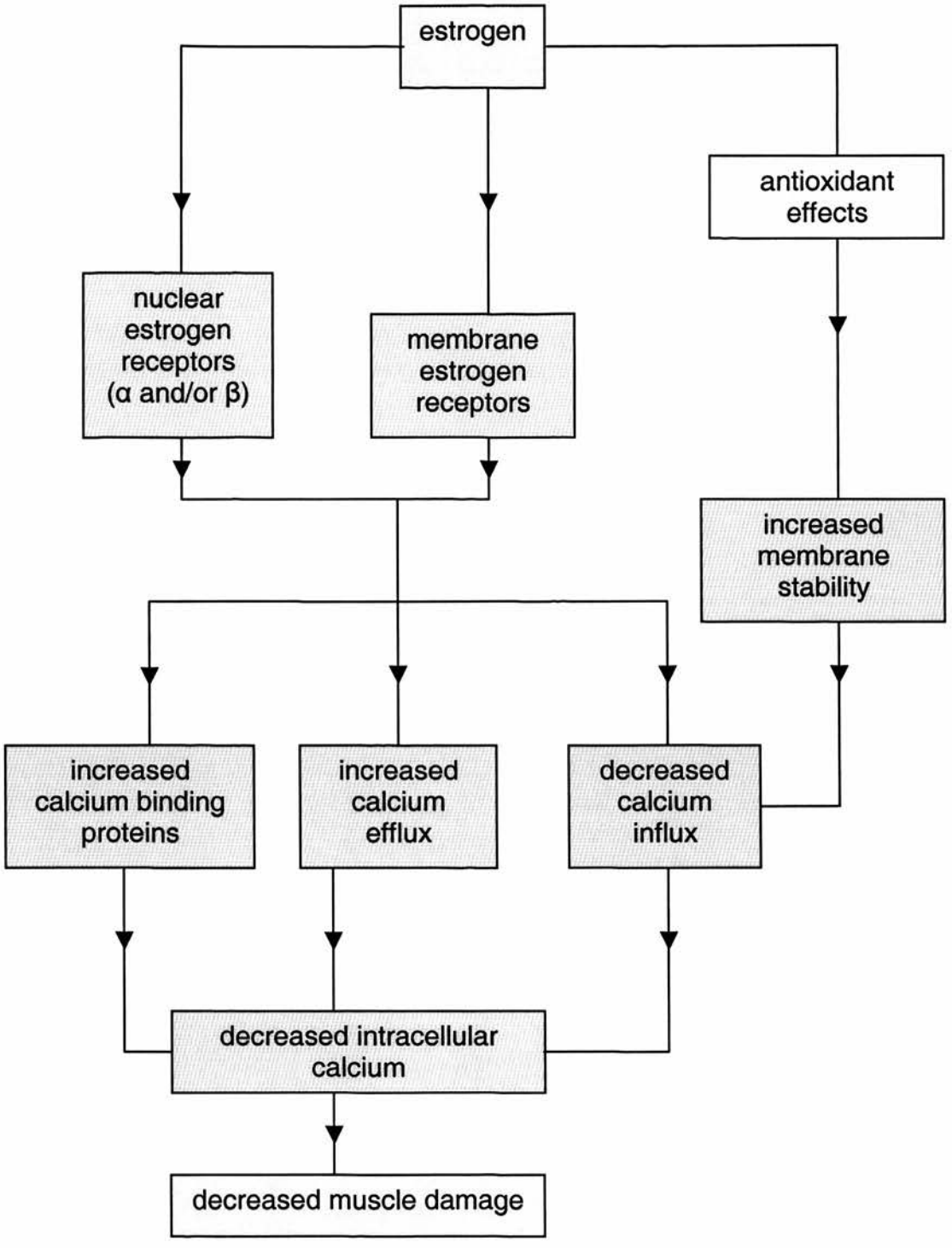



Figure 4.3 Flowchart summarising possible mechanisms through which estrogen may exert a myoprotective effect.

## 5.1 Introduction

Chickens are endotherms - they have a body temperature principally dependent on their internally generated metabolic heat. Both physiological and behavioural thermoregulatory mechanisms may be used to achieve a constant body temperature. The thermal exchange between a chicken and the components of its environment is proportional to the difference in their temperatures. In any physical system the basic principles of heat exchange will involve conduction, convection and radiation (Wilmer, Stone and Johnston, 2000). Adaptations to facilitate heat exchange include the vasodilation of unfeathered extremities such as the comb, wattles and feet, and increased capillary blood flow to the extremities (Hillman, Scot and van Tienhovwen, 1985). Birds also have the option of heat control by evaporative loss of water (EHL), which can occur cutaneously due to passive diffusion of water vapour through the skin and by EHL from the upper respiratory tract through panting (Hillman *et al*, 1985).

The equation below demonstrates how the thermal balance of a chicken is determined by the net exchanges of heat together with metabolic heat production (M) (Wilmer *et al*, 2000). Ta is ambient temperature; Tb is body temperature; Ts is surface temperature; Tsur is temperature of the surrounding surfaces; E is evaporative loss of water; S is heat storage and the *h*-values are various heat transfer coefficients, which are similar for the three main temperature exchange routes. If metabolic heat production exceeds heat loss, heat is stored and body temperature rises.

$$\mathbf{M = h_{conduction}\,(Tb\text{-}Ta) + h_{convection}\,(Ts\text{-}Ta) + h_{radiation}\,(Ts\text{-}Tsur) + E + S}$$

It has been proposed that the growth associated myopathy apparent in chickens of divergent production traits described in Chapter 3, may predispose birds to further heat stress induced myopathy (Mitchell and Sandercock, 1995a). Heat stress is

commonly experienced by broiler chickens in the commercial environment, during handling, transportation and holding (Mitchell and Kettlewell, 1998). Profound hyperthermia accompanied by increased muscle damage has been demonstrated in broilers exposed to elevated thermal loads (Mitchell and Sandercock, 1995b). However, in a series of controlled environmental studies on turkeys, there was no increase in muscle damage that could be attributable to heat stress (Mills, 2001).

In mammals there is a well-documented pattern of elevated serum enzyme activity in response to acute muscle trauma or unaccustomed exercise (Noakes, 1987; Van Der Meulen *et al*, 1991). The typical pattern for creatine kinase, lactate dehydrogenase and aspartate aminotransferase in serum is a fairly rapid rise from control levels to a maximum occurring between 8 and 36 hours post trauma/exercise, followed by a slower return to normal levels (Van Der Meulen *et al*, 1991). Secondary histological changes a few hours after injury induction include inflammatory processes, with prevalent necrosis occurring by 48 hours (Armstrong, 1990). However, a number of studies have reported discrepancies between the amount of histological muscle damage observed compared to muscle enzyme release (Van Der Meulen *et al*, 1991; Komulainen and Vihko, 1994).

## 5.2 Experimental aim

To determine the profile of elevated plasma enzyme markers of muscle damage and structural muscle damage over the 48 hour period following the exposure of broiler chickens to acute heat stress.

## 5.3 Methods

### 5.3.1 Experimental protocol

30 broiler chickens were grown under standard conditions to six weeks of age. The birds were crated at a density of 5 birds per crate and placed in controlled environment chambers. The birds were crated in order to simulate commercial conditions. 15 birds were exposed to an acute heat stress (chamber programmed to 30°C and 75% relative humidity) for three hours and 15 control birds were exposed to a control climate (chamber programmed to 21°C and 50% relative humidity) for three hours.

The original design had involved 36 broiler chickens. However, despite rearing of 50 chicks from 1 day old, only 32 birds survived to 6 weeks of age. Subsequent post-mortem reports (received after the experiment) revealed that the mortalities were due to an outbreak of necrotic enteritus within the flock, which is caused by infection with the bacterium *Clostridium perfringens*. The clinical characteristics of necrotic enteritus include severe necrosis of the intestinal mucosa; shortened villi; mononuclear cell infiltration; congested or occluded blood vessels and the presence of cellular debris in the lumen (Ficken, 1991).

### 5.3.2 Samples

Rectal temperatures and blood samples were taken from all birds before the experiment (T0) and immediately after exposure to the heat stress/control conditions (T1). Thereafter, at 12 (T2), 24 (T3) and 48 (T4) hours after the exposure to the heat stress/control conditions, rectal temperatures and blood samples of 5 heat stressed and 5 control birds were taken. At these time intervals the birds were killed. Samples of the breast muscle *Pectoralis major* and the thigh muscle *Biceps femoris* were removed from standardised regions of the left hand side of the bird. The muscle samples were frozen at -70°C pending cryostat sectioning and

histopathological assessment. Plasma samples were prepared from the blood samples and stored at -20°C pending analysis.

### 5.3.3 Measurements

#### 5.3.3.1 Blood samples

The blood samples were analysed for pH, $pCO_2$ and sodium and potassium electrolyte concentrations immediately after collection, using clinical analysers. Due to mechanical problems with the blood gas analyser, it was not possible to measure blood pH and $pCO_2$ at T4.

#### 5.3.3.2 Plasma samples

Plasma activities of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were determined. These enzymes are commonly employed markers of muscle damage.

#### 5.3.3.3 Muscle samples

The frozen muscle samples were sectioned and stained for Haematoxylin and Eosin (H&E), Masson's Trichrome, NADH, ATPase and non-specific esterase. Characterisation of muscle morphometry involved the measurement of the minimum fibre diameter of 100 fibres and the estimation of connective tissue content from *Pectoralis major* and *Biceps femoris* muscle sections taken from each bird at each T2, T3 and T4. A qualitative, descriptive evaluation of muscle pathology was also performed on *Pectoralis major* and *Biceps femoris* muscle sections taken from each bird at each T2, T3 and T4, using adapted human biopsy evaluation forms.

### 5.3.4 Statistics

Exploratory data analysis incorporating the Anderson-Darling Normality Test was performed to assess the normality of data distributions, and if necessary data

transformation was undertaken. The natural log (ln) transformation was applied to the plasma CK, LDH and AST activities data. $Log_{10}$ transformation was applied to the fibre diameter measurements. The arcsin transformation for proportions was applied to the percentage connective tissue data.

#### 5.3.4.1 Body temperature, blood and plasma data analysis

All 30 broilers were sampled at both T0 and T1. Due to the repeated sampling of the same bird, a paired t-test was performed to examine the differences between paired observations in the groups exposed to the heat stress and control conditions. Two-way analysis of variance (ANOVA) was performed on the data obtained from the 5 different birds sampled at T2, T3 and T4, in order to determine the effect of treatment (heat stress/control) and time. General Linear Model analysis incorporating pairwise comparisons using Tukey's Test was undertaken to compare groups within the ANOVA model (analysis advised by Dr. C McCorquodale, statistician at Roslin Institute).

#### 5.3.4.2 Fibre size and connective tissue content data analysis

Differences in mean minimum fibre diameter and connective tissue content due to treatment and time were determined using split-plot ANOVA, to account for the comparison of the different muscles (individual bird = plot, muscle = split-plot).

## 5.4 Results

### 5.4.1 Environmental conditions

The two climate chambers used in this experiment were programmed to a temperature of 30°C and 75% relative humidity (RH) (heat stress conditions), and 21°C and 50% RH (control conditions). Tiny-Talk data loggers that continuously recorded temperature and humidity were attached to a crate in each room. In the chamber programmed to 30°C and 75% RH, the mean temperature and humidity inside an empty crate was 26.9°C and 71.5% RH, compared to 31.4°C and 59.5% RH when the crate contained five broilers. In the control chamber, the mean temperature and humidity inside an empty crate was 19.5°C and 40.0% RH, compared to 24.7°C and 43.0% RH when five birds were in the crate (Table 5.1).

| | heat stress chamber | | control chamber | |
|---|---|---|---|---|
| index | crate only | crate with birds | crate only | crate with birds |
| temperature (°C) | 26.9 (0.42) | 31.4 (0.43) | 19.5 (0.30) | 24.7 (0.32) |
| relative humidity (%) | 71.5 (1.34) | 59.5 (2.17) | 40.0 (0.14) | 43.0 (0.67) |

Table 5.1 The temperature (°C) and relative humidity (%) inside a standard crate when empty and containing chickens in the heat stress and control chambers, with 1 standard deviation in parentheses.

### 5.4.2 Body temperature

At T0 there was no significant difference in mean body temperature between the heat stress and control birds (Table 5.2 and Figure 5.1). From T0 to T1, the mean body temperature of the heat stress birds significantly increased ($P<0.001$) from 40.8°C to 42.5°C. At T1 there was a significant difference ($P<0.001$) in mean body temperature between the heat stress and control birds (42.5°C and 40.6°C

| index | T0 | | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S | C | S | C | S | C | S | C | S | C |
| body temperature (ºC) | 40.8 (0.2) | 40.8 (0.2) | 42.5 (0.6) | 40.6 (0.3) | 40.6 (0.2) | 40.5 (0.2) | 40.5 (0.2) | 40.5 (0.2) | 40.4 (0.3) | 40.4 (0.1) |
| blood $pCO_2$ (mmHg) | 55.1 (5.8) | 55.3 (6.0) | 44.1 (4.5) | 47.5 (5.5) | 58.0 (6.0) | 49.6 (2.1) | 50.2 (5.8) | 53.4 (5.2) | - - | - - |
| blood pH | 7.28 (0.05) | 7.27 (0.06) | 7.34 (0.05) | 7.32 (0.03) | 7.28 (0.04) | 7.31 (0.03) | 7.30 (0.04) | 7.30 (0.05) | - - | - - |
| blood [sodium] ($mmoll^{-1}$) | 143.7 (2.0) | 143.5 (2.6) | 144.4 (2.4) | 144.3 (2.2) | 145.4 (1.3) | 144.8 (2.5) | 148.0 (1.6) | 146.6 (1.3) | 148.8 (0.8) | 149.0 (2.4) |
| blood [potassium] ($mmoll^{-1}$) | 5.05 (0.38) | 5.30 (0.71) | 4.70 (0.44) | 4.87 (0.28) | 5.20 (0.40) | 4.79 (0.20) | 5.02 (0.50) | 5.13 (0.34) | 5.24 (0.18) | 5.31 (0.32) |

Table 5.2 Mean values of body temperature (ºC), blood $pCO_2$ (mmHg), blood pH, blood sodium concentration ($mmoll^{-1}$) and blood potassium concentration ($mmoll^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5), with 1 standard deviation in parentheses.

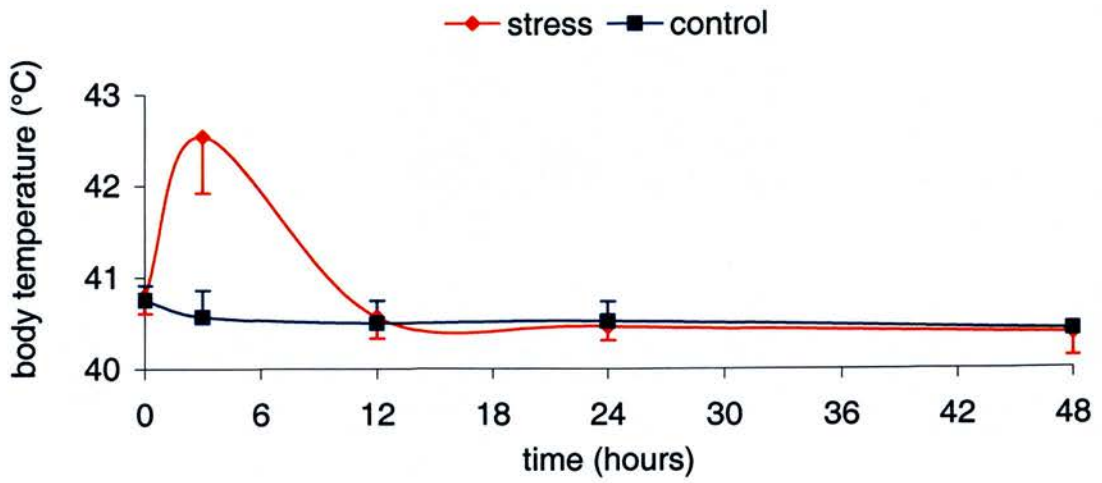



Figure 5.1 Rectal body temperature (°C) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

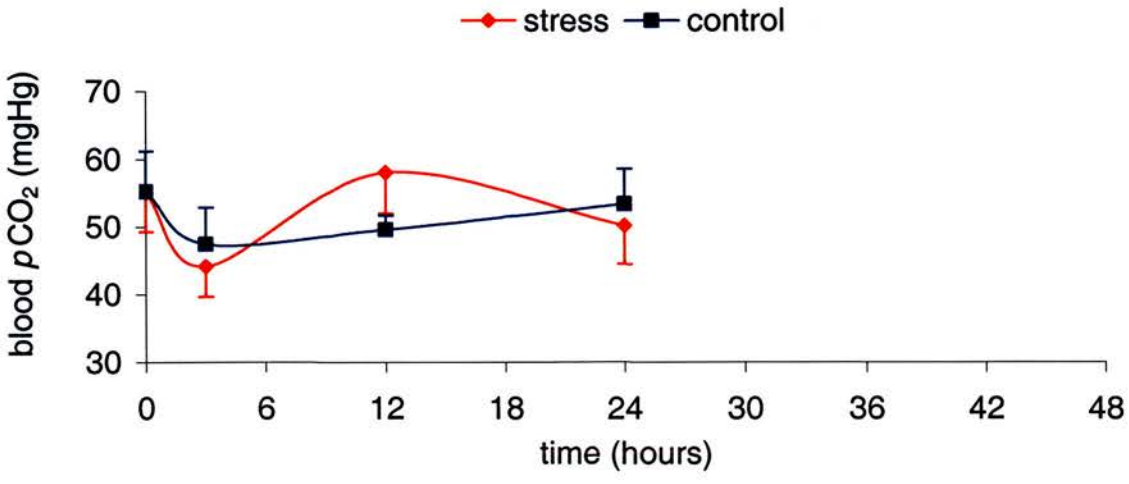



Figure 5.2 Blood $pCO_2$ (mmHg) of the heat stressed (S) and control (C) birds at T0*, T1*, T2** and T3** (*n=15, **n=5) (mean values +/- 1 standard deviation).

respectively). By T2, the mean body temperature of the heat stress birds had fallen to 40.6°C, with no significant difference in the mean body temperature between the heat stress and control birds. From T2 to T4, there was no significant effect of treatment or time.

### 5.4.3 Blood analysis

#### 5.4.3.1 Blood $pCO_2$

There was no significant difference in mean blood $pCO_2$ between the heat stress and control birds at T0 (Table 5.2 and Figure 5.2). From T0 to T1, the mean blood $pCO_2$ of both the heat stress and control birds significantly decreased ($P<0.001$), from 55.1 mgHg to 44.1 mgHg and from 55.3 mgHg to 47.5 mgHg respectively. By T3 the mean blood $pCO_2$ was 53.4 mgHg and 50.2 mgHg in the control and heat stress birds respectively, with no significant difference between the two groups.

#### 5.4.3.2 Blood pH

At T0 there was no significant difference in mean pH between the heat stress and control birds (Table 5.2 and Figure 5.3). From T0 to T1, the mean blood pH of both the heat stress and control birds significantly increased ($P<0.001$), from 7.28 to 7.34 and from 7.27 to 7.32 respectively. By T3 the mean blood pH was 7.30 in both the control and heat stress birds, with no significant difference between the two groups.

#### 5.4.3.3 Blood sodium concentration

At T0 there was no significant difference in the mean blood sodium concentration between the heat stress and control birds (Table 5.2 and Figure 5.4). From T0 to T1, the mean sodium concentration of both the heat stress and control birds did not significantly alter. However, from T2 to T5 there was a significant increase in the mean sodium concentration of both the heat stress and control birds ($P<0.05$), with no significant difference between the two groups. During this period, the mean

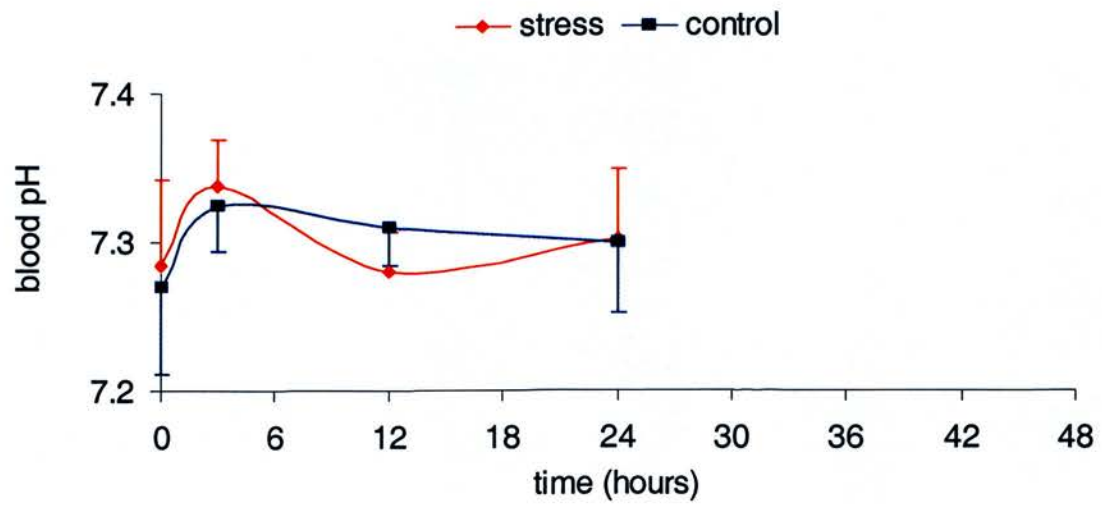



Figure 5.3 Blood pH of the heat stressed (S) and control (C) birds at T0*, T1*, T2** and T3** (*n=15, **n=5) (mean values +/- 1 standard deviation).

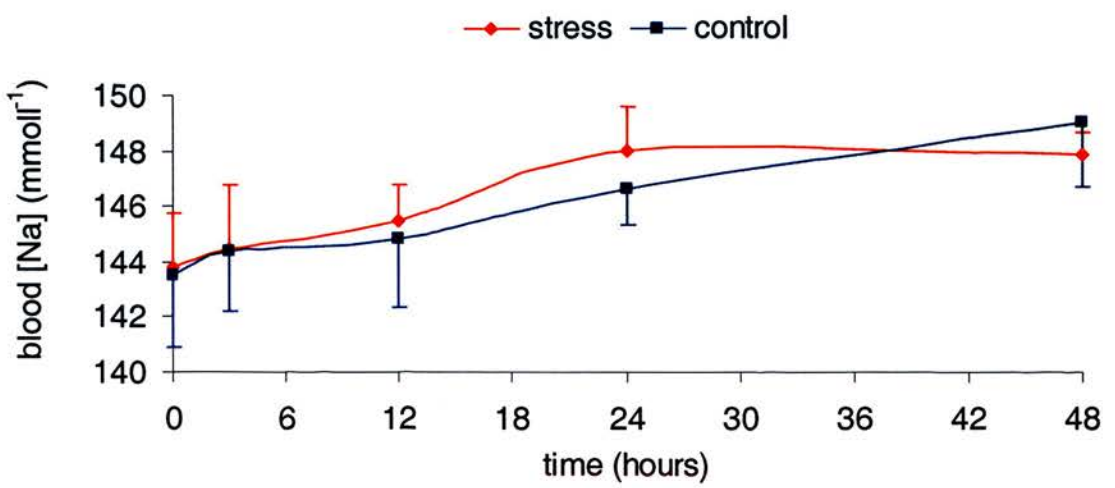



Figure 5.4 Blood sodium concentration [Na] ($mmoll^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

sodium concentration of the heat stress birds increased from 145.4 $mmoll^{-1}$ at T2 to 148.8 $mmoll^{-1}$ at T4. The mean sodium concentration of the control birds increased from 144.8 $mmoll^{-1}$ at T2 to 149.0 $mmoll^{-1}$.

#### 5.4.3.4 Blood potassium concentration

At T0 there was no significant difference in the mean blood potassium concentration between the heat stress and control birds (Table 5.2 and Figure 5.5). From T0 to T1, the mean potassium concentration of both the heat stress and control birds significantly decreased ($P<0.001$), from 5.05 $mmoll^{-1}$ to 4.70 $mmoll^{-1}$ and from 5.30 $mmoll^{-1}$ to 4.87 $mmoll^{-1}$ respectively. By T3 the mean blood potassium concentration was 5.13 $mmoll^{-1}$ and 5.02 $mmoll^{-1}$ in the control and heat stress birds respectively, with no significant difference between the two groups.

### 5.4.4 Plasma analysis

#### 5.4.4.1 Plasma creatine kinase (CK) activity

At T0 there was no significant difference in mean plasma CK activity between the heat stress and control birds (Table 5.3 and Figure 5.6). From T0 to T1, the mean plasma CK activity of both the heat stress and control birds significantly increased ($P<0.05$), from 455 $IUl^{-1}$ to 778 $IUl^{-1}$ and from 440 $IUl^{-1}$ to 665 $IUl^{-1}$ respectively. At T3 the mean plasma CK activity of the heat stress birds peaked at 1074 $IUl^{-1}$. At T2 the mean plasma CK activity of the control birds peaked at 1047 $IUl^{-1}$. By T4, the mean plasma CK activity had fallen to 690 $IUl^{-1}$ in the heat stress birds, and to 399 $IUl^{-1}$ in the control birds.

#### 5.4.4.2 Plasma lactate dehydrogenase (LDH) activity

At T0 there was no significant difference in mean plasma LDH activity between the heat stress and control birds (Table 5.3 and Figure 5.7). From T0 to T1, the mean plasma LDH activity of the control birds significantly decreased ($P<0.05$), from 193

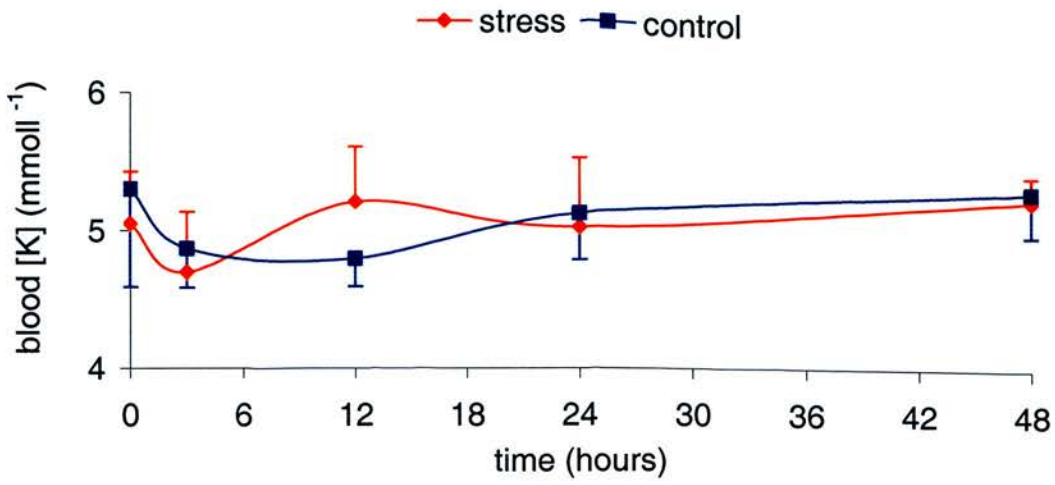



Figure 5.5 Blood potassium concentration [K] ($mmoll^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

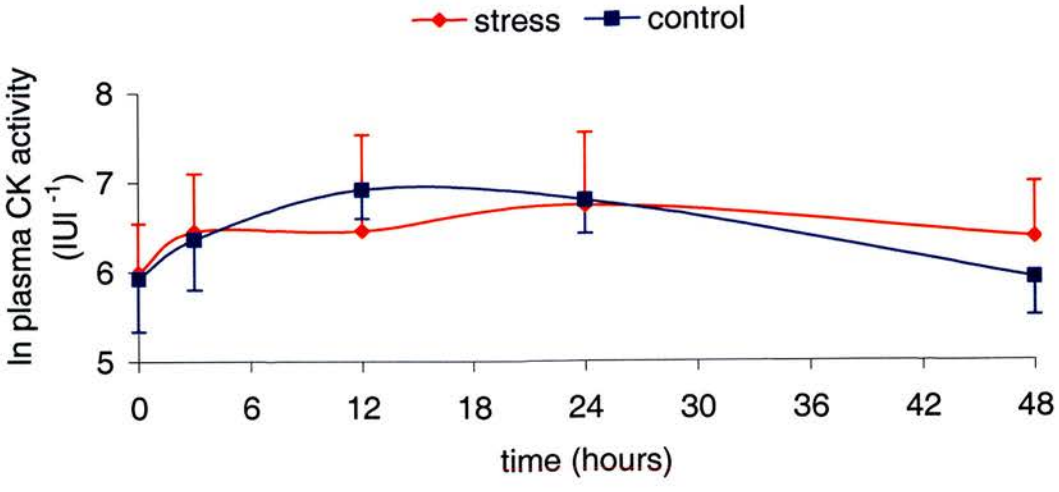



Figure 5.6 ln plasma creatine kinase (CK) activity ($IUl^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

| plasma enzyme | T0 | | T1 | | T2 | | T3 | | T4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S | C | S | C | S | C | S | C | S | C |
| creatine kinase ($IUl^{-1}$) | 455 (253) | 440 (270) | 778 (593) | 665 (351) | 914 (724) | 1047 (301) | 1074 (805) | 942 (319) | 690 (485) | 399 (162) |
| lactate dehydrogenase ($IUl^{-1}$) | 209 (47) | 193 (39) | 201 (62) | 168 (50) | 229 (65) | 212 (34) | 222 (84) | 223 (64) | 203 (58) | 165 (32) |
| aspartate aminotransferase ($IUl^{-1}$) | 74.0 (9.0) | 69.2 (6.9) | 77.3 (11.2) | 74.8 (9.5) | 76.0 (9.2) | 72.5 (4.7) | 77.0 (14.3) | 78.8 (13.5) | 79.5 (10.2) | 78.4 (7.1) |

Table 5.3 Mean activities of plasma creatine kinase ($IUl^{-1}$), lactate dehydrogenase ($IUl^{-1}$) and aspartate aminotransferase ($IUl^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5), with 1 standard deviation in parentheses.

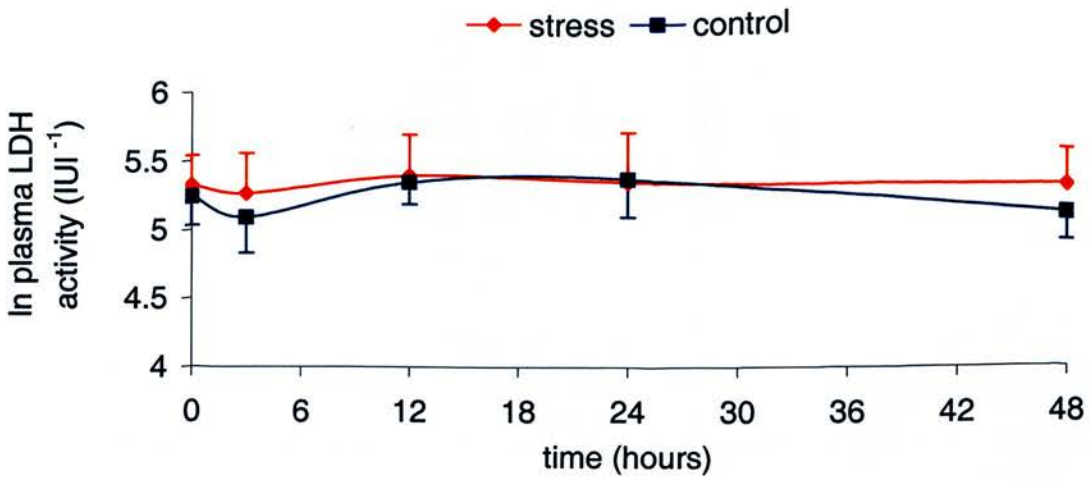



Figure 5.7 ln plasma lactate dehydrogenase (LDH) ($IUl^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

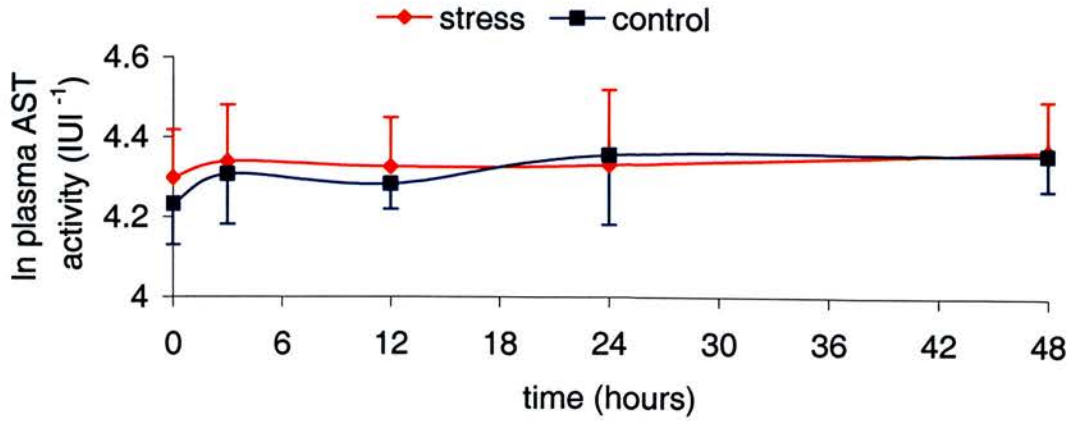



Figure 5.8 ln plasma aspartate aminotransferase (AST) ($IUl^{-1}$) of the heat stressed (S) and control (C) birds at T0*, T1*, T2**, T3** and T4** (*n=15, **n=5) (mean values +/- 1 standard deviation).

$IUl^{-1}$ to 168 $IUl^{-1}$. There was no significant difference in mean plasma LDH activity from T0 to T1 in the heat stress birds. At T2 the mean plasma LDH activity of the heat stress birds peaked at 212 $IUl^{-1}$. At T3 the mean plasma LDH activity of the control birds peaked at 223 $IUl^{-1}$. By T4, the mean plasma LDH activity had fallen to 203 $IUl^{-1}$ in the heat stress birds, and to 165 $IUl^{-1}$in the control birds.

#### 5.4.4.3 Plasma aspartate aminotransaminase (AST) activity

At T0 there was no significant difference in mean plasma AST activity between the heat stress and control birds (Table 5.3 and Figure 5.8). The mean plasma AST activity of the heat stress and control birds did not significantly change from T0 to T1. At T3, the mean plasma LDH activity of the control birds peaked at 78.8 $IUl^{-1}$. At T4, the mean plasma LDH activity of the control birds peaked at 78.4 $IUl^{-1}$.

### 5.4.5 Muscle characterization by fibre typing and morphometric assessment

NADH and myofibrillar ATPase staining demonstrated that the *Pectoralis major* breast muscle was almost entirely made up of fast, 'glycolytic', Type IIb fibres (Figure 3.5 And Figure 3.6). The *Biceps femoris* leg muscle contained slow, oxidative Type I fibres as well as type II muscle fibres (Figure 3.7 And Figure 3.8).

The mean Minimum Fibre Diameter (MFD) (n=100) of the *Pectoralis major* muscle (41.5 μm) was significantly greater than the mean MFD of the *Biceps femoris* muscle 36.1 μm ($P<0.05$) (Table 5.4). The mean connective tissue content of the *Pectoralis major* (14.5%) was significantly lower than that of the *Biceps femoris* (18.5%) ($P<0.05$) (Table 5.4).

### 5.4.6 Muscle pathology assessment

The *Biceps femoris* muscle sections generally contained more structural abnormalities than the *Pectoralis major* sections. Fibre size variation, fibre splitting and 'tiny' fibres (Figure 3.22 and Figure 3.23) were recorded in many of the

| muscle | connective tissue content (%) | Minimum Fibre Diameter (MFD) | | | | | |
|---|---|---|---|---|---|---|---|
| | | mean (µm) | standard deviation | coefficient of variation | minimum (µm) | maximum (µm) | range (µm) |
| *Pectoralis major* | 14.5 (1.5) | 41.5 | 5.8 | 14.0 | 5.1 | 80.7 | 75.6 |
| *Biceps femoris* | 18.5 (2.1) | 36.1 | 3.4 | 9.3 | 5.1 | 72.0 | 66.9 |

Table 5.4 Connective tissue content (%) (1 standard deviation in parentheses), (n=6), mean minimum fibre diameter (MFD) (µm) (n=600 per muscle), standard deviation and coefficient of variation, and overall minimum MFD (µm), maximum MFD (µm) and MFD range (µm) values of the *Pectoralis major* and *Biceps femoris* muscle fibres of randomly selected heat stressed (S) and control (C) birds at T2, T3 and T4.

sections. Other abnormalities observed included necrosis (Figure 3.24 and Figure 3.25), hyalinisation (Figure 3.27), fibres containing NADH negative cores or NADH rich rims (Figure 3.28), extensive basophilia (Figure 3.28; Figure 5.9 and Figure 5.10) and inflammation (Figure 5.11).

*Pectoralis major*

No more than 10 sections from the heat stress and control birds at T2, T3 and T4 showed fat present within and between some fascicles. A low incidence of basophilic fibres (<5 per section) was present in sections from 2-3 birds from the control groups at T2, T3 and T4 and the heat stress group at T2 and T3. A greater incidence of basophilic fibres (10-20 per section) was present in sections from 1-2 birds from the control groups at T2 and T4 and the heat stress group at T2, T3 and T4. At T2, a very high incidence of basophilic fibres (>30) was present in the section from 1 control bird. One or two hyaline fibres were present in a few fields of view of a section of 1 control bird at T2, and in sections from at least 3 birds from both the heat stress and control groups at T3 and T4. A low incidence of necrotic fibres (<5) was present in sections from 2 birds from the control groups at T2, T3 and T4 and the heat stress group at T2. At T3, a greater incidence of necrotic fibres (>15) was present in the section from 1 control chicken. All of the sections from the heat stress group showed a low incidence of necrotic fibres at T4. Inflammatory reactions indicated by the presence of macrophages in the muscle fibres were observed in many of the sections.

*Biceps femoris*

The sections from all of the birds showed fat present between some fascicles. Fat was present within the fascicles of 15 sections. A low incidence of basophilic fibres and hyaline fibres was present in the majority of sections from the heat stress and control birds at T2, T3 and T4. At T2, hyaline fibres (<5) were present in most fields of view in sections from 1 heat stress and 3 control chickens. At T4, hyaline fibres (<5) were present in most fields of view in sections from 1 control chicken. Also, a large number (>5) of hyaline fibres were present in a few fields of view in

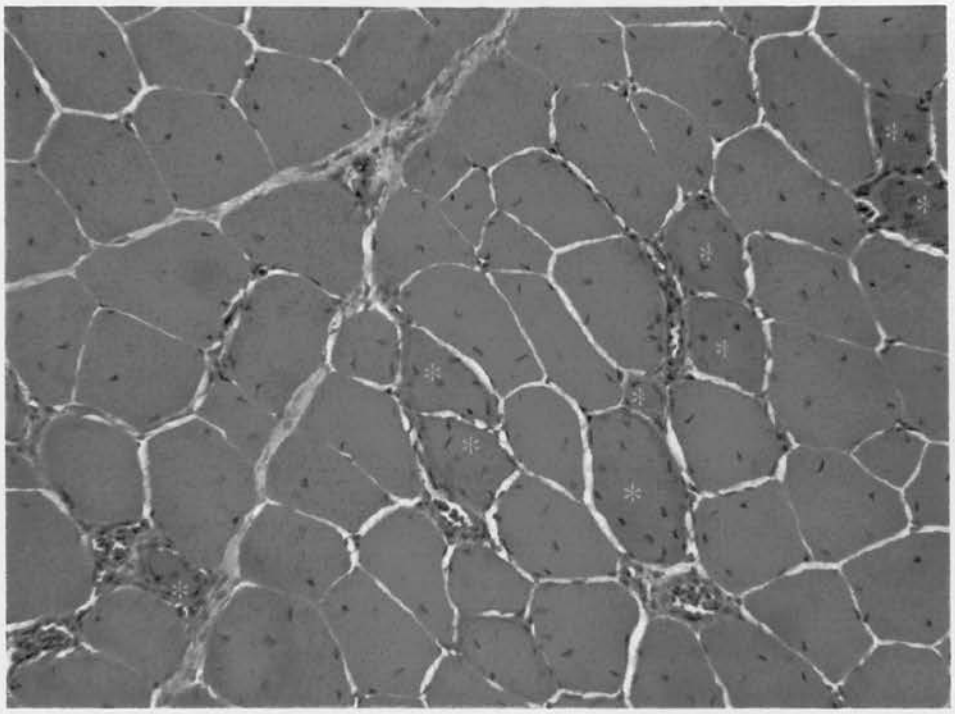


Figure 5.9 Basophilic fibres (*) in a section from a 6 week old broiler *Pectoralis major* muscle sample removed at T3 following exposure to heat stress (H&E stain, magnification x25).

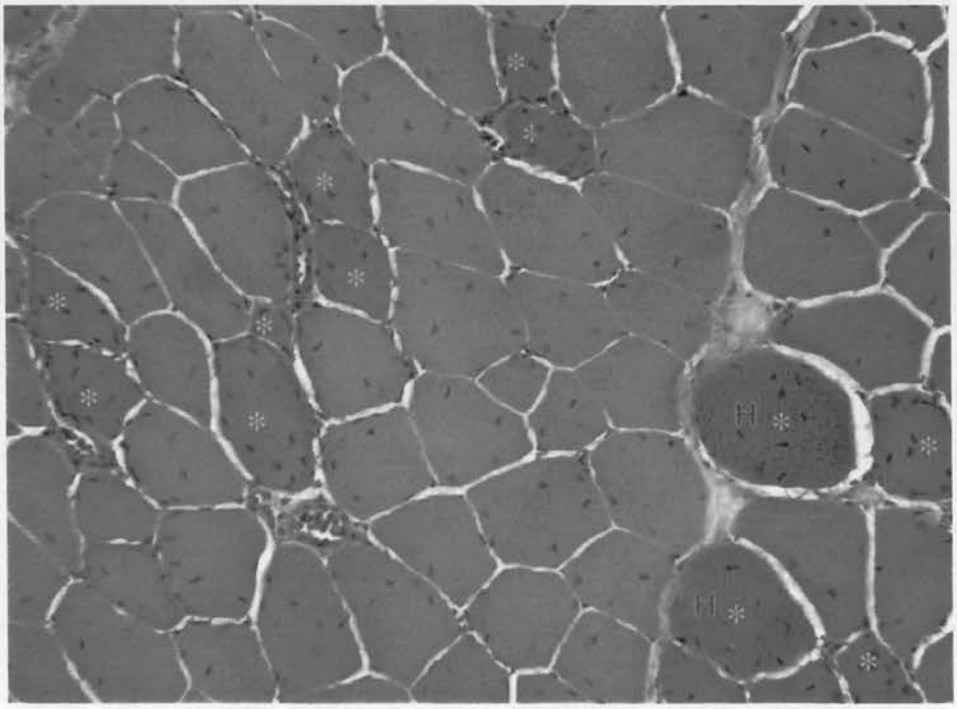



Figure 5.10 Basophilic (*) and hyaline (H) fibres in a section from a 6 week old broiler *Pectoralis major* muscle sample removed at T3 following exposure to heat stress (H&E stain, magnification x25).

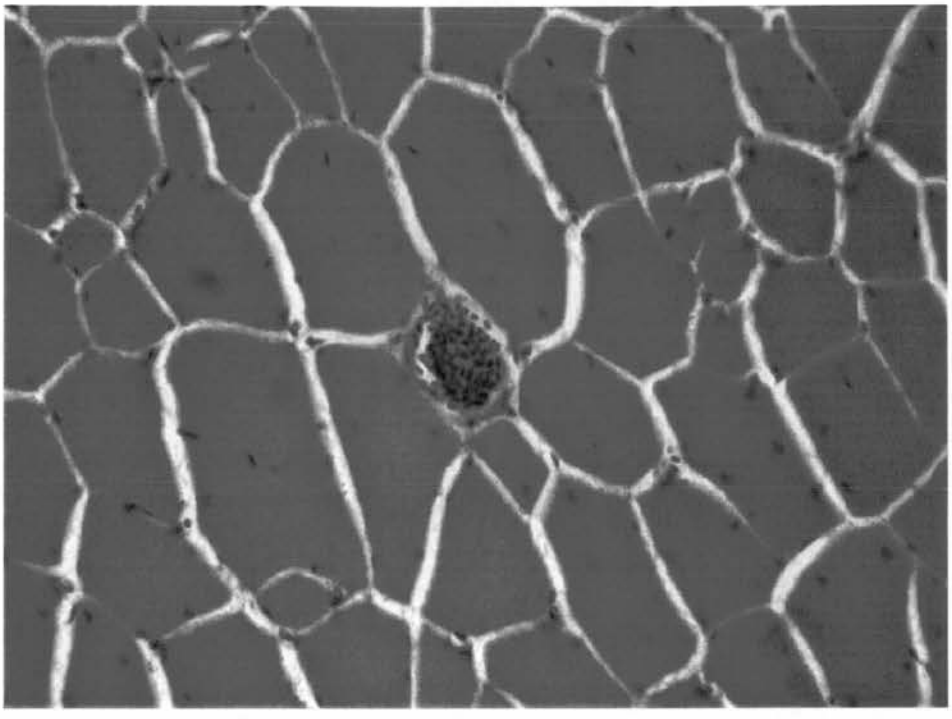


Figure 5.11 Inflammatory reaction indicated by macrophages present inside a muscle fibre. Section is from a 6 week old broiler *Biceps femoris* muscle sample removed at T3 following exposure to heat stress (H&E stain, magnification x40).

sections from 1 heat stress and 1 control chicken at T4. A low incidence of necrotic fibres (<5) was present in sections from 1-3 birds from both the heat stress and control groups at T2 and T3, and in all of the sections at T4. At T2, a high incidence of necrotic fibres (>30) was present in the section from 1 control chicken. Fibres containing cores were only observed in sections from heat stressed birds (1 bird at T2 and T4; 2 birds at T3). At T2, T3 and T4, rims were observed in 3-4 sections from the heat stressed birds and in 0-2 sections from the control birds. Inflammatory reactions were seen in many of the sections.

## 5.5 Discussion

Continuous recordings from the TinyTalk dataloggers revealed that the temperature inside a crate filled with chickens increased by 4-5°C, compared to when the crate was empty. This was because the air inside the crate gained heat from the birds through conduction, convection, radiation and evaporative heat loss. Absolute humidity is the mass of water vapour divided by the mass of dry air in a volume of air at a given temperature (Monteith, 1973). The hotter the air is, the more water it can contain. Relative humidity is the ratio of the current absolute humidity to the highest possible absolute humidity, which depends on the current air temperature (Monteith, 1973). In the chamber programmed to heat stress conditions, the relative humidity inside a crate filled with chickens was more than 10% lower than that inside an empty crate, due to the increased temperature caused by the presence of the birds.

At T0, prior to the experiment, there were no significant differences in body temperature, blood chemistry (pH, $pCO_2$ and electrolyte concentrations) or plasma enzyme markers of muscle damage. Therefore any subsequent alterations in these indices were caused by the procedures of the experiment. The birds placed in the chamber programmed to heat stress conditions exhibited hyperthermia with an increase in mean body temperature from 40.8°C at T0 to 42.5°C at T1 (3 hours after heat stress exposure initiation). There was no change in the mean body temperature of the birds exposed to control conditions between T0 and T1. Previous studies have also induced hyperthermia by exposing broilers to heat stress (Mitchell and Sandercock, 1995a; Sandercock *et al*, 2001). However, these previous heat stress studies induced greater elevations in body temperature of 44.5°C (Mitchell and Sandercock, 1995a) and 43.5°C (Sandercock *et al*, 2001) compared to the present experiment.

Blood gas analysis revealed that between T0 and T1, both groups of birds (heat stress and control) had been flushing out carbon dioxide during hyperventilation (severe panting), which lead to hypocapnia (reduced blood $pCO_2$). This in turn

influenced blood acid-base balance leading to respiratory alkalosis (increased blood pH). Previous studies have demonstrated significant alterations in blood pH and blood $pCO_2$ only in birds exposed to heat stress conditions, and not in birds exposed to control conditions (Mitchell and Sandercock, 1995a; Sandercock *et al*, 2001). The concomitant reduction in blood potassium concentration observed in the heat stress and control birds may reflect the disturbances in blood acid-base chemistry. By T3 (24 hours after exposure to heat stress/control conditions), the chicken blood acid-base chemistry had recovered, although blood sodium concentration of both groups of birds significantly increased from T2 to T5. This may be indicative of dehydration due to water loss as a consequence of evaporative heat loss during panting (Hillman *et al*, 1985), and appeared to exert effects for a long time following exposure to the heat stress/control conditions. It therefore appears that in this study, all or a combination of catching, handling, blood sampling and crating procedures induced the hypocapnic alkalosis response in the broilers. Exposure to heat stress did not exacerbate this response, in contrast to previous studies (Mitchell and Sandercock, 1995a; Sandercock *et al*, 2001).

The activity profile of each plasma marker of muscle damage was also similar for the group of birds exposed to heat stress and the group exposed to control conditions, suggesting an association with the hypocapnic alkalosis response but not with exposure to heat stress conditions. The activity profiles of the plasma markers of muscle damage examined in this study differed between each enzyme. Plasma CK and LDH activity peaked at T2-T3 (12-24 hours following initiation of exposure to heat stress/control conditions), whereas plasma AST activity peaked at T3-T4 (24-48 hours following initiation of exposure to heat stress/control conditions). Studies on human exercise-induced muscle damage have revealed that serum LDH activity peaks within 8 hours of exercise and serum AST and CK activities peak 24 to 48 hours following prolonged exercise (Noakes, 1987). Activities return to baseline levels at varying times depending on the enzyme, how much its activity increased with exercise, and the individual studied (reviewed in Noakes, 1987).

The differences in enzyme profiles observed in the broiler chickens and the comparative human studies may have been a consequence of varying rates of release and clearance for the different enzymes, resulting in different enzyme profiles. Plasma enzyme activity represents a balance between the rate of tissue release and removal from the bloodstream. Enzymes differ in the times at which they peak, and the rates at which they return to control levels (Noakes, 1987). The non-specificity of the AST and LDH enzymes may have also contributed to enzyme profile differences. Elevated plasma/serum AST activity may be suggestive of liver dysfunction as well as muscle damage (Harris, 2002). Plasma/serum LDH activity may increase due to erythrocyte haemolysis or cardiac muscle, liver, kidney or bone tissue damage, as well as muscle damage (Harris, 2002). In contrast to AST and LDH, plasma CK activity is a very specific marker of stress-induced muscle damage in broilers (Mitchell & Kettlewell, 1998; Mitchell and Sandercock, 1995a). Also, the serum CK assay is the most common and useful serum test obtained in the muscle clinic (Hilton-Jones and Kissel, 2001). However, a problem with the use of CK as an indicator of muscle damage, is that CK demonstrates a large inter-subject variability in man (Clarkson and Ebbeling, 1988) and chickens (Mitchell, 1999a).

The profiles of the enzyme markers suggest that the respiratory alkalosis induced by the catching, handling, sampling and/or crating procedures may have compromised muscle cell function. Subjecting laboratory rats to acute respiratory alkalosis has been shown to induce a fatal malignant hyperthermia-like syndrome, characterised by generalised tetanic muscle spasms (Belonje, Wislon, Berman and Human, 1995). Therefore in the present study, respiratory alkalosis may have resulted in disruptions in plasma muscle membrane integrity and the subsequent leakage of CK, LDH and AST into the plasma during the 12-48 hour period following the initiation of the exposure to heat stress/control conditions.

Assessment of muscle sections confirmed the findings in Chapter 3 that (i) the *Pectoralis major* and *Biceps femoris* muscle fibre sizes have diverged, with the *Pectoralis major* muscle fibres being larger than the *Biceps femoris* muscle fibres,

and (ii) the connective tissue content of the *Pectoralis major* muscle fibres is lower than that of the *Biceps femoris*.

The most notable observation made when assessing muscle pathology was the presence of hyaline fibres in *Pectoralis major* and *Biceps femoris* muscle samples at T2, T3 and T4 (12, 24 and 48 hours following the initiation of the exposure to heat stress/control conditions). During normal growth (Chapter 3), hyaline fibres did not appear until 16 weeks of age in the broiler *Biceps femoris* muscle, and not at all in the *Pectoralis major* muscle. It is possible that the catching, handling, blood sampling and/or crating procedures induced muscle fibre hypercontraction, which would have resulted in muscle cell membrane disruption, marker enzyme leakage into the plasma and the development of necrosis, which was also observed. A greater frequency of fibres containing cores and rims were observed in the *Biceps femoris* sections from the heat stressed compared to the control birds. The heat stress may have increased the fibres' oxygen requirements, resulting in altered mitochondrial distribution within the fibres to reduce diffusion distances.

In the present study there was no obvious relationship between the degree of structural damage and the plasma activities of the enzyme markers. This finding is in agreement with previous studies that have induced damage in mammalian muscle (Van der Meulen *et al*, 1991; Komulainen and Vihko, 1994). The relationship between exercise-induced muscle damage and enzyme release has been examined in rats (Van der Meulen *et al*, 1991). In this study, the amount of structural damage observed 72 hours after the exercise protocol was significantly lower than the estimated amount of damage, based on the total amount of CK, LDH and AST released into the vascular compartment. Assessment of structural muscle damage and serum CK activity in rats over the four days following an exercise protocol also concluded that serum CK activity was an inaccurate estimate of exercise induced muscle damage as regards interpretation of the degree and the time course of pathological events in the muscle (Komulainen and Vihko, 1994). The histological and enzyme marker data described in Chapter 3 showed that plasma enzyme markers of muscle damage are only indicative of the really large changes in

pathology incidence, with little or no correspondence between relatively low levels of structural abnormalities and marker enzyme activities. The structural abnormalities observed in this study generally correspond to the 'lower end' of the spectrum of pathology incidence observed in the study described in Chapter 3. Therefore the lack of correspondence between the incidence of structural abnormalities and marker enzyme activities in the present study is not surprising. The environmental conditions of the present study (30°C and 75% RH) did not appear to be severe enough to cause heat-stress induced muscle damage in the broiler chickens. A more challenging environment that causes broiler body temperatures to approach the upper lethal limit of 46°C (Sturkie, 1986) may induce muscle damage characterised by a closer correspondence between the activities of the plasma markers of muscle damage and the structural abnormalities observed in the muscle tissue.

Inflammatory responses including frequent basophilia and the presence of macrophages within muscle fibres were observed in many of the muscle samples. These responses are typical clinical symptoms of secondary inflammatory myopathies, which can occur in association with bacterial, viral or parasitic infections (Dalakas and Karpati, 2001). It is therefore likely that the inflammatory responses observed were a consequence of the outbreak of necrotic enteritus, which is caused by infection with the bacterium *Clostridium perfringens*. Consequently, it is difficult to confidently interpret the effect of the experimental procedures on muscle structure, as some of the observed abnormalities may have been induced by the inflammatory response.

It therefore appears that in this study, all or a combination of catching, handling, blood sampling and crating procedures, and not exposure to heat stress induced a hypocapnic alkalosis response in the broilers, which induced muscle damage. This is contrary to previous studies that used comparable environmental conditions (Mitchell and Sandercock, 1995a) and surprising, as the birds exposed to heat stress conditions exhibited hyperthermia, with a mean body temperature of 42.5°C at T1 (3 hours after heat stress exposure initiation). It is possible that the ongoing intensive

genetic selection programme has reduced broilers' susceptibility to heat stress induced muscle damage. However, it is likely that in the present study, the environmental conditions of 30°C and 75% RH were not severe enough to produce the rise in broiler body temperature required to cause heat-stress induced muscle damage.

Although the temperature and humidity settings were the same as those of previous studies that have resulted in heat-stress induced muscle damage, the present and previous studies were undertaken in different models of climate chambers. The present study was undertaken using chambers built in 2002. Unlike the previous model, the new chambers are well ventilated with good air movement throughout the chamber. Recently a new design of poultry transport vehicle with controlled forced fan ventilation was launched, following studies that had demonstrated a 'thermal core' of high temperature and humidity in the conventional transport vehicles (Mitchell, 1999b). It is possible that the high quality ventilation system of the new chambers may reduce the heat stress experienced by the birds. In the present study, the body temperatures of the heat stressed birds did not approach the upper lethal limit of 46°C (Sturkie, 1986), whereas in previous studies the body temperature has risen to 44.5°C (Mitchell and Sandercock, 1995a).

Exposure to heat stress induces the expression of heat shock proteins (HSPs) in mammalian skeletal muscle (Oishi, Taniguchi, Matsumoto, Ishihara, Ohira and Roy, 2002). It has been proposed that the HSPs provide protection against the muscle damage that occurs by a pathological increase in intracellular calcium or uncoupling of the mitochondrial respiratory chain (Maglara, Vasilaki, Jackson and McArdle, 2003). Therefore, the heat stress may not have been severe enough to disrupt the protective actions of the HSPs. Alternatively, genetic selection of broilers over the years may have inadvertently selected for an improved HSP response to heat stress.

Detrimental effects of blood sampling, catching, handling, and crating procedures are well documented in the literature. The repetitive blood sampling procedure may have contributed to the observed increases of the enzyme markers of muscle damage

(Bacou and Bressot, 1976; Lefebvre, Jaeg, Rico, Toutain and Braun, 1992). Catching the birds in their home pen immediately prior to the experiment may have resulted in exercise and trauma induced muscle damage akin to capture myopathy (Spraker *et al*, 1987). Severe wing flapping during handling and/or capture may have lead to skeletal muscle bruising (Wilson and Brunson, 1968). Handling stress can be reduced in broilers by the use of upright, rather than inverted handling (Jones, 1992; Kanan and Mench, 1996), which was the method used in this study. However, crating, and the duration of crating has been shown to be more potent stressors than handling (Kanan and Mench, 1996). Commercial catching, handling and crating methods are far rougher than the procedures used in this study and may therefore induce greater levels of muscle damage. Possible solutions include using mechanical harvesters to handle birds (Duncan, Slee, Kettlewell, Berry and Carlisle, 1986; Prescott, Berry, Haslam and Tinker, 2000) and training the handling crews regarding humane aspects of pre-slaughter management and their implications for poultry welfare and economics (Kanan and Mench, 1996).

The stress-induced muscle damage observed in this study due to all or a combination of the catching, handling, sampling and crating procedures may influence meat quality post mortem. Associations between stress, muscle damage and meat quality attributes are investigated in Chapter 6.

## 6.1 Introduction

Genetic selection of broiler chickens for increased breast yield and reduced abdominal fat has been extremely successful. This focus is in response to consumer desire for healthier meat and an increased demand for portioned and processed products (Barton, 1994). However, selection for increased muscle mass may have detrimental effects on meat quality attributes (Dransfield and Sosnicki, 1999; Le Bihan-Duval, Millet and Remignon, 1999).

Exposure to pre-slaughter stressors including heat stress exposure and handling are also recognised to affect broiler meat quality (Wood and Richards, 1975; Northcutt, Foegeding and Edens, 1994; Holm and Fletcher, 1997). Acute pre-slaughter stress induces an increased rate of post-mortem glycolysis, which accelerates tissue pH reduction and increases the likelihood of Pale Soft Exudative (PSE) meat. PSE or a PSE like condition may be becoming more prevalent in modern poultry (Barbut, 1997). Alterations in cell calcium homeostasis, $PLA_2$ activity, lipid peroxidation and sarcolemmal permeability predispose pigs to PSE (Klont, Lambooy and Van Logtestijn, 1994), however it is unknown if this occurs in poultry.

Acute heat stress and handling stress may be experienced by broiler chickens during commercial transportation and pre-slaughter holding (Kettlewell, Mitchell and Meehan, 1993; Warriss, Kestin, Brown, Knowles, Wilkins, Edwards, Austin and Nicol, 1993). During a simulated transport study, marked pre-slaughter hyperthermia was associated with hypocapnic alkalosis and increased plasma creatine kinase activities, possibly reflecting calcium-mediated alterations in muscle membrane integrity. Increased water loss, elevated haemorrhage scores and paler broiler breast fillets were observed post slaughter (Sandercock *et al*, 2001). The authors proposed that heat-stress induced antemortem alterations in muscle membrane permeability and concomitant changes in muscle metabolism in broilers may influence postmortem meat quality. Recent comparative studies have shown

that broiler great-grandparent lines exhibit differences in muscle membrane integrity, heat stress sensitivity (Gonet, Mitchell, Sandercock, Hunter and Carlisle, 2000) and meat quality attributes (Gonet, Sandercock, Hunter & Mitchell 2001). However, no study to date has examined the heat-stress induced alterations in muscle structure using the histological 'gold standard' (Bar *et al*, 1997) and the associated changes in the meat quality of broiler-type lines. Alterations in muscle structure were observed in the study described in Chapter 5.

## 6.2 Experimental aims

(i) To determine changes in muscle damage following exposure to acute heat stress using enzyme markers and histological assessment in three lines of great-grandparent broiler chickens.

(ii) To assess whether there are associations between muscle damage (determined by changes in enzyme markers and structural abnormalities) and alterations in meat quality attributes.

This study compared three commercial great-grandparent lines (Lines A, B and C). These lines were different to those examined in comparable previous studies (Gonet *et al*, 2000, 2001), and each line differed in genetic origin and recent selection history.

## 6.3 Methods

### 6.3.1 Experimental protocol

Whilst the three great-grandparent lines examined in this study have all undergone intensive selection for growth related production traits, distinct differences in their genetic make-up exist (Introduction, section 1.1). For example, Line A is a female line that has been selected for an earlier peak in egg production, but does not lay for as long a time period as Line B, which is a female line with a later egg production peak. Overall, both lines produce the same number of eggs. Line C is a male line, and has been selected primarily for growth related traits. 12 female broiler great-grandparent birds from Line A, B and C (36 birds in total) were grown under standard conditions to six weeks of age. At this age the mean body weights of Lines A, B and C were 2.3 kg, 2.3 kg and 2.6 kg respectively. The mean body weight of Line C was significantly greater ($P<0.05$) than that of Lines A and B.

The conditions in the heat stress chamber of Chapter 5 may not have been severe enough to induce muscle damage, therefore in the present study the temperature that the heat stress chamber was programmed to was increased from 30°C to 32°C. Two crates were filled with 6 birds (one bird from lines A, B and C and 3 non-experimental birds, which were used as ballast to maintain an appropriate stocking density) and placed in controlled environment chambers. One crate was placed in a heat stress environment (chamber programmed to 32°C and 75% relative humidity) for three hours, and the other crate was placed in a control environment (chamber programmed to 21°C and 50% relative humidity) for three hours. This was undertaken 6 times (3 times a day over 2 consecutive days). On the second day, the birds used as ballast in the heat stress crate were replaced by one bird from line A, B and C, which were implanted with Tinytalk dataloggers.

### 6.3.2 Samples

Rectal temperatures and blood samples were taken from all birds before the experiment (T0), immediately after exposure to the heat stress/control conditions (T1) and at 48 hours after the exposure to the heat stress/control conditions (T2). Plasma samples were prepared from the blood samples and stored at -20°C pending analysis.

At 48 hours after the exposure to the heat stress/control conditions (T2) the birds were killed. This time point was selected to allow any stress-induced pathology to develop. Samples of the breast muscle *Pectoralis major* were removed from standardised regions of the left hand side of the bird. The muscle samples were frozen at -70°C pending cryostat sectioning and histopathological assessment. The carcasses were then chilled for 24 hours at 6°C. The right breast fillets were then excised from the carcass, weighed and frozen at -20°C. Fillets were thawed over 24 hours at 6°C.

### 6.3.3 Measurements

#### 6.3.3.1 Tinytalk dataloggers

A small number of birds (3 per line) were implanted to act as sentinels to provide additional information on the body temperature profiles. The implanted dataloggers had been programmed to record body temperature every five seconds during (a) a 48 hour control period before the heat stress exposure (b) the 3 hour heat stress and (c) a 48 hour recovery period after the heat stress exposure. The following indices were calculated from the continuous temperature recordings.

Time period (a): mean body temperature.

Time period (b): mean body temperature; maximum body temperature; time to reach maximum body temperature; rate of increase in

temperature from 0-30 minutes; 'adapted' body temperature and time to reach adapted body temperature.

Time period (c): mean body temperature; rate of decrease in temperature from 0-30 minutes; over-compensatory body temperature; time to reach over-compensatory body temperature and time to return to mean body temperature.

#### 6.3.3.2 Blood samples

The blood samples were analysed for pH, $pCO_2$ and sodium and potassium electrolyte concentrations immediately after collection.

#### 6.3.3.3 Plasma samples

Plasma activities of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were determined. These enzymes are commonly employed markers of muscle damage.

#### 6.3.3.4 Muscle samples

The frozen muscle samples were sectioned and stained for NADH, ATPase, Haematoxylin and Eosin (H&E), Masson's Trichrome and non-specific esterase. The minimum fibre diameter of 100 fibres and the connective tissue content were estimated from *Pectoralis major* muscle sections taken from each bird at T2. A descriptive evaluation of muscle pathology was performed on the *Pectoralis major* muscle sections, using adapted human biopsy evaluation forms.

#### 6.3.3.5 Meat quality

The color and degree of haemorrhaging of the breast fillets were measured following excision from the chilled carcasses. The thawing of the frozen breast fillets allowed

estimates of water loss (thaw loss) to be obtained from the breast fillets. The fillets were then cooked in a water bath for 30 minutes at 90°C and left to cool for 5 hours. Assessment of meat texture was performed on standard sections of meat. Indices measured were hardness, work done during the first compression of a simulated bite, springiness index, cohesivess and adhesive force.

### 6.3.4 Statistics

The normality of data distributions was assessed using exploratory data analysis incorporating the Anderson-Darling Normality Test. Data transformation was undertaken if necessary. The natural log (ln) transformation was applied to the plasma CK, LDH and AST activities data. $Log_{10}$ transformation was applied to the fibre diameter measurements. The arcsin transformation for proportions was applied to the percentage connective tissue data.

#### 6.3.4.1 Data logger recordings

Statistical analysis was not appropriate as only 3 birds per line were implanted with data loggers and there was notable inter-bird variation. Therefore a descriptive evaluation of the data was performed.

#### 6.3.4.2 Body temperature, blood and plasma data analysis

General Linear Model analysis incorporating pairwise comparisons using Tukey's Test was performed on the data obtained at T0, T1 and T2, in order to determine the effect of line, treatment (heat stress/control) time and batch, and to compare groups within the model.

### 6.3.4.3 Characterising fibre size and connective tissue content

Differences in mean minimum fibre diameter and connective tissue content due to treatment, line and batch were determined using General Linear Model analysis incorporating pairwise comparisons using Tukey's Test.

### 6.3.4.4 Meat quality measurements

The effects of treatment, line and batch on body weight, breast yield, fillet thaw loss, fillet colour and indices of texture due to treatment and line were determined using General Linear Model analysis incorporating pairwise comparisons using Tukey's Test. The haemorrhage score assigned to each fillet was subjective on a scale of 1-5 and was measured on the ordinal scale. Therefore the non-parametric Kruskal-Wallis test was used to compare the medians of each group of 6 birds.

## 6.4 Results

### 6.4.1 Environmental conditions

The heat stress chamber was programmed to a temperature of 32°C and 75% relative humidity (RH). The control chamber was programmed to 21°C and 50% RH. Tiny-Talk data loggers that continuously recorded temperature and humidity were placed (i) in the chamber and (ii) attached to a crate containing chickens. The mean temperature and humidity inside the heat stress chamber was 31.8°C and 69.9% RH, compared to 33.1°C and 46.9% RH inside a crate containing broilers. In the control chamber, the mean temperature and humidity was 19.8°C and 48.5% RH, compared to 23.0°C and 47.8% RH inside a crate holding birds (Table 6.1).

| | heat stress chamber | | control chamber | |
|---|---|---|---|---|
| index | room | crate with birds | room | crate with birds |
| temperature (°C) | 31.8 (0.21) | 33.1 (0.35) | 19.8 (0.67) | 23.0 (0.46) |
| relative humidity (%) | 69.9 (1.5) | 46.9 (0.77) | 48.5 (1.48) | 47.8 (1.90) |

Table 6.1 The temperature (°C) and relative humidity (%) inside the heat stress and control chambers and inside a crate containing broilers, with 1 standard deviation in parentheses.

### 6.4.2 Body temperature

#### 6.4.2.1 Rectal probe data

There was a significant effect of treatment ($P<0.05$) and time ($P<0.05$) on body temperature, but no significant effect of line or batch over the 48 hour period studied (Table 6.2 and Figure 6.1). Between T0 and T1, the mean body temperatures of the heat stress birds from lines A, B and C increased significantly ($P<0.05$) to 43.2°C,

| index | time interval | line A | | line B | | line C | |
|---|---|---|---|---|---|---|---|
| | | control | stress | control | stress | control | stress |
| body temperature (°C) | T0 | 40.5 (0.2) | 40.6 (0.2) | 41.0 (0.3) | 40.8 (0.4) | 40.6 (0.2) | 40.9 (0.2) |
| | T1 | 40.5 (0.1) | 43.2 (1.2) | 40.8 (0.2) | 42.8 (0.7) | 40.7 (0.2) | 42.7 (0.7) |
| | T2 | 40.0 (0.1) | 40.6 (0.4) | 40.4 (0.2) | 40.5 (0.6) | 40.6 (0.7) | 40.5 (0.2) |
| blood $pCO_2$ (mmHg) | T0 | 59.8 (8.6) | 61.3 (8.0) | 58.3 (5.7) | 58.7 (8.8) | 61.3 (2.8) | 61.5 (5.9) |
| | T1 | *49.0 (4.6)* | *41.8 (7.8)* | *51.0 (7.9)* | *41.2 (5.6)* | *61.2 (9.8)* | *38.7 (6.6)* |
| | T2 | 53.6 (5.0) | *57.0 (4.0)* | *59.8 (11.3)* | *56.3 (5.2)* | *70.7 (14.4)* | *54.2 (6.1)* |
| blood pH | T0 | 7.24 (0.07) | 7.22 (0.07) | 7.22 (0.05) | 7.27 (0.04) | 7.22 (0.05) | 7.20 (0.02) |
| | T1 | 7.34 (0.03) | 7.33 (0.10) | 7.33 (0.06) | 7.39 (0.05) | 7.28 (0.07) | 7.37 (0.03) |
| | T2 | 7.30 (0.07) | 7.30 (0.04) | 7.28 (0.04) | *7.29 (0.03)* | *7.22 (0.08)* | *7.28 (0.02)* |
| blood [sodium] ($mmoll^{-1}$) | T0 | 145.0 (1.4) | 145.3 (0.8) | 146.0 (1.4) | 144.7 (3.6) | 145.3 (2.1) | 145.8 (1.7) |
| | T1 | 145.3 (1.6) | 147.2 (1.8) | 145.0 (2.4) | 147.2 (1.5) | 145.2 (2.0) | 143.5 (2.1) |
| | T2 | 145.4 (2.1) | 146.4 (3.8) | 145.0 (2.5) | 146.2 (1.9) | 145.0 (2.0) | 142.3 (7.1) |
| blood [potassium] ($mmoll^{-1}$) | T0 | 5.6 (0.6) | 5.9 (0.7) | 5.7 (0.2) | 5.7 (0.3) | 5.6 (0.3) | 5.9 (0.2) |
| | T1 | 4.8 (0.3) | 4.8 (0.4) | 5.0 (0.4) | 5.0 (0.4) | 5.6 (0.6) | 5.1 (0.6) |
| | T2 | 5.1 (0.5) | 5.2 (0.3) | 5.4 (0.4) | 5.3 (0.4) | 5.5 (0.5) | 5.5 (0.6) |

Table 6.2 Mean values of body temperature (°C), blood $pCO_2$ (mmHg), blood pH, blood sodium concentration ($mmoll^{-1}$) and blood potassium concentration ($mmoll^{-1}$) of the heat stressed (S) and control (C) birds before the experiment (T0), and at 3 hours (T1) and 48 hours (T2) after exposure to the heat stress/control conditions (n=6), with 1 standard deviation in parentheses.

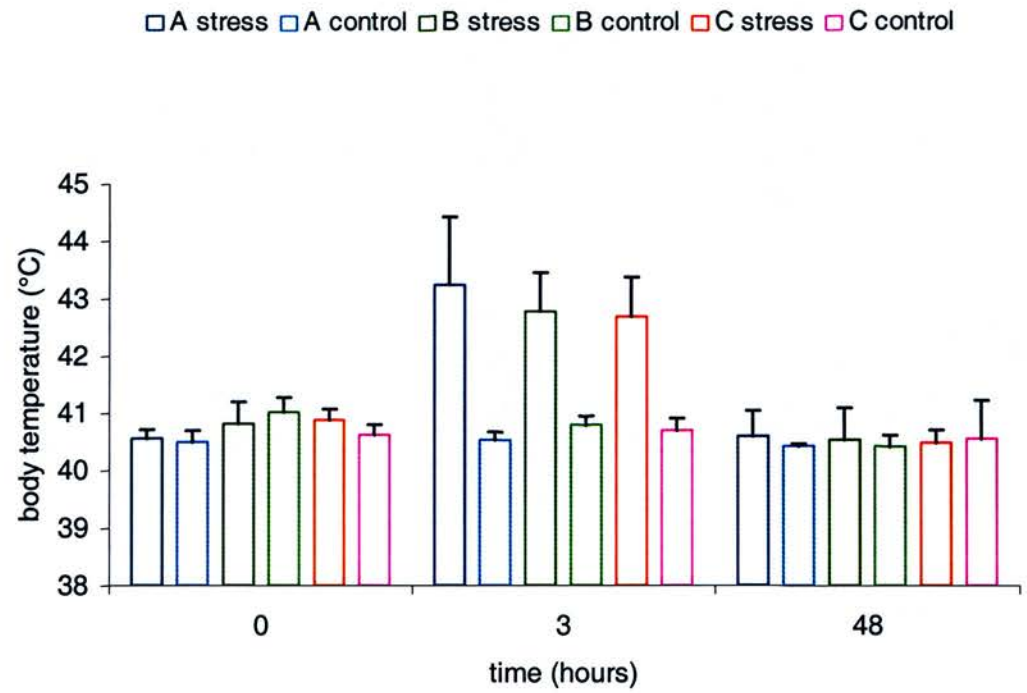



Figure 6.1 Rectal body temperature (°C) of the heat stressed (S) and control (C) birds from Lines A, B and C at 0 (T0), 3 (T1) and 48 (T2) hours following initiation of exposure to heat stress/control conditions.

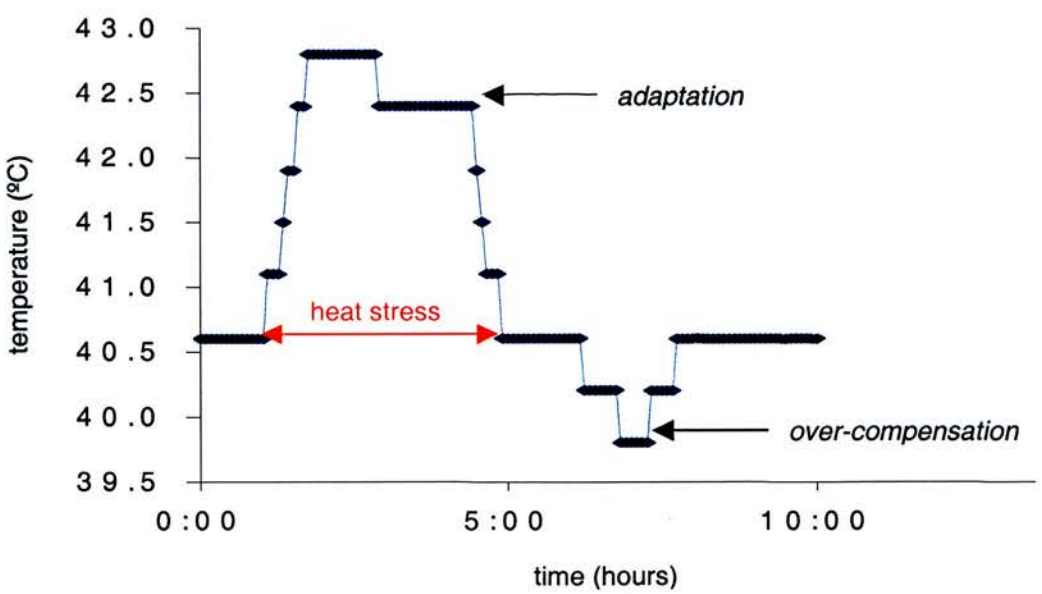



Figure 6.2 Profile of body temperature recordings (°C) showing adaptation during exposure to heat stress conditions and over-compensation following removal from the heat stress conditions.

42.8°C and 42.7°C respectively. The mean body temperature of the heat stress birds from each line was not significantly different from that of the appropriate control birds at T2.

### 6.4.2.2 Datalogger recordings

The mean body temperatures of lines A, B and C ranged from 42.2°C to 43.1°C, 41.9°C to 42.5°C and 41.8°C to 42.6°C during the heat stress (Table 6.3). The mean body temperature of each implanted bird increased by at least 1°C during the heat stress. Following a 48 hour recovery period, the mean body temperature of each implanted bird was very similar to/the same as the temperature recorded during the 48 hour control period.

During the first 30 minutes of heat stress exposure the rate of increase in body temperature of the birds from lines A, B and C ranged from 0.043 to 0.060, 0.030 to 0.057 and 0.027 to 0.060 respectively (Table 6.4). The maximum body temperature reached by the implanted birds from Line A ranged from 42.8°C to 43.7°C, and that of Lines B and C both ranged from 42.4°C to 42.8°C. The time taken to reach the maximum body temperature ranged from 45 to 75 minutes, 35 to 70 minutes and 35 to 55 minutes in Lines A, B and C respectively.

After reaching their maximum body temperature, 4 birds exhibited a reduction in body temperature to 42.0°C during the heat stress period (Table 6.5 and Figure 6.2). Two birds were from Line A, and it took 115 and 125 minutes respectively for their body temperatures to reach the adapted temperature value. One bird was from Line B, and it took 65 minutes for it's body temperature to attain the adapted temperature value. The fourth bird was from Line C, and it took 100 minutes for it's body temperature to reach the adapted temperature of 42.0°C.

During the first 30 minutes following removal from the heat stress chamber, the rate of decrease in body temperature of the birds from lines A, B and C ranged from 0.043 to 0.060, 0.043 to 0.073 and 0.030 to 0.073 respectively (Table 6.6). During

| line | bird number | mean body temperature (°C) | | |
|---|---|---|---|---|
| | | 48 hour control period | 3 hour heat stress period | 48 hour recovery period |
| A | 1 | 40.9 | 42.2 | 40.6 |
| | 2 | 40.7 | 42.4 | 40.6 |
| | 3 | 41.2 | 43.1 | 41.2 |
| B | 1 | 40.6 | 42.5 | 40.6 |
| | 2 | 40.6 | 42.2 | 40.5 |
| | 3 | 40.4 | 41.9 | 40.4 |
| C | 1 | 40.9 | 42.3 | 40.8 |
| | 2 | 40.2 | 41.8 | 40.2 |
| | 3 | 40.4 | 42.6 | 40.3 |

Table 6.3 Mean body temperature (°C) of 3 birds implanted with data-loggers from Line A, B and C during the 48 hour control period, 3 hour heat stress period and 48 hour recovery period.

| line | bird number | maximum body temperature (°C) | time to reach maximum body temperature (s) | rate of increase from 0-30 minutes ($°Cs^{-1}$) |
|---|---|---|---|---|
| A | 1 | 42.8 | 60 | 0.060 |
| | 2 | 42.8 | 45 | 0.043 |
| | 3 | 43.7 | 75 | 0.043 |
| B | 1 | 42.8 | 70 | 0.057 |
| | 2 | 42.4 | 55 | 0.030 |
| | 3 | 42.4 | 35 | 0.030 |
| C | 1 | 42.4 | 35 | 0.027 |
| | 2 | 42.4 | 55 | 0.043 |
| | 3 | 42.8 | 35 | 0.060 |

Table 6.4 Maximum body temperature (°C), time to reach maximum body temperature (s) and rate of increase from 0 -30 minutes ($°Cs^{-1}$) of 3 birds implanted with data-loggers from Line A, B and C during the 3 hour heat stress period.

| line | bird number | 'adapted' body temperature (°C) | time to reach 'adapted' body temperature (mins) |
|---|---|---|---|
| | 1 | 42.0 | 125 |
| A | 2 | 42.0 | 115 |
| | 3 | - | - |
| | 1 | - | - |
| B | 2 | - | - |
| | 3 | 42.0 | 65 |
| | 1 | - | - |
| C | 2 | 42.0 | 100 |
| | 3 | - | - |

Table 6.5 'Adapted' body temperature (°C) and the time taken to reach the adapted temperature following placement in the heat stress chamber.

| line | bird number | rate of decrease from 0-30 minutes ($°Cs^{-1}$) | over-compensatory body temperature (°C) | reach time (mins) | return time (mins) |
|---|---|---|---|---|---|
| | 1 | 0.057 | 39.8 | 25 | 125 |
| A | 2 | 0.060 | 39.8 | 110 | 200 |
| | 3 | 0.043 | - | - | 120 |
| | 1 | 0.073 | 40.2 | 55 | 185 |
| B | 2 | 0.060 | - | - | 20 |
| | 3 | 0.043 | 40.2 | 90 | 145 |
| | 1 | 0.030 | - | 95 | 45 |
| C | 2 | 0.043 | - | - | 75 |
| | 3 | 0.073 | 39.4 | 50 | 420 |

Table 6.6 The rate of decrease from 0 -30 minutes ($°Cs^{-1}$) following removal from the heat stress chamber, the time taken to reach the over-compensatory body temperature (s) (if applicable) and the time to return to mean body temperature (°C) in 3 birds implanted with data-loggers from Line A, B and C during the 48 hour recovery period.

the recovery period, an over-compensatory body temperature that fell below the mean control body temperature (Figure 6.2) was recorded in 5 birds. Two birds were from Line A and it took 25 and 110 minutes respectively for their body temperature to attain an over-compensatory temperature of 39.8°C. It took 125 and 200 minutes respectively for their body temperatures to return to control values. Two birds were from Line B and it took 55 and 90 minutes respectively for their body temperature to reach an over-compensatory temperature of 40.2°C. It took 185 and 145 minutes respectively for the body temperatures of these birds to return to control values. The fifth bird was from Line C and it took 50 minutes for it's body temperature to reach an over-compensatory temperature of 39.4°C, and 420 minutes for the body temperature to return to the control value.

### 6.4.3 Blood analysis

#### 6.4.3.1 Blood $pCO_2$

There was a significant effect of treatment ($P<0.05$) and time ($P<0.05$) on blood $pCO_2$ and a significant interaction between treatment and time ($P<0.05$) over the 48 hour period studied (Table 6.2 and Figure 6.3). There was no significant effect of line or batch. Between T0 and T1, the mean blood $pCO_2$ of the heat stress birds from lines A, B and C fell significantly ($P<0.05$) to 41.8 mmHg, 41.2 mmHg and 38.7 mmHg respectively. There was no significant change in mean blood $pCO_2$ of the control birds between T0 and T1. At T2, the mean blood $pCO_2$ of the heat stress birds from each line was not significantly different from that of the appropriate control birds.

#### 6.4.3.2 Blood pH

There was a significant effect of treatment ($P<0.05$), time ($P<0.05$) and line ($P<0.05$) on blood pH over the 48 hour period studied (Table 6.2 and Figure 6.4). There were no significant interactions and no effect of batch. The mean blood pH of the heat stress birds from lines A, B and C increased significantly between T0 and

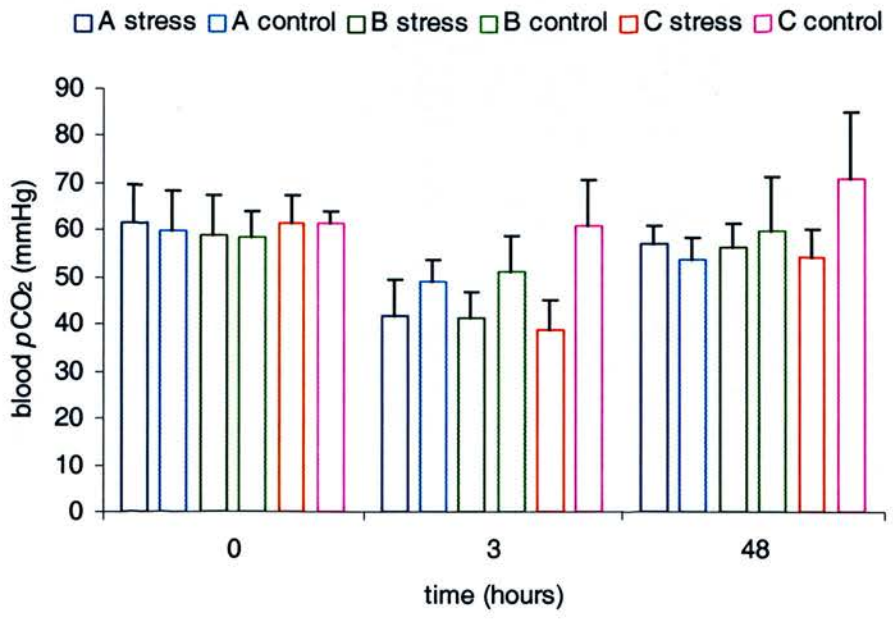



Figure 6.3 Blood $pCO_2$ (mmHg) of the heat stressed (S) and control (C) birds from Lines A, B and C at 0 (T0), 3 (T1) and 48 (T2) hours following initiation of exposure to heat stress/control conditions.

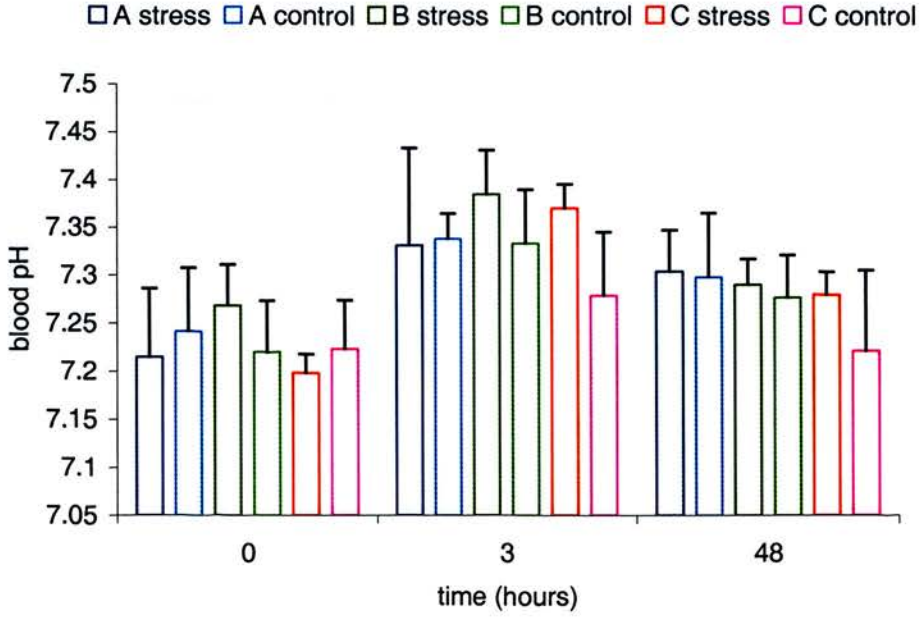



Figure 6.4. Blood pH of the heat stressed (S) and control (C) birds from Lines A, B and C at 0 (T0), 3 (T1) and 48 (T2) hours following initiation of exposure to heat stress/control conditions.

T1 ($P<0.05$) to 7.33, 7.39 and 7.37 respectively. Between T0 and T1, there was no significant change in mean blood pH of the control birds. The mean blood pH of the heat stress birds from each line and the corresponding control birds was not significantly different at T2. Although there was a significant effect of line, no appropriate significant pairwise differences were observed.

#### 6.4.3.3 Blood sodium concentration

There was no significant effect of treatment, time or line on blood sodium concentration (Table 6.2). However, there was a significant batch effect ($P<0.05$). The mean blood sodium concentration of batch 1 (143.2 $mmoll^{-1}$) was significantly lower than that of batch 2 (146.0 $mmoll^{-1}$) and batch 4 (145.7 $mmoll^{-1}$).

#### 6.4.3.4 Blood potassium concentration

There was a significant effect of time ($P<0.001$) and line ($P<0.05$) on blood potassium concentration (Table 6.2). There were no significant interaction and no effect of treatment or batch. The mean blood potassium concentration of Line A was significantly greater at T0 (5.7 $mmoll^{-1}$) than at T1 (4.8 $mmoll^{-1}$) ($P<0.05$). The mean blood potassium concentration of Line B was also significantly greater at T0 (5.7 $mmoll^{-1}$) than at T1 (5.0 $mmoll^{-1}$) ($P<0.05$).

## 6.5 Plasma analysis

### 6.5.1 Plasma creatine kinase (CK) activity

There was a significant effect of treatment ($P<0.05$), time ($P<0.05$), line ($P<0.05$) and batch on plasma creatine kinase activity (Table 6.7). There was a significant interaction between batch and line ($P<0.05$). Exposure to the control treatment resulted in significantly greater plasma CK activities compared to the heat stress treatment ($P<0.05$). Line A (1506 $IUl^{-1}$) had significantly higher plasma CK activities compared to Lines B (1120 $IUl^{-1}$) and C (1117 $IUl^{-1}$) ($P<0.05$). Batches 2

| plasma enzyme | time interval | line A | | line B | | line C | |
|---|---|---|---|---|---|---|---|
| | | control | stress | control | stress | control | stress |
| creatine kinase ($IUl^{-1}$) | T0 | 1368 (254) | 1342 (601) | 995 (311) | 1058 (321) | 982 (398) | 893 (357) |
| | T1 | 1807 (562) | 1658 (879) | 1305 (680) | 1076 (299) | 1561 (309) | 998 (340) |
| | T2 | 2227 (707) | 1430 (604) | 1497 (579) | 1239 (428) | 1533 (363) | 1434 (909) |
| lactate dehydrogenase ($IUl^{-1}$) | T0 | 228 (43) | 208 (48) | 218 (44) | 214 (45) | 231 (38) | 244 (37) |
| | T1 | 258 (50) | 237 (49) | 222 (51) | 211 (53) | 277 (67) | 234 (19) |
| | T2 | 305 (48) | 247 (83) | 255 (57) | 233 (81) | 248 (49) | 220 (52) |
| aspartate aminotransferase ($IUl^{-1}$) | T0 | 48.2 (11.3) | 52.3 (14.7) | 53.5 (6.2) | 52.1 (4.2) | 58.7 (18.4) | 66.6 (18.5) |
| | T1 | 52.8 (9.0) | 50.0 (11.6) | 52.7 (11.1) | 48.9 (4.4) | 52.4 (5.1) | 54.5 (15.5) |
| | T2 | 56.3 (16.1) | 51.2 (11.6) | 55.8 (8.7) | 54.8 (5.9) | 56.6 (8.0) | 63.6 (18.9) |

Table 6.7 Mean activities of plasma creatine kinase ($IUl^{-1}$), lactate dehydrogenase ($IUl^{-1}$) and aspartate aminotransferase ($IUl^{-1}$) of the heat stressed (S) and control (C) birds before the experiment (T0), and at 3 hours (T1) and 48 hours (T2) after exposure to the heat stress/control conditions (n=6), with 1 standard deviation in parentheses.

(928 $IUl^{-1}$) and 6 (1001 $IUl^{-1}$) had significantly lower plasma CK activities compared to batches 1 (1205 $IUl^{-1}$), 3 (1359 $IUl^{-1}$), 4 (1425 $IUl^{-1}$) and 5 (1620 $IUl^{-1}$) ($P<0.05$).

Pairwise comparisons did not reveal any significant changes in the mean plasma CK activity of the heat stressed or control birds from Lines A, B and C between T0 and T1, T0 and T2 or T1 and T2. However, large numerical increases in mean plasma CK activity were observed between T0 and T1 in the control birds from Lines A (1368 to 1807 $IUl^{-1}$), B (995 to 1305 $IUl^{-1}$) and C (982 to 1561 $IUl^{-1}$), and between T1 and T2 in the control birds from Line A (1807 to 2227 $IUl^{-1}$). The heat stressed birds from Line A showed a numerical increase in mean plasma CK activity between T0 and T1 (1342 to 1658 $IUl^{-1}$). An increase in mean plasma CK activity was also observed between T1 and T2 in the control birds from Line C (998 to 1434 $IUl^{-1}$), although the standard deviation of the latter value was very high (+/-909).

### 6.5.2 Plasma lactate dehydrogenase (LDH) activity

There was a significant effect of treatment ($P<0.05$) and batch ($P<0.001$) on plasma LDH activity and a significant interaction ($P<0.05$) (Table 6.7). The effect of time was just short of significance ($P = 0.057$). Exposure to the control treatment resulted in significantly greater plasma LDH activities compared to the heat stress treatment ($P<0.05$). The mean plasma LDH activity of the heat stressed birds in batch 1 (196 $IUl^{-1}$), were significantly lower that of batch 3 (251 $IUl^{-1}$) and 4 (276 $IUl^{-1}$). The mean plasma LDH activity of the heat stressed birds of batch 4 (276 $IUl^{-1}$) was significantly larger that of batch 6 (220 $IUl^{-1}$).

Between T0 and T1, notable numerical increases in mean plasma LDH activity were observed in the control birds from Lines A (228 to 258 $IUl^{-1}$) and C (231 to 277 $IUl^{-1}$). Increases in mean plasma LDH activity were observed in the control birds from Lines A and B between T1 and T2 (258 to 305 $IUl^{-1}$ and 222 to 255 $IUl^{-1}$). The heat stressed birds from Line A showed a large numerical increase in mean plasma LDH activity between T0 and T1 (208 to 237 $IUl^{-1}$).

### 6.5.3 Plasma aspartate aminotransaminase (AST) activity

There was no significant effect of treatment, time or line on plasma AST activity. However, there was a significant batch effect ($P<0.05$). The mean plasma AST activity of batch 6 (48.7 $IUl^{-1}$) was significantly lower than that of batch 3 (58.7 $IUl^{-1}$).

## 6.6 Characterising muscle fibre types and morphometry

NADH and myofibrillar ATPase staining of a selection of sections demonstrated that the *Pectoralis major* breast muscle of Lines A, B and C were almost entirely made up of fast, 'glycolytic', Type IIb fibres (Figure 3.5 and Figure 3.6). There was no significant effect of line or treatment on the mean Minimum Fibre Diameter (MFD) or connective tissue content of the *Pectoralis major* muscle (Table 6.8).

## 6.7 Muscle pathology assessment

Qualitative evaluations of pathology revealed fibre size variation, fibre splitting and 'tiny' fibres (Figure 3.22 and Figure 3.23) in many of the sections. Hyaline fibres were not observed in any of the sections. Sections from one or two birds from each group of six contained a low incidence (1-9) of basophilic fibres (Figure 3.26). Necrotic fibres were present in varying numbers in most sections assessed. Two sections contained >30 necrotic fibres (Figure 3.24 and Figure 3.25) per section (out of approximately 45,000 fibres). One section was from a muscle sample from a bird from Line A that had been exposed to heat stress. The other was from a muscle sample from a bird from Line B that had experienced control conditions. A further three sections contained 20-29 necrotic fibres per section. These were from muscle samples from a bird from each line (A, B and C) that been exposed to heat stress. There appeared to be a slightly greater incidence of necrosis amongst many of these sections from the Line A and Line C birds that were exposed to heat stress compared to the other groups.

| index | line A | | line B | | line C | |
|---|---|---|---|---|---|---|
| | control | stress | control | stress | control | stress |
| mean MFD (μm) | 38.6 (4.2) | 38.1 (5.9) | 41.6 (1.8) | 37.3 (5.2) | 41.0 (4.5) | 39.8 (2.7) |
| minimum MFD (μm) | 4.2 | 4.0 | 3.9 | 2.1 | 5.2 | 5.3 |
| maximum MFD (μm) | 72.7 | 81.5 | 84.0 | 71.5 | 85.5 | 83.4 |
| MFD range (μm) | 68.6 | 77.5 | 80.1 | 69.4 | 80.3 | 78.5 |
| connective tissue content (%) | 15.1 (0.9) | 15.2 (1.0) | 14.8 (0.7) | 15.9 (1.2) | 15.1 (1.1) | 15.1 (1.1) |

Table 6.8 Mean Minimum Fibre Diameter (MFD) (μm) (n=600 fibres), and connective tissue content (%) (n=6) and values for the MFD minimum, maximum and range (μm) of the *Pectoralis major* muscle from the heat stressed (S) and control (C) birds from Lines A, B and C, with 1 standard deviation in parentheses where appropriate.

| index | line A | | line B | | line C | |
|---|---|---|---|---|---|---|
| | control | stress | control | stress | control | stress |
| body weight (kg) | 2.3 (0.1) | 2.3 (0.3) | 2.3 (0.2) | 2.3 (0.2) | 2.7 (0.2) | 2.5 (0.3) |
| breast fillet weight (g) | 169 (18) | 174 (27) | 207 (12) | 190 (27) | 235 (31) | 214 (45) |
| breast yield (%) | 6.9 (0.7) | 8.0 (1.4) | 8.9 (0.5) | 8.3 (0.7) | 8.7 (0.9) | 8.5 (0.9) |

Table 6.9 Mean body weight (kg), breast fillet weight (g) and breast yield (%) of the heat stressed and control birds from Lines A, B and C (n=6), with 1 standard deviation in parentheses.

## 6.8 Meat quality analysis

### 6.8.1 Breast fillet yield

There was a significant effect of line ($P<0.05$) on breast fillet yield (Table 6.9). There was no effect of treatment or batch. The mean breast fillet yield of Line A (7.4%) was significantly lower ($P<0.05$) than that of Line B (8.6%) and Line C (8.6%). There was no significant difference in mean breast fillet yield between Lines B and C.

### 6.8.2 Breast fillet thaw loss

There was a significant effect of line ($P<0.05$) and batch ($P<0.05$) on breast fillet thaw loss (Table 6.10). There was no effect of treatment and no interaction. The mean breast fillet thaw loss of Line A (6.8%) was significantly lower ($P<0.05$) than that of Line B (9.7%). There was no significant difference in mean thaw loss between Lines B and C. The mean breast fillet thaw loss of batches 2 (6.8%) and 4 (6.3%) were significantly lower ($P<0.05$) than that of batch 6 (10.8%).

### 6.8.3 Breast fillet colour

There was no significant effect of line, treatment or batch on the white (L*) red (a*) or yellow (b*) colour of the breast fillet (Table 6.10).

### 6.8.4 Breast fillet haemorrhage score

There was a significant effect of line ($P<0.05$) on breast fillet haemorrhage score (Table 6.10). There was no significant effect of treatment or batch. The median haemorrhage score of the breast fillets from Line A birds (2) was significantly lower than that of the breast fillets from Line B (3) and C (3) birds.

| index | | line A | | line B | | line C | |
|---|---|---|---|---|---|---|---|
| | | control | stress | control | stress | control | stress |
| haemorrhage score | | 2 (0.5) | 2 (0) | 3 (0.75) | 3.5 (1.0) | 3 (0.5) | 3 (1.4) |
| fillet colour | L* (white) | 56.4 (3.0) | 54.1 (2.1) | 56.6 (4.2) | 57.6 (2.6) | 58.2 (2.4) | 56.7 (4.7) |
| | a* (red) | 3.5 (2.0) | 3.8 (1.4) | 3.3 (1.6) | 4.3 (1.8) | 3.3 (1.4) | 3.9 (1.6) |
| | b* (yellow) | 0.9 (0.4) | 0.6 (0.8) | 1.3 (1.4) | 1.6 (0.6) | 1.7 (0.6) | 0.9 (1.0) |
| thaw loss (%) | | 7.1 (1.9) | 6.3 (3.1) | 8.2 (4.6) | 9.6 (1.6) | 9.1 (3.6) | 7.1 (2.8) |

Table 6.10 Median haemorrhage scores, mean white, red and yellow fillet colour, and thaw loss (%) of the *Pectoralis major* breast muscle samples removed from the heat stressed and control birds of Line A, B and C, (n=6), with 1 standard deviation in parentheses.

| index | line A | | line B | | line C | |
|---|---|---|---|---|---|---|
| | control | stress | control | stress | control | stress |
| hardness (N) | 21.0 (5.0) | 19.5 (4.9) | 23.1 (7.3) | 23.1 (5.3) | 22.1 (8.1) | 16.7 (2.2) |
| work done (Nmm) | 175 (29) | 151 (42) | 186 (36) | 199 (33) | 181 (65) | 131 (22) |
| springiness index | 0.63 (0.19) | 0.56 (0.20) | 0.51 (0.24) | 0.70 (0.48) | 0.63 (0.44) | 0.62 (0.29) |
| cohesiveness | 0.047 (0.017) | 0.072 (0.026) | 0.051 (0.025) | 0.047 (0.01) | 0.043 (0.026) | 0.062 (0.033) |
| adhesive force (N) | 2.4 (4.0) | 4.6 (4.8) | 3.9 (5.9) | 6.3 (3.3) | 6.0 (3.8) | 3.9 (2.5) |

Table 6.11 Values obtained for hardness (N), work done (Nmm), springiness index, cohesiveness and adhesive force during the texture analysis of the *Pectoralis major* breast muscle samples removed from the heat stressed and control birds of Line A, B and C, (n=6), with 1 standard deviation in parentheses.

### 6.8.5 Texture analysis

There was no significant effect of line, treatment or batch on any of the measured indices of texture (hardness, work done, springiness index, cohesiveness and adhesive force) (Table 6.11).

*Meat quality summary*

Exposure to heat stress conditions did not induce changes in any of the meat quality attributes measured. Line differences were observed in mean breast fillet yield, mean breast fillet thaw loss and median haemorrhage score of the breast fillets.

## 6.5 Discussion

Distinct differences in the genetic make-up exist between the three great-grandparent lines examined in this study. Lines A and B are female lines which are selected for differing egg production traits. Line C is a male line, and has been selected primarily for growth related traits. The mean body weight of the male line, Line C (2.6 kg) was significantly greater compared to the two female lines, Lines A and B (2.3 kg). However the mean breast yield of both Lines B and C (8.6%) was significantly greater than that of Line A (7.4%). Line B is therefore notable in its ability to match the meat yield of the male line, whilst maintaining a high reproductive capacity.

Continuous recordings from the TinyTalk dataloggers (Table 6.1) revealed that in the chamber programmed to heat stress conditions, the temperature inside a crate filled with chickens was 1°C higher compared to room temperature. In the control chamber, room temperature was approximately 3°C lower than that of the crate containing the birds. The increased temperature inside the crates was due to the air gaining heat from the birds. In the chamber programmed to heat stress conditions, the relative humidity inside a crate filled with chickens was more than 20% lower than that of the chamber, due to the increased temperature caused by the presence of the birds. The changes in the temperature and relative humidity inside the crate placed in the heat stress chamber that were induced by the presence of birds are consistent with the continuous recordings of Chapter 5.

The mean body temperature (determined by rectal probes) of all three lines (Table 6.2 and Figure 6.1) increased following exposure to the heat stress conditions (T1). Blood gas analysis revealed that between T0 and T1, the birds had been flushing out carbon dioxide during hyperventilation, that lead to respiratory hypocapnic alkolosis, with recovery by T2 (48 hours following initiation of heat stress exposure) (Table 6.2; Figure 6.3 and Figure 6.4). In contrast to the study outlined in Chapter 5, these changes in blood gas chemistry were only significant in the birds exposed to heat stress conditions.

Qualitative examination of the continuous body temperature recordings from the dataloggers implanted inside the birds revealed that the body temperature of all of the birds greatly increased during the heat stress period (Table 6.3). There were no obvious differences between the three lines in the range of body temperatures recorded during the control, stress or recovery periods; the rate of change in body temperature when the heat stress conditions were initiated and terminated; the ability to adapt during the heat stress and reduce body temperature and in overcompensation following removal from the heat stress conditions to a body temperature below the mean control body temperature. However, there was considerable variation between individual birds within a line. Therefore, it appears that the thermoregulatory ability of no one line has been inadvertently compromised or improved following selection for different traits. This is in contrast to a previous comparative study of three great-grandparent lines (different lines to the present study), where marked line differences in thermoregulatory capacity were observed (Gonet *et al*, 2000). Despite rigorous commercial genetic selection procedures, large variation in thermoregulatory ability still exists within each of the lines examined in the current study. This variation could be reduced by the incorporation of selection criteria for thermoregulatory ability into the current genetic programmes of each line

The significant increase in the activities of the CK and LDH enzyme markers at T1 in the birds exposed to control conditions (Table 6.7) only was surprising, as the control birds exhibited a less severe hyopcapnic alkalosis of the blood compared to the birds exposed to heat stress at this time point. These results suggest that other factors are associated with induction of muscle damage as well as respiratory alkalosis. The findings of the present study are contradictory to previous studies on broiler chickens, where heat stress induced respiratory alkalosis and increases in plasma CK activity were observed in the birds exposed to heat stress conditions only (Mitchell and Sandercock, 1995b). Activities of enzyme markers were not determined at 12 or 24 hours following initiation of exposure to heat stress/control conditions in this study. It is likely that the activities of the enzyme markers

increased during this period in one or both of the groups (heat stress/control) giving a similar profile to that observed in Chapter 5.

Qualitative evaluations of muscle pathology did not reveal any overt effects of heat stress or differences between lines, although a slightly greater incidence of necrosis in the sections from birds exposed to heat stress from Lines A and C was recorded. The corresponding plasma activities of the enzyme markers were not elevated. The lack of a relationship between the degree of structural damage and the plasma activities of the enzyme markers was also observed in Chapter 5, and in previous studies (Van der Meulen *et al*, 1991; Komulainen and Vihko, 1994).

The morphometric and stereological data from the muscle section analysis demonstrate the role of the number of fibres set an embryonic development in muscle growth potential (Table 6.8). The mean breast fillet weight of the line A (169g) was significantly lower than that of Lines B (198g) and C (225g). Assessment of muscle sections revealed no differences between lines in either mean fibre size or connective tissue content. Therefore, additional fibres must provide the additional weight in the breast fillet of Lines B and C, compared to Line A. Lines B and C would have attained a given breast weight in a shorter time period than Line A, and have a greater overall growth potential. Again, Line B is notable in its ability to match the growth potential of the male line (Line C), whilst maintaining a high reproductive capacity. Larger fibre numbers at hatch have also been reported in commercial broilers (Remignon *et al*, 1995) and turkeys (Mills, 2001), compared to more traditional lines.

Effects of heat stress observed in previous studies on poultry meat quality attributes include a reduced pH (Holm and Fletcher, 1997; Sandercock *et al*, 2001), increased water loss (Northcutt *et al*, 1994; Sandercock *et al*, 2001), increased haemorrhaging (Sandercock *et al*, 2001), paler fillet colour (Babji, Froning and Ngoka, 1982) and tougher meat (Holm and Fletcher, 1997). Exposure to heat stress is associated with PSE-like (pale, soft and exudative) changes in poultry breast meat quality (Northcutt *et al*, 1994; McKee and Sams, 1997). If such muscles are used for further

processing, the product on the supermarket shelf often presents high package exudates, poor texture, cohesiveness and juiciness (World Poultry, 1996).

In the present study, there was no effect of the heat stress treatment on any of the meat quality indices assessed (Table 6.10 and Table 6.11). This may be because the birds were slaughtered 48 hours following initiation of exposure to heat stress/control conditions. This was to optimise visualising any histopathological alterations in muscle structure induced by the heat stress. The heat stress may have induced increased muscle glycolytic activity and an associated drop in pH, but the muscle metabolism may have recovered by the time the birds were slaughtered.

The pH of meat generally declines after slaughter. This is due to lactate accumulating as a by-product of glycogenolysis and anaerobic glycolysis processes that continue in the cytosol post-slaughter. Water binding capacity is the ability of meat to bind its own water and is modified by pH. Water binding capacity is high at pH 10, and decreases to a low capacity at pH 5.0 and 5.1 (the isoelectric point of meat proteins) (Swatland, 1994). Towards their isoelectric point, thick and thin filaments in myofibrils move closer together and reduce the water space between them. Thus, as the pH declines post mortem, filaments move closer together, myofibrils shrink and the volume of sarcoplasm increases. Eventually, muscle membranes are no longer able to confine the cell water and fluid is lost from the muscle fibre, contributing to exudate lost from the meat (Swatland, 1994). In the present study water holding capacity was estimated by measuring the water loss from the fillet during muscle thawing. The reduced mean breast fillet percentage thaw loss of Line A compared to Line B (Table 6.10) may reflect a reduced rate of pH decline in the breast muscle of Line A post-slaughter.

It has been proposed that haemorrhages in chicken breast fillets are the result of a local rise of blood pressure because of accumulation of blood causes the rupture of small, thin walled venules and collecting veins (Kranen, Lambooy, Veerkamp, Kuppevelt and Veerkamp, 2000). The median haemorrhage score of the breast fillets from Line A (2/5) were significantly lower than that of Lines B and C (3/5)

(Table 6.10). This may be attributable to the smaller size of the Line A breast muscles compared to those of Lines B and C and the smaller forces generated when flapping during capture and restraint (Sandercock *et al*, 2001), and during the simultaneous excitation of all of the breast muscle fibres immediately following slaughter (Kranen *et al*, 2000).

A number of studies have suggested that genetic selection for rapid growth and breast muscle development has had a detrimental effect on indices of poultry breast meat quality including paler colour (Le Bihan-Duval *et al*, 1999; Sandercock, Hunter, Mitchell, Robertson and Hocking, 1999; Sante, Bielicki, Renerre and Lacourt, 1991), increased haemorrhaging (Sandercock *et al*, 1999) and accelerated muscle pH decline post-slaughter (Sante *et al*, 1991). It is therefore possible that selection for the increased breast yield of lines B and C is associated with the increased haemorrhages and reduced water holding capacity of breast fillets from these lines. However, whilst the breast fillets of the great-grandparent lines differed in appearance and functionality, no between lines differences were observed in the texture analysis (Table 6.11).

The different genetic selection strategies imposed on Lines A, B and C has resulted in alterations in some, but not all indices of meat quality. Although it is difficult to rank the indices in order of importance to the consumer, meat texture is probably the single most critical quality factor associated with the customers' ultimate satisfaction with a poultry meat product (Fletcher, 2002). With uniformity of breast fillet texture across the pedigree lines, there should be little textural variation between the fillets of the descendant broilers.

Genetic selection for production traits such as growth rate, slaughter weight and feed conversion efficiency has been extremely successful in poultry. However, the traditional selection for these traits may have been almost fully exploited; at 42 days of age an average broiler chicken may reach a body weight of 2.2 kg having consumed only 1.82 g of food per 1.0 g of body weight gain (Mitchell, 1998). However this intense selection may have lead to an increased susceptibility to growth and stress induced myopathies in broiler chickens, which may have implications for both birds welfare and meat quality (Sandercock *et al*, 2001).

A comparison of growth in three chicken lines selected for divergent production traits (Chapter 3), revealed that by 25 weeks of age the mean body weights reached by the broiler and great-grandparent lines (5.1 and 5.2 kg respectively) was more than twice that of the layer line (1.9 kg). It is likely that the greater body weights observed in the broiler and great-grandparent lines are due to increased muscle yield, particular of the *Pectoralis major* breast muscle, which in turn may be related to increased muscle fibre sizes (estimated by measurements of minimum fibre diameter (MFD)). This is supported by the following findings of this study.

- By 25 weeks of age the mean *Pectoralis major* breast muscle fibre sizes reached by the broiler and great-grandparent lines (65.9 μm and 59.8 μm respectively) were 1.5 times greater than that of the layer line (38.1 μm).

- There was divergence in muscle fibre growth between the *Pectoralis major* breast muscle fibres and the *Biceps femoris* leg muscle fibres of the broiler and great-grandparent lines, but not the layer line during the growth period. This was reflected in the mean *Pectoralis major* MFD: *Biceps femoris* MFD ratios over the eight age intervals, which were considerably greater than 1 for the broiler and great-grandparent lines (1.33 and 1.27 respectively) and close to one 1 for the layer line (1.06).

- At 5 weeks of age the broiler *Pectoralis major* breast muscle fibres were considerably larger than the fibres of the other muscles assessed (*Coracobrachialis*, *Biceps femoris*, *Gastrocnemius* and *Peroneus longus*).

- The fibres of the broiler *Biceps femoris*, *Peroneus longus* and *Gastrocnemius* leg muscles did not differ in size at 5 weeks of age. However, between 16-23 weeks of age the *Biceps femoris* muscle fibres were smaller than both the *Peroneus longus* and *Gastrocnemius* fibres. The divergence in size after the commercial slaughter age may be an adaptation to the larger body weights that the muscles are supporting at the older age groups.

However, increased longitudinal fibre growth, increased fibre number (hyperplasia) and a greater fibre number set in the embryo could have also contributed to muscle growth, and should be investigated in further studies (discussed in Chapter 8).

The large muscle fibres of the broiler and great-grandparent lines may be reaching the maximum functional size constraints. The increased oxygen diffusion distances of large fibres may reduce oxidative capacity and alter mitochondrial distributions within the fibre. Cores and rims were observed in the leg muscle sections from older broiler and great-grandparent lines, and may result from a combination of larger fibre size and inadequate development of the supporting capillary supply. The split fibres observed could be an adaptive response to the metabolic stress associated with the increased diffusion distances for oxygen, metabolites and waste products in larger fibres (Mahon, 1999).

Whilst internal nuclei in human muscle fibres are indicative of myopathy (Dubowitz and Brooke, 1973), this is not the case in poultry (Pizzey and Barnard, 1983). At least one internal nucleus per fibre was observed in the majority of *Pectoralis major* fibres, but in fewer of the corresponding (smaller) *Biceps femoris* fibres. Furthermore, there was a linear relationship between the percentage of fibres containing at least one internal nucleus and the corresponding fibre size of the

*Biceps femoris* for the great-grandparent and layer lines. Therefore, the centralised nuclei may play a specialised role in fibre size regulation in specific avian muscles.

Serial determinations of enzyme markers of muscle damage between 5 and 25 weeks of age were indicative of a growth associated myopathy in the broiler, great-grandparent and layer lines. However, the extent of the myopathy was greater in the broiler and great grandparent lines, which are primarily selected for growth related production traits. Histopathological assessments of muscle sections also revealed muscle damage. Abnormal structural features occurred primarily in the broiler muscles, to a lesser extent in the great-grandparent muscles, and at a comparatively very low incidence in the layer muscles. The type of structural abnormality observed seemed to be related to bird age, muscle type and ovarian estradiol secretion.

Necrotic and basophilic fibres were the predominant types of abnormal fibres observed in sections of *Pectoralis major* muscle samples, and were prominent in the 5 week old broilers. This may be a consequence of muscle fibre growth outstripping that of the connective tissue and capillary support systems. (Kranen *et al*, 2000; Swatland, 1990) The absence of structural abnormalities present in the corresponding unselected *Coracobrachialis* muscle sections supports this hypothesis. In contrast hyaline and basophilic fibres were the predominant types of abnormal fibres observed in sections of the leg muscles of older broilers. The hyaline fibres may be associated with the large body weights, which may have overloaded the weight bearing leg muscles.

Marker activities peaked and started to decrease before the increase in egg yolk precursor production and calcium intake in all three lines. Therefore, a mechanism through which estrogen that protects muscle function in the face of the calcium requirements for egg-shell synthesis appears to be in place before the induction of intestinal adaptations to absorb a greater amount of calcium. This myo-protective effect was also observed in the muscle sections. Solely regenerative rather than degenerative, necrotic processes were associated with the large numbers of hyaline

fibres in the broiler leg muscles. The increased levels of ovarian estrogen secretion may induce increased satellite cell activity and fibre repair and regeneration following muscle damage. Further studies examining this hypothesis would be informative (discussed in Chapter 8).

The protective action of estrogen on avian skeletal muscle may involve receptor mediated gene regulation. This hypothesis is supported by the purely antagonistic action of tamoxifen on the myo-protective action of estrogen administration to birds (Carlisle *et al*, 1997), coupled with the presence of both the alpha and beta estrogen receptor mRNA in chicken skeletal muscle (Chapter 4). However, the ways in which estrogen exerts myo-protective effects are likely to be complex. As well gene regulation through intracellular estrogen receptors, this may also involve plasma membrane estrogen receptors, membrane stabilisation and free radical reduction as well as other, as yet unknown, mechanisms. It would be useful to undertake further research on the myo-protective actions of estrogen in birds (discussed in Chapter 8).

A wide range of factors can induce muscle damage, primarily through the loss of calcium homeostasis, the loss of energy supply to the cell or over-activity of oxidising free radical-mediated reactions (McArdle and Jackson, 1997). These factors include exercise (Apple, Rogers, Casal, Sherman and Ivy, 1985; Amelink and Bar, 1986); hyperthermia (Mitchell and Sandercock, 1995a; Sandercock *et al*, 2001); toxic substances such as monensin (Dowling, 1992) and dietary deficiencies (Hassan *et al*, 1990). The growth-associated myopathy apparent in broiler chickens (Chapter 3) may predispose birds to further heat stress induced myopathy (Mitchell and Sandercock, 1995a). Heat stress is commonly experience by broiler chickens in the commercial environment, during handling, transportation and holding (Mitchell and Kettlewell, 1998). A study was undertaken to determine the profile of elevated plasma enzyme markers of muscle damage and structural muscle damage in the 48 hour period following exposure of broiler chickens to acute heat stress.

All or a combination of catching, handling, blood sampling and crating procedures induced a hypocapnic alkalosis response in the blood of the broilers. The heat stress

did not exacerbate this response, despite an increase in the body temperature of the birds exposed to heat stress from 40.8°C to 42.5°C. Previous studies have also induced hyperthermia by exposing broilers to heat stress (Mitchell and Sandercock, 1995a; Sandercock *et al*, 2001). However, these previous heat stress studies induced greater elevations in body temperature of 44.5°C (Mitchell and Sandercock, 1995a) and 43.5°C (Sandercock *et al*, 2001) compared to the present experiment. The enzyme marker profiles of muscle damage were also similar for the birds exposed to heat stress and control conditions, with activities peaking at least 12 hours after the initiation of exposure to heat stress/control conditions. Therefore all or a combination of the catching, handling, blood sampling and crating procedures induced muscle damage.

The presence of hyaline fibres in *Pectoralis major* and *Biceps femoris* muscle samples may be indicative of stress-induced damage. However inflammatory responses to the necrotic enteritus infection were also observed in the muscle tissue, making it difficult to confidently interpret the effect of the experimental procedures on muscle structure.

The absence of heat-stress induced muscle damage is contrary to previous studies (Mitchell and Sandercock, 1995, Sandercock *et al*, 2001) and surprising, as the birds exposed to heat stress conditions exhibited hyperthermia. It is likely that the imposed heat stress was not severe enough to cause muscle damage. However, the stress-induced muscle damage response due to all or a combination of the catching, handling, blood sampling and crating procedures, is directly relevant to the poultry industry. Commercial catching, handling and crating methods are far rougher than the procedures used in this study and may therefore induce greater levels of muscle damage. Further studies examining the individual contributions of the catching, handling, sampling and crating procedures to stress induced muscle damage would be beneficial (discussed in Chapter 8).

The growth associated myopathy (Chapter 3) and stress induced myopathy (Chapter 5) observed in broilers may have detrimental effects on meat quality. A study was

undertaken to assess whether there are associations between stress-induced muscle damage (determined by changes in enzyme markers and structural abnormalities) and alterations in meat quality attributes in three lines of great-grandparent broiler chickens (Chapter 6). Again, the stressor was heat exposure, but at an increased temperature of 32°C.

The continuous recording of body temperature during control, heat stress and recovery periods revealed that the body temperature of all of the birds greatly increased during the heat stress period. There was considerable between-bird variability within a line. However, genetic selection for the line-specific traits has not lead to between-line differences in thermoregulatory ability.

In contrast to the study described in Chapter 5, the acute heat stress induced a greater hypocapnic alkalosis compared to the control conditions, although this was not accompanied by elevated activities of plasma markers of muscle damage immediately after the heat stress. The birds were only subsequently sampled at 48 hours (but not 12 or 24 hours) after the initiation of exposure to heat stress/control conditions. From the profile of the enzyme markers of muscle damage determined in Chapter 5, the activities of the enzyme markers may have peaked during this period in the birds subjected to one or both of the treatments. The birds were slaughtered 48 hours following initiation of exposure to heat stress/control conditions. This was to optimise visualising any histopathological alterations in muscle structure induced by the heat stress. Histopathological analysis revealed a slightly greater incidence of necrosis in the muscles of birds exposed to heat stress from Lines A and C.

The histological and enzyme marker data described in Chapter 3 showed that plasma enzyme markers of muscle damage are only indicative of the really large changes in pathology incidence, with little or no correspondence between relatively low levels of structural abnormalities and marker enzyme activities. The structural abnormalities observed in Chapters 5 and 6 generally correspond to the 'lower end' of the spectrum of pathology incidence observed in the study described in Chapter 3.

Therefore the lack of correspondence between the incidence of structural abnormalities and marker enzyme activities in the present study is not surprising. The environmental conditions of Chapters 5 and 6 (30°C and 75% RH; 32°C and 75% RH respectively) did not appear to be severe enough to cause heat-stress induced muscle damage in the broiler chickens. A more challenging environment that causes broiler body temperatures to approach the upper lethal limit of 46°C (Sturkie, 1986) may induce muscle damage characterised by a closer correspondence between the activities of the plasma markers of muscle damage and the structural abnormalities observed in the muscle tissue.

The study detailed in Chapter 6 also demonstrated the role of the number of fibres set an embryonic development in muscle growth potential. There were no differences between lines in either mean fibre size or connective tissue content. However, the mean breast fillet weight of Lines B (198g) and C (225g) were greater than that of line A (169g). Therefore additional fibres must be providing the additional fillet weight, giving Lines B and C a greater overall growth potential.

There was no effect of the heat stress treatment on any of the meat quality indices assessed (colour, haemorrhage score, thaw loss and texture). Any alterations in muscle metabolism induced by the heat stress may have recovered in the 48 hour period between heat stress and slaughter. There were lines differences in some meat quality indices. Line A, with the smallest body weight and breast yield, produced breast fillets with the lowest median haemorrhage score and percentage thaw loss. Therefore this study is in agreement with previous work that has suggested that selection for increased breast yield may be associated with alterations in meat quality (Dransfield and Sosnicki, 1999; Remignon *et al*, 1996; Le Bihan-Duval, Millet and Remignon, 1999). However, breast fillet texture, the most critical quality factor associated with the customer satisfaction (Fletcher, 2002), was uniform across the pedigree lines.

This project has demonstrated that genetic selection for growth related production traits has lead to a marked growth associated myopathy in the broiler chicken. The predominant type of structural muscle damage observed is determined by the age and muscle of the bird. Increased ovarian estrogen secretion appears to ameliorate this myopathy, possibly through satellite cell activation and fibre repair and regeneration. This myoprotective action may involve estrogen receptor α and or estrogen receptor β. The growth associated myopathy apparent in immature broiler chickens may predispose birds to further stress induced myopathy as a result of commercial catching, handling and crating procedures. The three broiler great-grandparent lines studied were equal in their thermoregulatory ability during exposure to an acute heat stress. Heat stress appeared to induce a slight increase in structural muscle damage in two of the great-grandparent lines, but did not lead to any alterations in meat quality 48 hours post heat stress. However, this does not preclude changes in meat quality immediately after heat stress.

## Longitudinal fibre growth

It would be useful to study longitudinal as well as radial growth of breast and leg muscle fibres during the growth and development of the broiler, great grandparent and layer lines. Studies in turkeys have demonstrated that the degree of longitudinal compared to radial growth varies between muscles (Swatland, 1979a; 1979b), but no comparable work has been undertaken in chickens.

## Number of muscle fibres set at embryonic development

The characterisation of the number of fibre numbers set at embryonic development in the broiler, layer and 3 great-grandparent lines would be valuable, as this may contribute to muscle growth potential post-hatch. It has been proposed that an increased number of fibres contribute to muscle mass in commercial meat-type chickens (Remignon *et al*, 1995) and turkeys (Mills, 2001). In the present study, the fibre sizes and connective tissue content of the *Pectoralis major* breast muscle of 3 great-grandparent lines were the same, despite differences in breast fillet weights. Therefore a greater number of fibres (set in the embryo) must be providing the additional fillet weight. Selection for increased embryonic muscle fibre number, rather than for increased radial fibre growth could improve growth potential. It may also alleviate muscle damage due to fibre growth outstripping the support systems and large fibres splitting due to metabolic stress.

## The role of satellite cells in muscle growth and regeneration

Post hatch muscle growth and nuclear accretion occurs through the fusion of satellite cells to the growing muscle cells. It has been proposed that the capacity for satellite cell proliferation differs between heavy- and light- weight strains of turkey (Merley *et al*, 1998). Furthermore, satellite cells appear to have a greater activity in response to mitogenic stimuli in commercial compared to unselected turkey lines (McFarland

*et al*, 1993) and fast growing compared to slow growing chicken lines (Duclos *et al*, 1996). Satellite cells derived from the broiler *Biceps femoris* muscle are more responsive to mitogenic stimuli compared to satellite cells derived from the broiler *Pectoralis major* muscle. However, satellite cells derived from the broiler *Pectoralis major* muscle fuse more rapidly to form multinucleated myofibres than those derived from the broiler *Biceps femoris* muscle (Kocamis, McFarland and Killefer, 2001). It would be useful to assess satellite cell activity *in vivo* in leg and breast muscles of the broiler, layer and three great grandparent lines at commercially relevant ages, body weights and muscle yields. Active satellite cells are identifiable by labelling skeletal muscle sections with fluorescently tagged antibodies to show both the incorporation of bromodeoxyuridine (BrdU) and the presence of the basal lamina component laminin (Brotchie, D., Davies, I., Ireland, G. and Mahon, 1991). The study of the cellular factors controlling satellite cell activity in chicken muscle, such as muscle-specific transcription factors (MyoD, myogenin, myf5 and MRF4), growth factors, hormones and neurotransmitters (Olsen, 1993) would also be informative. In particular, it would be interesting to examine the interactions between increased levels of ovarian estrogen secretion, the associated muscle regeneration observed in Chapter 3, and satellite cell activity. Characterising satellite cell activity and understanding the controlling cellular pathways could lead to improvements in broiler and great-grandparent muscle growth and its ability to regenerate following muscle damage (McFarland, 1999).

**The myo-protective effect of estrogen**

To reinforce the demonstration of ERα and ERβ mRNA in mature chicken skeletal muscle, it would be useful to demonstrate the presence of the respective proteins in muscle tissue. This could be investigated using antibodies specific to ERα and ERβ using either immuno-histochemical staining techniques on muscle sections or western blotting (protein electrophoresis followed by blotting onto a nitrocellulose membrane and labelling with specific antibodies). It would also be valuable to assess changes in ERα and ERβ expression in immature and mature birds. An up-regulation of the ERα and/or ERβ would imply involvement in the myoprotective

effect of estrogen associated with the onset of sexual maturity observed in Chapter 3. This could be investigated using immuno-histocytochemical or real-time PCR techniques to quantify the protein and mRNA respectively.

Using similar molecular and immunological techniques, it would be interesting to study the expression of calcium binding proteins calbindin $D_{28k}$ and calbindin $D_{9k}$ in skeletal muscle of immature and mature chickens. Calcium transport is thought to involve estrogen interacting with estrogen receptors to promote the expression of calbindin $D_{28k}$ in the small intestine, which protects the enterocytes from the increased calcium load associated with the onset of sexual maturity (Wu *et al*, 1994). A parallel protective mechanism may exist in the skeletal muscle fibres. $1,25(OH)_2D_3$ induces the synthesis of calbindin $D_{9k}$ in chick embryo myoblasts (Lila, Susana and Ricardo, 1994), therefore this protein may also mediate protective mechanisms in the intestine and skeletal muscle.

Studies in mammals have shown that estrogen inhibits the activation of calpain, a protease involved in degenerative processes in the muscle following damage. It would be informative to determine whether this mechanism is also in place in avian skeletal muscle. Again, molecular and immunological techniques could be employed to quantify calpain expression in immature and mature chickens.

**Stress-induced muscle damage**

All or a combination of catching, sampling, crating and handling procedures caused stress-induced muscle damage. It would be interesting to assess the effect each of these procedures in isolation have on blood acid-base chemistry, enzyme markers of muscle damage and changes in muscle structure.

It would also be useful to repeat the study that examined the profile of muscle damage following exposure to heat stress (Chapter 5), using higher temperature and humidity combinations. This would determine whether the heat stress conditions the

birds were exposed to in the present study were not severe enough to induce muscle damage.

Heat shock proteins (HSPs) are induced in cells by heat or several other types of stresses (including exercise), and are often used as markers of stress and adaptation in a variety of physiological systems (reviewed in Kregal, 2002; Moseley, 1997). It has been proposed that the HSPs provide protection against stress-induced muscle damage (Maglara *et al*, 2003; Oishi *et al*, 2002). It would be interesting to measure the expression of the most stress-inducible HSPs - HSP60, HSP70, HSP72 and HSC73 (Oishi *et al*, 2002), following exposure to stress (heat / catching / handling / sampling / crating) using molecular and immunological techniques. Muscle type-specific responses of the expression of HSPs during recovery after elevation of muscle temperature have been demonstrated in mammalian studies (Oishi *et al*, 2002). Therefore it would be informative to compare HSP expression in the predominantly glycolytic *Pectoralis major* breast muscle with a more oxidative leg muscle (e.g. *Biceps femoris*, *Gastrocnemius* or *Peroneus longus*). Furthermore, it has been proposed that the greater HSP70 induction in males compared to females after physiological stress is mediated through a protective effect of estrogen (Paroo, Dipchand and Noble, 2002). Therefore, it would also be useful to compare HSP expression in immature and mature chickens, in conjunction with the further studies investigating the myo-protective effects of estrogen proposed earlier.

**Meat quality**

The results of these two latter studies would allow further studies on the associations between stress (heat / catching / handling / sampling / crating), muscle damage and meat quality indices in broiler and great-grandparent lines.

**Microarray technology**

mRNA profiling methods using microarrays allows thousands of genes to be assayed for. Expression profiling studies on mammalian muscle have investigated

the molecular diversity of muscle fibre types; physiological plasticity and adaptation of muscle; muscle atrophy; muscular dystrophy and muscle regeneration (reviewed in Hoffman, DuBois, Hoffman and Almon, 2003). This technique could be employed to undertake comparable studies in chicken muscle, examining changes in the muscle mRNA profile induced by fast growth, stress and increased ovarian estradiol secretion. However, the majority of biochemical pathway regulation occurs at the protein level, not during gene transcription, therefore this technology is limited by the lack of a parallel protein dataset.

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