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GENETICS OF MUSCLE AND MEAT QUALITY IN CHICKEN

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Table of Contents

List of Figures

List of Tables

List of Appendices

Preface

The work contained within this thesis is my own and has not been done in collaboration, except where otherwise stated. The text does not exceed 70,000 words. No part of this thesis has been submitted to any other university in application for a higher degree.

Imran Zahoor

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Dedication

I dedicate my thesis to my country, Islamic Republic of Pakistan.

Abstract

Skeletal muscles in broilers are generally characterised by pathological muscle damage, indicated by greater plasma creatine kinase (CK) activity, higher incidence of haemorrhages, lighter and less coloured breast muscles, compared with layers and traditional breeds of chicken. Muscle damage is further exacerbated by exposure to stressful conditions such as high ambient temperatures which results in a further decrease in the quality of broiler meat and leads to the production of pale, soft and exudative (PSE) meat**.** This growing incidence of poor quality poultry meat is causing substantial losses to the meat industry. However, in contrast to pork the genetics of poor muscle and meat quality in chicken is unknown. The present project was conducted to identify the underlying genetics of this low quality meat by using heat-stress as a tool to amplify muscle damage and expression of the relevant genes. Whole-genome expression studies in broiler and layer breast muscles were conducted before and after heat-stress and some phenotypic data were also recorded. From the gene expression studies, 2213 differentially expressed genes ($P<0.05$) were found. About 700 of these genes had no gene ontology (GO) terms associated with them for biological process or function. The significant gene set was analysed in BioLayout Express and interesting clusters of the genes, based on their positive correlation with each other, were selected for further investigation. Genes were grouped together in 6 different categories or clusters, on the basis of their expression pattern. The genes in the selected clusters were analysed in Ingenuity Pathway Analysis (IPA) software, for each category separately, and relevant biological pathways and networks for those genes were studied. Similarly, the genes filtered out

by BioLayout Express at a Pearson threshold of 0.80 were also analysed in IPA separately and interesting pathways and networks were selected. From the pathways and networks analyses of these genes, it was discovered that genes involved in inflammatory, cell death, oxidative stress and tissue damage related functions were up-regulated in control broilers compared with control and similar to heat-stressed layers. After exposure to heat-stress the expression levels of these genes were further increased in broilers. These results led us to develop the hypothesis that breast muscles in broilers are under stress-related damage even under the normal rearing conditions. This hypothesis was tested by rearing the broilers birds at normal/conventional and comparatively low ambient temperature and its effects on breast muscle quality and meat quality were studied. Significant improvement of breast muscle redness was observed. Additionally substantial numerical improvements for other meat and muscle quality traits like breast muscle lightness and histopathology were observed. From the key positions of interesting significant pathways and networks, candidate genes were selected for further investigation. In total, 25 candidate genes were selected for SNP genotyping: 19 genes were selected from the interesting pathways and networks and 6 genes were selected on the basis of their GO terms. For each gene 4-5 SNPs were selected, where possible, that were present in exons and promoter regions of the candidate genes. The selected SNPs were genotyped for muscle and meat quality traits in 34 breeds of chicken and significant causative SNPs for each trait including plasma CK activity, pHi and pHu for breast muscles, colour (L*, a*, and b*) traits for breast and thigh muscles were found. These SNPs were responsible for explaining a moderate to high (15-55%)

percentage of phenotypic variance for these traits. To our knowledge this is the first study in which gene-expression in chicken breast muscle was conducted in response to heat-stress and additionally, for the first time, a set of novel SNPs for all of these traits were identified. Some of the significant causative SNPs were lying in the protein coding sequences and some were present in the promoter regions of the candidate genes.

Chapter 1 Introduction

1.1 Poultry Industry

Poultry are a major source of providing high-quality protein to people around the globe, at consistently low prices. The poultry industry is also one of the world's fastest growing industries, in spite of a number of constraints such as rising feed prices, diseases like avian influenza (bird flu), and a number of management and environmental stress factors such as catching, crating, transportation, cold stress and especially heat stress in the tropical and sub-tropical parts of the world.

Between 1995 and 2005, there was a remarkable increase in the consumption and production, globally, of poultry products such as (percentage increase) chicken meat (53%), turkey meat (13%), duck meat (67%), goose meat (53%), chicken eggs (39%), and other eggs (27%). The top ranking countries with the largest increases for chicken meat production were India (217%), Brazil (112%), and China (67%); the increases for the USA and Europe were 38% and 30% respectively during this period (Scanes 2007).

The total world poultry meat production was less than 5 kg per capita in 1965, and it has grown to more than 13 kg per capita in 2006. In 2006 the world poultry meat production was 81 Million tons (Mt), the major portion of which (70 Mt) was produced by broiler chickens. The production of 71 Mt of chicken meat needs a crop of at least 40 billion broilers, per year. Comparative production statistics also show a higher growth rate for the poultry sector (53% for poultry meat), compared with other areas/sectors of meat production (Scanes 2007; McKay 2009).

1.2 Genetic Selection and Modern Broiler Industry

In the broiler industry, about 3% annual improvement in the efficiency of meat production has been observed, as a result of genetic selection for growth, body composition, feed efficiency, reproduction, health and welfare-related traits (Havenstein et al. 2003; McKay 2009). Genetic selection has resulted in reducing 1 day per generation in the market age of commercial broilers, between 1970 to 1990 (Anthony, 1998). In the 1940s, broiler birds took about 16 weeks to reach 2.0-2.5 kg body weight and in 1990s, modern broiler strains attained the same weight in less than 40 days (Griffin and Goddard, 1994; McKay 2009). Toyomizu et al., (2011) investigated the effects of feed conversion efficiency on mitochondrial bioenergetics (oxidative phosphorylation) in skeletal muscle mitochondria of both types (broiler and layer) chickens. It was reported that mitochondria of meat-type chickens showed greater efficiency of oxidative phosphorylation than layers. This greater efficiency of oxidative phosphorylation may partially be responsible for higher feed efficiency in meat type chicken.

Genetic selection for meat-related traits in broiler chicken has had deleterious effects on health, welfare and their ability to cope with different stresses (Sandercock *et al.* 2006; MacRae *et al.* 2007; Sandercock *et al.* 2009a). Additionally, this genetic selection has resulted in a greater incidence of spontaneous and stress-induced skeletal muscles abnormalities in broilers (MacRae *et al.* 2007; Sandercock *et al.* 2009b).

The increased body weights of modern broilers are due to increased muscle yield, particularly of the *Pectoralis major* (Pm) breast muscle. The increase in muscle size of present day broiler birds may be due to the hypertrophy of their muscle fibre sizes (MacRae *et al.* 2007; Zheng *et al.* 2009). MacRae, *et al.,* (2006) compared the skeletal muscle fibre growth and growth related myopathy in broiler and layer chicken. It was reported that the fibre size of Pm muscle was 1.5 times greater in commercial broilers (65.9 mm) and great-grand (GGP) parent lines of broiler chicken (59.8 mm) than in layer birds (38.1 mm), at 25 weeks of age. It was also found that growth of the Pm fibres was greater than those of the *Biceps femoris* (Bf) muscle in the broiler and GGP birds; fibre growth of the Pm and Bf muscle was not much different in layer birds. However, in all lines connective tissue contents were generally higher in the Bf than in the Pm. Muscle fibres in commercial broilers and turkeys are characterised by their larger diameter compared with their unselected counterparts at the same age (Mills *et al.* 1998; Mills *et al.* 2000). It is possible that there is a certain limit for the maximum size of the muscle fibres beyond which fibres cannot safely grow due to the greater diffusion distances for oxygen, nutrients and metabolic wastes (Mills *et al.* 1998; MacRae *et al.* 2006). However, it has also been hypothesised that growth of the connective tissue in skeletal muscle does not keep pace with muscle fibre radial growth and the fibres outgrow the supporting connective tissue, leading to muscle damage or myopathy in poultry selected for meat production (Mitchell 1999; Kranen *et al.* 2000; Sandercock *et al.* 2006). Connective tissue content in Pm muscle of broilers are much lower compared with Bf muscle of broilers at 5 weeks of age. These might be negative consequences of

genetic selection for lean breast meat in broilers (MacRae *et al.* 2006). Thigh muscles are mainly comprised of the type I, oxidative fibres that need oxygen for their metabolism. This might be the reason for their comparatively lower diameter compare with breast muscles so that oxygen can diffuse properly for their normal functioning. On the other hand, the fast, glycolytic type IIb muscle fibres of the breast muscles are used for high-force transient movements. As these myofibers are metabolic in nature they may be more responsive to genetic selection for increased fibre size and ultimately lead to larger breast muscles (Goldspink 1996; Hughes & Schiaffino 1999).

In broiler chickens and turkeys these improvements in productive performance may be associated with some detrimental effects not only upon skeletal muscles but also on meat quality. Sandercock *et al.,* (2009) studied meat quality parameters in 34 broiler, layer and traditional lines of chicken at 8 weeks of age. They observed that breast muscles from broilers were lighter in colour and less red and yellow compared with breast muscles of layers and traditional lines. It was also found that initial pH (pHi) and final pH (pHu) were lower in broiler breast muscles compared with layer and traditional breeds and the rate of pH decline was also faster in broilers. They argued that the reason for the low ante-mortem and post-mortem muscle pH and greater rate of fall in pH might be the higher level of cations (Na^{+} and Ca^{+2}) in broiler muscles.

1.3 Anatomy and Physiology of Muscles

There are rod-like structures in the muscle fibre, which are about 1 μm in diameter and are called myofibrils. These myofibrils are the structural units of the myofibre which in turn are units of the muscle. Myofibrils are made up of protein filaments which are arranged in units called sarcomeres. Sarcomeres consist of two types of myosin filaments, thick filaments and actin, thin filaments. Actin filaments are attached to the Z-discs, that form the boundaries of the sarcomere, and myosin filaments are in the centre of the sarcomere, as shown in Figure 1.1 (b). Each myosin molecule consists of two heavy chains and myosin cross-bridges. These cross-bridges are finally attached with actin filaments and pull them towards the centre of the sarcomere during the process of muscle contraction, so that each sarcomere shortens and generates force. This process of shortening and contraction is mediated by ATP (Goldspink 1996).

The heads of myosin cross-bridges, having an actin-binding site and an ATPase site, are attached to the myosin molecule by a lever arm. The two light chains, one essential and the other an alkaline light chain of myosin molecules are attached with each lever arm. These light chains are involved in effective transduction of the force. Different types of muscles, i.e. fast and slow skeletal muscle and cardiac and smooth muscle, have different types of light chains (Goldspink 1964; Goldspink 1996).

1.3.1 Types of Skeletal Muscle Fibres

There are three main types of skeletal muscle fibres, Type I, Type IIa, and Type IIb.

Type I muscle fibres are known as slow oxidative fibres. In these myofibers, the myosin hydrolyses ATP very slowly resulting in a slow contraction of the muscle fibre and generation of less force. This characteristic makes these fibres more suitable for producing slow repetitive movements and sustaining isometric force (Goldspink 1984). Type IIa fibres are also known as fast, oxidative, glycolytic fibres. They are suitable for producing high power for a longer period of time. Type IIb fibres are known as fast, glycolytic fibres. Their speciality is to produce high power output but for a shorter time period than type IIa fibres. Myosin and other contractile proteins of type II fibres have the ability to hydrolyse the ATP very quickly so they can produce a fast cross-bridge cycle and develop force rapidly. But the reason for the difference in these two types of fibre is that the type IIa fibres have more mitochondria and a more oxidative metabolism, and as a consequence these muscle fibres are able to produce a high power output for a remarkably long duration. At the start of any activity, slow fibres become activated and when demand for power is not fulfilled by these fibres the fast type II fibres are activated to generate more force and muscle contraction (Goldspink 1996).

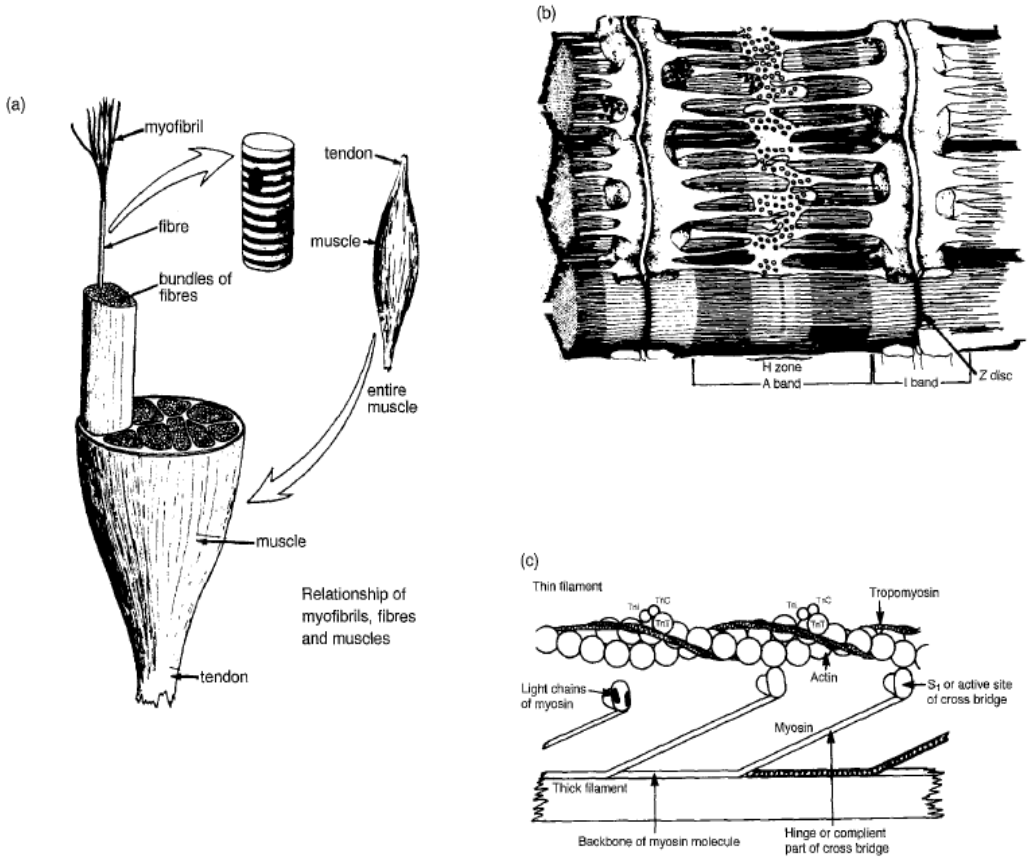


Figure 1-1 The different levels of organisation of skeletal muscle. (a) Section through the belly of the muscle showing bundles of striated muscle fibres. Within each fibre there are many myofibrils which are the contractile elements and which are also striated. (Goldspink 1996). (b) An electronmicrographic reconstruction showing the thick (myosin) and thin (actin) filaments organised in units called sarcomeres. The release of calcium from the sarcoplasmic reticulum activates the myosin cross-bridges which are the independent force generators that move the thin filaments (Goldspink 1996). (c) The molecular organisation of the thick and thin filaments. The thin filaments are decorated by the regulatory proteins, the tropomyosin/troponin complex. When activated by calcium the tropomyosin is pulled to one side, exposing active sites on the filament to which the myosin cross-bridge attach. The cross-bridges are part of the double myosin heavy chain molecules. Part of the myosin heavy chain is the rod structure which is embedded in the thick filament (Goldspink 1996).

Generally, at the end of embryogenesis the final numbers of muscle fibres are fixed in the body. Myogenic precursor cells originating from somites first give rise to myoblasts that undergo further proliferation and migrate to their final locations and finally fuse into multinucleated myotubes, which finally differentiate into mature muscle fibres. Muscle growth after birth is achieved by increase in fibre size, which is the consequence of satellite cells fusing to existing fibres (Smith 1963). Mature muscle fibres exhibit a remarkable plasticity, in response to external stimuli or disease. Physical exercise and mechanical loading are capable of producing a significant increase in muscle mass by muscle fibre hypertrophy (Lumini *et al.* 2008; Radak *et al.* 2008).

The molecular mechanisms controlling the divergent muscle growth rates and muscle mass in broiler and layer chicken were studied by Zheng *et al* (2009) during different developmental stages by a microarray hybridization experiment. They identified 543 differentially expressed genes. It was also reported that divergent muscle growth rates of the two chicken lines was controlled by differential regulation of slow-type muscle gene expression, satellite cell proliferation and differentiation, protein degradation rate and by genes involved in different metabolic pathways. The correlation of expression profiles of differentially expressed genes with growth rates of broilers and layers, reflect their involvement in regulating muscle growth during development. Complicated molecular networks controlling the regulation of chicken muscle growth were found.

1.3.2 Creatine Kinase

Creatine kinase (CK), also known as phosphocreatine kinase or creatine phosphokinase, is an enzyme or type of protein that is found in several tissues, including the muscle and the brain. The enzyme is involved in the reversible transphosphorylation of adenosine diphosphate (ADP) and creatine and is central to the maintenance of intracellular energy supplies by the spatio-temporal buffering of adenosine triphosphate concentrations (Bruton *et al.* 2003).

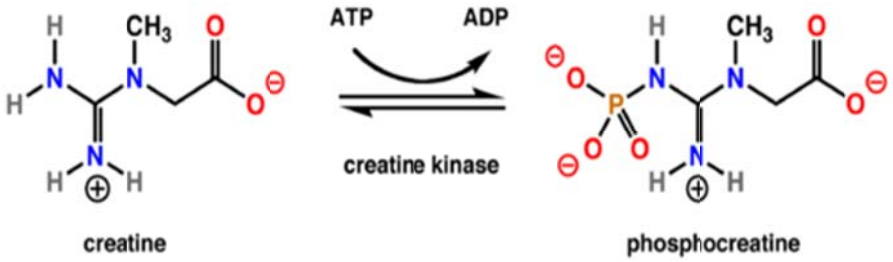


Figure 1-2 The chemical structures of the creatine kinase molecule (right) and its phosphorelated counterpart phosphocreatine (left) (Clark 1996).

CK activity is present in the cytosol and may be bound to specific intracellular structures such as the plasma membrane, the endoplasmic or sarcoplasmic reticulum, nuclei, mitochondria and myofilaments representing the major sites of production and utilisation of ADP (Mitchell & Sandercock 1995a; Mitchell *et al.* 1999a).

In vertebrate tissues, at least four subunit isoforms of CK are expressed in a tissue-specific manner: two 'cytosolic' forms, MM-CK (muscles) and BB-CK (brain), and two mitochondrial Mi-CK isoforms (mitochondrial) (Wallimann *et al.* 1998; Wallimann *et al.* 2011). M-CK and B-CK subunits combine to give the three typical dimeric 'cytosolic' MM-CK, MB-CK and BB-CK isoenzymes identified as skeletal muscle, cardiac muscle and brain types, respectively, due to their apparent distribution among these tissues. Interestingly, during muscle cell differentiation *in vitro* and *in vivo*, a developmental transition from BB-CK via the transitory MB-CK hybrid to the MM-CK homodimer has been observed (Wallimann *et al.* 1998).

The intracellular compartmentalisation of these major isoenzymes of CK and the more recently described isoforms and subtypes promotes the functional coupling of the production and consumption of energy and the integration and control of cellular metabolism. Isoenzyme activity and profiles of CK in human plasma or serum samples are used as an important diagnostic technique in a number of pathologies including myopathies and dystrophies, myocardial infarction, lesions of the central nervous system and neoplastia (Chiu *et al.* 2009; LaFramboise *et al.* 2009; Sandercock *et al.* 2009a; Xu *et al.* 2009). The diagnostic interpretation is based upon the assumption that the relatively tissue-specific and distinguishable isoenzymes would be released in response to cellular damage and result in characteristic blood profiles. In a number of avian species, the changes in total plasma CK activity which occur in response to various pathologies, acute heat stress and transportation (Mitchell & Carlisle 1992) have been reported but without reference to the

associated isoenzyme profiles. Although the MM-CK and BB-CK isoenzymes in the skeletal and nervous tissues of birds may be analogous to those identified in mammals, it has been suggested that the CK-M monomer is not expressed in post embryonic avian cardiac muscle and that as a result the MB-CK dimer does not appear in this tissue, which contains only BB-CK (Mitchell & Sandercock 1995a). The relative distribution of the activities of the isoenzymes of CK in the plasma of birds may therefore differ markedly from that described in mammals, in both normal and diseased or stressed individuals. Muscle isoenzyme (MM-CK) is the predominant form in plasma (99 per cent), and an increase in its plasma activity in response to acute heat stress is a consequence of muscle damage. Thus measurements of plasma CK activities in poultry are a useful tool to measure the extent and nature of muscle damage induced by different treatments, environments and challenges (Mitchell and Sandercock 1995).

1.4 Modern broilers and susceptibility to pre-slaughter stressors

Stressful conditions including acute exposure to elevated ambient temperature or crating, and transportation lead to increased plasma activity of the intracellular muscle enzyme, creatine kinase in chicken (Mitchell & Sandercock 1995b; Sandercock *et al.* 2001; Sandercock *et al.* 2006; Nadaf *et al.* 2007b; Zulkifli *et al.* 2009). It was considered that the release of CK from muscles to the extracellular fluid and plasma is due to alterations in the integrity of the skeletal muscle cell membrane, which lead to the deleterious effects of stress upon skeletal muscle

structure and functions (Mitchell & Sandercock 1995b). This increase in plasma CK activities can be considered as a useful indicator of "physiological stress"(Mitchell *et al.* 1994). Genetic selection in broilers that has increased body weight is also associated with a higher concentration of cations and CK activities, lower initial and final muscle pH and to paler, and less red meat (Sandercock *et al.* 2009a). Variations in these attributes of the muscle are important commercially and are related to aspects of muscle and meat quality (see below).

1.4.1 Effect of pre-slaughter stress conditions on physiological parameters

Pre-slaughter stress conditions like catching, crating, transportation and exposure to high ambient temperature are known to have harmful effects on bird health, production and welfare. Stressful stimuli elicit a series of consequences such as an increase in plasma corticosterone concentrations (which ultimately increase plasma glucose), a high heterophil : lymphocyte ratio and higher plasma activities of some enzymes like lactate dehydrogenase (LDH) and CK, good indicators of muscle damage and stress (Sandercock *et al.* 2006). Effects of heat stress are more severe in their extent as compared to the effects of other stressors. In addition to these effects, heat stress causes hyperthermia, hypocapnic alkalosis (due to panting), disturbances in acid-base balance, changes in electrolytes levels, and heat-stress-induced myopathy (Mitchell *et al.* 1994; Sandercock *et al.* 2001).

Mitchell and Sandercock, (1995) studied the effects of acute heat stress on the plasma profile of creatine kinase isoenzymes in broiler chicken. They subjected the broilers to acute heat stress (32.6°C and 94%RH) for 90 minutes and observed an increase of 2.8°C in deep body temperature and 24% increase in plasma CK activity. They further reported that the comparatively greater increase in the plasma level of MM-CK isoenzyme may be due to a higher proportion of muscles in the body or to a higher sensitivity of muscles to higher ambient temperatures, effects that may be related to limited sarcolemmal integrity (Mitchell & Sandercock 1995b; Sandercock *et al.* 2009a).

High environmental temperature causes a decrease in breast muscle glycogen level, but increases plasma concentrations of glucose and albumin. Crating broilers at high temperature also increases the impact of heat stress, indicated by increases in heterophil: lymphocytes (H:L ratio), an indicator of heat stress. Crating temperature has no effect on tonic immobility (TI) duration, a measure of fearfulness, and TI has no relationship with meat quality (Aksit *et al.* 2006). High ambient temperature during rearing and crating reduces the moisture content of broiler breast muscles and glycaemia and glycolytic potential of thigh muscle (Debut *et al.* 2005).

Yalcin *et al.,* (2004) investigated the age-related effects of catching, crating, and transportation at different seasons on body temperature and physiological blood parameters in broiler chickens. They reported that increases in age, (mainly due to the associated increases in body mass) have positive effects on physiological responses of birds to pre-slaughter stress conditions. Crating caused the highest

increase in rectal temperature, in parallel with stocking density, as compared to catching and transportation. It was also found that young broilers (<42 d of age) were more susceptible to transportation stress, whereas crating appeared to be a major stressor in older birds (>49 d of age).

Debut *et al.,* (2009) studied the effects of shackling and acute heat stress on behavioural and physiological responses in a slow-growing line, '*French Label Rouge*', (SGL), a fast-growing standard line (FGL) and a heavy line (HL). Birds were slaughtered at the same body weight and exposed to three pre-slaughter treatments, shackling for 2 minutes, exposure to high ambient temperature at 35°C and 60% humidity for 3.5 h and then shackled for 2 min or only shackling for 10 seconds before stunning (Control). They found higher plasma corticosterone levels in treated groups as compared to controls, irrespective of genotype. The struggling activity on the shackle line was much higher in SGL birds compared with FGL and HL. They also reported that wing flapping duration has negatively correlated with blood pH and bicarbonate concentration but positively correlated with the lactate contents of breast muscles. Finally, they concluded that pre-slaughter acute heat stress and shackling were important stressors for all types of broilers.

1.5 Heat Stress and muscle damage in broiler chicken

Although there are numerous pre-slaughter stressors, as mentioned above, heat stress is the most detrimental for the bird's physiology, welfare and meat quality. Birds are

"heat stressed" if they have difficulty in achieving a balance between body heat production and body heat loss (Ali *et al.* 2008). In the 'thermoneutral zone', birds can lose heat at a controlled rate using normal behaviour. When the environmental temperature rises above the thermoneutral zone, the bird experiences a stress condition (Kranen *et al.* 1998), which can generate acute hyperthermia, the production of reactive oxygen species (ROS) that are involved in creating muscle damage, along with a reduction in food consumption, lower growth rate, and increased mortality (Khan *et al.* 2011).

1.5.1 Role of Heat-Stress in amplifying muscle damage through production of Reactive Oxygen Species

Acute heat stress was suggested to be an environmental factor responsible for stimulating ROS production (Zuo *et al.* 2000; Mujahid *et al.* 2007c) because of similarities in gene expression patterns observed following heat stress compared with that following exposure to oxidative stress (Salo 1991). Zuo *et al.* (2000) used laser scan confocal microscopy with ethidium fluorescence as a probe for intracellular ROS and found that heat stress stimulated intracellular and extracellular ROS production, particularly superoxide (O^{+2}) formation, in mouse diaphragm muscle. However, there is no direct evidence for the source(s) of ROS production in animals exposed to heat stress. Given that the mitochondria serve as the principal source of ROS in cells, it is likely that in heat-treated animals ROS are mainly produced in the skeletal muscle mitochondria. It is known that heat stress perturbs the balance between the production of free radicals of oxygen and their elimination by the

antioxidant systems of the body (Lin *et al.* 2006; Lin *et al.* 2008). In addition to this, heat stress is known to negatively affect the capability of the antioxidant system to cope with the overproduction of ROS (Lin *et al.* 2008). In meat type chickens, heat stress enhanced superoxide production in skeletal muscle mitochondria compared with laying chickens (Mujahid *et al.* 2005). In broilers, this increase in superoxide production is associated with heat-induced increments in rectal and muscle temperatures, leading to significant body weight loss (Mujahid *et al.* 2005).

Percentage increases of superoxide production in the presence of carboxyatractylate, a specific inhibitor of adenine nucleotide translocator (ANT), were the same for skeletal muscle mitochondria from meat-type and laying-type chickens from the control or heat-treated group. This indicates the irrelevance of ANT in the regulation of reactive oxygen species flux under heat stress conditions (Mujahid *et al.* 2005; Mujahid *et al.* 2006; Mujahid *et al.* 2007a). In another study, Mujahid *et al.,* (2007) used 3-week-old male broiler chickens and exposed them to acute heat stress (34^{o}C for 18 h) while thermo-neutral conditions (25^{o}C) were given to control chickens. They isolated skeletal muscle subsarcolemmal mitochondria to study mitochondrial malondialdehyde (MDA) and protein carbonyl groups. It was finally reported that in heat-stressed chickens, mitochondrial MDA was 2.7 fold higher, and 82 mitochondrial proteins were oxidized compared with that of control chickens. These results indicated that increased mitochondrial ROS production leads to oxidative damage to mitochondrial lipids and proteins.

In a similar study Mujahid *et al.,* (2006) observed that oxidative stress in heat-treated broilers was coupled with the down-regulation of avian uncoupling protein (avUCP) in broiler skeletal muscles. The results indicate that the overproduction of ROS by mitochondria can be minimised by the appropriate expression of avUCP in broiler muscles. This anti-oxidant property of avUCP could be helpful in reducing the negative effects of heat-stress on muscle function and metabolism (Mujahid *et al.* 2007a). Azad *et al.* (2010) studied the effects of chronic heat-stress on performance and oxidative damage in different strains of chicken. They observed that exposure of broilers to chronic heat-stress resulted in greater depression of growth, than layer chickens, due to greater oxidative stress in broilers. But it was also reported that the extent of oxidative stress was not as severe as in the case of acute heat-stress.

Although chronic heat stress was involved in lipid peroxidation and the MDA level was augmented in broiler breast muscles it was also observed that mtDNA copy number was not affected by chronic heat exposure (Azad *et al.* 2010a). Similarly, it was also reported that gene expression and the protein content of uncoupling protein (avUCP) are generally lower in skeletal muscle mitochondria of meat-type than laying-type chickens (Toyomizu *et al.* 2011).

Normally there is a defence system in the body against the oxidative stress that comprises both enzymatic and non-enzymatic antioxidants. These antioxidants normally scavenge free radicals of oxygen and maintain a stable redox state. Superoxide dismutase (SOD) and glutathione peroxidise (GPx) are the enzymes mainly involved in antioxidant functions in the body. Superoxide dismutase

catalyzes the conversion of O^2 (superoxide) to hydrogen peroxide (H_2O_2), which is then reduced to water by some scavenging enzymes (such as catalase and glutathione peroxidase) (Finkel & Holbrook 2000; Chang *et al.* 2007). Chronic heat stress resulted in a significant increase in the activity of both SOD and catalase enzymes, but no significant change was observed in the activity of GPx (Azad *et al.* 2010a; Azad *et al.* 2010b). Thus, these finding suggest that during the phase of chronic heat stress, the antioxidant scavenging system is built up and activated to some extent which might be responsible for reducing the extent of oxidative stress and ultimate damage to the muscles (Azad *et al.* 2010a).

1.6 Effect of Acute Heat Stress on Heat Shock Protein 70 (Hsp 70)

Exposure of organisms to different thermal and non-thermal stressors, for example exposure to heavy metals, oxidants, toxins, bacterial and viral infections (Morimoto 1993) and feed deprivation, reduce the synthesis of most proteins, but at the same time rapid increases in the synthesis of a group of highly conserved proteins, known as heat shock proteins (HSP), have been observed (Al-Aqil & Zulkifli 2009). The most important functions of HSP is to protect organisms from the toxic effect of heating (Katoh *et al.* 2004). HSPs play crucial roles in protein synthesis, transport, protein folding and unfolding (Salo 1991) and the refolding of damaged proteins (Zhen *et al.* 2006).

In the synthesis of HSP, a transcription factor known as heat shock factor (HSF) in eukaryotic cells is involved in transcriptional activation of heat shock genes. In normal circumstances (unstressed cells), HSF is distributed in both the cytoplasm and nucleus in a monomeric form, which is inactive and has no DNA binding activity. In response to heat stress and other physiological stresses, HSF molecules unite and assemble into a trimer and accumulate within the nucleus (Morimoto 1993). Activation and binding of HSF to the heat shock element (HSE), a specific DNA recognition sequence located in the 5-flanking sequences of heat shock-responsive genes, is completed very quickly upon exposure to high temperature. This binding between HSF and DNA does not always correlate with transcriptional activity, which suggests the presence of multiple steps in the activation process. HSF also undergoes a stress-dependent phosphorylation that may affect its activity. Prolonged exposure of cells to intermediate heat shock temperature (42^{o}C), or on returning to physiological temperature (37^{o}C) results in attenuation of the heat shock transcriptional response; this attenuation leads to the conversion of the active trimeric form of HSF to the inactive monomeric form, and finally a return to the normal subcellular distribution. However, prolonged exposure to elevated temperature (43^{o}C) results in sustained transcription of the heat shock genes (Fujimoto & Nakai 2010; Hao *et al.* 2012; Yuan *et al.* 2012) and prolonged HSF DNA binding activity, as shown in Figure 1.3 (Morimoto 1993). Of the many expressed HSPs, those with a molecular weight of approximately 70 kDa appear to be most closely associated with heat tolerance. It is also reported that the heat shock response occurs in a large number of tissues. Amongst all members of the 70 kDa

family, the one that has attracted most attention is heat shock protein 70 (Hsp 70) (Yu *et al.* 2008).

Yu and Bao (2008) studied the expression and localization of heat shock protein 70 (Hsp 70) and its mRNA in the heart, liver, and kidney of broiler chicken exposed to acute thermal challenge (37°C) for various times. During the exposure to heat stress, the heart, liver and kidney of broiler chickens exhibited maximum expression of Hsp 70 mRNA after a 2 hour heat stress. A significant increase in the level of Hsp 70 was observed after 2, 3 and 5 hours of exposure to high temperature in the heart, liver and kidneys, respectively, indicating the variations in stress-induced responses of different tissues.

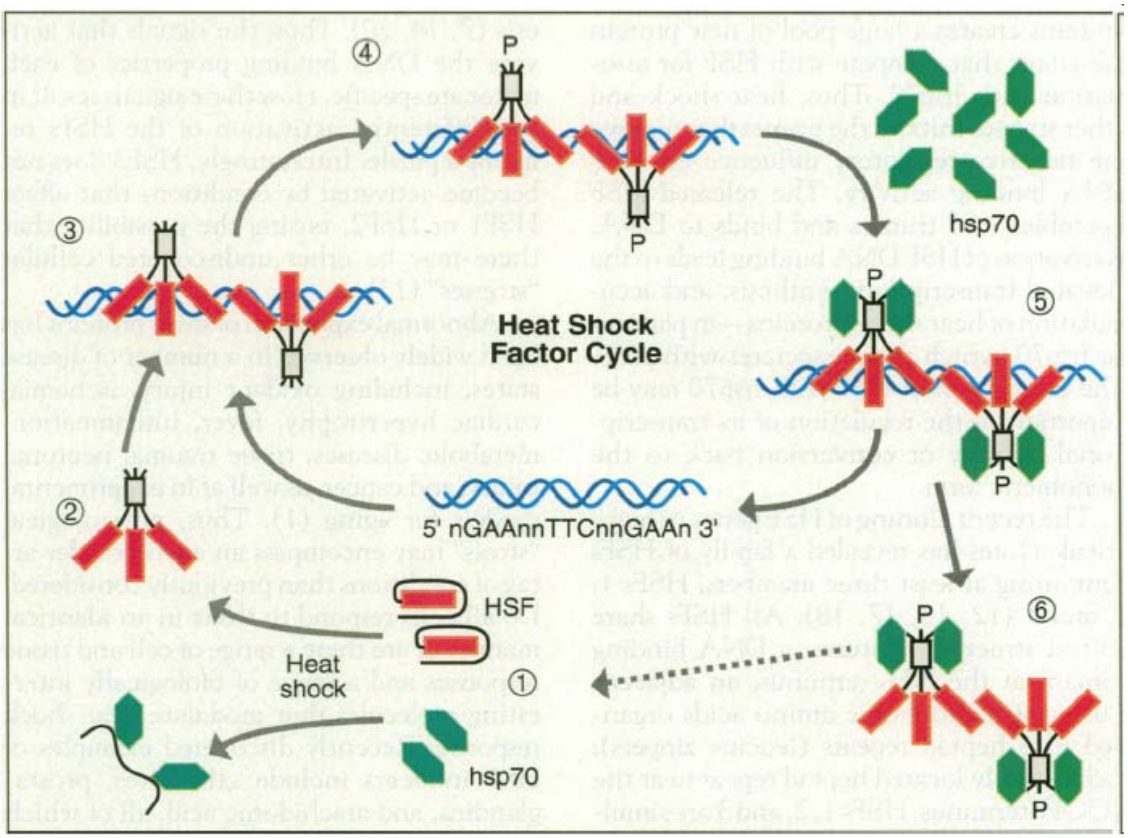


Figure 1-3 A model of Heat Shock Factor (HSF) regulation. In the unstressed cell, HSF is maintained in a monomeric, non-DNA binding form through its interactions with hsp70. Upon heat shock or other forms of stress, HSF assembles into a trimer, binds to specific sequence elements in heat shock gene promoters (3), and becomes phosphorylated (4). Transcriptional activation of the heat shock genes leads to increased levels of hsp70 and to formation of an HSF-hsp70 complex. Finally, HSF dissociates from the DNA and is eventually converted to non-DNA-binding monomers (Morimoto 1993).

Zulkifli *et al.* (2009) investigated the effects of acute heat stress and crating on blood parameters and Hsp 70 expression in broiler chickens differing in their fear levels. They subjected the birds to either crating or heat challenge (34°C) for 3 hours and observed raised levels of heterophil/lymphocyte ratios in both low fear (LF) and high fear (HF) birds whereas plasma corticosterone concentrations were higher in HF than

LF broilers. There was no difference in Hsp 70 before heat stress between the two groups but after exposure to heat stress (3 h) a greater response was observed for the HF as compared to the LF group.

Effects of housing systems and early age feed restriction on Hsp 70 expression and blood parameters in broiler chickens were studied by Al-Aqil and Zulkifili (2009). The birds were subjected to crating and transportation for 6 h on day 42 and birds raised in open-sided house and feed restricted had smaller increases in heterophil:lymphocyte ratios and plasma corticosterone concentrations than those in a controlled environment and fed *ad-libitum*. Greater expression of Hsp 70 was observed for the open housed birds after transportation than control housed birds, and similarly the feed restricted birds exhibited greater Hsp 70 expression than *ad-libitum* fed birds. Finally, they concluded that the increased tolerance to transport stress in open housed and feed restricted chicks may be associated with greater Hsp 70 expression (Zulkifli *et al.* 2009).

1.7 Role of cations in muscle damage in broiler

Calcium ions (Ca^{2+}) have an important role in skeletal muscle functions. A physiological increase in mycoplasma free Ca^{2+} affects numerous cellular processes (e.g., excitation-contraction coupling). Sustained and unchecked increases in myoplasma Ca^{2+} activate several degenerative processes within the cell, which cause cellular damage in skeletal muscles (Duncan and Jackson, 1987; Jackson, 1993). Raised intracellular calcium inhibited mitochondrial oxidative phosphorylation and

increased lactate production. The CK efflux from skeletal muscle cells in these situations is known to be a result of disruption in sarcolemmal integrity, caused by increase in mycoplasma Ca^{2+} (Sandercock and Mitchell, 1998; Sandercock *et al.,* 2001). A raised level of myocellular sodium concentration causes an increase in the mycoplasma Ca^{2+} concentration, which then leads to alterations in sarcolemmal integrity and efflux of CK. The Na^{+}-induced increase in myocellular calcium may be mediated via direct extracellular Ca^{2+} entry or redistribution from internal Ca^{2+} stores (Sandercock & Mitchell 2004).

Raised intracellular calcium may induce metabolic disturbances and cellular damage including changes in membrane integrity by a number of mechanisms and mediators (Mitchell, 1999). One such mediator, causing alteration in sarcolemmal integrity may be the activation of the Ca^{2+}-dependent membrane associated enzyme phospholipase A_2 (PLA_2) (Jackson, 1993; Jackson *et al.,* 1984). An increase in intracellular Ca^{2+} leads to the activation of PLA_2 enzyme (Mitchell *et al.* 1994; Sandercock & Mitchell 2003) which ultimately results in hydrolysis of lipid, present in cell membrane, and generation of pro-inflammatory intermediates such as prostaglandins, thromboxanes, and membrane-damaging lysophospholipids (Kramer and Sharp, 1997). Inhibition of phospholipase activity decrease the protein loss from metabolically inhibited mouse skeletal muscle (Jackson *et al.* 1984).

The role of Ca^{2+}-activated PLA_2 in the mechanism of skeletal muscle damage in broiler chickens was investigated by Sandercock and Mitchell (2003) *in vitro* using a novel, synthetic, PLA2-specific inhibitor Ro31. They found that treatment with the

specific Ca^{2+}-ionophore resulted in a 72% increase ($P< 0.05$) in muscle $^{45}Ca^{2+}$ accumulation, which led to a 7.6-fold increase ($P< 0.001$) in muscle CK efflux. However, incubation of ionophore treated muscles with Ro31 (50 μM) resulted in a 45% reduction ($P< 0.001$) in CK efflux, but no effect on $^{45}Ca^{2+}$acumulation was observed.

Disturbances in cellular sodium (Na^{+}) homeostasis are considered to be involved in the development of cellular damage, mainly by interacting with Ca^{+2} ions. Depending on the Na^{+} gradient, the membrane-localized Na^{+}/Ca^{2+} exchange pump can cause translocation of Na^{+} and Ca^{2+} in opposite directions (Sandercock & Mitchell 2004). Mitchell and Sandercock, (1994) studied myotoxicity by monensin (a polyether carboxylic-ionophore antibiotic) and its relationship with monensin-induced sodium influx and calcium accumulation in skeletal muscle cells. They concluded that monensin myotoxicity is mediated by disturbances in intracellular calcium and sodium homeostatic levels subsequent to enhanced sodium entry, possibly through sodium-calcium exchange and disruption of sarcolemmal integrity due to lipid degradation. They also reported a high positive correlation ($r^2=0.91$) between CK efflux and intracellular calcium concentrations.

1.8 Meat Quality

The quality of poultry meat can be divided into several attributes, namely the sensory (colour, tenderness, flavour, juiciness) and the physical (muscle yield, water-holding capacity, and cooking loss) attributes of meat. These quality criteria vary with

growth rate and body composition and are mainly determined by post-mortem metabolism and its effect on the colour and water-holding capacity of the product (Duclos *et al.* 2007).

The rate and the extent of decrease in pH have a strong effect on both organoleptic and technological parameters of meat quality. After slaughtering, the muscles have to rely on the anaerobic glycolytic pathway to use the intramuscular glycogen stores for ATP regeneration, which leads to the accumulation of lactic acid and protons. In this way, the acidification process depends mainly upon the amount of glycogen stores (estimated by the glycolytic potential) and the rate of the glycolysis (Duclos *et al.* 2007).

In the chicken, normal pH values at 15 min post-slaughter (pH_{15}) are around 6.2 to 6.5, and normal ultimate pH (pHu) values are around 5.8 (Fletcher 1999). If the pH_{15} value is less than 6.0, when the muscles are still warm, the proteins are subjected to denaturation, which leads to poor water-holding capacity and decolouration of the meat. These types of meats are often characterised as pale, soft and exudative (PSE) meats and have poor technological yield (Owens *et al.* 2009). Although PSE meats, in the raw form, exhibit a soft texture, after cooking they tend to be less tender, due to excessive exudation (McKee & Sams 1997). Acid muscles are characterized by a low ultimate pH (pHu < 5.7), which induces structural changes in the muscles with a decrease in the technological processing ability. Artificially acidifying turkey meat induces a destruction of the myofibrillar network, which also induces a marked decrease in water-holding capacity. On the other hand, muscle with high ultimate pH

also show defects in their colour, texture, and water-holding capacity. Muscles with high pHu are considered as dark, firm, and dry (DFD) and show enhanced water-holding capacities, and an increased sensitivity for microbial development (Barbut 1997).

1.8.1 Genetic selection and meat quality

Genetic selection has an important role in controlling meat quality represented by a number of meat related traits such as $pH_{15,}$ pHu, lightness (L^*), redness (a^*), yellowness (b^*), drip loss (water retention ability), thawing and cooking loss, and tenderness. It is generally considered that genetic selection for growth-related traits is associated with some negative effects on meat quality in broiler chicken. Sandercock *et al.,* (2009) measured meat quality parameters in broilers, layers and traditional breeds in a multi-strain experiment. They observed that the breast muscles from broiler lines were lighter and less red and had greater haemorrhages compared with breast muscles of layer and traditional breeds, which were similar. Moreover, moderate (total) heritability estimates (averaging 0.3), were found for the above mentioned traits. Glycogen reserves of the breast muscles are highly heritable (h^2 =0.43) and a strong negative correlation (-0.97) was measured between pHu and glycolytic potential that suggests a common genetic control for these traits; breast weight was negatively correlated with glycogen reserves and had a positive correlation with fibres size (0.76) and pHu (0.84) (Le Bihan-Duval *et al.* 2008a).

Genetic parameters of meat characteristics and their correlation with growth and body composition were studied by Le Bihan-Duval *et al.* (2007) in an experimental broiler line. They found that colour parameters of meat were the most heritable traits, with a heritability range from 0.50 to 0.57; the estimated heritability for drip loss (DL) was 0.39 and for the pH of the meat (ranging from 0.35 to 0.49). They also reported extremely low estimates for the genetic correlation between pH_{15} and extent of decline of final pH suggesting that they are controlled by different genes. In contrast, the very high negative correlation of pHu with lightness (-0.91) and water holding capacity (-0.83) suggests relevant selection criteria for better quality meat (Aksit *et al.* 2006; Chabault *et al.* 2012); it has been reported that remarkable improvement in meat quality can be achieved by using pHu as a selection criteria in future breeding programmes (Chabault *et al.* 2012). Similarly the negative genetic correlation of breast muscle weight with L^* values, tenderness and a negative correlation between pHu and L^* values have been reported by Yalcin *et al.* (2005).

However, there are quite a few reports in the literature which conflict with these findings. Le Bihan-Duval *et al.* (2001) reported that genetic selection for growth-related traits in chicken was not found associated in having adverse effect on breast meat quality; though it has caused a decrease in colour intensity of breast meat. Consistent with this Berri *et al.*, (2007) investigated the effects of selection for improved body composition on quality of broiler meat and reported that selection has resulted in higher protein but lower moisture contents and pigment in breast meat, leading to paler colour of breast fillet. A lower rate of fall and extent of pHu decline

was found in the selected line, as compared to the respective control, due to decreased glycolytic potential. They also rejected the hypothesis that selection for improved body composition has negative effects on meat quality. These results are in agreement with the finding by Le Bihan-Duval *et al.* (2007) who found a poor genetic correlation of body weight and breast meat yield with pH at 15 min and 24 hours post-mortem, indicating that improvements in body weight are not related to any decrease in meat quality. In another study, Berri *et al*., (2005b) reported that faster growing birds are more suitable for further processing in terms of their low water holding capacity, texture, and tenderness.

However, in contrast with these reports Le Bihan-Duval *et al.,* (1999) found that genetic selection for body weight resulted in paler (less red and yellow) meat in selected lines of chicken compared with the control lines. Berri *et al.,* (2001) also reported that selection for increase growth rate and body weight resulted in decreased haem pigments of breast muscles and to a less red and more pale colour.

Genetic selection is also considered to be associated with muscle defects such as focal myopathy and susceptibility to heat-stress induced myopathy in meat-type birds compared with egg-type birds which may be related to the greater diameter of myofibres in broilers. Genetic selection for increased embryonic muscle fibre numbers, rather than for increased radial fibre growth, could help in improving growth potential and in preventing muscle damage (MacRae *et al.* 2007).

In recent years several quantitative trait loci (QTLs) for traits related to meat quality have been reported. Nadaf *et al.,* (2007) identified some QTLs for meat quality traits by crossing two divergently selected lines for body weight and abdominal fat (fast growing line × slow growing line). The fast growing line showed low values for pH_{15}, pHu, redness and yellowness of breast meat but higher values for lightness compared with the slow growing line. They found 5 significant QTLs, 2 QTLs for pH_{15}, on linkage groups GGA1 and GGA2, 1 for drip loss on GGA1, 1 for redness and 1 for yellowness of breast meat, both on GGA11. In addition to this, they also found 4 suggestive QTLs for yellowness, pH_{15}, pHu and drip loss (DL) on linkage groups GGA1, GGA4, GGA12 and GGA14, respectively.

The refinement of the yellowness QTL, on chromosome 11, along with the combination of gene expression QTL technique led to the identification of a candidate gene BCMO1, encoding β-carotene 15 (Le Bihan-Duval *et al.* 2011). Moreover, it was also found that 2 single nucleotide polymorphisms (SNP) in the promoter region were responsible for a 3-fold increase in the expression of BCMO1 gene causing a substantial difference in the meat yellow colour, by altering the carotenoid contents of the breast meat (Le Bihan-Duval *et al.* 2011).

However, it has also been reported that there is a significant amount of variation in the colour of breast filets in the different broiler lines. Keeping in view the economic importance of these meat quality traits, their moderately high heritability estimates and the rapidly increasing body of literature about meat quality traits, it seems logical that genetic selection could be helpful in reducing the variability of these

traits among different lines of broiler leading improved meat quality (Sandercock *et al.* 2009b).

1.8.2 Effect of pre-slaughter stress on meat quality

Ante-mortem stressors like exposure to high temperature, catching, crating, transportation, and struggling on the shackle lines are major stressful factors affecting (negatively) the physiology and meat quality of chickens, especially broiler birds (Petracci *et al.* 2001). Crating, transportation and heat stress are associated with an increase in rectal temperature, plasma uric acid and glucose concentrations, heterophil: lymphocyte ratio and creatine kinase activities, especially in older birds (perhaps due to higher body mass) (Yalçin *et al.* 2004) but transportation has more adverse effects in younger birds (Yalcin *et al.* 2005). Crating and transportation of broiler chickens, prior to slaughter, resulted in an increase in plasma corticosterone concentration and also affected thigh meat colour, but no effect was found on breast meat quality (Kannan *et al.* 1997b). The stress, whether induced hormonally (by ACTH) or by exposure to elevated temperature, causes losses in the form of decreased meat yield and poor meat quality (Tankson *et al.* 2001).

Acute heat stress caused greater reduction in thigh muscle quality compared with breast muscle, especially in fast growing lines of chicken (Debut *et al.* 2005). This reduction in meat quality is attributed to lower final pH, paler meat and lower cooking yield of thigh muscles. Breast (glycolytic), muscle on the other hand, were more susceptible to bird activity on the shackle line, such as wing flapping. High

bird activity led to more rapid fall in pH due to accelerated post mortem glycolysis, which suggest that breast meat quality of fast growing birds should be better compared with slow growing birds, due to their increased struggling during slaughtering (Debut *et al.* 2003; Debut *et al.* 2005). In contrast to this, Nadaf *et al.*, (2007a) reported that fast growing birds were more active on the shackle line that might exacerbate the effects of heat-stress and lead to pale, soft, and exudative (PSE)-type meat in broilers. Struggling of the birds on the shackle line has a strong negative relationship with pH at 15 min post-slaughter of the breast muscles. The decline in pH_{15} also has a moderate negative correlation with the glycolytic potential of breast muscles. High glycolytic potential is associated with lower pHu, higher L* and drip loss (Berri *et al.* 2005a). Shackling time increased the plasma corticosterone concentration and also affected the colour of the breast fillet (Kannan *et al.* 1997a).

In addition to shackling stress, there are some other stressors which are known to be involved in significantly reducing meat quality. High ante-mortem temperature is more detrimental to chicken meat quality than low ante-mortem holding temperature. Exposure of broiler birds to acute heat stress causes hyperthermia, respiratory alkalosis, disturbances in acid/base balance and reduction in muscle membrane integrity, indicated by higher plasma CK activities (Sandercock *et al.* 2001; Sandercock *et al.* 2006). An increase in the age of birds increases the magnitude of these adverse effects by changing the metabolism of breast muscles, indicated by lower pHi (immediately after slaughter), higher water loss and more haemorrhages in breast muscles (Sandercock *et al.* 2001). Haemorrhage severity is not related to

growth rate and body weight, and there are multi-factorial causes of haemorrhages (Kranen *et al.* 1998).

Petracci *et al.,* (2001) studied the effects of ante-mortem holding temperatures on live shrink losses, processing yields, and breast meat quality in broiler chickens, at six weeks of age. Before slaughtering birds were held at three different temperatures 25°C, 29.5°C, and 34°C for 12 hours and it was found that birds held at 34°C exhibited significantly greater live shrink losses and lower processed carcass yield. After 2 and 24 h post-mortem, the breast meat was removed in order to determine meat pH, R-value (an estimate of status of rigor mortis), sarcomere length, meat colour (lightness, redness, and yellowness), cooked yield, and tenderness (shear force). They reported higher redness, yellowness and R-values but lower cooked meat yield and shear value for the breast meat harvested at 2 h post-mortem, for the birds held at 25°C, whereas breast meat removed 24 h after slaughter, exhibited higher pH, R-values, and redness at the same temperature conditions. Pre-slaughter holding of broiler birds at high temperature increased live shrink losses and reduced breast meat quality (Holm & Fletcher 1997).

Pale, soft, exudative meat is a growing challenge for the poultry industry. It is the result of rapid post-mortem pH decline and loss of protein functionality, which is due to accelerated post-mortem glycolysis especially when carcass temperatures are still high (Owens *et al.* 2000; Petracci *et al.* 2009). It has been reported that meat with low pH (initial and ultimate), excessive water loss, and more light/pale colour represent from 5 to 40% of the total meat production of the poultry industry and its

incidence increased with the increase ambient temperature to which birds were exposed (Petracci *et al.* 2009). It has also been estimated that due to the rising incidence of PSE meat, a single turkey processing plant could be losing $2 to 4 million per year, resulting in a loss in excess of $200 million by the turkey industry alone in the USA (Owens *et al.* 2009). No evidence of the genetic basis for the PSE syndrome has been found for the turkey or broiler chicken. PSE meat in poultry presents the same characteristics as those described for pigs like very pale colour, higher tenderness and poor water-holding capacity (Remignon & Le Bihan-Duval 2003) but is not thought to be affected by the ryanodine receptor, as in case of pig (Prof M.A. Mitchell, personal communication).

1.9 Strategies to minimize the effects of heat-stress on muscle damage and meat quality in chicken

It is considered that heat-stress results in production of ROS in skeletal muscle mitochondria which ultimately leads to muscle damage and reductions in meat quality in chicken. Vitamin C (ascorbic acid) and vitamin E (α-tocopherol) are well known for their antioxidant role (Nain *et al.* 2008) and they also act synergistically in the body (Sahin *et al.* 2002). Due to this property they are commonly used as feed supplements in diets of human and many animal species to reduce oxidative stress and inflammation induced damaged to tissues (Huey *et al.* 2008). Vitamins C, α-tocopherol and dantrolene sodium are also involved in reducing the stress-induced increase in creatine kinase efflux and calcium accumulation, and ultimately muscle damage. It is proposed that α-tocopherol may offer a valuable protective role against

stress-induced skeletal muscle damage in broiler chickens (Mitchell *et al.* 1994; Sandercock & Mitchell 1998; Mitchell 1999).

Voljc *et al.,* (2011) investigated the effects of different levels of vitamin E supplementation and bioactivity of different isomers of α-tocopherol in broiler diets on oxidative stress and oxidative stability of broiler meat. Different groups of broilers were fed high fat diets with or without supplementation of vitamin E to contain in total 85 or 200 IU of vitamin E. DNA damage and MDA level in plasma, liver and breast muscles were measured to determine the extent of oxidative stress *in vivo*. MDA levels were also measured in fresh, stored, and heat-treated breast meat to determine the oxidative stability of the meat. It was concluded that concentrations of both isomers of vitamin E were not sufficient to prevent all deleterious effects of lipid peroxidation on muscle physiology *in vivo* and on breast meat. Therefore, higher levels of vitamin E supplementation were proposed to ensure the good stability of meat lipids especially after heat treatment. Supplementation of vitamin E and vitamin C resulted in substantial decreases in serum levels of glucose, cholesterol and ACTH (adrenal corticotrophin hormone), an indicator of stress in the body. It also significantly reduced the MDA level in serum of layer chickens reared under high ambient temperature (Sahin *et al.* 2002).

Huey *et al.,* (2008) studied the effects of vitamin E supplementation on the expression of pro-inflammatory cytokines in mouse skeletal muscles challenged by inflammation causing substances. They reported that vitamin E resulted in reduced oxidation of proteins and the extent of inflammatory response (production of inflammatory cytokines) was also decreased. Chang *et al.,* (2007) also demonstrated

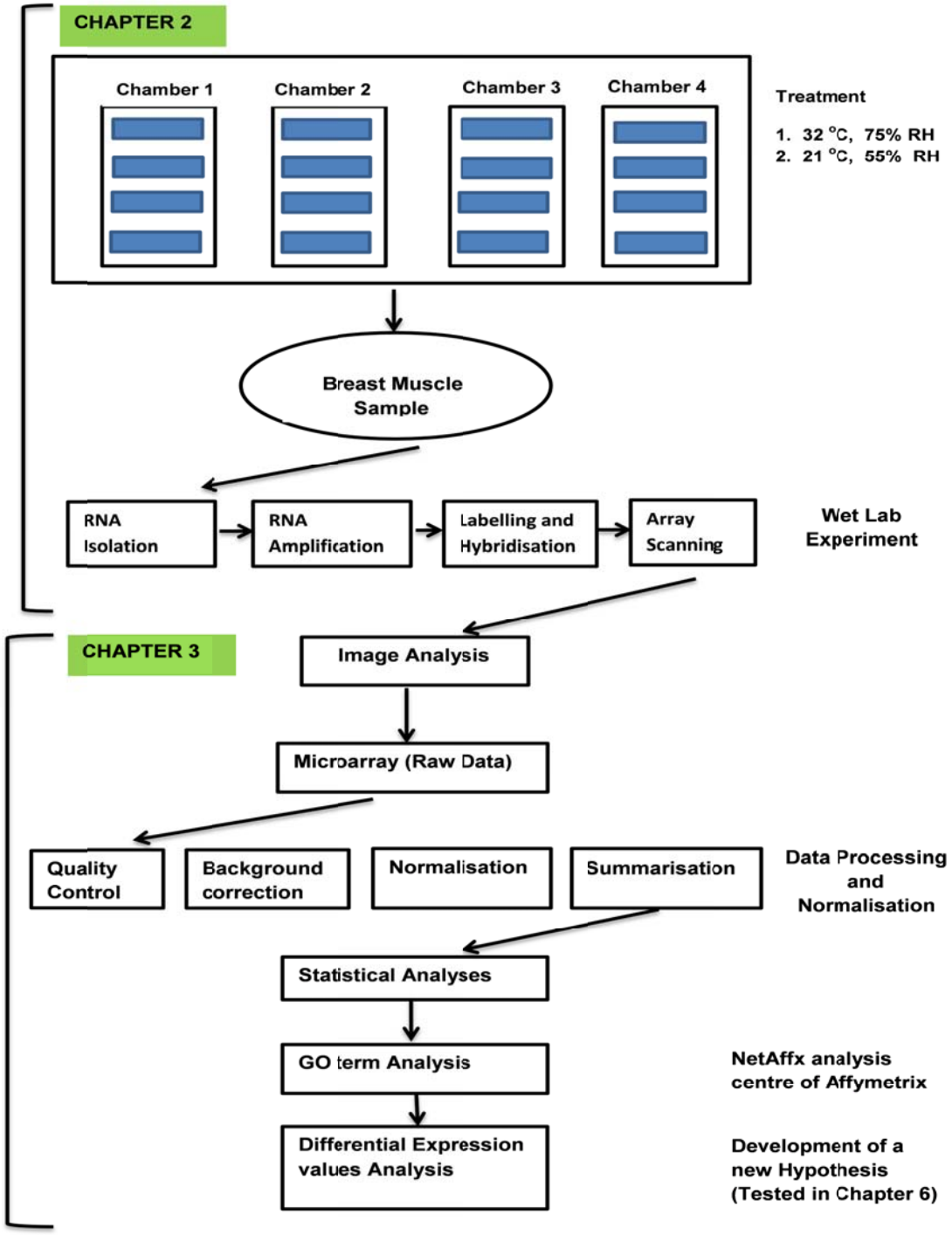
that vitamin E supplementation augmented the activity of naturally occurring antioxidant enzymes in the body like SOD in the skeletal muscle to reduce oxidative stress. However, it was also reported that high doses of vitamin E supplementation are related to some negative effects and can increase mortality (McGinley *et al.* 2009). Similarly, Mujahid *et al.,* (2009) reported that an olive-oil supplemented diet resulted in augmented expression of avUCP in skeletal muscle mitochondria and resulted in reduced oxidative stress induced muscle damage. Vitamin C, vitamin E, dantrolene sodium and olive-oil are known to have some anti-stress and anti-oxidant effects on skeletal muscles of heat-stressed broiler birds but are not ideal and sustainable solutions of the problem. It is not always possible to supplement broiler feed with these ingredients and even if these supplements have been added to broiler diet then, in addition to increasing the cost of production, they might not prevent muscle damage and improve meat quality in all cases.

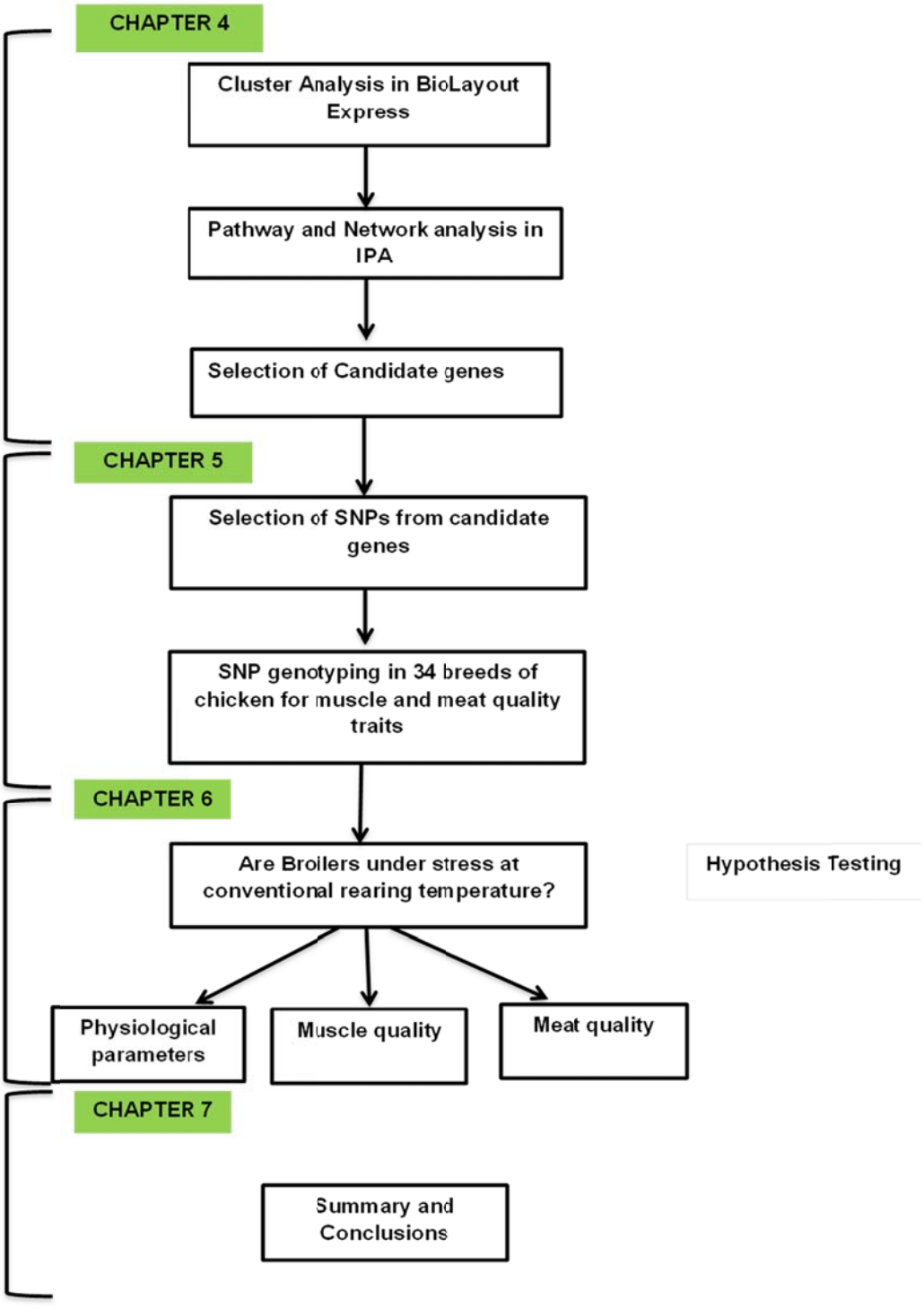
1.10 Objectives of the Project

The presence of higher incidence of muscle damage in meat-type chicken and its further exacerbation in response to various pre-slaughter stressors especially acute heat-stress suggest that genetic selection for growth-related traits has resulted in reducing the muscle and meat quality of broilers. Therefore, in the present project, it was decided to use heat stress as a tool to further amplify the expression of the genes involved in muscle pathology and to help identify candidate genes. The candidate genes were subsequently used to select a list of genetic markers (SNPs), responsible for causing this pathology and affecting meat quality, which could be used in future

breeding programmes to ameliorate muscle damage and improve meat quality in chicken. An outline of the thesis chapters is given in Figure 1-4.

Figure 1-4: A brief work flow diagram of thesis chapters. Detailed description along with the parts of the flow diagram is given in the relevant chapters.




CHAPTER 4
Cluster Analysis in BioLayout Express
Pathway and Network analysis in IPA
Selection of Candidate genes
CHAPTER 5
Selection of SNPs from candidate genes
SNP genotyping in 34 breeds of chicken for muscle and meat quality traits
CHAPTER 6
Are Broilers under stress at conventional rearing temperature?
Hypothesis Testing
Physiological parameters
Muscle quality
Meat quality
CHAPTER 7
Summary and Conclusions

Chapter 2 Measurement of Physiological Parameters and Sampling for Microarray Experiment

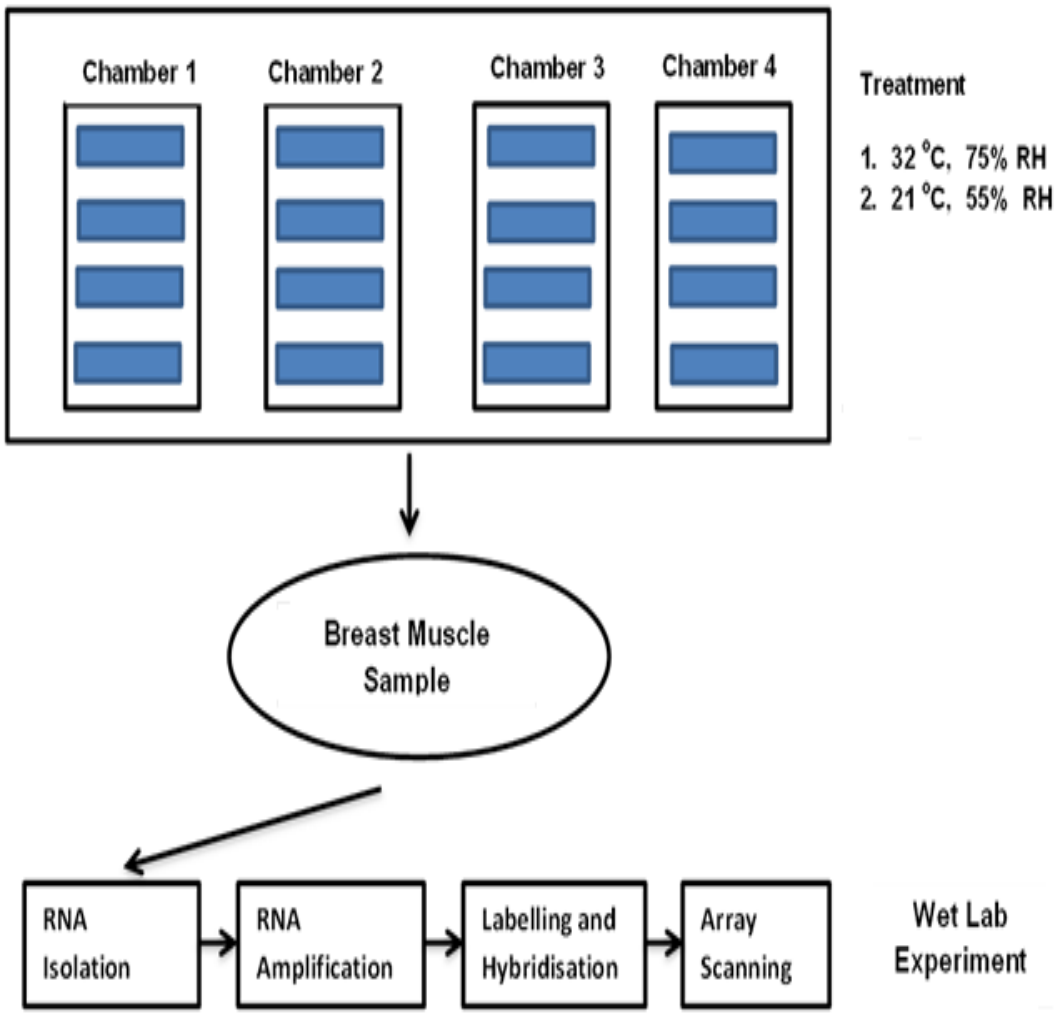
2.1 Introduction

Modern broilers are characterised by their faster growth rate and better feed conversion ratio (FCR) compared with layers and traditional breeds. As a result of genetic selection for growth-related traits the current broiler strains show at least a three-fold improvement in their growth rate and FCR relative to broilers fifty years ago. Current broilers can reach a body weight of 3 kg at market age but fifty years ago broilers could only achieve one fifth of this on the same feed (Havenstein *et al.* 2003). The increase in body weight of modern broilers is attributed mainly to the growth of breast muscles, *pectoralis muscles* (Pm), and to some extent to the thigh muscles, *bicep femoris* (Bf) (MacRae *et al.* 2006; Sandercock *et al.* 2009b). However, skeletal muscles of modern broilers are characterised by higher levels of pathological muscle damage indicated by their higher plasma creatine kinase activity compared with their genetic predecessors and egg-type chickens (Mitchell *et al.* 1994; Sandercock *et al.* 2009a; Sandercock *et al.* 2009b).

There is a higher incidence of spontaneous and stress-induced myopathies in broiler skeletal muscles (Mitchell *et al.* 1999a; MacRae *et al.* 2007), which may partly be due to the greater diameter of muscle fibres so that the cells are unable to absorb sufficient oxygen and nutrients from blood and similarly are unable to get rid of their waste products. This muscle damage is further exacerbated by different pre-slaughter stressors like catching, crating, shackling and heat stress; of these stressors, heat-stress is the one which can be controlled most readily in an experimental setting. Therefore, it was decided to use heat-stress as a tool to amplify the muscle pathology together with gene expression profiling in order to identify the genes underlying

these changes. Some pilot projects were run before starting the actual project in order to optimise the protocol for the final experiment (Details in Appendix-1.1) and an outline of the steps described in this chapter is presented in Figure 2-1.

Figure 2-1 Work Flow Diagram of Chapter 2 showing the experimental outline, with 4 chambers for exposing the birds to 2 treatments randomly on alternative days. Each chamber had 4 crates, each holding 2 birds of each breed and sex (4 x 2). RNA samples extracted from the breast muscles were used in the microarray experiment.



2.2 Materials and Methods

2.2.1 2.2.1 Rearing of birds and data collection

In this experiment, 80 broiler chicks (male line Ross-308, from a commercial hatchery) half male and half female, and 74 layer chicks (White Leghorn), hatched at the Poultry Unit, The Roslin Institute, were obtained. For the first two weeks, birds were randomly reared in groups of 20 birds per pen; broiler birds were reared with the different sexes in separate pens but layer birds were reared in mixed sex groups. At the end of week 2, layer birds that had been genotyped for gender (the sexing protocol is detailed in Appendix-2.2) were separated into pens by sex in a completely randomised design. Birds were reared to 6 weeks of age, fed on a layer starter diet on an *ad libitum* basis, and given a daily photoperiod of 16 hours light and 8 hours darkness.

Feed intake and body weight of the birds were measured on a weekly basis. After six weeks half the birds were subjected to heat stress and the other half to "cool" normal conditions as described below over 4 days from 42 to 46 days of age.

There were 16 pens in total, each with 8-10 birds; 4 pens were randomly selected on each day and the birds were transferred into controlled environment chambers. There were 4 chambers in total and out of these, 2 chambers were used for the heat treatments (32°C, 75% RH) and 2 for the control (21°C, 50% RH) on each day. Treatments were randomised to chambers on 4 successive days (Appendix-2.1, Table-1). Each chamber had 4 crates, each containing 2 birds (from the same pen)

each line and sex and the order of the pairs (crates) in each room was also randomised (Appendix-2.1, Table-2) crates were at the same floor level (on a wooden pallet).

About 30 minutes before the birds were transferred to the chambers, the relevant chamber was turned on so that it could get to the required temperature and humidity before birds were placed into the chamber for 2 hours. Each chamber was turned on and populated with birds with an interval of 45 min between successive chambers to allow for sampling the birds placed in the former chamber at the end of the treatment period. In this way, 32 birds were subjected to treatments on each of the 4 days and in total 128 birds were used in the experiment.

After completing the 2 h treatment, birds were taken out and their rectal temperatures were measured using a thermistor probe (Model 612-849; RS Components Limited., Corby, Northants, UK). A blood sample (2 ml) was taken from the brachial vein of each bird, using a sterilised and disposable 5 ml syringe, for subsequent determining plasma creatine kinase (CK) activity. Birds were euthanised by an intravenous injection of sodium pentobarbitone into the wing vein. The birds were deemed dead when there was cessation of visible respiratory movements and then birds were weighed. Two tissue samples each with a weight of 100-120 mg were taken from left pectoral muscle of each bird for subsequent RNA extraction.

For RNA extraction, samples were randomised prior to extraction and 8 of the randomised samples were used in one extraction. The protocol for RNA extraction is outlined in Appendix-2.3. After extracting the RNA for all of the 128 birds, the

concentrations of RNA solutions of the broiler males (to be used on the microarray) were determined using the Nanodrop procedure. The RNA samples were subjected to a Quality Control test (Appendix-2.4). The RNA samples were diluted to have the same concentration (50 ng/µl) and 20 µl aliquots from each sample were used for pooling the samples, in pairs, on the basis of same day, chamber and crate.

2.2.2 Statistical analysis

The experiment (comparing two breeds at the same age) was a 2 × 2 × 2 factorial design (breed × sex × treatment), with day/chambers/crates as blocking factors. Standard analysis of variance methods were used to analyse the data, with body temperature and body weight as the Y-variate (one by one), using GenStat (http://www.vsn-intl.com/genstat/).

2.3 Results

The data were analysed in GenStat with ANOVA using a balanced design with Day, Chamber and Crate as strata and testing for breed (broiler, layer) sex (male, female) and treatment (control, heat stressed) as fixed effects as well as their interactions. The assay for creatine kinase activity did not provide reliable results and the data are not presented.

2.3.1 Body Temperature

An increase in body temperature was recorded at the end of experiment for the heat stressed birds (H) that was greater in the broilers than in the layers. The body

temperatures of broiler and layer birds in the Control treatment (C) were 40.9°C and 42.0°C respectively while in the High Temperature treatment (H) they were 43.5°C and 42.3°C.

Exposure to a high thermal load significantly ($P<0.001$) increased the deep body temperature in birds of both breeds. Body temperature showed a significant ($P<0.001$) interaction between breed and treatment (Table 2.1) but other interactions (breed x sex) were not significant.

2.3.2 Body Weight

Average body weight of broiler and layer birds at 6 weeks of age showed significant ($P<0.001$) Breed x Sex interaction and other interactions (Breed x Treatment) were not significant. Average body weight of broiler and layer birds respectively were 4384 g (M) and 3694 g (F) and 693 g (M) and 559 g (F) at 6 weeks of age. By running ANOVA after log transformation, the breed x sex interaction was no longer significant, indicating that this interaction was due to a scale effect.

Table 2-1 Breed x Treatment interaction of body temperature (^{o}C) of male line (Ross-308) and White Leghorn layer birds at 6 weeks of age (*P*<0.001, SED=0.1030)

Treatment	Breed	
	Broiler	Layer
Control	40.931	41.975
Heat Treated	43.556	42.312

Table 2-2 Breed x Sex interaction for average body weight (g) of male line (Ross-308) and White Leghorn layer birds at 6 weeks of age (*P*<0.001, SED=69.1)

Sex	Breed	
	Broiler	Layer
Female	3694	559
Male	4384	693

2.4 Discussion

Body temperature for broilers in the control treatment was 1.04°C less than that of layer birds. After exposure to acute heat stress there was a significant increase in deep body temperature of about 2.63°C in broiler and 0.34°C in layers. The post heat-stress body temperature of broiler birds was 1.244°C higher than layer birds. These results confirm that genetic selection for broiler traits has decreased the ability of broiler birds to cope with the acute thermal challenge that may ultimately lead to detrimental consequences for normal body function and can also result in poor muscle and meat quality and be associated with adverse effects on their welfare. A lower body temperature in broilers compared with layers, in the control treatment, has not been observed before and may indicate that the broilers have a lower basal metabolic rate. Alternatively the layer may have been more active than the broilers as the latter could not move around freely in the crates. However, Debut *et al.,* (2005) studied the physiological responses to shackling and heat-stress in 3 lines (slow-growing (SGL), fast-growing line (FGL) and a heavy line (HL) of chicken for Breed, Treatment, Breed x Treatment. In agreement with us, they reported that muscle (breast and thigh muscles) temperature in SGL was significantly higher compared with FGL and HL.

Results of the present study are in agreement with the findings of Sandercock *et al.* (2001) who observed a significant increase in deep body temperature in broiler birds at 35 and 65 days of age compared with layers. Consistent with this, Mitchell and Sandercock (1995b) also observed similar results by exposing the birds to a high thermal load of 32.6°C and relative humidity 94%. This induced a highly significant

increase in deep body temperature of 2.8°C ($P<0.001$) which was accompanied by a 24 per cent increase ($P<0.05$) in total plasma CK activity, indicating the susceptibility of broilers to heat-stress and also the role of heat-stress in inducing muscle damage.

However, the results for body weight show that programmes of genetic selection for different quantitative traits (meat and egg-related traits) in these two types of chicken has been very successful in terms of its divergent effect not only on body weight and also in increasing the susceptibility to different ante-mortem stressors such as heat stress.

2.5 Conclusion

These results confirmed that broilers are more susceptible to heat stress compared with layers. RNA samples obtained in this chapter from heat stress and control broilers and layers were used for the Microarray Experiment (Chapter 3).

Chapter 3 Microarray Data Analyses

3.1 Introduction

Gene expression analysis has been a subject matter of interest to biologists over the past few decades to investigate the involvement of genes of interest under certain given conditions. Gene expression of a given set of genes is the measurement of the transcription level, transformation of DNA sequence information into RNA sequences, for that set of genes. Over the past years, different techniques have been employed to study gene expression including northern blot, real-time quantitative RT-PCR for a few genes of interest; the advent of microarray technology has made it possible to simultaneously monitor the genome-wide expression levels of genes, in a given tissue or organ at a given time point.

The major purpose of designing and using a whole-genome array is to detect the differentially expressed genes (DEGs), in relevant tissue types and treatments in any given organism, by statistical analysis, and to measure the extent of expression of these genes. A microarray slide (also commonly known as a gene chip, DNA chip, or biochip) is a glass slide or silicon chip that has a collection of microscopic DNA spots (single strand DNA fragments or probes) attached to a solid surface (Leung & Carvalieri 2003). The number of DNA probes varies from a few thousand in the early cDNA arrays to over a hundred thousand in modern oligonucleotide arrays. (Baird 2010).

There are two types of microarray, one-colour (Affymetrix) and two-colour microarray. The sample/target cDNA is labelled with a fluorescent dye or an

antibody that binds to a dye. In two colour microarrays, two dyes, a red (Cy5) and a green (Cy3) dye are used, whereas in Affymetrix chips an antibody to biotin is used (Baird 2010). The intensity of the scanned measurement shows the result of the competitive hybridization between the two samples.

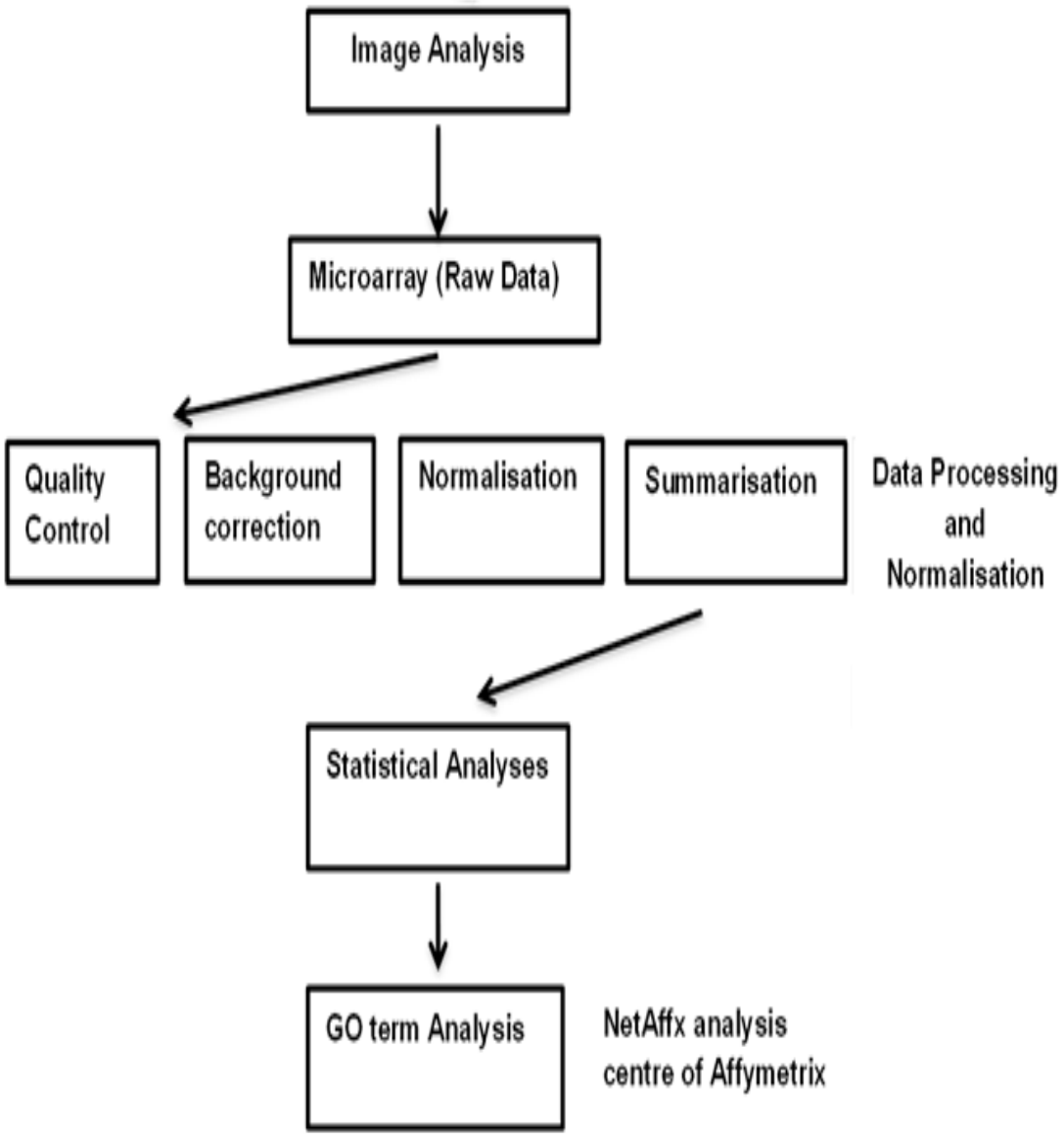
The layout of Affymetrix chips is more complex than a spotted array or cDNA array. On average there are 11 probe pairs for each gene and this set of 11 probe pairs is called a probe set for that gene. Each probe pair consists of one perfect match oligonucleotide (PM) and a mismatch oligonucleotide (MM). Both of these probes are 25-mer in their length. The mismatch probe differs from the perfect match probe by a single base substitution in the centre (13^{th} bp) (Jiang *et al.* 2008a). Probe sequences are chosen from the consensus sequence for that gene in publically available databases (Robert *et al.* 1999). The aim of the mismatch probe is to determine the background and non-specific hybridization that contributes to the signal measured for the perfect match probe.

3.2 Objectives

From the review of the relevant literature (Chapter 1) it is clear that many physiological mediators are involved in the responses to heat stress including various ions, enzymes, ROS etc. It was, therefore, decided to use microarray technology to get a comprehensive picture of gene expression in broiler breast muscles, in response to heat-stress, in order to help identify the gene candidates for muscle quality and

subsequent meat characteristics. An outline of the experimental procedures described in this chapter is presented in Figure 3-1.

Figure 3-1 Work Flow Diagram of Chapter 3 showing steps of microarray data processing and normalisation and their subsequent analyses.



3.3 Materials and Methods

3.3.1 Rearing of Birds

In total 128 birds were reared, that were equally divided into two breeds and two sexes (2 x 2 x 32). For the microarray experiment only male birds were used (32 broiler males and 32 layer males). Details of rearing and treating the birds are described in Chapter 2 section 2.2.1.

3.3.2 Tissue sample collection and data recording

Details of tissue sample collection and data recording are given in Chapter 2, section 2.2.1. After the extraction of RNA from the breast muscle samples, the concentration of RNA samples were measured, by using the Nanodrop, and they were diluted with deionised and RNAase free water to the final concentration in 50µl for each sample individually (details in section 2.2.2). Finally, the RNA samples for the 32 broiler male and 32 layer males were pooled on the basis of their treatment, day (sampling time), chamber and crate to give 8 replicates of each treatment (breed x treatment).

3.3.3 Microarray Slides

The Affymetrix chicken array chips (38.5K) employed in the current experiment were comprised of over 38,000 probe sets representing 32,773 transcripts corresponding to over 28,000 chicken genes. This Chicken Genome Array also contains 689 probe sets for detecting 684 transcripts from 17 avian viruses. In this

array 11 pairs of probes were used per gene and all of them were 25-mer in length. For hybridization controls three types of biotin (bioB, bioC, bioD from *Escherichia coli*) were used. Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), beta-actin and GAPDH were used as control genes in the assay. The detection sensitivity of this array was 1:100,000 (AffymetrixDatasheet 2009), meaning that it can detect 1 molecule out of 100,000 molecules (http://users.soe.ucsc.edu/~sugnet/microarray). The microarray hybridisation was completed by Miss Alison Downing in the ArkGenomics laboratory from the RNA samples provided by the author.

The mRNA was converted to complementary RNA (cRNA) and labelled with biotin. The biotinylated cRNA was fragmented prior to hybridisation. After hybridisation the arrays were washed, stained and scanned to visualise the spots (Appendix-2.4). After scanning, all of the 32 microarray slides were translated to so-called Cel files. The 32 Cel files were processed in GenStat to obtain expression values in 4 batches 8 slides/batch. Each batch contained slides from birds treated on the same day. The Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003) was used to extract the gene expression data. RMA combines the intensity values from the CEL files and the chip information from a CDF file.

This processing method (RMA), which is also called probe level model, only uses perfect match (PM) information and transforms these values according to a kernel density estimate of the PM distribution. The processing procedure of RMA comprises 3 steps: i) Background correction, ii) Quantile normalization, and iii) Summarisation. Finally, the program computed the expression values against each

probe on every slide and summarises the expression value for every gene on every slide. These steps are explained briefly in Appendix 3-1.

After processing the Cel files, RMA returned a spreadsheet with 4 columns (Slides, Probes, Expression, SE) and 308,280 rows for each set of 8 slides, having expression and standard error (SE) for all the 38,535 genes against each slide. All of the 32 slides were filtered for expression levels greater than 1 which resulted in the reduction of the number of probes from 38,535 to 19,038 on each slide. The detailed procedure is outlined in Appendix-3.3. Filtered data were used to create a new combined spreadsheet for all the 32 slides.

Another spreadsheet was created within GenStat with 32 rows (one row for each slide) and 5 columns containing information on the category of each slide, the slide identification, the treatment, day and chamber. The slide, treatment, breed, day and chamber were used as factors in an ANOVA (Appendix-3.1).

The normalised data were analysed by using Microarray One-Channel ANOVA. Breed x treatment was used as the treatment structure and the hierarchical structure day/chamber/breed was used as the blocking factor. From these ANOVA results the false discovery rate (FDR) was calculated for the three probability values for the effects of treatment, breed and their interaction, respectively. FDR was calculated using the Mixture Model within GenStat. Maximum iteration cycles were set to 300 in the FDR calculation. Significant genes showing a breed x treatment interaction (*P*-value <0.05) were used for subsequent investigation. The gene ontology terms (GO

terms) for this significant set of genes were downloaded from the *NetAffx* Analysis Centre of Affymetrix (www.affymetrix.com).

3.4 Results

A total of 19,038 genes were statistically analysed in GenStat. From the ANOVA analysis 1,873 genes were significant ($P< 0.05$) for treatment (heat stress vs control); 10,731 genes were significant for the breed comparison (broiler vs layer); and 2,213 genes were significant for the breed x treatment interaction, as shown in Table 3.1. The results for the interaction showed that 93 genes were significant at $P<0.001$, 635 at $P<0.01$ and 2,213 at $P<0.05$ levels of significance respectively. The false discovery rate (FDR) for 2,213 statistically significant genes ($P<0.05$) was less than 31.5% (Appendix-3.2). While the FDR for the significant genes for treatment was 44% at $P<0.05$ of significance and for the significant genes for the breeds was 3% at $P<0.05$ level of significance.

Table 3-1 Numbers of significant genes (*P*<0.05) for treatment, breed, and breed x treatment interaction

Level of significance	Treatment (Heat-stress vs Control)	Breed (Broiler vs Layer)	Breed x Treatment interaction
0.001	107	5,208	93
0.01	617	8,182	635
0.05	1922	10,733	2,213

The main objective of this study was to identify the genes involved in muscle damage in broilers that was further exacerbated by exposure to acute heat stress. Layers have no detectable muscle damage and exhibit greater resistance to heat stress compared with broilers. So, from the microarray results, the genes significant for 'breed x treatment' interaction were selected for further investigation in order to explore the genetics and comparative genetic differences between these two types of chickens for muscle damage with and without exposure to heat-stress.

3.4.1 Categorisation of candidate genes from significant breed x treatment interactions on the basis of their biological functions

Genes significant for the breed x treatment interaction were further divided into different categories on the basis of the biological functions in which they are involved. The total number (2,213) of differentially expressed genes was classified into 12 different categories, as shown in Table 3-2. More than half (1,321) of the genes had no Gene Ontology (GO) term for biological process or function. These genes are present in two major categories, i) 567 genes that have no known function and ii) 754 genes that are not involved in any biological process on the basis of their GO terms. The results indicate that there are a substantial number of genes which are involved in pathogenesis of heat-stress induced muscle damage and response to heat-stress but their function are not currently known.

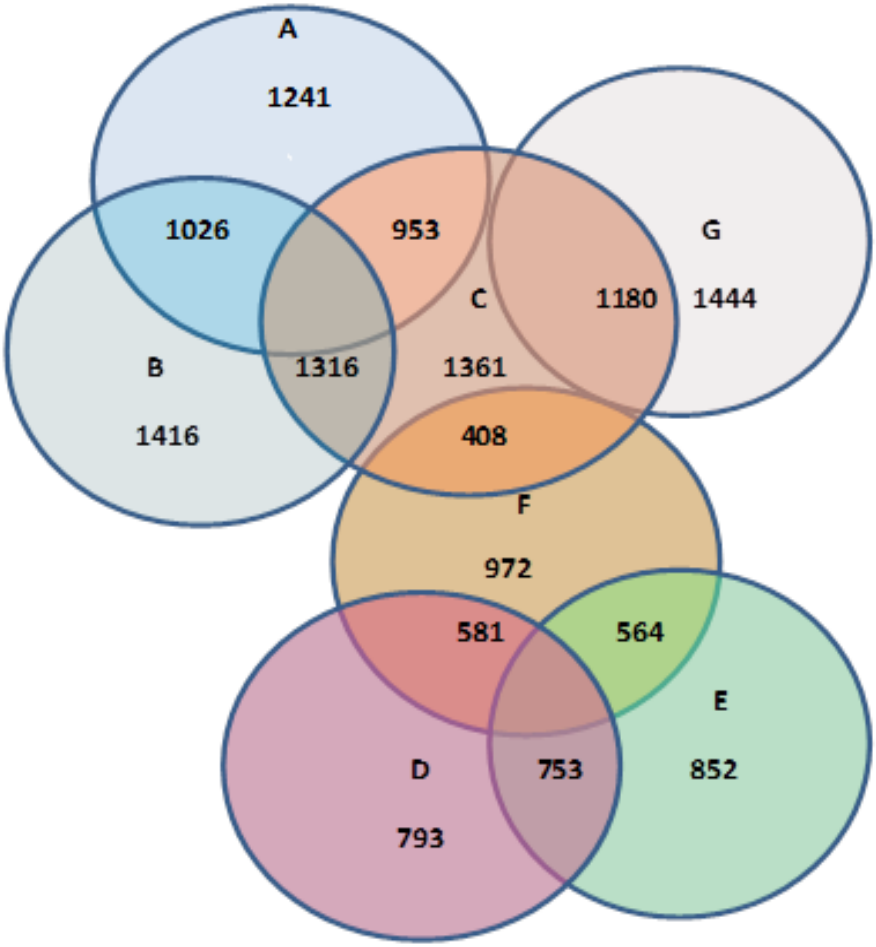
Table 3-2 Categories of significant genes for breed x treatment interaction (*P*<0.05) on the basis of their biological functions

Group	List of Categories	No. of genes
1	New transcripts (previously unidentified genes)	567
2	Genes with no GO term for biological functions	754
3	Signal transduction	130
4	Stress-related response, inflammatory, apoptotic and proteolytic functions	116
5	Inter and intra-cellular transport of substances and muscle contraction	128
6	Metabolic process	121
7	Protein phosphorylation, dephosphorylation, and folding	92
8	Transcription and translation	136
9	RNA processing and metabolism	25
10	Cytoskeleton organization and polymerization of filaments	23
11	DNA repair and metabolism	34
12	Genes involved in cellular proliferation, development-related functions and biosynthesis of various biological substances	82

3.4.2 Different comparisons (within and between breed and treatment) of genes significant for interaction

The selected 2,213 genes (significant for interaction) were further divided into up- and down-regulated categories for various comparisons within and between breed and treatment, that is, broiler heat-stress (BH) vs broiler control (BC); layer heat-stress (LH) vs layer control (LC); BC vs LC; BC vs LH; BH vs LC; and BH vs LH). The results showed that a large number of genes which were up and down regulated in various comparisons overlapped with each other, as shown in Figure 3-2.

Figure 3-2 Number of overlapping genes in different up and down regulated categories of the significant genes (*P*<0.05) for the breed x treatment interaction



A **Down regulated genes in BC vs LC**

B **Down regulated genes in LH vs LC**

C **Up regulated genes in BH vs BC**

D **Up regulated genes in LH vs LC**

E **Down regulated genes in BH vs BC**

F **Up regulated genes in BC vs LC**

G **Up regulated genes in BH vs LH**

3.5 Discussion

In the present study data were normalised by using RMA due to its robustness and superiority in terms of bias, variance, the ability to detect differential expression levels, and to produce reproducible and reliable results compared with other commonly used algorithms (Irizarry *et al.* 2003; Millenaar *et al.* 2006). The choice of processing methods has a significant impact on the results of microarray data analyses, therefore, the selection of the processing methods should be made according to the specific questions and needs of the projects (Shedden *et al.* 2005; Jiang *et al.* 2008a). The quantile normalisation methods (RMA, GCRMA-EB, GCRMA-MLE, PDNN) are superior and faster compared to other methods e.g. contrast methods and non-linear methods (Bolstad *et al.* 2003).

After normalisation and background correction the data were filtered for expression levels greater than 1 which resulted in the reduction in the number of genes from 38,535 to 19,038. It is generally observed that large numbers of genes on an array are either not expressed or expressed at very low levels or at such levels that are of no biological significance. Usually in any given tissue only 30–40% of the genes are expressed at array detectable levels under any given condition (Lu *et al.* 2011). In modern GeneChips, tens of thousands of genes are represented on each array and the number of true null hypotheses is very large. In these situations, multiple testing corrections are needed in order to reduce the number of false positives, to control False Discovery Rate (FDR) and to increase power (Hackstadt & Hess 2009). Due to this reason the data in this study were filtered out on the basis of overall expression

signals to remove the non-informative genes and reduce the number of null hypotheses. In filtering by signals, the genes having low signal/expression values (closer to background) are filtered out to reduce the noise and increase power. Finally, the genes that passed the filter were analysed leading to lower FDR and better power (van Iterson *et al.* 2010) in a two-stage approach (Bourgon *et al.* 2010). MAS detection (or Present/Absent) calls for Affymetrix arrays, the overall mean/signals and the variance are the commonly used filter statistics for microarray data. These filters are also known as non-specific filters because they are used across all samples by ignoring the treatment. Non-specific filters are preferably used to avoid the treatment bias and subsequent interference with down-stream statistical analyses (Bourgon *et al.* 2010) and a non-specific filter was chosen to remove the uninformative set of genes in the present study.

For the breed x treatment interaction, 93, 635 and 2,213 number of genes were differentially expressed at significant levels $P<0.001$, $P<0.01$ and $P<0.05$ respectively. All the statistically significant genes (2,213) were significant at a false discovery rate (FDR) less than 31.5%.

From the ANOVA results, out of a total of 19,038 genes, 5,208, 8,182 and 10,733 genes were significant at $P<0.001$, $P<0.01$ and $P<0.05$ levels of significance respectively, for the breed comparison. These results indicate that there were big differences between broilers and layers. On the other hand, comparatively few significant genes (107, 617 and 1,922 at $P< 0.001$, $P<0.01$ and $P<0.05$, respectively) were differentially expressed in the treatment comparison indicating that the

differences in gene expression of treated and control birds were not as high as the breed differences. The reasons for this may be that the birds were exposed to 32°C temperature and 75% relative humidity for 2 hours and this duration might not be sufficient to elicit the expression of a greater number of genes. However, it has been observed that during transportation of birds from farm to processing plant, which typically takes about two hours, broilers may suffer substantial heat-stress that ultimately leads to significant reduction in meat quality of broiler chickens (Petracci *et al.* 2006; Petracci *et al.* 2009). It was also in agreement with our finding from the phenotypic data of the present study showing that heat-stress resulted in 2.63°C increase in the body temperature of broilers and 0.34°C increase in the body temperature of layers, within just two hours. Alternatively, it is possible that there are a comparatively small number of genes which are involved in the heat stress response in breast muscles compared to the number of genes involved in the breed difference.

Lu *et al.,* (2011) reported that of all the genes present on any given array only a small percentage (<10%) of differentially expressed genes are true positives in a typical experiment. In the present study, 19,038 genes were analysed in total and we found 2,213 genes significant ($P<0.05$) for the breed x treatment interaction which are about 11.6% of the analysed genes and is in agreement with the finding reported by Lu *et al.,* (2011).

These 2,213 genes were further split into up and down-regulated genes for various comparisons. It is very interesting that out of 1,361 up-regulated genes in BH

compared with BC and 1,416 down-regulated genes in LH compared with LC, 1,316 genes were shared. In agreement with this, out of the 852 down-regulated genes in BH and 793 up-regulated genes in LH (compared with their respective controls) 753 genes were shared. These results show that these two types of chicken are almost opposite to each other in their heat-stress-induced gene expression.

Of the 1,444 genes that were up-regulated in the BH vs LH comparison, 1,180 were also represented in the 1,361 up-regulated genes from the BH vs BC comparison. Interestingly, out of the 1,416 down-regulated genes in the LH vs LC and 1,241 down-regulated genes in the BC vs LC comparisons, 1,026 genes were down-regulated in both. These results indicate that gene expression in breast muscles of BC and LH was similar. This was further confirmed by the presence of 753 common genes, among the total of 793 up-regulated genes in the LH vs LC comparison and 852 down-regulated genes in the BH vs BC comparison.

In the current study, gene expression results are different in broilers and layers in their response to heat stress, even though there was a significant increase (0.34^0C) in the body temperature of layers, suggests that there are different biological mechanisms which are initiated by heat stress in broilers and layers. Given that broilers are more susceptible to heat stress, as indicated by the body temperature data, down regulation of those genes in LH compared with LC which are up-regulated in BH (compared with their control counterpart) is surprising and merit further exploration.

The 2,213 differentially expressed genes in the interaction term were divided into different categories on the bases of their functions and biological processes in which they are involved. A total of 567 transcripts had no gene symbol and no gene name i.e. these are genes that are not yet characterised and they comprised 25.6% of the total number of significant genes. It indicates that there are many genes which are involved in heat-stress induced response in chicken skeletal muscle that have not been identified and merit further investigation. Similarly the largest group of genes, representing about 34% of the 2,213 significant genes, were found to have no GO term for biological function against them which indicates that these genes are not well-annotated as to their function at the time the GO terms for this gene set were retrieved (in March, 2012) from *NetAffx* Analysis Centre of Affymetrix.

Other major categories of genes were involved in various biological functions. About 5% of genes were directly involved in oxidative stress, stress response, inflammation, apoptosis, proteolytic functions and tissue damage, consistent with muscle pathology caused by heat-stress. Similarly 6% (129) of the total of 2,213 significant genes were involved in signal transduction and of these about 62% were directly involved in G-protein signalling, through the G-protein coupled receptor (GPCR) protein signalling pathways and small GTPase mediated signal transduction. GPCR are present in the cell membrane and upon activation by extracellular stimulus they transduce these signals to G-proteins (Brady & Limbird 2002; Krumins & Gilman 2006). G-proteins then transduce these signals to downstream genes and control various biological processes including oxidative stress, inflammation, muscle contraction, glycogen metabolism, biosynthesis of protein and cell proliferation, and

the concentrations of intracellular ions by coordinating the signals between GPCR, some enzymes and voltage-gated ion channels (Ashton *et al.* 2003; Amir *et al.* 2006; Allen *et al.* 2007; Lattin *et al.* 2007; Sprang *et al.* 2007; Baker *et al.* 2008). It indicates that in addition to the genes (Group 4, Table 3-2) involved in inflammatory and cell death related functions directly, the genes (Group 3, Table 3-2) involved in signal transduction are also involved indirectly in similar functions (Kuehn & Gilfillan 2007; Dietrich & Horvath 2010; Dagda *et al.* 2011; Jeon *et al.* 2011; Deng *et al.* 2012) and additionally they are also involved in controlling the intracellular levels of ions (Grieco *et al.* 2005; Currie 2010). In this study, a large proportion (5.73%) of significant genes was involved in inter- and intra-cellular transport of various substances, mainly ions (cations), and were ultimately involved in muscle contraction and muscle damage-related functions (Allen 2004; Allen *et al.* 2010; Buraei & Yang 2010). Genes involved in cellular proliferation, development-related functions and biosynthesis of various biological substances represented 3.7% of the significant genes. It is proposed that these genes might be involved in tissue repair and maintenance related functions to minimise the deleterious effects of heat stress. Additionally, it is also suggested that about 1.5% of significant genes that were present in the DNA damage repairing category, might be involved in repair of heat-stress associated pathology.

Protein folding and modification, mainly by phosphorylation, was also a major category representing 4.2% of genes. These genes are involved in carrying out various biological functions and a variety of biochemical reactions in the body

(Hebert & Molinari 2007). In addition, about 6% of the genes were involved in metabolic and redox functions. Stress is known to accelerate the metabolic rate, mainly through carbohydrate metabolism, to produce greater amounts of energy and to facilitate a 'fight or flight' responses (Kaiser *et al.* 2009; Shini & Kaiser 2009; Shini *et al.* 2009). Other categories include RNA processing and metabolism, cytoskeleton organization and polymerization of filaments which comprised 25 and 33 genes respectively and represent 2.6% of the total number of significant genes. Genes involved in the RNA processing category may be related to transcription and translation (Group 8, Table 3-2). However, the cytoskeleton is known to be responsible for cell shape, motility and its involvement in cell division (Maekawa *et al.* 1999; Krupp *et al.* 2006). Hence, it is suggested that genes present in this group (Group 10, Table 3-2) might be involved in the movement and division of leukocytes such as heterophils and macrophages. Therefore, it is likely that these genes serve as a secondary mediator of the genes present in categories 3, 4 and 5 to shape the stress and inflammatory response to heat stress induced muscle damage. Out of these significant genes, 49 are expressed in mitochondria. This reflects the fact that that the mitochondrion is the major organelle in the cell that is affected by heat-stress in skeletal muscles and leads to oxidative stress and triggering cell death. Similar to this, about 200 genes are those that directly affect the cell membrane. The results suggest that damage to mitochondria and the cell membrane are potentially important components of heat-stress induced pathogenesis in chicken breast muscles.

Taken together, these results suggest a picture of stress responses, inflammation and oxidative stress and tissue damage. However, as tens to hundreds of genes were present in each of these categories the selection of true candidate genes is difficult. The major objective of this study was to identify the candidate genes involved in muscle damage in broilers that is further exacerbated by exposure to acute-heat-stress. In order to select an accurate and reliable set of genes for further investigation, it was decided to use different bioinformatics tools to sift out the relevant clusters and pathways and identify suitable candidate genes by exploring underlying biological mechanism.

3.6 Conclusion

1. The present study is the first study of its kind in which genome-wide expression levels were studied in the breast muscles of heat-stressed chicken.
2. A reasonable number of differentially expressed genes were found.
3. A large set of differentially expressed genes were found having no gene symbol and no associated GO term indicating that the functions of these genes are not yet characterised.

Chapter 4 Bioinformatics Analyses of Gene Expression Differences between Broilers and Layers in response to Acute Heat Stress

4.1 Introduction

The use of high throughput technology has made it possible to monitor the expression of tens of thousands of genes simultaneously, in a given tissue or organ at a given time point; which is in turn useful in identifying differentially expressed genes under the effect of any specific treatment (Whitworth 2010). However, it is often difficult to assign biological significance to a large number of genes that are implicated in an experimental comparison. This problem can be solved when the number of differentially expressed genes are organised via hierarchical clustering methods (Lim *et al.* 2007). In the current study, BioLayout Express^{3D} and Ingenuity Pathways Analysis (IPA) software were used to detect underlying patterns in the gene expression data.

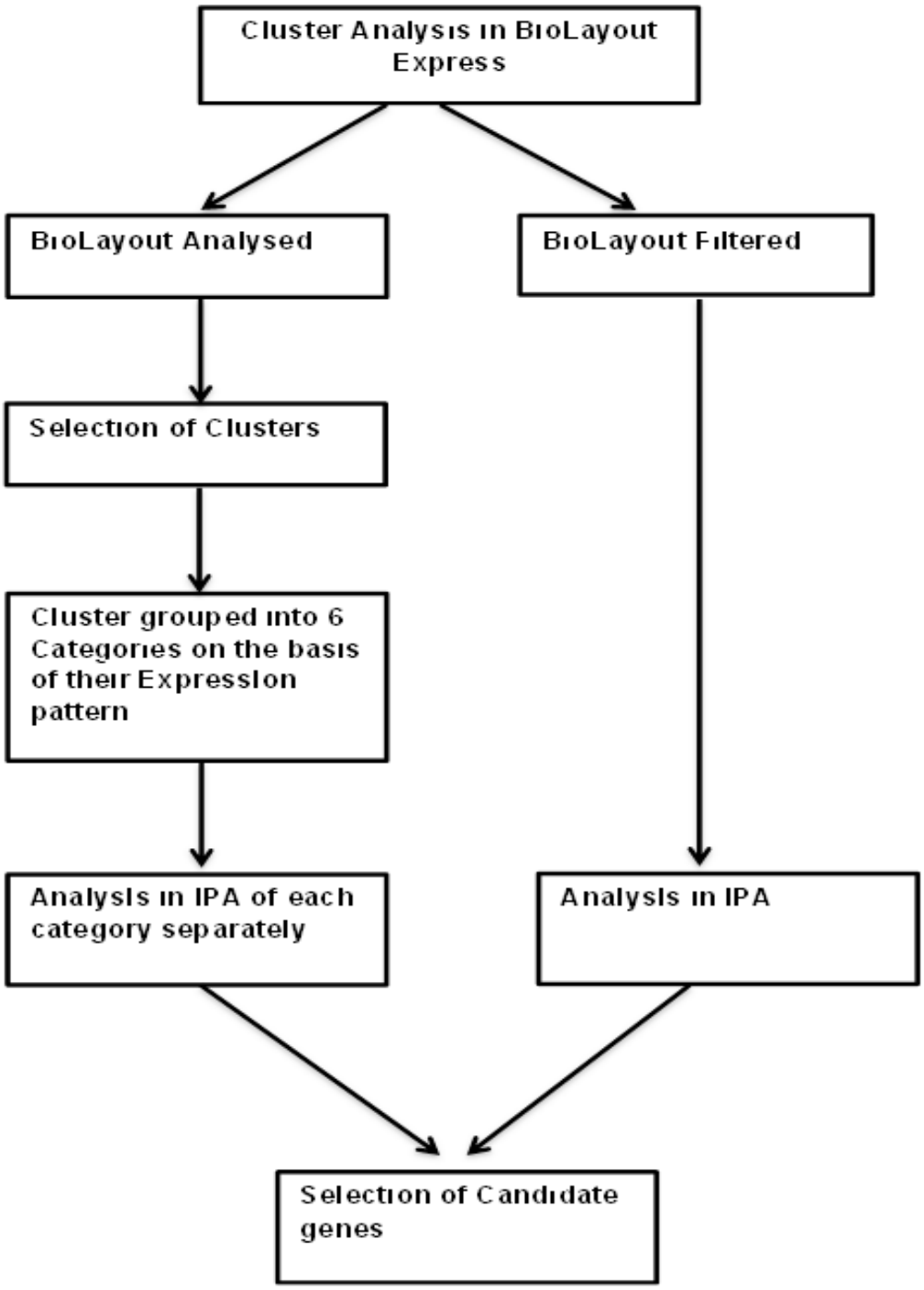
BioLayout Express^{3D} is a network visualization tool (BioLayout Java 1,2) used to visualize and manipulate graphs of different types of biological information generated from gene expression and protein data sets (Theocharidis *et al.* 2009). It visualizes and analyses the correlation between high dimensional data points, and also gives a view of clustering and analysis of large network graphs in two and three dimensional space, derived primarily from biological data (Freeman *et al.* 2007; Theocharidis *et al.* 2009).

Ingenuity Pathways Analysis (IPA) is a commercial bioinformatics application used to explore biological pathways and provide biological process information for a given set of genes. IPA can simultaneously compute multiple networks of pathways

using the Ingenuity Pathways Knowledge Base, which contains over 1 million findings extracted from peer-reviewed journal articles. These functional relationships such as gene regulation, protein-protein interactions, post-translational modifications and drug-target interactions are displayed on the following website (http://www.ingenuity.com/).

In this study, differentially expressed genes ($P<0.05$) from the microarray experiments (Chapter 3) were first analysed in BioLayout Express^{3D} to identify the clusters of the genes having positive correlations with each other. IPA was used to identify biological pathways and networks among the genes clustered by BioLayout to narrow down the number of differentially expressed genes on the basis of biological significance. On the basis of these pathways potential candidate genes for meat quality, differentially expressed as a result of heat stress, were selected. An outline of the structure of the chapter is presented in Figure 4-1.

Figure 4-1 Work flow diagram of Chapter 4. In this chapter significant genes from the Microarray experiment (Chapter 3) were analysed in Biolayout Express and Ingenuity Pathway analysis. Finally candidate genes were selected from the significant pathway and networks. Some candidate genes were also selected on the basis of their Go terms.



4.2 Material and Methods

This Chapter is based on the results of the microarray experiment that is described in Chapter 3. The genes that were significant at $P< 0.05$ for the breed x treatment interaction were used for post-microarray bioinformatics analyses. As described in the previous chapter the main objective of this study was to identify the candidate genes responsible for muscle damage in broilers that is exacerbated by heat-stress compared with layers that have no incidence of muscle damage and a greater resistance to heat-stress.

4.2.1 Cluster analysis in BioLayout

A single input file was developed for the combined data set in tab delimited text format, with Probe Ids in the left most column, followed by their respective *P*-values, GO terms and expression values for all of the slides. The annotations for these genes were downloaded from the NetAffx analysis centre of Affymetrix (http://www.affymetrix.com/analysis/index.affx). There were 32 slides in total, 8 slides for broiler control (BC), 8 for broiler heat-stress (BH), 8 for layer control (LC), and 8 for layer heat-stress (LH). As the RMA was used to get the expression values and were $\log_2$ transformed, the expression values were unlogged. The input file was loaded into BioLayout Express and a 0.80 Pearson correlation threshold was selected. The resulting 1,147 genes were only those which had a positive correlation >0.80 as the genes having a low or negative correlation with other genes were filtered out.

Clusters were viewed in the Class Viewer, after running the Markov Clustering Algorithm (MCL). For cluster size, a minimum threshold of 4 genes per cluster was selected to limit the size of the smallest clusters (Theocharidis *et al.* 2009). Clusters were selected for further study on the basis of their respective graphs, showing clear differences in the expression pattern (up and down-regulation) of the genes for different treatments and breeds. It was observed that these clusters could be grouped in different categories on the basis of similarities of their graph patterns with each other. Hence, the clusters that showed a very similar type of expression graph were grouped into 6 categories.

Finally the gene expression data for all clusters belonging to the same category were combined into a single Excel sheet that was used to calculate the differential expression values of the genes for different breed x treatment interactions (BC, BH, LC, and LH) for that category; in order to make the input files for analysis in IPA. Finally those input files (with expression values) were used for the pathways and network analysis in IPA.

4.2.2 Pathways and Networks analysis in IPA

Out of the total 2,213 genes ($P<0.05$), 1,147 genes were analysed and clustered by Biolayout Express at a Pearson correlation threshold of 0.80. The genes in each category were analysed in the Ingenuity Pathway Analysis (IPA) to determine their respective pathways and networks. IPA analyses the submitted gene lists by using Fisher's exact Test identify biological functions and pathways that are enriched in the data set. Genes were analysed in IPA by using 'Core Analysis' and they were

mapped against all the 'Tissues and Cell Lines' available in Ingenuity Pathway Analysis Knowledge Base (IPAKB). IPAKB has millions of findings extracted from peer-reviewed journals, books and other publically available databases. Because the IPA has the information mainly about mammals (Human, Mouse and Rat) in its Knowledge Base, the submitted gene lists were mapped against all the species and some information (different in avian) was changed in the significant pathways and networks e.g. Neutrophil to Heterophil. For network generation, a threshold of 35 molecules per network and 25 networks per analysis was selected; both direct and indirect relationships of molecules were considered for the network generation. After uploading the input file it was saved in IPA and instantly subdivided into 4 sub-datasets, (i) All IDs (having all the genes from the input file), (ii) Mapped IDs (genes that were successfully matched to their respective molecules), (iii) Unmapped IDs (genes which failed to map to genes available in IPAKB), and (iv) Analysis-ready IDs (genes able to proceed in pathways and networks analysis).

Gene sets of all the categories were analysed separately in IPA by using the methodology described above and significant pathways ($P<0.05$) and networks were selected, as shown in the Table 4.1 – Table 4.6. Similarly, the 1,066 genes filtered out by Biolayout Express were also analysed in IPA, by using the same procedure, to determine the interesting significant pathways and networks for this gene set, as shown in Table 4.7.

4.2.3 Selection of Candidate Genes

1. Genes were selected from all the categories on the basis of their key positions in the selected pathways and networks. In Pathways, priority was given to those genes that were present in the upstream and switching on/off the down-stream molecules through their signalling cascades.
2. In networks, the focus was on genes that were common in two or more networks and therefore involved in switching on more than one network at a time.
3. Genes which could not be mapped in IPA but were interesting on the basis of their GO terms were also selected, to address the particular questions of the present study.

4.3 Results

4.3.1 Selected clusters from BioLayout analysis

There were 54 clusters in total and 21 of them were selected on the basis of their clear expression patterns, as shown below, while other clusters in which the expression pattern was not clear were rejected. The 21 selected clusters included a total of 509 genes. The selected clusters could be grouped into 6 distinct categories depending on their specific graph patterns, based on the expression values of the genes.

Category I The expression values of genes in this category were higher in broilers than layers. Heat-stress resulted in a further increase in expression levels for broiler but a decrease in layers, compared to their respective controls (Figure 4-2). In total, 285 genes were present in clusters 01, 13, 14, 16, 18, 21, 31 and 41 grouped in category I.

Category II The expression level of genes in this category were higher for broilers than layers under control temperatures. After heat-stress expression levels were decreased in broilers compared with BC and conversely, increased in layers as compared to LC (Figure 4-2). Cluster 02 and 46 were part of this category, comprising 93 genes in total.

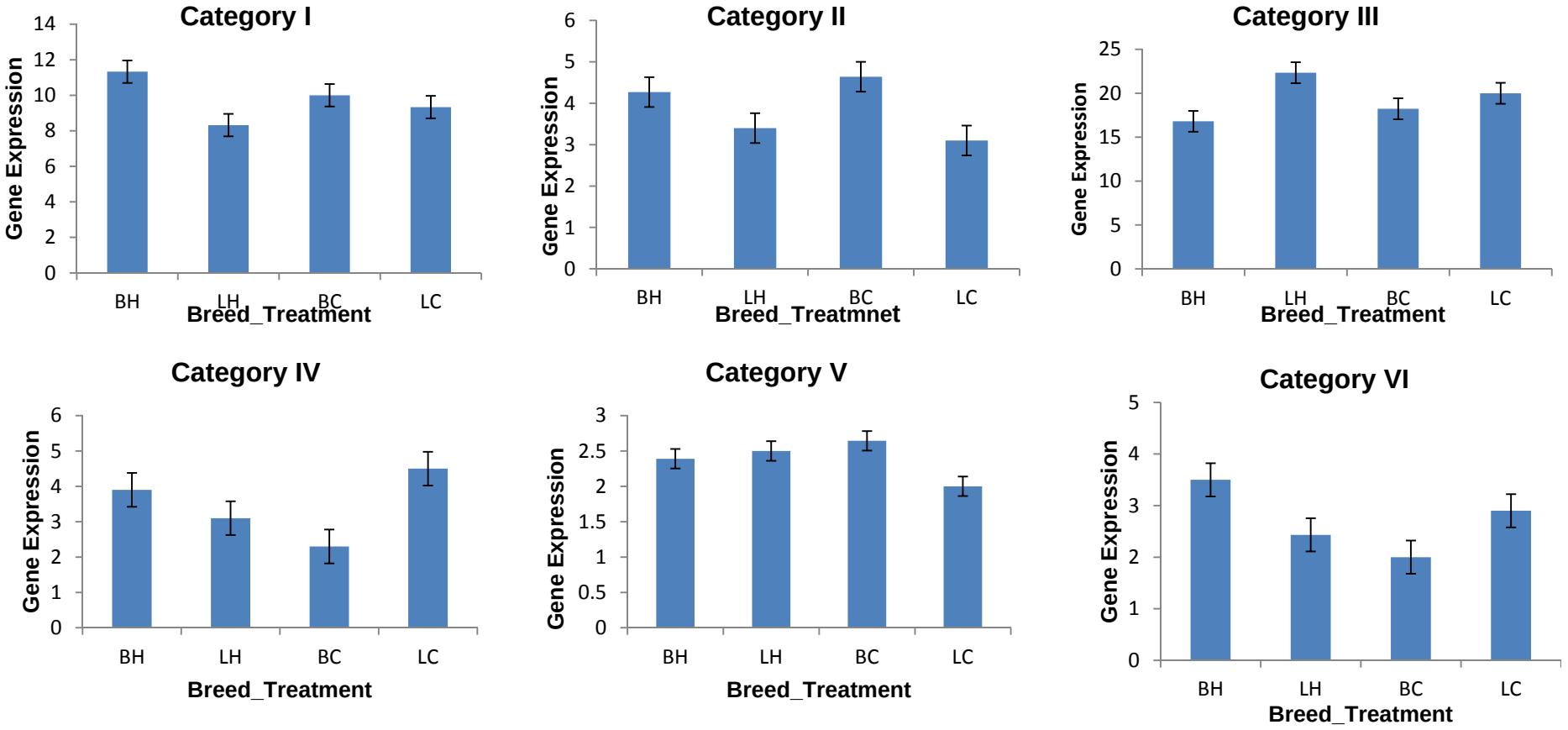
Category III Expression values of these genes were higher for layers than broilers in control but heat-stress resulted in a further increase in these expression levels in layers but a decrease for broilers compared with their control (Figure 4-2). Clusters 03, 06, 37 were present in this category, comprising 74 genes in total.

Category IV In this category, control layers had the highest expression compared with other groups. Heat-stress resulted in a decrease in gene expression levels in layers but an increase in broilers (Figure 4-2). There were 23 genes, in total, present in clusters 12, 20, 22 and 54 grouped in this category.

Category V Expression levels were highest in broiler controls but heat-stress resulted in a decrease in their expression and an increase in layers (Figure 4-2). In total, 10 genes in clusters 19 and 36 were present in this category.

Category VI Expression values for control layers were greater than respective broilers. After treatment the expressions of these genes were increased in broilers but decreased in layers (Figure 4-2). Clusters 07, 24 were grouped in this category having 18 genes in total (12 and 6 genes respectively).

Figure 4-2 Mean expression values of genes, (significant at *P*<0.05, Chapter 3) (y-axis) in comparisons of Breed x Treatment interactions (x-axis), for Categories I-VI. Each graph has 4 bars and each bar represents one group (BC, BH, LC, and LH). The expression values of each bar are the mean of the expression values of the genes, present in that cluster, over the 8 microarray slides for each group.



4.3.2 Selected Pathways and Network from IPA analysis

4.3.2.1 Pathways and Network from the analysis of category I genes

Among the 286 Category-I genes, 194 genes could be mapped to the corresponding identifiers. These 194 mapped genes included 14 duplicates that were removed. The analysis of the 180 unique genes revealed 35 significant (P<0.05) pathways and 23 networks in total. Out of these results, 9 interesting pathways were selected (Table 4-1) and similarly 5 networks were selected (Figure 4-3), on the basis of their relevance to skeletal muscles (functions, metabolism and damage). A complete list of significant pathways is given in Appendix 4-1. The score of each network is based on a P-value calculation, which determines the likelihood that the occurrence of Network Eligible Molecules in any given network is by random chance. (The score is the negative exponent of the right-tailed Fisher's exact test result (http://www.ingenuity.com).

It can be seen that all of the selected pathways and networks were related to stress response, inflammation, oxidative stress, cellular damage, connective tissue disorder, and skeletal and muscular disorder. Hence, the results suggest that stress and inflammation related tissue damage and muscular disorders are greater in broilers than layers, and further exacerbated by heat-stress in broilers.

Table 4-1 Selected pathways from Category I cluster analysis

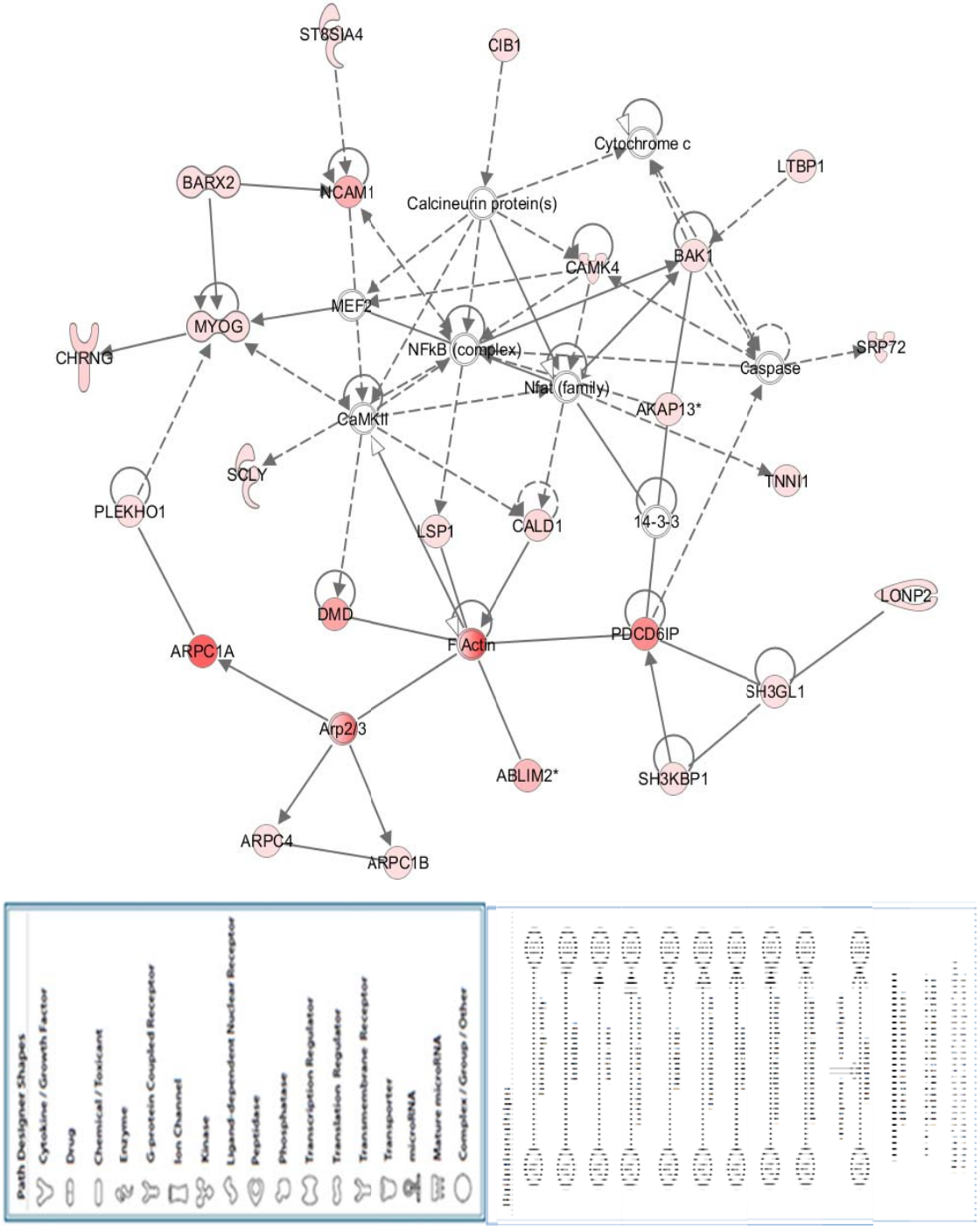
Sr. No.	Pathway titles	*P*-value[1]	Ratio[2]	Functions and diseases
1.	Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.000022	0.078	Inflammatory response (Caveggion *et al.* 2003); Cellular immune response (Kedzierska *et al.* 2002); Phagocytosis(Lee *et al.* 2007)
2.	fMLP Signalling in Neutrophils	0.000057	0.062	Involved in inflammatory diseases (Lehmann *et al.* 2008); cause degranulation and production of tissue-destructive oxygen-derived free radicals (Omori *et al.* 2008).
3.	CCR5 Signalling in Macrophages	0.0067	0.043	Inflammation; Calcium influx; activation of PYK2;
4.	CD28 signalling in T Helper Cells	0.0107	0.038	Cellular immune response (Appleman *et al.* 2002)
	CCR3 Signalling in Eosinophils	0.0186	0.039	Inflammation, tissue damage by release of reactive oxygen species (ROS) and toxic granule proteins (Borchers *et al.* 2002). Connective tissue diseases of unknown origin (Pease 2006).
5.	α-Adregnergic signalling	0.0228	0.038	Stress response, glycogenolysis; rise in intracellular Ca^{2+} (Stojkov *et al.* 2012)
6.	NRF2-mediated	0.0282	0.031	transactivates detoxifying and

	Oxidative Stress Response			antioxidant enzymes, such as heme oxygenase and superoxide dismutase (Dhakshinamoorthy *et al.* 2005)
7.	VEGF Signalling	0.0326	0.04	mediator of hypoxia-induced angiogenesis, proliferation of endothelial cells (Karkkainen & Petrova 2000)
8.	Role of MAPK Signalling in the Pathogenesis of Influenza	0.0374	0.046	Apoptosis, production of prostaglandin E2 (Lu *et al.* 2010)
9.	Chemokine signalling	0.0472	0.041	Inflammatory response (Lee *et al.* 2003)

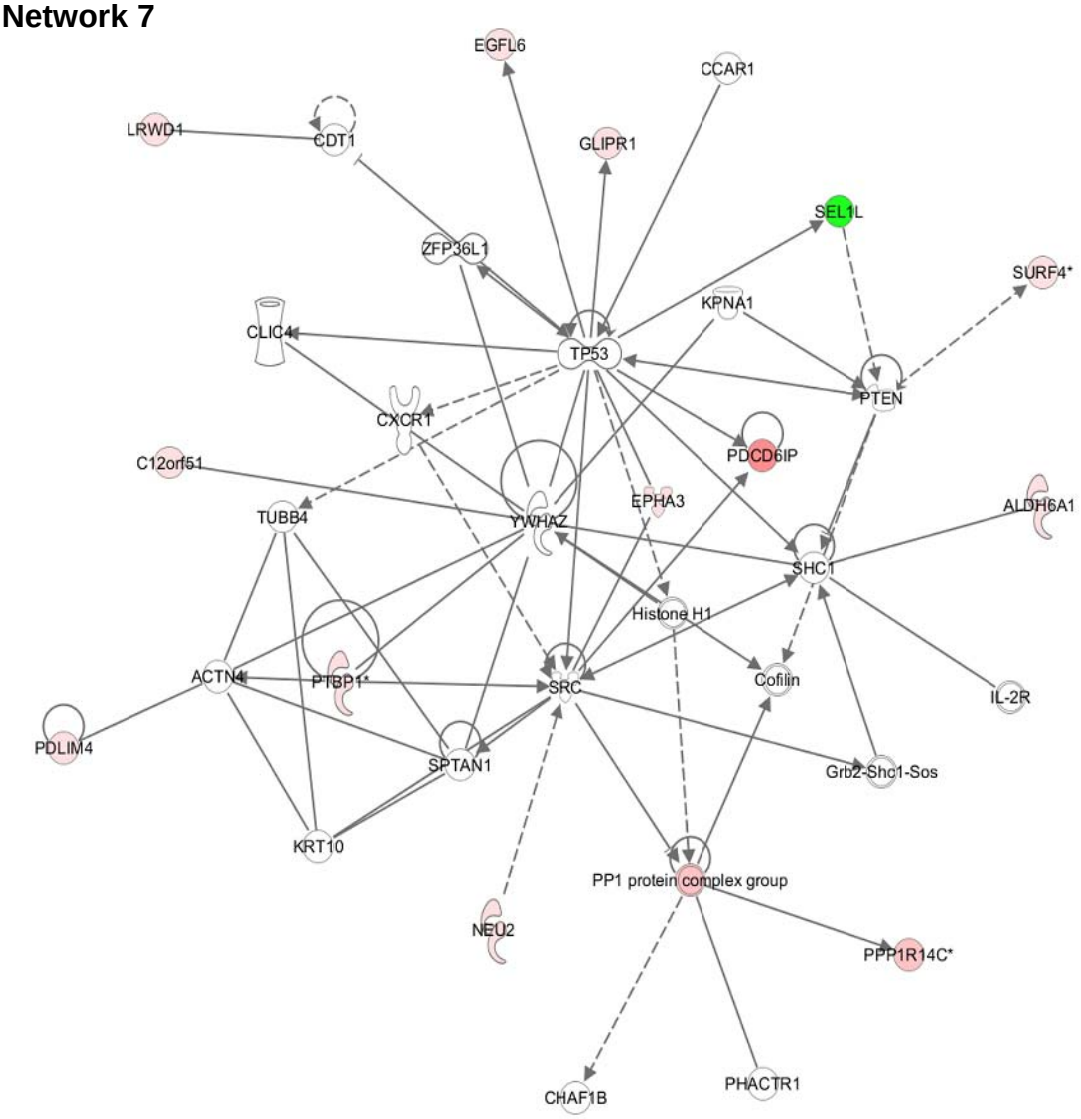
1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

Figure 4-3 Selected networks (1, 7, 9) from Category I analysis. Red colour genes are up-regulated and those in green colour are down-regulated. The following three networks are linked to each other by a single gene 'Programmed Cell Death 6 Interacting Protein' (PDCD6IP) that is involved in cell death and is up-regulated in these 3 networks.

Network 1



Network 7


EGFL6
CCAR1
LRWD1
CDT1
GLIPR1
SEL1L
ZFP36L1
SURF4*
KPNA1
CLIC4
TP53
PTEN
CXCR1
PDCD6IP
C12orf51
EPHA3
ALDH6A1
TUBB4
YWHAZ
SHC1
Histone H1
ACTN4
PTBP1*
SRC
Cofilin
IL-2R
PDLIM4
SPTAN1
Grb2-Shc1-Sos
KRT10
PP1 protein complex group
NEU2
PPP1R14C*
CHAF1B
PHACTR1


Path Designer Shapes
Cytokine / Growth Factor
Drug
Chemical / Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
microRNA
Mature microRNA
Complex / Group / Other

Network 9

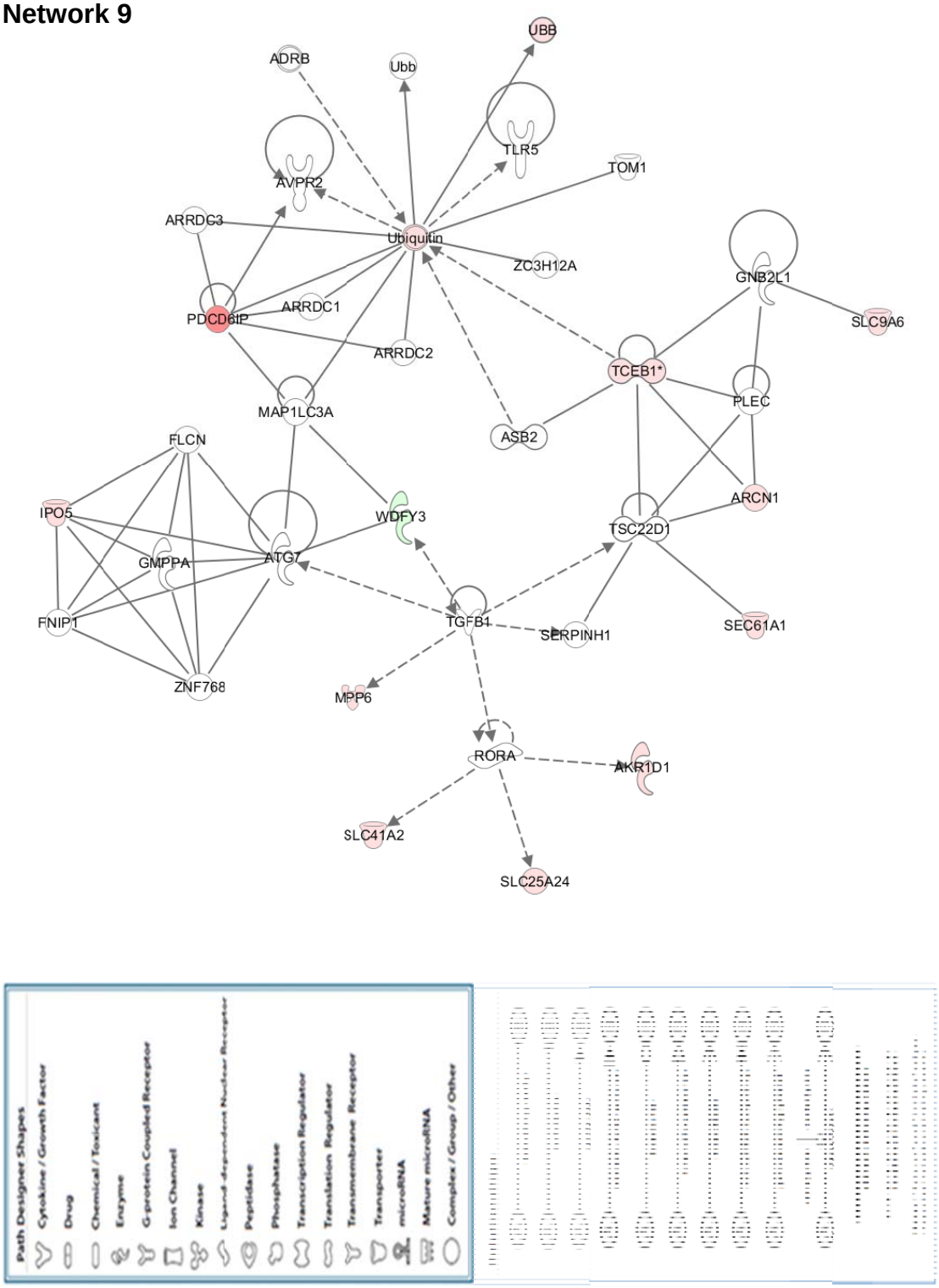
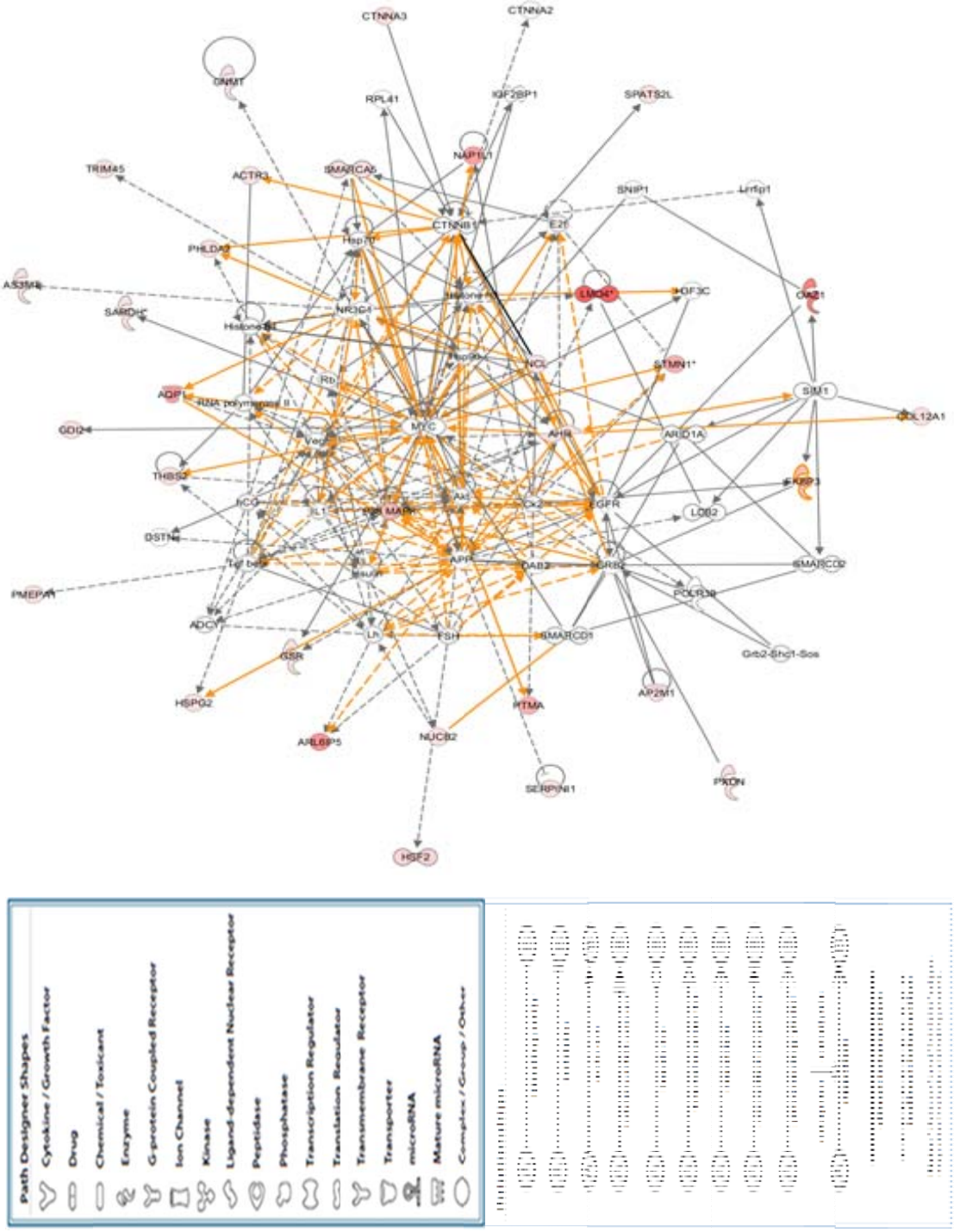

UBB
ADRB
Ubb
TLR5
TOM1
AVPR2
ARRDC3
Ubiquitin
ZC3H12A
GNB2L1
SLC9A6
PDCD6IP
ARRDC1
ARRDC2
TCEB1*
PLEC
MAP1LC3A
ASB2
FLCN
ARCN1
IPO5
WDFY3
TSC22D1
GMPPA
ATG7
FNIP1
TGFB1
SERPINH1
SEC61A1
ZNF768
MPP6
RORA
AKR1D1
SLC41A2
SLC25A24
Path Designer Shapes
Cytokine / Growth Factor
Drug
Chemical / Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
microRNA
Mature microRNA
Complex / Group / Other

Figure 4-4 Merging networks 4 and 6. Red colour genes are up-regulated and those in green colour are down-regulated. The following two networks are linked to each other by a single gene, FKBP3. They are involved in connective tissue and muscular disorders.



4.3.2.2 Pathways and networks from the analysis of category II genes

Out of the 93 genes in Category II, 75 were successfully mapped to their respective genes. Out of the 75 mapped genes, 1 was a duplicate. The analysis of the remaining 74 genes revealed 40 significant pathways (P<0.05) and 7 networks. A complete list of significant pathways is given in Appendix 4-2. Out of these results, 13 interesting pathways and 4 networks were selected on the basis of their relevance to the skeletal muscles physiology and pathology. Details of these pathways and networks are provided in Table 4.2 and Figure 4.4. These pathways and networks were up regulated in LH vs LC. In contrast to gene expression in broilers (category-I), most of the pathways and networks were related to cellular development, anti-apoptotic, anti-inflammatory and anti-stress related functions. The pathways and networks that were related to inflammation, stress and tissue-damage related functions are few. This is in agreement to the phenotypic data of the present study in which there was only a 0.34°C increase in the body temperature of layers after heat-stress but the increment in broilers was 2.56°C.

Table 4-2 Selected pathways from Category II cluster analysis

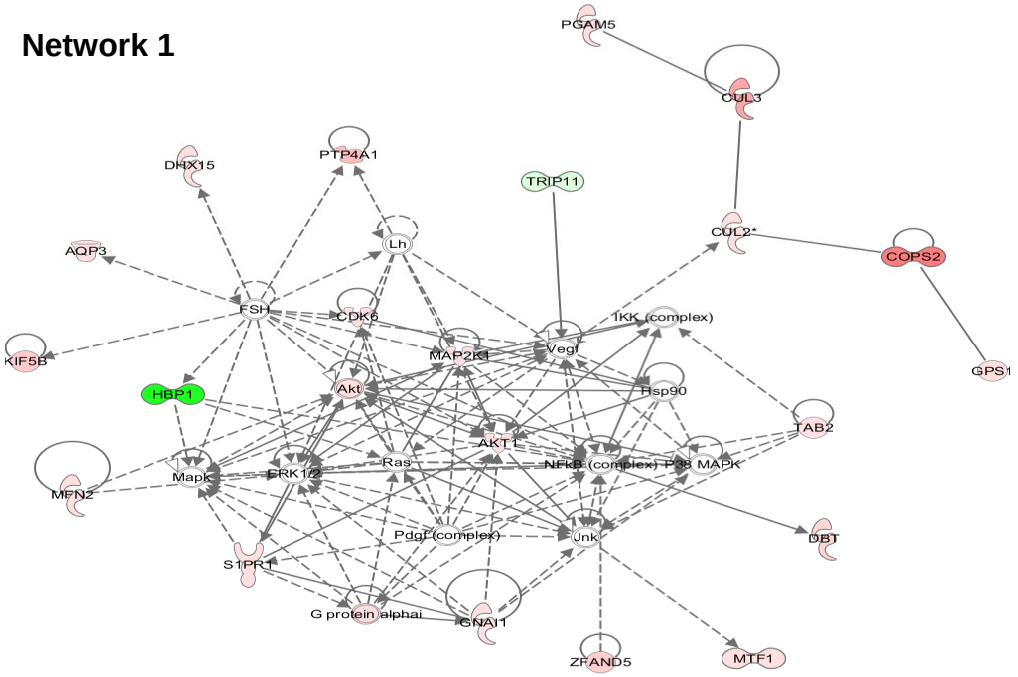
Sr. No.	Pathway titles	*P*-value[1]	Ratio[2]	Functions and disease
1.	Ceramide Signalling	0.0007	0.046	Apoptosis; Cell cycle regulation; Inflammatory response (Oh *et al.* 2006; Woodcock 2006)
2.	Regulation of eIF4 and p70S6K signalling	0.0024	0.03	Cellular growth and development; Protein synthesis (Cao *et al.* 2006)
3.	p70S6K signalling	0.0036	0.031	Cellular development; Protein synthesis (Karlsson *et al.* 2004)
4.	EIF2 signalling	0.0097	0.03	Protein synthesis (Jivotovskaya *et al.* 2006)
5.	HMGB1 signalling	0.0119	0.03	Inflammatory response,; Cellular immune response; Cytokine signalling; Proliferation of vessel-associated stem cells; Muscle tissue regeneration (Yang *et al.* 2005b; Park *et al.* 2006; Holmlund *et al.* 2007)
6.	Sphingosine-1-phosphate signalling	0.0172	0.025	Inhibition of caspases mediated apoptosis; Angiogenesis; Development and functions of cardiovascular system (Kono *et al.* 2004; Chalfant & Spiegel 2005; Alemany *et al.* 2007; Liu *et al.* 2012).
7.	Role of PI3K/AKT	0.0172	0.021	Involve in protein synthesis and angiogenesis and inhibition of

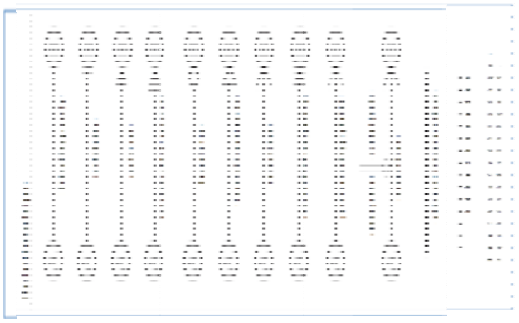
	Signalling			apoptosis (Garcia-Echeverria & Sellers 2008; Lu *et al.* 2010).
8.	Relaxin Signalling	0.0258	0.019	Inhibition of histamine release (Hossain et al. 2008); Connective tissue remodelling; Stimulation of angiogenesis; Modulation of blood pressure (Frost & Lang 2008)
9.	CNTF signalling	0.029	0.036	Enhance cell survival; Prevent degeneration of skeletal muscle cells (Yokogami *et al.* 2000; Grant 2008).
10.	Role of MAPK Signalling in the Pathogenesis of Influenza	0.0333	0.031	Cell death; Inflammatory response; production of prostaglandin E2 (Lu *et al.* 2010).
11.	Chemokine signalling	0.0395	0.027	Inflammatory response (Jaeschke & Hasegawa 2006; Hamal *et al.* 2010)
12.	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.0447	0.016	Cellular immune response; Production of NO and ROS in macrophages; Free radical scavenging (Vila-del Sol *et al.* 2007; Zhou *et al.* 2008).

1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

Figure 4-5 Selected networks (1, 5) from Category II Analysis. Networks are linked through a common gene, S1PR1. Red colour genes are up-regulated and those in green colour are down-regulated.

Network 1





Network 5

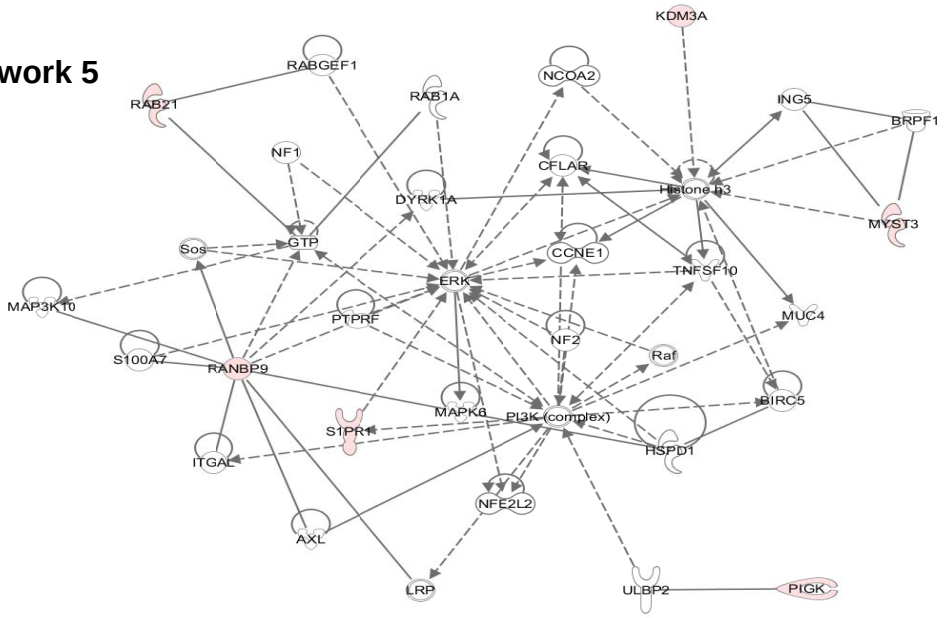
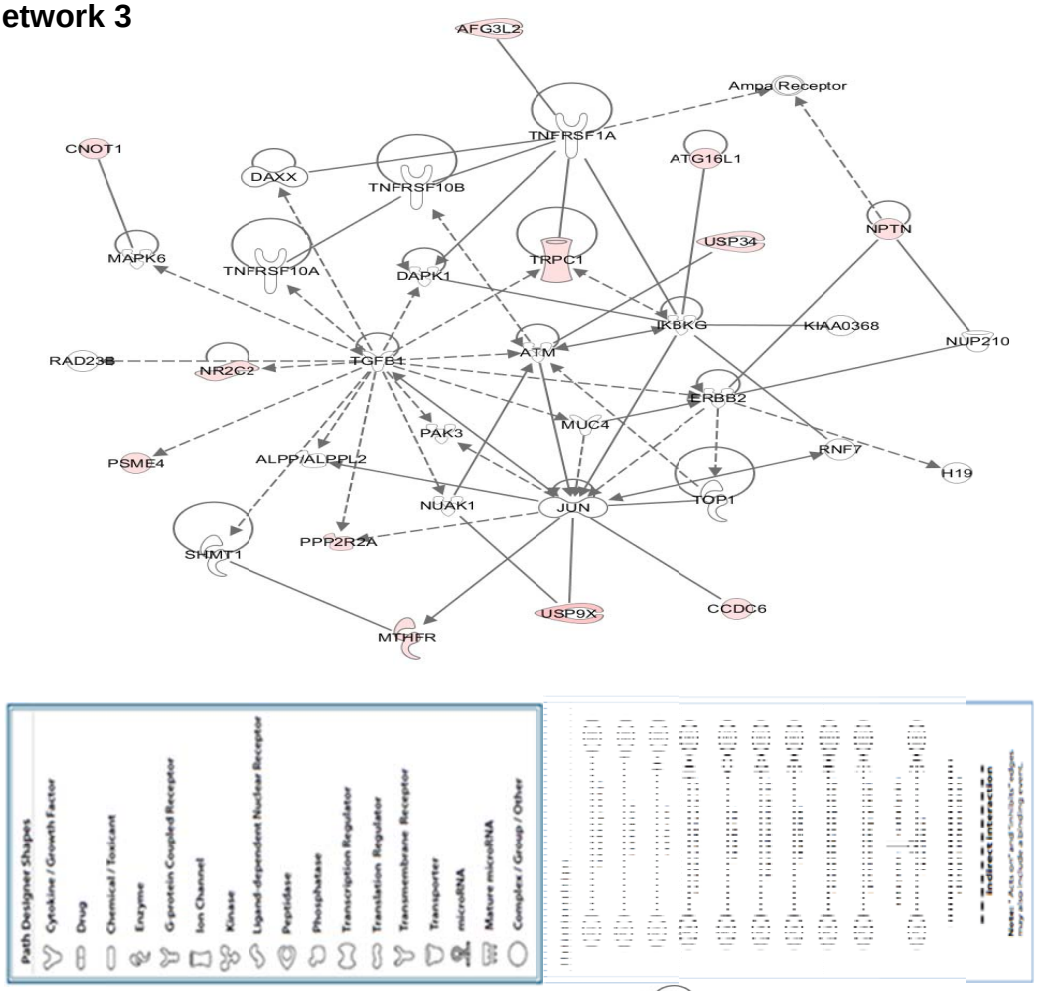
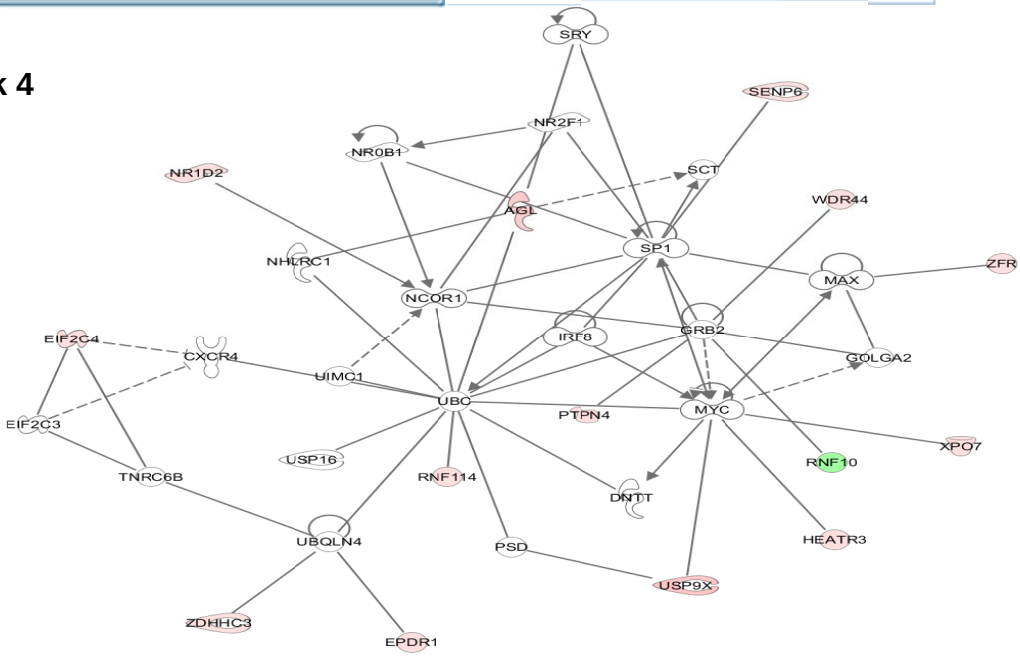


Figure 4-6 Selected networks (3, 4) from Category II analysis. Networks are linked through a common gene, USP9X. Red colour genes are up-regulated and those in green colour are the down-regulated.

Network 3



Network 4



4.3.2.3 Pathways and Network from the analysis of category III genes

In this category, 66 genes were analysed, out of which 9 were filtered out and 2 were duplicates. The differential expression values of these genes ranged from -0.176 to 20.347 in LH vs LC comparison. Analysis of these genes in IPA revealed 3 significant (P<0.05) pathways (Table 4-3) and 9 networks. A complete list of significant pathways is given in Appendix 4-3. Out of the total of 9 networks, 2 networks were selected (Figure 4-5) on the basis of their biological importance. The expression values of these genes were greater in layers than control and treated broilers but after exposure to heat-stress their expression levels were further increased in layers. The pathways and networks from this category were related to anti-apoptotic, anti-oxidant, anti-inflammatory and energy production related functions.

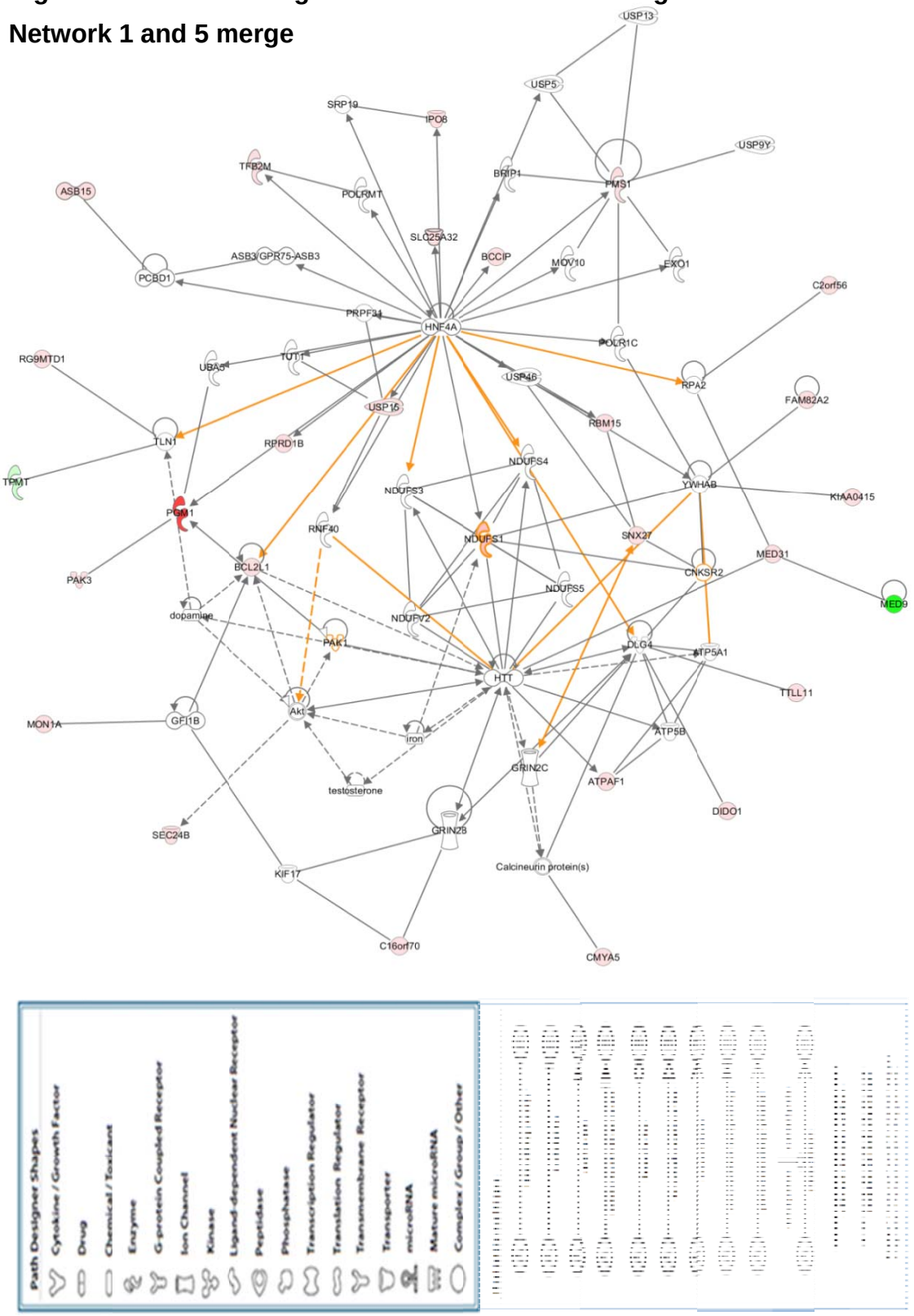
Table 4-3 Selected pathways from Category III cluster analysis

Sr. No.	Pathway titles	*P*-value[1]	Ratio[2]	Functions and diseases
1.	Estrogen receptor signalling	0.0378	0.015	Anti-oxidant functions, anti-apoptotic activities, anti-stress activity (Willoughby & Wilborn 2006; Chen *et al.* 2008; Enns & Tiidus 2008).
2.	Oxidative Phosphorylation	0.0468	0.013	Energy production (Toyomizu *et al.* 2011; Szendroedi *et al.* 2012)

1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

Figure 4-7 Selected networks (1 & 5) from Category III analysis. These two networks are linked together through, a common gene, NDUFS1 involved in electron transport chain and ATP production in mitochondrial respiratory chain complex I. Red colour genes up-regulated and those in green colour are the down-regulated.

Network 1 and 5 merge



4.3.2.4 Pathways and networks from the analysis of Category IV genes

The 4 clusters in this category had 23 genes in total. On their analysis in IPA, 13 genes were successfully mapped but 10 genes failed to map. From the results of this category, 9 relevant pathways were selected (Table 4-4) but no network of particular interest was found. A complete list of significant pathways is given in Appendix 4-4. The expression values of these genes increased in broilers after heat-stress but decreased in layers. Out of these 9 pathways, 5 were directly involve in stress, inflammatory and tissue damage related functions but the rest of them were involved in compensatory functions (anti-oxidant and wound healing etc.) to alleviate the negative effects of tissue damage related pathways.

Table 4-4 Selected pathways from Category IV cluster analysis

Sr. No.	Pathway Titles	*P*-value[1]	Ratio[2]	Functions and Diseases
1.	Melatonin signalling	0.000498	0.026	Scavenges oxygen-centred free radicals, especially the highly toxic hydroxyl radical (Masana & Dubocovich 2001). Also involved in the production of ROS.
2.	α-Adrenergic Signalling	0.000791	0.019	Stress response; glycogenolysis; elevation of intracellular Ca^{2+} (Stojkov *et al.* 2012)
3.	G Beta Gamma Signalling	0.000828	0.017	Control calcium signalling and Protein Kinase A signalling pathways in its down-stream effects (Ivanina *et al.* 2000; Qin *et al.* 2000).
4.	Corticotropin Releasing Hormone Signalling	0.00124	0.015	Stress response and secretion of glucocorticoids; cell death. Activate PKC -dependent and calcium-activated pathways respectively (Post *et al.* 2003; Thaxton *et al.* 2005; Mujahid & Furuse 2008) .
5.	P2Y Purigenic Receptor Signalling	0.00140	0.015	Angiogenesis; wound healing; cellular proliferation; ions transport (Datta *et al.* 1999; Burnstock 2002; Sak *et al.* 2003).
6.	Protein Kinase A Signalling	0.0094	0.006	Raised level of intracellular Ca2+; Proteasomal degradation; Glycolysis; Lipolysis (Seino & Shibasaki 2005; Wehrens *et al.* 2006)
7.	FcγRIIB Signalling in B	0.0202	0.017	Hypersensitivity Response; Inflammatory Response (Gardai *et*

	Lymphocytes			*al.* 2002; Yamasaki & Saito 2008)
8.	CCR5 Signalling in Macrophages	0.0383	0.011	Inflammatory response; Cytokine signalling; Cellular immune response (Balistreri *et al.* 2007; Shini *et al.* 2010a; Shini *et al.* 2010b)
9.	Chemokine signalling	0.0343	0.014	Inflammatory response; intracellular calcium release; production of reactive oxygen species (ROS) and changes in actin polymerization (Shini *et al.* 2010a; Shini *et al.* 2010b; Sun & Ye 2012; Viejo-Borbolla *et al.* 2012).
10.	Phospholipid degradation	0.0367	0.011	Production of PLA2; Phospholipid degradation (Ren *et al.* 2010; Schwartz & Reaven 2012).

1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

4.3.2.5 Pathways and networks from the analysis of Category V genes

There were 10 genes in total in this category of which 8 could be mapped to 7 unique genes. Their analysis in IPA revealed 9 pathways significant at $P<0.05$ (Table 4-5) and 4 networks. Out of these results 5 significant pathways were selected but no network was considered interesting for further analysis. A complete list of significant pathways is given in Appendix 4-5.

Table 4-5 Selected pathways from Category V cluster analysis

Sr. No.	Pathway titles	*P*-value[1]	Ratio[2]	Functions and diseases
1.	MIF-mediated Glucocorticoid Regulation	0.015	0.024	Counter-regulation of the anti-inflammatory effects of glucocorticoids; Cellular immune response; Organismal injuries and abnormalities (Daun & Cannon 2000; Roger *et al.* 2001; Calandra & Roger 2003).
2.	MIF Regulation of Innate Immunity	0.020	0.020	Proliferation and trafficking of immune cells; Cellular immune response (Ohkawara *et al.* 2006; Santos *et al.* 2008).
3.	Eicosanoid Signalling	0.024	0.013	Inflammation; vascular permeability, and allergic reaction (Nagata *et al.* 2002; Spik *et al.* 2005).
4.	Role of MAPK Signalling in the pathogenesis of Influenza	0.033	0.015	Oxidative stress and apoptosis through production of prostaglandin E2 (Lu *et al.* 2010).
5.	Phospholipid Degradation	0.039	0.011	PLA2, Phospholipid Degradation (Ren *et al.* 2010; Schwartz & Reaven 2012).

1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

4.3.2.6 Pathways and Network from the analysis of category VI genes

There were 18 genes in total in this category and on their analysis in IPA 16 genes were successfully mapped. The analysis of the 16 mapped genes returned 7 significant (P<0.05) pathways and 4 networks. Out of these results, 3 relevant pathways were selected (Table 4-6) but no network was found that was relevant to muscle damage. These pathways were involved in cell death, inflammatory and immune response, cellular development and haematopoiesis. The results suggest that these pathways were mainly involved in tissue damage and to some extent in repair and maintenance of the damaged tissues. Their expressions were highest in BH but conversely their expressions were decreased in LH. A complete list of significant pathways is given in Appendix 4-6.

Table 4-6 Details of selected pathways from Category VI analysis

Sr. No.	Pathway titles	*P*-value	Ratio	Functions and diseases
1.	Role of JAK1, JAK2 and TYK2 in Interferon Signalling	0.0221	0.037	Cytokine Signalling; Haematological System Development and Function; Haematopoiesis (Simoncic *et al.* 2002; Yang *et al.* 2005a)
2.	Interferon Signalling	0.0312	0.028	Inflammatory Response; Cellular immune response; Cytokine signalling; Organismal Injury and Abnormalities (Flammer *et al.* 2010; Y *et al.* 2010)
3.	TNFR1 Signalling	0.0438	0.019	Apoptosis; Cytokine signalling (Varfolomeev & Ashkenazi 2004)

1. *P*-value: Shows the strength of the association between a specific pathway and the uploaded dataset. Small *P* values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

4.3.2.7 Pathways and networks from the analysis of Biolayout filtered genes

In the analysis of 2,213 significant genes in BioLayout Express, only 1,147 genes could pass the correlation threshold of 0.80. These genes were analysed and grouped together in different clusters depending upon their positive correlation with each other. However, the remaining genes (1,066) which did not meet this correlation threshold were filtered out by BioLayout Express and separately analysed in IPA. In their analysis in IPA 644 were successfully mapped but 422 genes failed to map. From these 644 successfully mapped genes 37 were duplicates. The analysis of the remaining 607 genes in IPA revealed 32 significant pathways (P<0.05) and 25 networks. From these results, 8 relevant pathways (Table 4-7) and 5 networks (Figure 4-6) were selected for further exploration. A complete list of significant pathways is given in Appendix 4-7.

Table 4-7 Selected pathways from IPA analysis of Biolayout filtered genes

Sr. No.	Pathway titles	*P*-value	Ratio	Functions and diseases
1	Granzyme B signalling	0.00021	0.312	Oxidative stress; Cell death; Cellular immune response (Bots & Medema 2006; Jiang *et al.* 2008b)
2	Toll-like Receptor	0.00225	0.127	Apoptosis (Barton & Medzhitov 2003; Ding *et al.* 2010)
3	Type I Diabetes Mellitus Signaling	2.35E00	8.26E-02	Tissue damage and muscle weakness (Rolo & Palmeira 2006)
4	Cytotoxic T lymphocyte mediated apoptosis of target cells	0.00457	0.058	Cell death (Pinkoski *et al.* 2001) Jiang *et al.* 2008b)
5	Apoptosis signalling	0.00746	0.094	Apoptosis (Kim *et al.* 2005; Kuwano *et al.* 2005)
6	Role of MAPK signalling in the pathogenesis of influenza	0.00911	0.106	Oxidative stress; Apoptosis (Lu *et al.* 2010).
7	Lymphotoxin β receptor signalling	0.0175	0.098	Cell death (Kuai *et al.* 2003; You *et al.* 2006)
8	IL-1 signalling		0.075	Inflammation (Yang *et al.* 2010; Sigala *et al.* 2011; Shi *et al.* 2012)

1. *P-value*: Shows the strength of the association between a specific pathway and the uploaded dataset. Small P values suggest that the probability of a spurious association between the data and this pathway is unlikely (http://ingenuity.force.com/ipa/).
2. Ratio: The number of genes in a given pathway divided by total number of genes that make up that pathway (http://ingenuity.force.com/ipa/).

Figure 4-8 Selected networks from IPA analysis of Biolayout filtered genes. Red colour genes are up-regulated and those in green colour are down-regulated.

Network 4

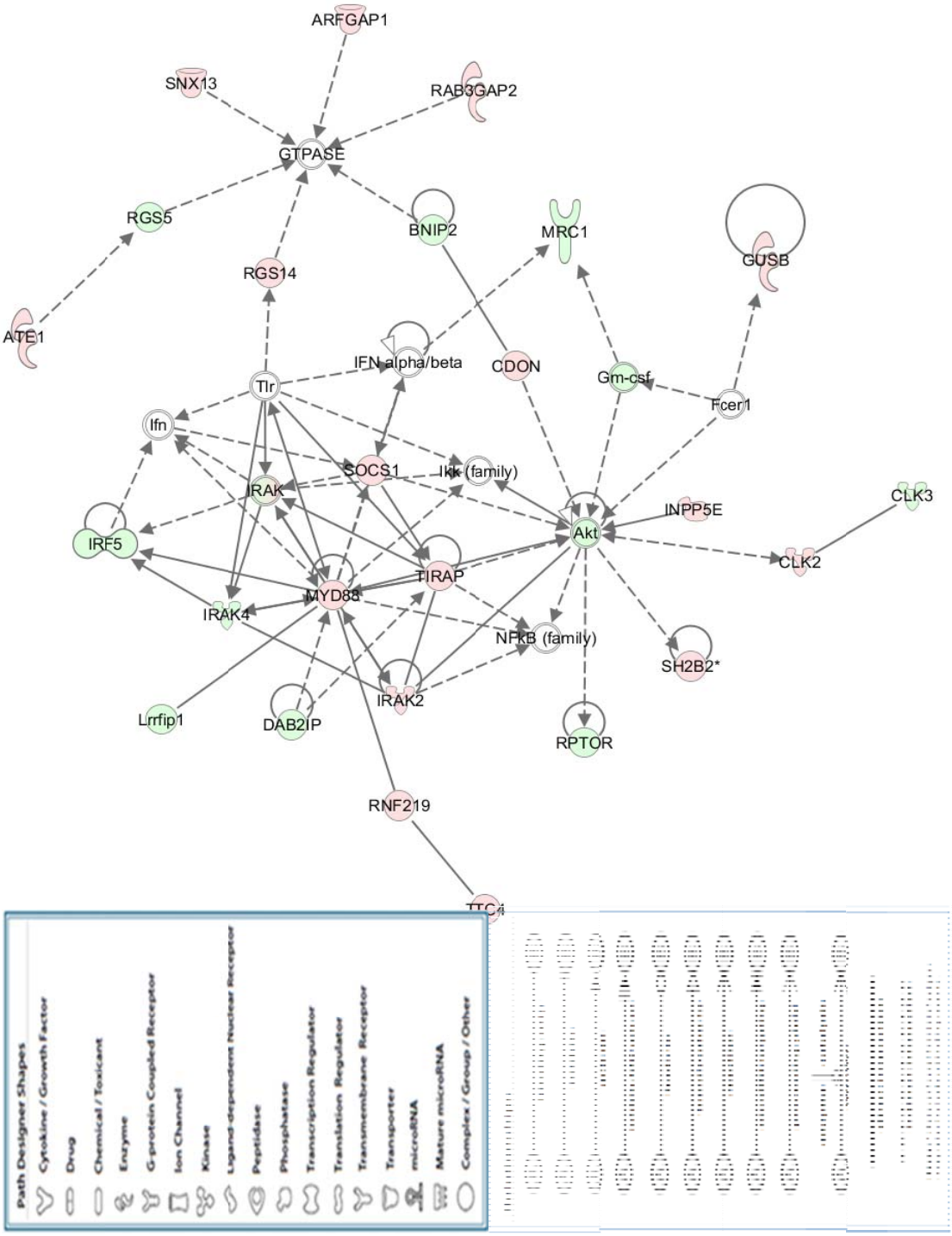


Figure 4-9 Merging networks 17 & 24 from IPA analysis of Biolayout filtered genes. These networks are linked to each other by a common gene COX6A1 that activates the COX family of genes. Red colour genes are up-regulated and those in green colour are down-regulated.

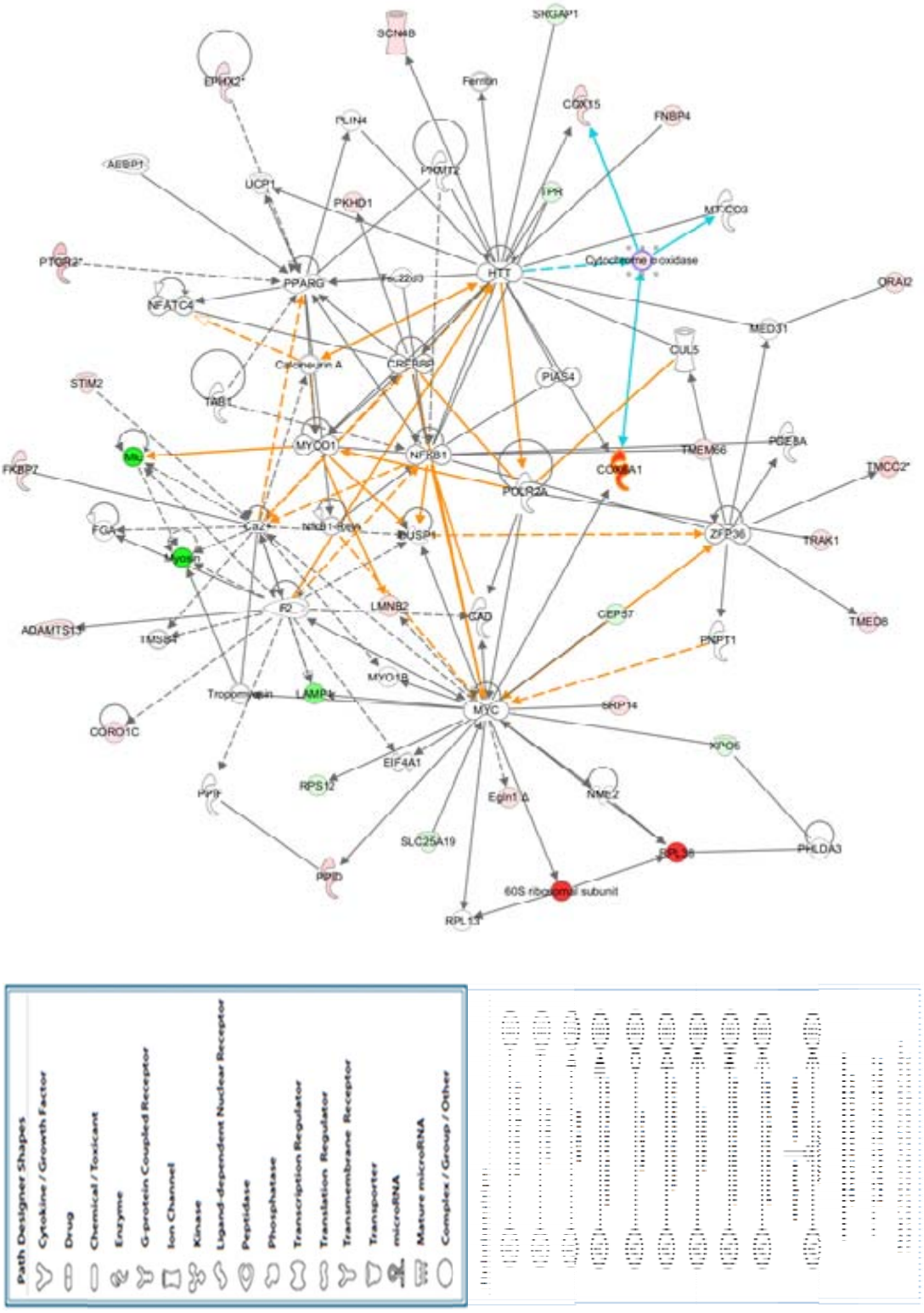
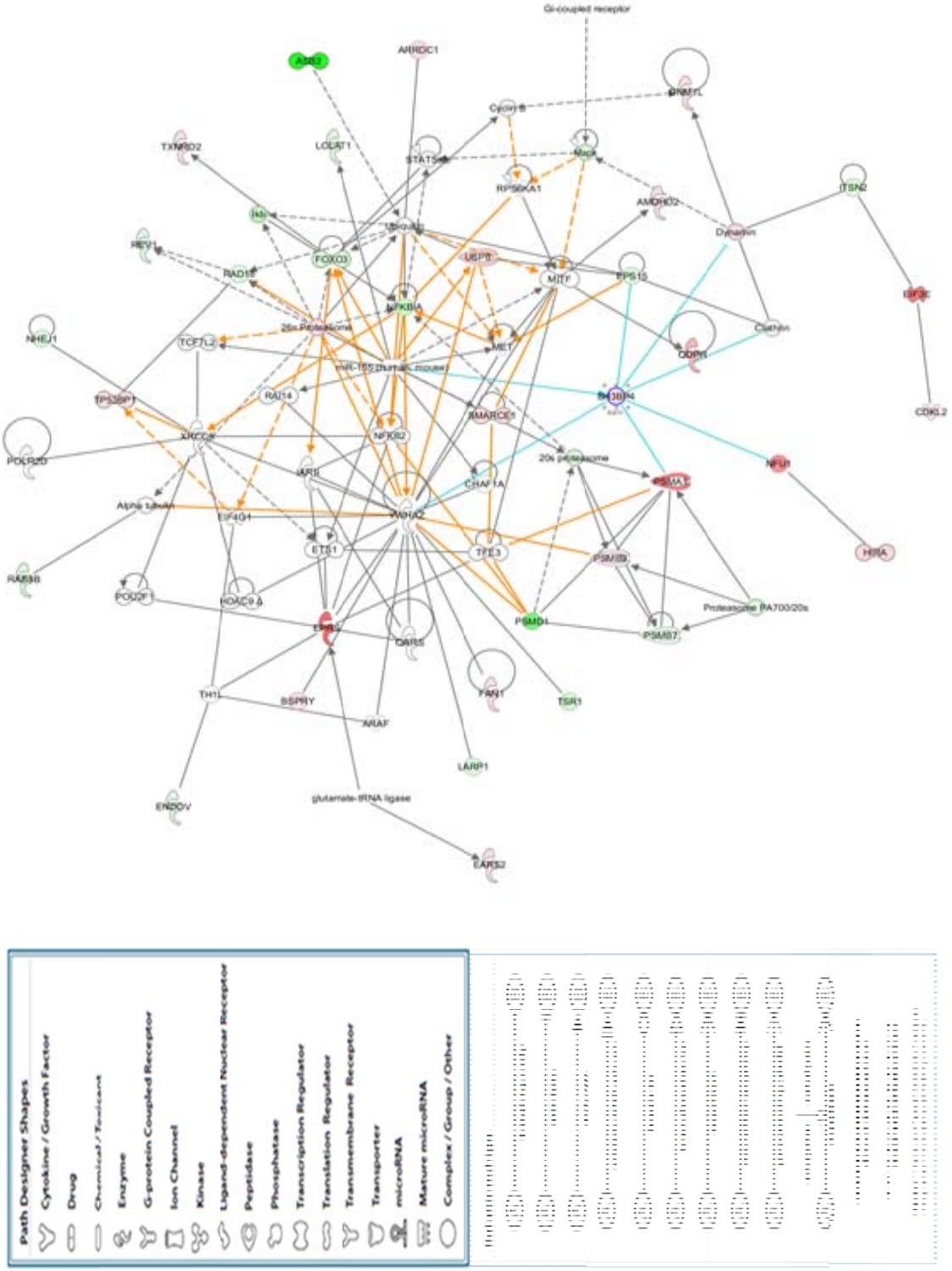


Figure 4-10 Merging networks 7 & 25 from IPA analysis of Biolayout filtered genes. These two networks are linked to each other by a single gene SH3BP1. Red colour genes are up-regulated and those in green colour are down-regulated.



4.3.3 Selection of Candidate genes

Genes were selected from all the categories on the basis of their key positions in the selected pathways and networks. Genes which could not be mapped in IPA but were interesting on the basis of their GO terms were also examined.

In total, 24 genes were selected, 10 genes from pathway analysis of Category I, II, IV, and VI, 5 from Pathway analysis of Biolayout filtered genes, 6 on the basis of their GO terms, 2 from Network analysis of biolayout filtered genes and 1 from network analysis of category I (Table 4-8).

In Category I pathway analysis, MAPK14, GNB5, GNG2, HRAS, PTGS2 were selected while PLCG2, PRKAG2 and IFNAR2 were selected from Category IV and VI pathway analysis. From Category II pathway analysis, AKT1 and MAP2K1 were selected (Table 4-8). All of these genes were present in the upstream positions in different pathways and switched on/off the down-stream genes through their signalling cascades. The selection of genes from the pathways is explained in detail below under section 4.4.4. However, the PDCD6IP gene was selected from network analysis of category I genes (Table 4-8). It connected the 3 networks (1, 7, & 9, Figure 4-3) all of which were involved in cell death and skeletal and muscular disorders. This gene activates several genes (Appendix 4-12) including the Caspase family of genes involved in cell death and tissue damage (Yeretssian *et al.* 2008; Wang & Youle 2009; Alway 2010).

From Pathway analysis of Biolayout filtered genes, ENDOG, LMNB2, CASP2, CASP3 and APAF1 were selected. However, COX6A1 and MTFR1 genes were

selected from Network analysis of biolayout filtered genes. The COX6A1 gene linked networks 17 and 24 that are involved in skeletal and muscular system development and functions, energy production and tissue damage related functions. This gene was up-regulated in both of these networks and activates the members of cytochrome C oxidase (COX) family (Figure 4-9) which are involved in mitochondrial dysfunction. CALM, HDAC4, PDCD6, CYP27A1, BCL2L1 and BCL10 genes were selected on the basis of their GO terms for biological functions.

Table 4-8 Final list of selected genes

Sr. No	Category[1]	Gene Symbol	Entrez gene name	Functions	Cellular position
1	GO terms	CALM	Calmoudlin	Cell death; chemokine expression (Chen *et al.* 2012)	Cytoplasm
2	Cat. 1 Path. Analysis	MAPK14	mitogen-activated protein kinase 14	Inflammatory response; cell death (Kyriakis & Avruch 2001; Fukao 2004)	Cytosol and mitochondria
3	Cat. 1 Path. Analysis	GNB5	guanine nucleotide binding protein (G protein), beta 5	G-protein coupled receptor signalling pathway (Thodeti *et al.* 2000; Wing *et al.* 2001)	Plasma membrane
4	Cat. 1 Path. Analysis	GNG2	guanine nucleotide binding protein (G protein), gamma 2	G-protein coupled receptor signalling pathway (Braun & Kelsall 2001; Yavropoulou & Yovos 2007; Teicher & Fricker 2010)	Plasma membrane
5	Cat. 1 Path. Analysis	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	Chemotaxis; apoptosis (Slack *et al.* 1999)	Plasma membrane
6	Cat. 4 Path. Analysis	PLCG2	phospholipase C, gamma 2	Elevation in intracellular Ca2+; apoptosis (Yamaoka *et al.* 2011)	Cytosol; plasma membrane
7	Cat. 4 Path. Analysis	PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Response to stress; regulation of glycolysis and intracellular cation level (Reiken *et al.* 2003)	cytoplasm
8	Cat. 2 Path. Analysis	AKT1	v-akt murine thymoma viral oncogene	Inflammatory response; apoptotic and also anti-	Cytoplasm

			homolog 1	apoptotic role (Majewski *et al.* 2004; Powell & Delgoffe 2010)	
9	Cat. 2 Path. Analysis	MAP2K 1	mitogen-activated protein kinase kinase 1	Cellular proliferation and differentiation; response to oxidative stress; regulation of transcription (Hu *et al.* 2004; Ahmad *et al.* 2011)	Cytoplasm, mitochondria, plasma membrane
10	Cat. 6 Path. Analysis	IFNAR2	interferon (alpha, beta and omega) receptor 2	type I interferon-mediated signaling pathway (LaFleur et al. 2001)	Plasma membrane
11	Path. analysis of BL filtered genes	ENDOG	endonuclease G	Cell death (Li *et al.* 2001)	Cytoplasm
12	Path. analysis of BL filtered genes	LMNB2	lamin B2	Cell death (Gottlieb 2001; Iredale 2001)	Nucleus
13	Path. analysis of BL filtered genes	APAF1	apoptotic peptidase activating factor 1	Cell death (Gogada *et al.* 2011)	Cytoplasm, golgi apparatus
14	GO terms	BCL2L1	BCL2-like 1	Oxidative stress; stress response (Gross *et al.* 1999; McClintock *et al.* 2002)	Mitochondria; cytoplasm
15	GO terms	CKMT1	creatine kinase, mitochondrial 1B	Indicator of muscle damage; involve in energy production (Steeghs *et al.* 1997; Ryu *et al.* 2005)	Mitochondria
16	Cat. 1 Path. Analysis	PTGS2	prostaglandin-endoperoxide	Heat hyperalgesia; synthesis of	Cytoplasm

			synthase 2	prostaglandin; inflammatory response (Dinarello 1996; Inglis *et al.* 2007)	
17	GO term	MTFR1	mitochondrial fission regulator 1	Mitochondrial fission	Mitochondria
18	GO term	PDCD6	programmed cell death 6	Apoptosis (Jang *et al.* 2002)	Cytoplasm
19	GO term	BCL10	B-cell CLL/lymphoma 10	Cell death (Cregan *et al.* 2004)	Cytoplasm, lysosome
20	Network analysis of BL filtered genes	HDAC4	histone deacetylase 4	Inflammatory response; muscle contraction by Ca^{2+} signalling (Kawabata *et al.* 2010; Joosten *et al.* 2011)	Nucleus
21	Network analysis of BL filtered genes	COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	Energy production (Fornuskova *et al.* 2010; Larsen *et al.* 2011)	Mitochondrial inner membrane
22	Network analysis of category I gene	PDCD6IP	programmed cell death 6 interacting protein	Cell death (Mahul-Mellier *et al.* 2006a)	Cellular membrane; cytoplasm
23	Path. analysis of BL filtered genes	CASP2	caspase 2, apoptosis-related cysteine peptidase	Cell death	Cytoplasm
24	Path. analysis of BL filtered genes	CASP3	caspase 3, apoptosis-related cysteine peptidase	Cell death	Cytoplasm

[1] BL = Biolayout, Path. = Pathway, Cat. = Category

4.4 Discussion

4.4.1 Clusters from Biolayout Express analysis

In total, 21 clusters were selected on the basis of their expression patterns and they were grouped into 6 categories due to the similarity of their expression patterns (graphs). This analysed set of genes was differentially expressed ($P<0.05$) for the Breed x Treat interaction from the microarray experiment (Chapter 3). Therefore, their occurrence in different (expression patterns) clusters is logical because heat-stress is known to affect the bird's physiology and create muscle damage, by disturbing various biological mechanisms of body, described in Chapter 1 (Introduction). These findings were also in agreement with our gene expression results, Chapter 3 (section 3.4.1), showing the presence of differentially expressed genes in relatively few categories, on the basis of their biological functions. Additionally, our results present some further categories that were previously not known, presenting a comprehensive picture of pathogenesis and pathology created by heat-stress.

4.4.2 Pathways and networks for Biolayout analysed genes in IPA

4.4.2.1 Category I

Pathways

For all the genes present in these clusters the expression levels of control broilers were higher than control layers. Heat-stress resulted in further increases in the expression of these genes in broilers. In this category, α-Adrenergic signalling (Table 4-1) that is involved in glycogenolysis in stressful conditions to provide energy for

muscle contraction was observed to be up regulated. However, it has been reported that stress hormones are known to alter the activities of immune cells, and lead to the production of various pro-inflammatory cytokines and chemokines (Shini *et al.* 2010a; Shini *et al.* 2010b).

In agreement with these findings there were several chemokine pathways in this category that were involved in cytokine signalling, tissue damage and related functions (Table 4-1). These pathways include, Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes, fMLP signalling in Neutrophils (Heterophil in chicken), CCR3 Signalling in Eosinophil, CCR5 Signalling in Macrophages, Role of MAPK Signalling in the Pathogenesis of Influenza, and Chemokine Signalling. They are involved in inflammatory responses (Hendey & Maxfield 1993; Lawson & Maxfield 1995; Sabri *et al.* 1998; Masiero *et al.* 1999; Garcia-Rodriguez & Rao 2000; Renieri *et al.* 2008), oxidative stress (Elsner *et al.* 1998; Kim *et al.* 2008), increase in intracellular calcium and apoptosis, connective tissue disorders, degranulation, phagocytosis and tissue damage. Similarly, the CD28 signalling in T Helper Cells pathway is involved in activation and regulation of cellular immune responses. Up-regulation of these pathways in control broilers indicate that breast muscles in broilers are under stress-related muscle damage that is further exacerbated by exposure to heat-stress.

However, the VEGF (vascular endothelial growth factor) signalling pathway, that was up-regulated, is also a significant mediator of hypoxia-induced angiogenesis and is usually up-regulated in hypoxia-like situations. Up-regulation of this pathway, in control broilers compared with layers, suggests that even in control conditions broiler muscle cells were under hypoxic-stress. The reason for this may lie in the greater muscle fibre size and inadequate capillary supply, that are in turn considered

to induce metabolic stress due to the larger diffusion distances for nutrients, metabolites and waste products (MacRae *et al.* 2006). Up-regulation of this pathway in control broilers explains the up-regulation of inflammation and cell death related pathways in this category. This is in agreement with the published reports that thermal insult leads to oxidative and muscle damage, indicated by higher plasma creatine kinase activity of heat-stressed broilers (Mitchell & Sandercock 1995b; Mujahid *et al.* 2005; Mujahid *et al.* 2006; Mujahid *et al.* 2007c). In agreement with these results, the NRF2-mediated Oxidative Stress Response pathway, which was up-regulated, is involved in the anti-oxidant functions of the body (Frost & Lang 2005; Vaarmann *et al.* 2008; Alamdari *et al.* 2010). The up- regulation of this pathway in broilers might represent a protective measure to minimise the damaging effects of heat-stress.

Networks

Out of 23 networks, 5 networks were selected (Figure 4-3 & 4-4 & Appendix 4-8), on the basis of their relevance to the particular interest of the study. Networks 1, 7, and 9 were involved in skeletal and muscular disorders, inflammatory and cell death related functions. They were linked to each other by a single gene 'Programmed Cell Death 6 Interacting Protein' (PDCD6IP) that was up-regulated in these 3 pathways (Figure 4-3). Protein from the PDCD6IP gene binds to the product of PDCD6 genes and initiates apoptosis in a calcium-dependent manner in skeletal muscles (Baietti *et al.* 2012; Bongiovanni *et al.* 2012). Furthermore, PDCD6IP affects (switching on/off) the 'Caspase' family of proteases (Figure 4-3) and is well-known for having a role in causing cell death/apoptosis (Alway 2010; Stegh & DePinho 2011; Teng *et al.* 2011). Additionally, this gene (PDCD6IP) is also involved in affecting the

'Cytochrome C gene' (Figure 4-3) which reflects its role in oxidative stress and cell death, ultimately leading to tissue damage.

Similarly, networks 4 and 6 were involved in inflammation, connective tissue and muscular disorders. These two pathways are linked through the 'Fk506 Binding Protein 3' (FKBP3) gene (Appendix 4-8). These findings are in agreement with the reports that connective tissues cannot keep pace with muscle fibre radial growth and, as a result, muscle fibre outgrows its support mechanisms and leads to myopathy-like conditions (Swatland 1990; Kranen *et al.* 2000). These results show that pathways and networks for this category were involved in tissue damage and related functions.

4.4.2.2 Category II

Pathways

In this category, 12 significant pathways were selected out (Table 4-2) of which 5 pathways (Ceramide signalling, HMGB1 signalling, Production of Nitric oxide and Reactive Oxygen Species in Macrophage, Role of MAPK signalling in the Pathogenesis of Influenza, and Chemokine signalling) were involved in inflammatory responses, (Abramson & Yazici 2006), oxidative stress (Aoi *et al.* 2004), elevation in intracellular Ca^{2+} levels, cellular immune response, cell death (Nencioni *et al.* 2009) and skeletal muscle damage (Yasuo & Yamamoto 2001; Andrea Cutrullis *et al.* 2009; Dinler *et al.* 2010). Except for these, all other pathways in this category were involved in protein synthesis, angiogenesis, anti-inflammatory and anti-oxidant activities. The expression values in this category of clusters were highest in BC and were decreased after heat-stress. Up-regulation of

protein synthesis and angiogenic pathways in BC is logical in the sense that broilers are meat-type birds and have many-fold greater growth rates and body mass than layers (Griffin & Goddard 1994). But exposure to heat-stress resulted in down-regulation of these pathways in broilers consistent with the negative effects of heat-stress on growth-related traits (Khan *et al.* 2011). Conversely, these pathways (inflammatory and anti-inflammatory) were up-regulated in layers after thermal stress. The up-regulation of inflammatory pathways might be due to deleterious effects of heat-stress and up-regulation of anti-inflammatory and survival-related pathways might be to protect the body from detrimental effects of HS. However, these results are in agreement with the physiological data (body temperature) from the present study where the increase in the layers' body temperatures was much lower compared with broilers. Consistent with this, Sandercock *et al.* (2006) reported that the effects of heat-stress on body temperature, and plasma creatine kinase values in layers were much less compared with broilers. Similarly, Mujahid *et al.* (2007b) have also observed that the extent of heat-stress induced oxidative stress in layer skeletal muscles was much less compared with broilers. Our data shows that the reason for this lower level of muscle damage in layers might be the up-regulation of the anti-inflammatory, anti-oxidant, and protein synthesis related pathways.

Networks

In agreement with the results of pathways analysis, 3 of the selected 4 networks were involved in cellular growth and proliferation, and skeletal and muscular system development and functions (Appendix 4-9). The results indicate that these networks might be involved in tissue repair and regeneration related functions and hence

reduce the damaging effects of heat-stress in layers. However, only one network was involved in cell death, and inflammatory response, suggesting that the extent of muscle damage in layer muscle on exposure to thermal challenge is limited, consistent with the phenotypic data and available literature.

4.4.2.3 Category III

Pathways: In this category the expression values of the genes were substantially greater for layers than broilers in control conditions and heat-stress resulted in further increases in layers but decreases in broilers. From the pathway analysis of this category only 3 pathways were significant, of which two (Table 4-3), Estrogen Receptor Signalling and Oxidative Phosphorylation were selected (Lannigan 2003; Padron *et al.* 2007). The Estrogen Receptor signalling pathway is mainly related to anti-oxidant, anti-stress and anti-apoptotic and a myo-protective role in various physiological and pathological conditions like oxidative stress and raised intracellular level of cations (MacRae *et al.* 2006). It is very likely that heat-stress induced up-regulation of this pathway in layers was a protective measure of the body to minimise the oxidative stress. However, the reason for the up-regulation of this pathway in LC is logical in the sense that layers are selected for egg and reproductive traits and hence have greater expression of estrogen and related genes. Ultimately, layers can lay more than 320 eggs in one production cycle (52 weeks) whereas broiler hens lay less than 200 eggs in a production cycle of 40 weeks. The reason for this lower expression of estrogen in broilers might be due to the negative correlation between growth-related traits and reproductive traits (egg numbers).

The Oxidative Phosphorylation pathway is involved in metabolism and energy production (Dumas *et al.* 2011; Szendroedi *et al.* 2012). The up-regulation of the Oxidative Phosphorylation pathway in layers compared with broilers, in control and stress conditions has not been reported previously, to our knowledge. The up-regulation of this pathway shows that layers have comparatively higher metabolic rate than broilers, which might be the reason for their higher body temperature under control conditions in this study. Hypoxia is known to decrease the efficiency of oxidative phosphorylation (Cerretelli & Gelfi 2011) and, therefore, the down-regulation of this pathway in broilers could be due to hypoxia-like conditions in skeletal muscles. In contrast to our finding, Toyomizu *et al.,* (2011) reported that broiler skeletal muscles had greater efficiency of oxidative phosphorylation compared with layers. But they compared these two types of chicken between 14-28 days of age when their body weights were about 1 kg and 0.2 kg for broilers and layers respectively. The greater efficiency of oxidative phosphorylation in broilers at that age is a logical outcome of selection for rapid growth. In the present study broilers were at 6 weeks of age were about 4 kg heavier and the occurrence of an hypoxia-like situation in their muscles is consistent with a greater muscle to capillary ratio and greater diffusion distances for nutrients and metabolic wastes (MacRae *et al.* 2006). Consistent with this conclusion, in category I some angiogenic pathways, like VEGF signalling, involved in the hypoxia-induced angiogenesis were up-regulated in broilers.

Networks

In the analysis of this category of genes 9 networks were analysed. Out of these, two networks, 1 and 5 (Figure 4-5), were selected on the basis of their relevance to the particular objective (muscle damage) of the study. These two networks were

involved in cell death, energy production and genetic disorders and were linked to each other through a common gene, 'NADH dehydrogenase (ubiquinone) Fe-S protein 1' (NDUFS1) (Table 4-3 and Appendix 4-10). This gene is present in (inner) mitochondrial membranes and is involved in the electron transport chain and ATP production in mitochondrial respiratory chain complex I (Iuso *et al.* 2006; Than *et al.* 2011). This gene is also involved in a mitochondrial dysfunction pathway and could lead to an accelerated rate of cell death in certain pathological situations (Ricci *et al.* 2004).

4.4.2.4 Category IV

Pathways: The up-regulation of Corticotropin Releasing Hormone Signalling and α-Adrenergic Signalling pathways in BH compared with BC (similar to Category I) shows that BH were more stressed than BC. In addition to these pathways, the G Beta Gamma Signalling pathway that is involved in the activation of Calcium Signalling and Protein Kinase A Signalling pathways in its downstream effects was also up-regulated (Table 4.4). In both of these pathways, PKA and PLC genes were up-regulated and are involved in increasing intracellular Ca^{2+} levels by mediating the release of Ca^{2+} from cellular stores (endoplasmic reticulum and sarcoplasmic reticulum) by switching on the RyR (calcium release channel of skeletal muscles sarcoplasmic reticulum) and IP3R genes in their down-stream signalling cascades.

Ziober *et al.* (2010) have reported an association between a SNP in the RyR1 gene and PSE-type meat in broilers. In contrast to this, it has also been reported that these Ca^{2+} release channels are normal in broilers and there is no defect in their structure and function (Malcolm Mitchell, person communication). Hence, up-regulation of PKA gene is responsible for greater expression of RyR gene and

ultimately higher levels of Ca^{2+} in broiler skeletal muscles that might be involved in altering muscle cell function and reduced meat quality (Sandercock *et al.* 2009a). Among other pathways, FcγRIIB Signalling in B Lymphocytes, CCR5 Signalling in Macrophages, Chemokine Signalling and Phospholipid Degradation pathways are involved in inflammatory and allergic response, oxidative stress, elevation in intracellular. Ca^{2+} level and lipid degradation (Table 4.7), mediated by the phopholipase A_2 (PLA_2) enzyme (Nishizuka 1992; Ren *et al.* 2010). These pathways were up-regulated in BH compared with BC, as in Category I, and are associated with muscle pathology in broiler breast muscles. Previous findings have shown that oxidative stress, raised intracellular Ca^{2+} level and poor sarcolemmal integrity have synergistic effects on muscle damage in combination with heat-stress in broilers (Mitchell & Sandercock 1995b; Mitchell *et al.* 1999a; Sandercock & Mitchell 1999; Sandercock *et al.* 2001; Mujahid *et al.* 2005; Sandercock *et al.* 2006; Mujahid *et al.* 2009).

4.4.2.5 Category V

Pathways: All of the 5 pathways in this category were involved in inflammation, vascular permeability, production of ROS and prostaglandin and, ultimately, cell death (Table 4-5) (Mitchell *et al.* 1999b; Alway 2010; Proskuryakov & Gabai 2010; Fukui *et al.* 2012; Tian *et al.* 2012). These pathways were up regulated in LH compared with LC, but were down regulated in BH compared with BC. The up-regulation of these inflammatory and cell death related pathways are logical in the sense that heat-stress is known to be involved in causing some oxidative stress and muscle damage in layers, though its extent is lower compared with broilers (Sandercock *et al.* 2006; Mujahid *et al.* 2007b). In agreement with these reports, our

results show that some inflammatory and apoptotic pathways in category V and some pathways in category II, were up-regulated in LH compared with LC. However, the reasons for the down-regulation of these pathways in BH might be that genetic selection for different quantitative traits has made these two types of chicken very diverse in terms of their response to various stressors.

4.4.2.6 Category VI

Pathways: In this category, 3 pathways were selected (Table 4-6) on the basis of their relevance to the skeletal muscles. Interferon Signalling and TNFR1 Signalling are involved in cytokine signalling, inflammation and cell death. The third pathway, Role of JAK1, JAK2, and TYK2 in Interferon Signalling are also involved in cytokine signalling but mainly in wound healing and tissue regeneration (Flammer *et al.* 2010). These pathways were up-regulated in BH compared with BC, in agreement with category I and IV, indicating that heat-stressed broilers have higher muscle damage compared with BC, LC and LH. However, these pathways were down-regulated in LH compared with LC. The reason for this might again be the same as mentioned under Category IV that more substantial up-regulation of survival-related pathways has resulted in decreasing the damaging effects in LH (Dogra *et al.* 2006).

4.4.3 Pathway and network analysis of Biolayout filtered genes in IPA

Pathways: In this group, 8 interesting pathways were selected on the basis of their relevance to tissue damage mainly caused by oxidative stress and accelerated rate of

cell death (Table 4-7); they were up-regulated in BH compared with BC, and in broilers compared with layers. They were all related to oxidative stress, cell death and tissue damage. The results are in agreement with available reports in chickens showing that heat-stress is involved in causing muscle damage in broilers that is mediated by oxidative stress (Sandercock & Mitchell 1999; Sandercock *et al.* 2001; MacRae *et al.* 2006; Sandercock *et al.* 2006).

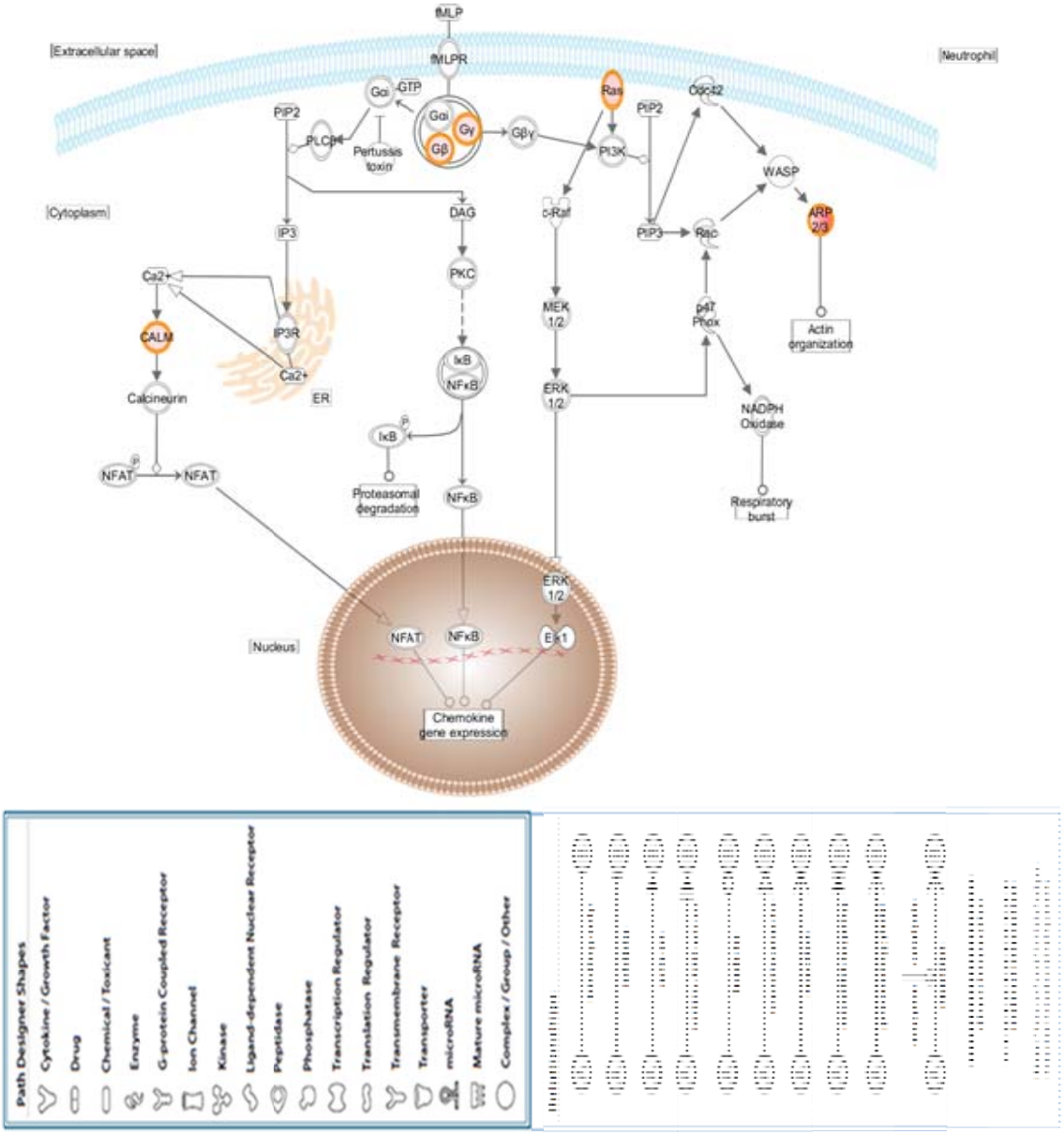
Networks: In this group of genes, 5 networks were selected (Figures 4-7, 4-8, 4-9) from the list of networks. All of the genes were involved in tissue damage, and skeletal and muscular disorders, consistent with the pathways results. Networks 17 and 24 were involved in skeletal and muscular system development and functions, energy production and tissue damage (Appendix 4-11). These two networks were linked to each other through a common gene, COX6A1, up-regulated in both pathways and involved in the activation of the cytochrome C oxidase (COX) family of genes which are involved in mitochondrial dysfunction. Similarly, networks 7 and 25 were involved in cell cycle, cancer and genetic disorders and linked together by a single gene, SH3BP4 (Appendix 4-11). These results show that breast muscles in broilers are subject to stress related damage that is further exacerbated on exposure to heat-stress (Mitchell & Sandercock 1995b; Sandercock & Mitchell 2001; Sandercock *et al.* 2006).

4.4.4 Selection of genes for further investigation

For subsequent studies (SNP genotyping) genes were selected from key positions of the selected pathways and networks. Priority was given to those genes which were present on the upstream position in the pathways and networks and switching on/off

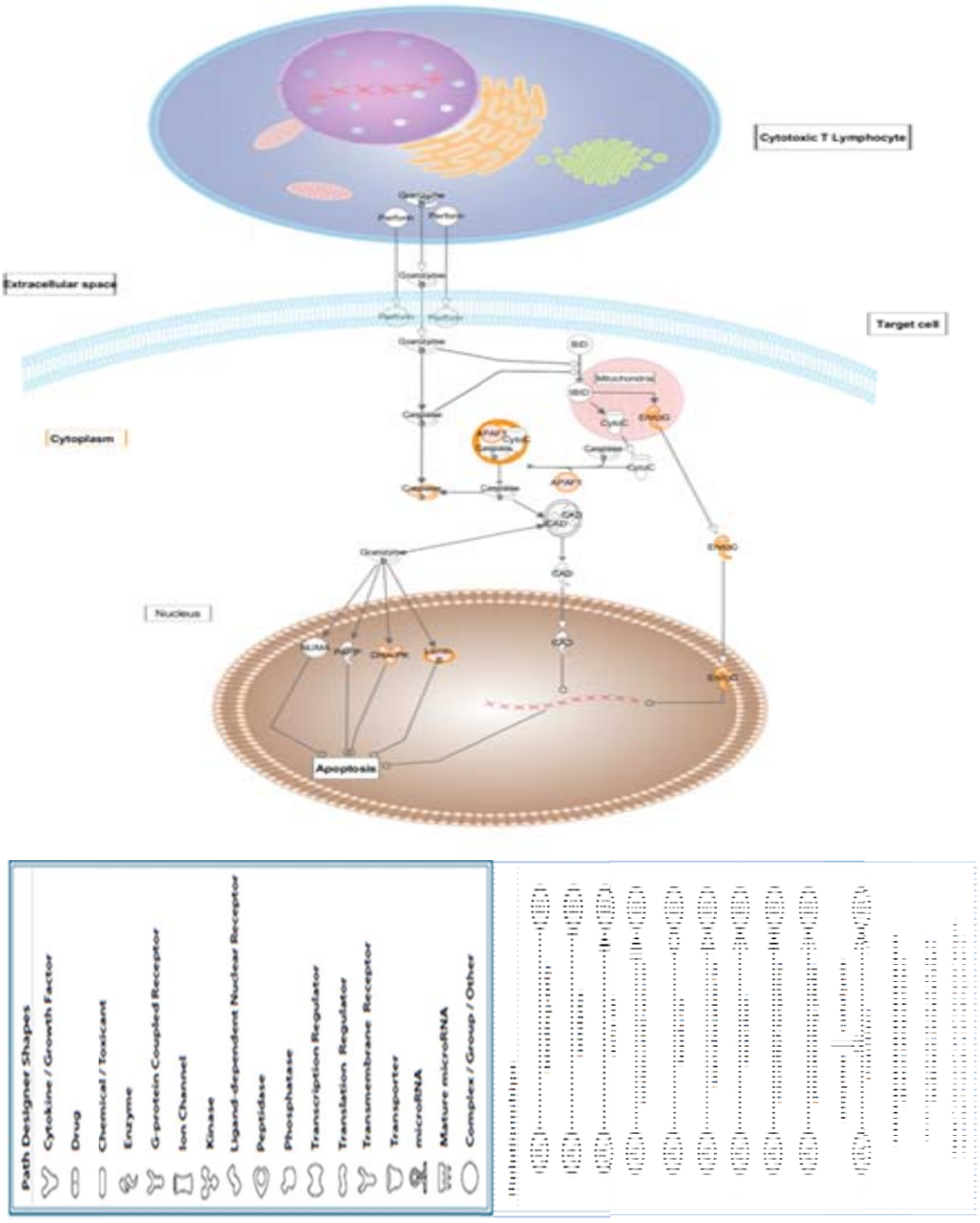
the down-stream genes. From the following pathways, fMLP signalling in Neutrophil (Figure 4-7), Gβ (GNB5), Gγ (GNG2) and HRAS genes were selected that are involved in switching on the down-stream genes and ultimately resulting in raised intracellular level of Ca2+, proteasomal degradation and enhanced expression of chemokine genes.

Figure 4-11 fMLP signalling pathway in neutrophils. This figure was created by using the tools of Path Designer of Ingenuity Pathway Analysis (IPA®). Red colour genes are up-regulated.



Similarly, from the following Granzyme B signalling pathway (Figure 4-8) the Endo G, APAF1 and LMNB2 genes were selected. They were up-regulated in this pathway and involved in DNA damage and accelerated rate of apoptosis as their down-stream effects.

Figure 4-12 Granzyme B signalling pathway. This figure was created by using the tools of Path Designer of Ingenuity Pathway Analysis (IPA®). Red colour genes are up-regulated.



In addition to these pathways, genes were also selected from the identified networks. Priority was given to those genes that were common in two or more networks and hence, involved in initiating more than one network. Genes which could not be mapped in IPA but were interesting on the basis of their GO terms were also selected, to address the particular questions of the present study (CKMT1, MTFRI, and PDCD6, Table 4-8). The biological functions of the selected genes in relation to muscle and meat quality are discussed in detail in Chapter 5.

4.5 Conclusion

1. By using the bioinformatics tools described in this study the gene list was reduced from more than 2,200 to only 18 genes, affecting a large number of other genes in down-stream signalling pathways. An additional 6 genes were selected on the basis of their GO terms for biological functions.
2. The results not only confirm most of the previous findings on muscle damage in broilers and in birds subjected to heat stress but also present novel insights into the genetics and pathogenesis of heat-stress induced muscle damage by reporting a large number of previously unknown pathways and networks.
3. Genetic selection for growth and reproductive traits has not only resulted in changing the body size and shape of these two types of chicken but has also made broilers and layers very different in terms of their response to stressors.
4. The results also show that broilers are not only under metabolic stress but genetic selection has made them more susceptible to heat stress compared with layers.
5. It is expected that these pathways and networks will be the subject of interest and contribute to unravelling some underlying biological mechanisms to

control or minimise the effects of thermal stress in chickens and improve muscle and meat quality in broilers.

Chapter 5 SNP genotyping of candidate genes for muscle and meat quality traits in chicken

5.1 Introduction

During the past few decades substantial progress has been made through the application of molecular genetics in the identification of chromosomal regions, using microsatellite markers, that affect traits of economic importance in animals (Andersson 2001). These genomic regions (quantitative trait loci, QTL) are usually very large, containing hundreds of genes and it was difficult to narrow down the region due to the requirement for a large number of animals/individuals and DNA markers. To date, (August 2012) 3,442 QTL for 286 traits have been reported in 172 publications in chicken, according to the statistics of animal genome QTL database (http://www.animalgenome.org) showing that genetic markers have been the subject of huge interest to discover genomic regions affecting quantitative traits (Fulton 2012).

Similarly, Marker Assisted Selection (MAS) has been widely used to assist in the selection of superior individuals, at an early age, in commercial breeding programmes (Fulton 2012). Nevertheless, there are several constraints in the application of MAS technology in commercial breeding that limited its use in practical breeding. These limitations include the presence of negative correlations between traits of interest and additionally requirements for huge amounts of capital and labour to develop and maintain crosses and data recording (Dekkers 2007) and lack of association between marker allele and phenotypic trait after a few generations (Fulton 2012).

The sequencing of the chicken genome and discovery and availability of about 2.8 million SNPs in the public domain in 2004 revolutionised the poultry breeding

industry and suddenly transformed it into the genomic era (Burt & White 2007) and made whole genome selection possible. The application of whole-genome selection in breeding programmes has made it possible to select the elite individuals on genomic information and lead to sustainable improvements in subsequent generations. In addition to these benefits it has also resulted in decreasing the number of animals selected for breeding, reducing the number of progeny to be phenotyped, and the identification of superior animal at very early age and thus decreasing the generation interval (Meuwissen *et al.* 2001; Fulton 2012). It is considered that most (up to 90%) of the genetic differences between individuals are explained by SNPs and, therefore, they are recognised as most preferable/desirable markers to use in breeding programmes. However, SNPs found within the exonic regions of DNA are of major interest because they can change the protein structure and its biological functions (Salem *et al.* 2012).

The arrival of SNP genotyping technology has made it possible to discover reliable genetic markers responsible for controlling different phenotypic traits in individuals (Dekkers 2012). Additionally, synergistic combination of this technology with modern statistical tools has enabled geneticists to predict the magnitude of phenotypic variance caused by SNPs and also to design breeding programs with better precision and accuracy.

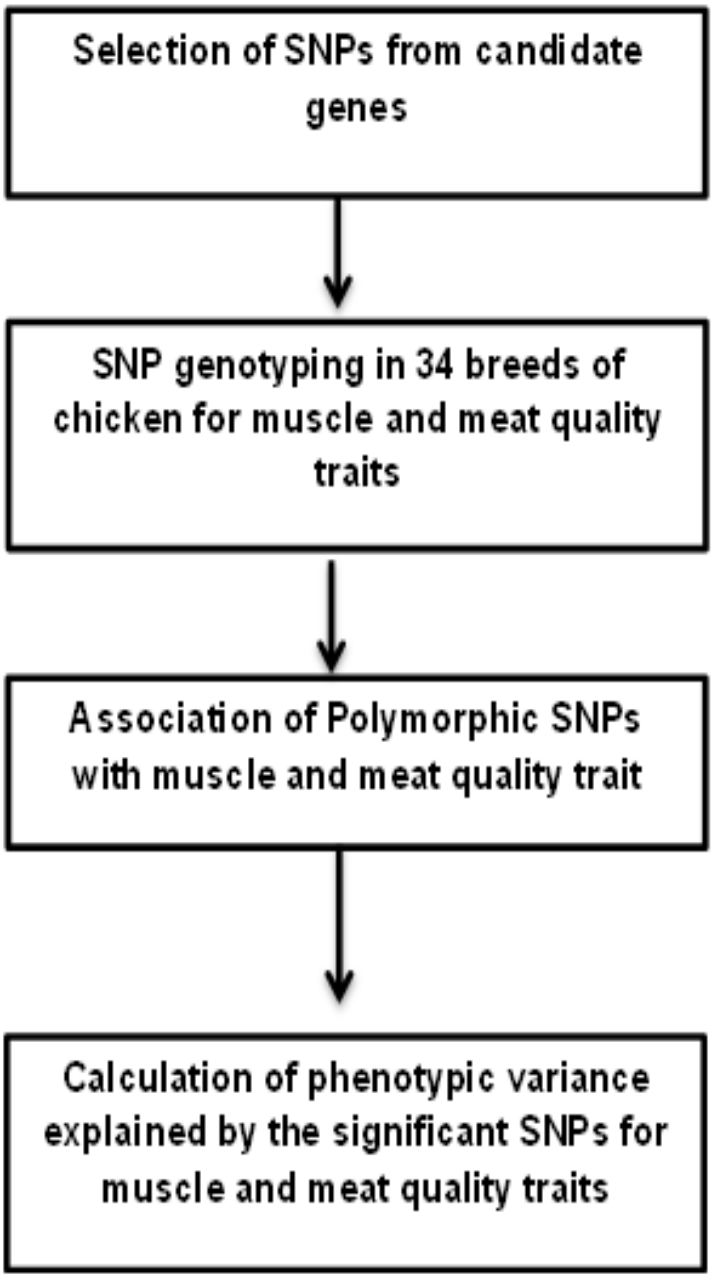
Through these developments genomic information has started to be used in poultry breeding programmes by different breeding companies, as indicated by the development of SNP chips by poultry breeders and commercial companies (Fulton 2012). However, it is essential that these SNPs should be associated with the

different phenotypic traits of interest. These new advances will lead the poultry breeders to apply genomic selection to enhance the accuracy of their programmes, especially for the traits where it is very hard to get the phenotypic data such as muscle and meat quality traits. These SNPs markers would be helpful in selecting superior birds for the trait of interest and also to update the existing SNP chips. An outline of the experimental procedures described in this chapter is presented in Figure 5-1.

5.2 Objectives

1. To identify SNP markers for muscle damage and meat quality traits in chicken.

Figure 5-1 Work Flow Diagram of Chapter 5. In this chapter SNPs were selected from the candidate genes selected in Chapter 4. Those SNPs were tested for muscle and meat quality traits in 34 breeds of chicken. Association of polymorphic SNPs were found with muscle and meat quality phenotypes by using several regression analyses. Finally, variance explained by significant SNPs for these traits were calculated.



5.3 Material and Methods

5.3.1 Husbandry and Phenotypic data

The data and traits were from previously published work as follows (Sandercock *et al.* 2009a; Sandercock *et al.* 2009b).

"In total more than 900 day-old chicks belonging to 34 lines (12 broiler, 12 layer, and 10 traditional) were obtained from 8 different sources (Table 5.1). The birds belonging to commercial lines, broilers (B), layers (L) and the traditional J-line (Brown Leghorn) were vent-sexed at the time of hatch and the males were housed in 4 large pens. Birds belonging to the remaining T lines were sexed by using a DNA method (Griffiths et al., 1998).

Birds from the commercial lines and the J-line were the offspring of at least 4 sires and 8 dams. While the other T line birds were the progeny of 2 males but the number of hens is not specified (the total number of sires was 125). The birds were randomly allocated to different pens, however, it was made sure that there was equal representation of each sire in each pen and chicks were identified with their unique wing band number for each bird.

The birds were provided with *ad libitum* access to water and feed; birds were offered a commercial broiler starter diet from 0 to 5 week and a commercial finisher diet from 5 to 10 week. A constant photoperiod of 16L:8D was maintained throughout the experiment and light intensity averaged 10 lux at the height of the head of the bird. The experiment was conducted under a United Kingdom Government Home

Office-approved license after review by the Institute's Animal Welfare and Ethics Committee".

Table 5-1 Genetic line (Breed), Category, Source and number of birds used for the collection of phenotypic trait data (Sandercock et al. 2009a; Sandercock et al. 2009b).

Line	Category[1]	Source[2]	No. of birds
Araucana	T	1	4
Barnevelder	T	1	4
Brown Leghorn	T	1	4
Buff Orpington	T	1	4
Friesian Fowl	T	1	4
Ixworth	T	2	4
J-line	T	3	4
Maran	T	1	4
White Dorking	T	1	4
White Sussex	T	2	4
Broiler 1	B	6	4
Broiler 2	B	6	4
Broiler 3	B	6	4
Broiler 4	B	6	4
Broiler 5	B	7	4
Broiler 6	B	7	4
Broiler 7	B	7	4
Broiler 8	B	7	4
Broiler 9	B	8	4
Broiler 10	B	8	4
Broiler 11	B	8	4
Broiler 12	B	8	4
Layer 1	L	4	4
Layer 2	L	4	4
Layer 3	L	4	4
Layer 4	L	4	4
Layer 5	L	4	4
Layer 6	L	4	4
Layer 7	L	5	4
Layer 8	L	5	4
Layer 9	L	5	4
Layer 10	L	5	4
Layer 11	L	5	4
Layer 12	L	5	4

[1]T = Traditional line; L = Commercial layer line; B = Broiler line

[2]Lines sharing the same number came from the same breeder (T lines) or commercial breeding company (B and L lines)

5.3.2 Physical traits studied

In this study, muscle and meat quality traits were studied. Plasma creatine kinase activities were used as a measure of muscle quality. While for the meat quality, pH and colour traits (lightness, redness, and yellowness) of breast and thigh muscle were studied.

5.3.3 Phenotypic data collection

Plasma creatine kinase (CK) levels were measured at 6 and 10 week of age, on 4 birds from each line (at least 1 progeny of each sire) by (Sandercock *et al.* 2009a; Sandercock *et al.* 2009b) . In the case of meat quality, colour traits lightness (B*L), redness (B*a), and yellowness (B*b) were measured form the ventral side of the right breast fillet using reflectance colorimetry [Minolta CR-300, CIELab, Minolta (UK) Ltd., Milton Keynes, UK]. The left breast fillet was used to measure initial and ultimate pH (pHi and pHu) by (Sandercock *et al.* 2009a; Sandercock *et al.* 2009b). Data for colour traits for thigh muscles (T*L), redness (T*a), and yellowness (T*b) was also collected in the same study by (Sandercock *et al.* 2009a; Sandercock *et al.* 2009b).

5.3.4 Selection of SNPs

SNPs were selected in the candidate genes, selected from pathways and networks analysis of Biolayout analysed (Category I-VI) and Biolayout filtered out genes separately (Chapter 4, section 4.3). SNPs were selected from the Ensembl genome browser using 'Biomart' option for the Broiler and Layer

(http://www.ensembl.org/biomart) in comparison with Red Jungle Fowl. SNP were also selected in the promoter regions of these genes.

For each gene, 4-5 SNPs were selected on average and preference was given to those SNPs present in exons. In total, 100 widely spaced SNPs were selected for genotyping.

5.3.5 Genotyping of SNPs

All of the 100 selected SNPs, from the candidate genes, were tested in 136 birds belonging to 34 breeds (4 birds/breed) mentioned in Table 5-1. These SNPs were genotyped by Kbioscience by using the KASP^{TM} technology. The KASP technology is a PCR-based assay that enables highly accurate bi-allelic scoring of SNPs across a wide range of genomic DNA samples (http://www.lgcgenomics.com/kasp-genotyping-reagents). Out of these 100 SNPs, 89 SNPs were subsequently found to be polymorphic.

5.3.6 Statistical analyses

All of these 89 polymorphic SNPs markers were analysed against 9 traits including CK, breast muscle lightness, breast muscle redness, breast muscle yellowness, thigh muscle lightness, thigh muscle redness, thigh muscle yellowness and initial and ultimate pH (pHi and pHu). Analyses were conducted in GenStat (www.vsni.co.uk/software/genstat/) using the Regression procedure which analysed the data using a randomized block model with SNP and CAT (broiler, layer or traditional) as fixed effects and Pen as the random effect separately for each gene. After running this analysis results were obtained for the following 3 steps (i) SNP

ignoring category (ii) SNP eliminating category (iii) SNP x Category. From these analyses, results for the SNP eliminating category were only used for subsequent analysis in order to eliminate the effects of CAT and to identify the minimal set of SNPS affecting each trait.

In the second step, multiple SNPs significant for one gene were further analysed jointly using the 'RSEARCH procedure' of Generalised Linear Model (GLM). In this procedure, the 'Backward Elimination' method was used to obtain the most significant SNP (from the fitted model) for that gene. In this step, the term 'Pen+CAT' was included in the model (Reduced model) whereas the terms 'Pen+CAT+SNP1+SNP2----+SNPn was used as the free formula (Full model).

In the third step, all the significant SNPs across all the genes, (including the most significant SNP from the 'Backward Elimination' step) for one phenotypic trait were analysed jointly using the 'Backward Elimination' method of RSEARCH procedure to obtain the most significant subset of SNPs from the fitted model for that trait. 'Pen+CAT' was used in the FORCED formula (Reduced model) whereas term 'Pen+CAT+SNP1+SNP2----+SNPn was used as the free formula (Full model). Finally, the SNPs returned by the GenStat as 'Final Model' were used to estimate the variance explained by these SNPs jointly, by using the FIT method of GLM. In this step the term 'Pen+CAT' was fitted and its effects on variance was estimated and then the model 'Pen+CAT+SNP1+SNP2+----------+SNPn' was fitted and its effect on variance was estimated (Table 5-3). Finally, the effects of SNPs on residual variance was measured by using the following formula

= [(Res. variance without SNPs – Res. variance with SNPs) / Res. variance without SNPs)] x 100.

5.4 Results

The 86 polymorphic SNPs markers in or close to 24 genes present on 14 different chromosomes were genotyped in 34 different breeds (12 Layers, 12 broilers, 10 traditional) and tested for associations against each of the 9 traits. After running the several regression analyses (section 5.3.6) and by using stringent criteria the following SNPs were found to be most strongly associated with the traits of interest and the *P*-values mentioned below under different traits are from the last (3rd regression analysis).

5.4.1 Creatine Kinase (CK)

For creatine kinase 2 SNPs, rs14604079 in HDAC4 on chromosome 7 and rs14216459 in gene PDCD6 on chromosome 2 were the most significant (P<0.05) SNPs affecting this trait (Table 5.3). These two SNPs jointly explained 15% of residual variance among the breeds (Table 5.3). The SNP rs14604079 was present in 5th exon of HDAC4 gene and was a synonymous mutation while the rs14216459 was present in the down-stream region of the PDCD6 gene.

5.4.2 Breast muscle lightness (B*L)

In total, 7 SNPs were significantly (P<0.001) associated with this trait and together they explained 48% of the variation for breast muscle lightness (B*L). These SNPs include rs14330673 in the intronic region of CALM gene on chromosome 3, a

synonymous SNP rs15093639 in the PDCD6IP gene on chromosome 2, rs13803200 in the intronic region of the MTFR1 gene present on chromosome 2, a non-synonymous mutation rs14868825 in the IFNAR2 gene on chromosome 1, rs13704016 in the MAP2K1 gene on chromosome 10 and two synonymous SNPs, rs14027061 and rs14702610, in the coding sequence of the PLCG2 gene on chromosome 11 were found most strongly associated with the for B*L (Table 5.2 and 5.3).

5.4.3 Breast muscle redness (B*a)

In the case of breast muscle redness, 4 SNPs were significantly (P<0.001) associated with B*a and together they explained the 28% of the residual variance (Table 5.2 and 5.3) for this trait. These SNPs include an intronic SNP rs14645300 in the BCL10 gene on chromosome 8, a synonymous SNP rs10727941 in the CALM gene on chromosome 3, rs15031083 in the upstream promoter of ENDOG gene on chromosome 17, and rs15126679 present in exon-intron junction of the PDCD6 gene on chromosome 2.

5.4.4 Breast muscle yellowness (B*b)

For breast muscle yellowness 3 SNPs were significantly (P=0.038) associated with the trait. These SNPs include rs10726982 in the upstream promoter of ENDOG gene on chromosome 17, rs13535756 at the intron-exon junction of PRKAG2 gene on chromosome 2, and a SNP rs14416824 also present almost the junction of intron and exon in SLC26A11 gene on chromosome 18 respectively. These 3 SNPs were jointly

responsible for explaining 20% of the residual variance (Table 5.2 and 5.3) for this trait.

5.4.5 Thigh muscle lightness (T*L)

Eight SNPs were strongly (*P*=0.001) associated with thigh muscle lightness and jointly they explained 45% of the residual variance for this trait among broilers, layers and traditional breeds (Table 5.2 and 5.3). These variations include two intronic SNPs, rs13803200 and rs14695299, in the MTFR1 gene on chromosome 2, two intronic SNPs, rs14330673 and rs14330679, in the CALM gene on chromosome 3, an intronic SNP rs14604083 in the HDAC4 gene on chromosome 7, an intronic variation rs13704016 in the MAP2K1 gene on chromosome 10, a synonymous SNP rs14702610 in the PLCG2 gene on chromosome 11 and similarly one synonymous SNP rs14305263 in the LMNB2 gene on chromosome 28.

5.4.6 Thigh muscle redness (T*a)

In the case of T*a three SNPs were found significantly associated (*P*=0.002) with this trait. These SNPs include an intronic SNP rs15671752 in HRAS gene on chromosome 5, rs14695299 in the upstream/promoter region of MTFR1 gene on chromosome 2, and one intronic SNP rs13591700 in AKT1 gene on chromosome 5 (Table 5.2 and 5.3). These 3 SNPs jointly explained the 19% of the residual variance for the T*a trait.

5.4.7 Thigh muscle yellowness (T*b)

A total of 6 SNPs including an intronic SNP rs14330679 in CALM gene on chromosome 3, an intronic SNP rs13595564 in CYP27A1 gene on chromosome 7, rs14604080 present almost at the intron-exon junction in HDAC4 gene on chromosome 7, a synonymous SNP rs14027066 in PLCG2 gene on chromosome 11, similarly one synonymous SNP rs14416825 in SLC26A11 gene on chromosome 18, and one synonymous SNP rs14305263 in LMNB2 gene on chromosome 28 showed significant (P<0.001) association with thigh muscle yellowness (Table 5.2 and 5.3). These SNPs explained 46% of residual variance for thigh muscle yellowness.

5.4.8 Initial pH (pHi)

For pHi, 5 SNPs namely rs15305228 at the intron-exon junction in CALM gene on chromosome 3, rs13803200 almost at the intron-exon junction in MTFR1 gene on chromosome 2, rs15031083 in the promoter region of ENDOG gene on chromosome 17, rs15126679 at the intron-exon junction in PDCD6 gene on chromosome 2, and rs13535812 in the intronic region of PRKAG2 gene on chromosome 2 were associated with the trait (P<0.001). These 5 significant SNPs jointly explained 41% of the residual variance for pHi.

5.4.9 Ultimate pH (pHu)

Seven SNPs, were very strongly (P<0.001) associated with ultimate pH (pHu) (Table 5.2 and 5.3). These 7 significant SNPs jointly explained the 55% of residual variance for pHu. These SNPs were an intronic SNP rs14645300 in BCL10 gene on

chromosome 8, a SNP rs15177275 in the intronic region of BCL2L1 gene on chromosome 20, rs14615869 in the intronic region of CYP27A1 gene on chromosome 7, rs15031083 in the promoter region of ENDOG gene on chromosome 7, a synonymous SNP rs14946390 in GNB5 on chromosome 10, similarly one synonymous SNP rs14305263 in LMNB2 gene on chromosome 28, and rs14695299 in the intronic regions of MTFR1 gene on chromosome 2.

Table 5-2 Results for individual SNPs in a model including breed type

Category	Gene Name	SNP Id	Position	CHR	CK	B*L	B*a	B*b	T*L	T*a	T*b	pHi	pHu
Cat 2 Pathway	AKT1	rs13591699	Upstream	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs13591700	Intronic	5	NS	NS	NS	NS	NS	**	NS	NS	NS
		rs14548962	Upstream	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs14548963	Upstream	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
Biolayout filtered	APAF1	rs13651273	Upstream	1	N.S.	N.S.	N.S.	NS	N.S.	N.S.	N.S.	N.S.	N.S.
		rs13861269	Upstream	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs13861271	Upstream	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs13861273	Upstream	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs13861293	Upstream	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Biolay fil. Path	BCL10	rs14645300	Intronic	8	*	NS	**	NS	NS	NS	NS	NS	***
		rs14645301	Intronic	8	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs15917771	Intronic	8	NS	NS	NS	NS	NS	NS	NS	NS	NS
GO term	BCL2L1	rs14278113	Intronic	20	*	NS	NS	NS	NS	NS	NS	NS	*
		rs15177274	Intronic	20	**	NS	*	NS	NS	NS	NS	NS	*
		rs15177275	Intronic	20	NS	NS	NS	NS	NS	NS	NS	NS	*
		rs15177278	Intronic	20	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cat 1 Pathway	CALM	rs10727941	Synonymous	3	*	NS	***	NS	NS	NS	NS	NS	NS
		rs14330673	Intron	3	NS	*	NS	NS	**	NS	NS	***	NS
		rs14330679	Intron	3	NS	NS	NS	NS	*	NS	*	***	*
		rs14330680	Upstream	3	*	NS	*	NS	NS	NS	NS	NS	NS
		rs15305228	Edge of exon	3	NS	NS	NS	NS	NS	NS	NS	***	NS
GO term	CKMT1	rs14955550	Intronic	10	NS	NS	NS	NS	**	NS	NS	NS	NS
		rs14955553	Intronic	10	NS	*	NS	NS	NS	NS	NS	NS	NS
		rs14955554	Upstream	10	NS	*	NS	NS	NS	NS	NS	NS	NS
Cat 3	COX6A1	rs15024046	Upstream	15	*	NS	NS	NS	NS	NS	NS	NS	***

		rs15024047	Upstream	15	NS	NS	NS	NS	NS	NS	NS	NS	NS
GO term	CYP27A1	rs13595564	Intronic	7	NS	NS	NS	NS	NS	NS	*	*	NS
		rs13595565	Intronic	7	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs13595566	Intronic	7	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs14615869	Intronic	7	*	NS	NS	NS	0.052	NS	NS	NS	**
Biolay fil. Path	ENDOG	rs10726982	Promoter/coding	17	NS	NS	NS	*	NS	NS	NS	NS	NS
		rs15031083	Promoter	17	NS	NS	**	NS	0.056	NS	*	***	***
		rs15031084	Intronic	17	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cat 1 Pathway	GNB5	rs14004719	Intronic	10	NS	NS	NS	NS	*	NS	NS	NS	NS
		rs14004720	Intronic	10	NS	NS	NS	NS	*	NS	NS	NS	NS
		rs14004752	Synonymous	10	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs14946390	Synonymous	10	NS	NS	NS	NS	*	*	NS	NS	NS
Cat 1 Pathway	GNG2	rs13594872	Upstream	5	*	NS	NS	NS	NS	NS	NS	0.053	NS
		rs13594873	Upstream	5	*	NS	NS	NS	*	NS	NS	**	NS
		rs14556386	Upstream	5	*	NS	NS	NS	***	NS	NS	*	NS
		rs14556387	Upstream	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs15746863	Intronic	5	**	NS	NS	NS	*	NS	NS	**	NS
Cat 1 Network analysis	HDAC4	rs14604079	Synonymous	7	***	NS	*	NS	NS	NS	NS	NS	NS
		rs14604080	Edge of exon	7	NS	NS	NS	*	NS	NS	0.056	NS	NS
		rs14604082	Upstream	7	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs14604083	Intron	7	NS	NS	NS	NS	*	NS	NS	NS	NS
		rs14604084	Upstream	7	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cat 1 Pathway	HRAS	rs15671745	Intronic	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs15671750	Intronic	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs15671752	Upstream	5	NS	NS	NS	NS	NS	*	NS	NS	NS
		rs15671757	Intronic	5	*	NS	NS	NS	NS	NS	NS	NS	NS
Cat 6	IFNAR2	rs14868816	5 Prime_UTR	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs14868817	Intronic	1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

		rs14868825	Non-synonymous	1	N.S.	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	*
Biolay fil. Path	LMNB2	rs14305263	Synonymous	28	NS	NS	*	*	**	0.053	*	NS	0.054
Cat 2 Pathway	MAP2K1	rs13704016	Intronic	10	NS	**	NS	NS	**	NS	NS	NS	NS
		rs14013207	Intronic	10	*	NS	NS	NS	NS	NS	NS	NS	NS
		rs14953751	Intronic	10	NS	NS	NS	NS	*	NS	NS	NS	NS
		rs14953769	Synonymous	10	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cat 1 Pathway	MAPK14	rs13724831	Intronic	26	NS	NS	NS	NS	NS	NS	NS	**	NS
		rs14416336	Upstream	26	NS	NS	NS	NS	NS	NS	NS	***	NS
		rs14416337	Intronic	26	NS	NS	NS	NS	NS	NS	NS	NS	NS
GO term	MTFR1	rs13803200	Edge of exon	2	N.S.	**	**	N.S.	*	*	N.S.	**	0.053
		rs14695294	Synonymous	2	NS	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs14695299	Intronic	2	N.S.	N.S.	0.055	N.S.	*	**	N.S.	N.S.	0.053
GO term	PDCD6	rs14216459	Intron	2	*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs15126679	Edge of exon	2	NS	N.S.	*	N.S.	N.S.	0.052	N.S.	*	N.S.
		rs15126686	Downstream	2	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Cat 1 Network analysis	PDCD6IP	rs14174893	Intronic	2	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs14174902	Intronic	2	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs15093639	Synonymous	2	NS	*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Cat 4 Path	PLCG2	rs14027061	Synonymous	11	NS	**	NS	*	NS	NS	NS	NS	NS
		rs14027066	Synonymous	11	NS	NS	NS	*	*	NS	*	NS	NS
		rs14702610	Synonymous	11	*	**	NS	NS	*	NS	NS	NS	***
Cat 4	PRKAG2	rs13535756	Edge of exon	2	*	N.S.	*	*	N.S.	N.S.	N.S.	N.S.	N.S.
		rs13535812	Intronic	2	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**	N.S.
		rs13535831	Upstream	2	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
		rs14133173	Upstream	2	NS	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Cat 4 Path	PTGS2	rs15910128	Intronic	8	NS	NS	NS	NS	NS	NS	NS	NS	NS
		rs15910130	3 prime UTR	8	NS	NS	NS	NS	*	NS	NS	NS	NS

GO term	SLC26A11	rs14416822	Upstream	18	NS	NS	NS	*	*	NS	NS	NS	NS
		rs14416824	Edge of exon	18	NS	NS	NS	**	**	NS	**	NS	NS
		rs14416825	Synonymous	18	***	NS	NS	NS	NS	NS	***	0.053	NS
		rs15469299	Promoter	18	NS	***	NS	NS	NS	NS	NS	NS	NS

*** Significant at $P<0.001$

** Significant at $P<0.01$

* Significant at $P<0.05$

NS Non significant

Table 5-3 Results for joint SNPs analyses following backward elimination, including breed-type

Gene Name	SNP Id	CH	CK	B*L	B*a	B*b	T*L	T*a	T*b	pHi	pHu
AKT1	rs13591699	5									
	rs13591700		---	---	---	---	---	rs13591700	---	---	---
BCL10	rs14645300	8									
	rs14645301		---	---	rs14645300	---	---	---	---	---	rs14645300
	rs15917771										
BCL2L1	rs14278113	20									
	rs15177274										
	rs15177275		---	---	---	---	---	---	---	---	rs15177275
	rs15177278										
CALM	rs10727941	3									
	rs14330673										
	rs14330679		---	rs14330673	rs10727941	---	rs14330673	---	rs14330679	rs15305228	---
	rs14330680						rs14330679				
	rs15305228										
CKMT1	rs14955550	10									
	rs14955553		---	---	---	---	---	---	---	---	---
	rs14955554										
COX6A1	rs15024046	15									
	rs15024047		---	---	---	---	---	---	---	---	---
CYP27A1	rs13595564	7									
	rs13595565										
	rs13595566		---	---	---	---	---	---	rs13595564	---	rs14615869
	rs14615869										
ENDOG	rs10726982	17									
	rs15031083		---	---	rs15031083	rs10726982	---	---	---	rs15031083	rs15031083
	rs15031084										
GNB5	rs14004719	10									
	rs14004720										
	rs14004752		---	---	---	---	---	---	---	---	rs14946390
	rs14946390										
GNG2	rs13594872	5									
	rs13594873		---	---	---	---	---	---	---	---	---
	rs14556386										

	rs14556387										
	rs15746863										
HDAC4	rs14604079	7									
	rs14604080		rs14604079								
	rs14604082			---	---	---	rs14604083	---	rs14604080	---	---
	rs14604083										
	rs14604084										
HRAS	rs15671745	5									
	rs15671750										
	rs15671752		---	---	---	---	---	rs15671752	---	---	---
	rs15671757										
IFNAR2	rs14868816	1									
	rs14868817		---	rs14868825	---	---	---	---	---	---	---
	rs14868825										
LMNB2	rs14305263	28	---	---	---	---	rs14305263	---	rs14305263	---	rs14305263
MAP2K1	rs13704016	10									
	rs14013207										
	rs14953751		---	rs13704016	---	---	rs13704016	---	---	---	---
	rs14953769										
MAPK14	rs13724831	26									
	rs14416336		---	---	---	---	---	---	---	---	---
	rs14416337										
MTFR1	rs13803200	2					rs13803200				
	rs14695294			rs13803200			rs14695299	rs14695299		rs13803200	rs14695299
	rs14695299										
PDCD6	rs14216459	2									
	rs15126679		rs14216459	---	rs15126679					rs15126679	
	rs15126686										
PDCD6IP	rs14174893	2									
	rs14174902		---	rs15093639	---	---	---	---	---	---	---
	rs15093639										
PLCG2	rs14027061	11									
	rs14027066			rs14027061							
	rs14702610			rs14702610	---	---	rs14702610		rs14027066	---	---
PRKAG2	rs13535756	2									
	rs13535812		---	---	---	rs13535756	---	---	---	rs13535812	---

PTGS2	rs13535831 rs14133173 rs15910128 rs15910130		---	---	---	---	---	---	---	---	---	---
SLC26A11	rs14416822 rs14416824 rs14416825 rs15469299	18	---	---	---	rs14416824	---	---	rs14416825	---	---	---
% of variance explained by the SNPs jointly[1]			15	48	28	20	45	19	46	41	55	

5.5 Discussion

Several regression analyses were run using very stringent criteria to evaluate the association of SNPs with variation in the 9 phenotypic traits. Finally, a number of SNPs were found that very strongly associated with the traits of interest and responsible for explaining a substantial amount of variation in these traits. It is worth mentioning that all of these SNPs are novel and have not been reported to date for their association with any trait in chicken. However, the data in the current study do not provide enough evidence of a causal relationship between a SNP and these traits; hence, more investigation is recommended to confirm if there is any causal relationship between any of the identified SNP and a muscle or meat quality trait.

5.5.1 Muscle quality traits

5.5.1.1 Creatine Kinase

For creatine kinase 2 SNPs, a synonymous SNP rs14604079 in the HDAC4 gene and an intronic SNP rs14216459 in the PDCD6 gene were the most strongly associated ($P<0.05$) SNPs for this trait (Table 5.3). The presence of significant SNPs in the HDAC4 and PDCD6 genes for creatine kinase shows the association of these genes in breast muscle metabolism and damage in chicken. Though the HDAC4 gene is known to have a crucial role in regulating the genes involved in muscle metabolism, functions and development in human (Du *et al.* 2008a; Choi *et al.* 2012); it is the first time that any SNP affecting muscle damage in this gene has been identified in chicken. Consistent with this, McGee and Hargreaves (2010) reported that inhibition of HDAC4 could be an effective therapeutic tool in treating various metabolic

diseases like insulin resistance, obesity, and Type 2 diabetes. In agreement with this Du *et al.* (2008b) reported that HDAC4 inhibited the differentiation of skeletal muscle cells in response to its stimulation by Protein Kinase A (PKA). These finding were further confirmed by Chen *et al.* (2006) who discovered micro-RNAs (microRNA-1) that could promote myogenesis by reducing/inhibiting the effects of the HDAC4 gene on skeletal muscle genes. It seems likely that this synonymous variation/mutation has enhanced the effect of HDAC4 on muscle damage in chicken, possibly by its association with some other variations which could not be checked/studied in this experiment, and segregating together.

Similarly, though the PDCD6 gene is known for its role in cell death and tissue damage, no SNP has been reported in this gene for its association with any muscle and meat quality trait, to date. However, in agreement to our results Rho *et al.* (2012) have reported that the PDCD6 gene is not only involve in promoting cell death but it also suppress the angiogenesis, stimulated by vascular endothelial growth factor (VEGF), and protein synthesis. Consistent with this Park *et al.* (2012) reported that PDCD6 further augments the rate of cell death caused by TNF-alpha mediated by NFkB.

5.5.2 Meat quality traits

5.5.2.1 Breast muscle lightness (B*L)

In total, 7 SNPs were significantly associated with breast muscle lightness (B*L) and they jointly explained 48% variation for this trait (Table 5.2 & 5.3). An intronic mutation rs13803200 in the MTFR1 gene was found significantly associated with the trait. To date, no SNP in the MTFR1 gene in any species has been associated with

any trait and this is the first time that a SNP from this gene has been found associated with breast muscle lightness. Gu *et al.* (2009) have described the MTFR1 gene as a candidate gene for muscle strength and athletic-performance traits in thoroughbred horses. Whereas, Monticone *et al.* (2007) have reported that the MTFR1 gene has an anti-oxidant role and protects the tissues against oxidative stress which is in agreement with its role in enhancing muscle strength because oxidative stress causes tissue damage and weakness of the muscles (Yoda *et al.* 2010). However, the SNP reported in the present study might be involved (associated) in compromising the anti-oxidant function of this gene. Nevertheless, this SNP and gene merit further exploration to discover its actual role for this trait.

Similarly, a novel synonymous mutation rs15093639 in the PDCD6IP/Alix gene has been found associated with B*L. However, Strappazzon *et al.* (2010) have reported that Alix in combination with ALG2 (apoptosis-linked gene-2) is involved in Ca^{2+} induced activation of the caspases 9 gene which in turn is involved in causing cell death. In our case, this gene was up-regulated in heat stressed broilers compared with broiler controls, and in broilers compared with layers, suggesting a greater rate of cell death in broilers than layers (see chapter 4). Secondly, this gene linked the three networks (Network 1, 7, & 9) (see section 4.3.2.1) all of which were involved in cell death, and in genetic and muscular disorders (Appendix 4-8). Consistent with this, Mahul-Mellier *et al.* (2006b) have also showed that the Alix gene is involved in promoting cell death.

Similarly, one intronic mutation, rs14330673, in the CALM gene on chromosome 3 was also found to be significantly associated with this trait. The calmodulin (CALM)

gene is known to be involved in controlling the intracellular Ca^{2+} level by regulating the activity of ryanodine receptors (RyR) (Chen *et al.* 1997). Al-Shanti and Stewart (2009) have reported the involvement of CALM in various neuromuscular disorders like Huntington's disease, and Parkinson disease characterised by greater muscle contraction and cell death. The intronic mutation rs13704016 in the MAP2K1 gene reported in the present study is novel and, to date, no SNP in this gene has been reported to have an association with any trait in any farm animal species. However, the literature shows that it is involved in repressing the differentiation of muscles cells. Consistent with this, Fubini and Hubbard (2003) have reported that oxidative stress resulted in the activation of the MAP2K1 gene which led to up-regulation of inflammatory cytokines (TNF-alpha, IL-1) and finally cell death. It shows that the MAP2K1 gene was also involved in promoting cell death and hence, affecting B*L in chicken.

A non-synonymous mutation (A/G) rs14868825 in the IFNAR2 gene was associated (P<0.001) with B*L. This mutation was involved in changing the amino acid asparagine to aspartic acid, both of which belong to hydrophilic class of amino acids. However, the biological significance of the association of this non-synonymous mutation with B*L is not known and merits further exploration.

A mutation in IFNAR2 has been reported to be associated with multiple sclerosis in human (Leyva *et al.* 2005). Similarly, 2 synonymous SNP, rs14027061 and rs14702610 in the coding regions of the PLCG2 gene were found significantly associated with B*L. The PLCG2 gene is known to be involved in various auto-inflammatory and immune disorders (Bergholdt *et al.* 2012; Ombrello *et al.* 2012),

hence it might be speculated that these SNPs are involved in causing the inflammatory reaction and tissue damage in the present experiment. Consistent with this, mutations in this gene in mouse have been reported to cause immune disorders, inflammatory arthritis, and metabolic problems, mediated by increased uptake of extracellular Ca^{2+} (Yu *et al.* 2005; Abe *et al.* 2011). Therefore, it is speculated that synonymous SNP discovered in this gene in the present study could be a marker for inflammation and tissue damage, by raising intracellular level of Ca^{2+}, and this hypothesis was further supported by Sandercock *et al.* (2009a) who reported that modern broilers have higher levels of Ca^{2+} and other cations in their plasma and skeletal muscles compared with layers and traditional breeds. Consistent with this, SNPs in 3 genes (PDCD6IP, CALM and PLCG2) might also be involved in affecting B*L by promoting cell death and tissue damage mainly through excessive uptake of extracellular Ca^{2+} (Lee *et al.* 2005; Abe *et al.* 2011; Huang *et al.* 2011; Kramerova *et al.* 2012; Ombrello *et al.* 2012; Zhou *et al.* 2012).

5.5.2.2 Breast muscle redness (B*a)

Four SNPs, rs14645300, rs10727941, rs15031083, and rs15126679 belonging to the BCL10, CALM, ENDOG and PDCD6 genes respectively were found significantly associated with breast muscle redness (B*a) (Table 5.2 & 5.3). These SNPs jointly explained 28% of the residual variance for this trait. The BCL10 gene is a transcription regulator that mainly regulates the apoptotic process within the cell by regulating the activation of cysteine-type endopeptidase activator (members of caspases family). However, a SNP in 3' UTR of the BCL10 gene in pork has been reported to be significantly associated with immune cell, red blood cell count and

haemoglobin level in blood (Huang *et al.* 2010). Consistent with these, it has also been reported that a SNP in BCL10 gene is associated with tumorigenesis in human (Hayashi *et al.* 2009) showing that it is involved in enhancing angiogenesis, blood supply and proliferation of blood vessels. It is likely that the SNP in BCL10 gene in present study is involved in the similar type of functions. However, this gene was down-regulated in broilers compared with layers (Chapter 3 and 4) which is in agreement with the hypoxia-like situation of broiler breast muscles possibly due to insufficient capillary supply (MacRae *et al.* 2006). This hypoxia-like state also suggests that there is a lack of myoglobin, responsible for imparting red colour to meat and also transportation of oxygen in muscles. This hypoxia-like situation further leads to the further production of ROS from the affected cells (Solaini *et al.* 2010) and can cause more tissue damage. Similarly, CALM gene was also involved in apoptotic functions, as discussed under the B*L (section 5.5.2.1) and hence, may lead to tissue damage and affect the colour of meat.

A SNP rs15031083 in the promoter region of the ENDOG gene was found significantly associated with B*a. Though ENDOG is a nuclear gene, its protein localises in mitochondria and is involved in controlling mitochondrial biogenesis, respiration and metabolism (Yakovlev & Faden 2004). McDermott-Roe *et al.* (2011) found an insertion in exon 1 of this gene as a primary cause of cardiac hypertrophy and abnormal heart function mediated by mitochondrial depletion and impaired functions. Consistent with these results, we also found some up-regulated pathways and networks involved in cardiac hypertrophy, and oxidative stress. It is commonly considered that the cardiac hypertrophy is involved in heart failure and sudden death syndrome. However, keeping in view the association of variation in

ENDOG gene with cardiac hypertrophy, it might be speculated that the discussed variation/mutation in the ENDOG gene might also be involved in sudden death syndrome in broilers; a major problem of modern broilers. The SNP rs15126679 in the PDCD6 gene was present close to an exon-intron junction and might be involved in splicing and also in augmenting the anti-angiogenic role of this gene (section 5.5.2.2) (Rho *et al.* 2012); hence will trigger hypoxia-like situation, and cell death leading to pale and less red meat colour.

Taken together, all of these 4 SNPs are likely to be involved in oxidative stress, cell death and anti-angiogenic functions.

5.5.2.3 Breast muscle yellowness (B*b)

Three SNPs, rs13535756 in PRKAG2, rs10726982 in ENDOG and rs14416824 in SLC26A11 gene, were found strongly associated with B*b. These 3 SNPs jointly explained 20% of the residual variance for this trait (Table 5.3).

All of these 3 SNPs are novel and have been reported here for the first time. The SNP (rs10726982) in the ENDOG gene was present in the promoter region and likely to be involved in causing oxidative stress and cell death through impaired mitochondrial functions, possibly by altering the expression of this gene, as discussed under the B*a in section 5.5.2.2. To date, no SNP association in the SLC26A11 gene have been reported, however, the few published reports about the function of this gene show that it is involved in the transport of various anions, mainly sulphate and chloride and in facilitating acid secretion in collecting ducts (Vincourt *et al.* 2003; Kere 2006). The SNP rs14416824 in the SLC26A11 gene was

present very close to the coding sequence and might be involved in mRNA splicing. However, the physiological relation of this variation/mutation to B*b is not known and merits further investigation.

Similarly, rs13535756 SNP in the PRKAG2 gene was also present close to an exon-intron junction and hence, might be involved in RNA splicing. However, some mutations in the PRKAG2 gene in humans are known to have associations with diabetes and hypertrophic cardiac myopathy (Jablonski *et al.* 2010; Kim *et al.* 2012; Wang *et al.* 2012). Diabetes is responsible for causing muscle weakness mediated mainly by oxidative stress and reduced glucose uptake. It suggests that oxidative stress is involved in causing muscle damage and might be involved in affecting the yellowness of meat. This hypothesis was further supported by Choi *et al.* (2010) who supplemented the broiler diet with some anti-oxidants (garlic powder and alpha-tocopherol) and observed significant improvement for redness and yellowness of meat.

5.5.2.4 Thigh muscle lightness (T*L)

In case of T*L, after all 3 regression analysis (section 5.3.6), 8 SNPs were found to be strongly associated (P=0.001) with this trait. These 8 SNP markers jointly explained 45% of the residual variance for this trait among broilers, layers and traditional breeds (Table 5.3). The SNPs found in the CALM, MTFR1, MAP2K1, and PLCG2 genes were also found significant for B*L and it is very likely that they might be affecting the thigh muscle lightness in a similar manner as they were for B*L (section 5.5.2.1). Similarly, the SNP in the HDAC4 gene might also be

involved in accelerating cell death and tissue damage (Du *et al.* 2008a; Choi *et al.* 2012), as discussed under the CK trait (section 5.5.1.1).

The synonymous SNP rs14305263 in the LMNB2 gene has also been reported for the first time for its association with any trait in chicken. Munoz-Alarcon *et al.* (2007) have reported that a mutation in the LMNB2 gene in Drosophila caused muscle defects and surviving adults were unable to fly but could walk like aged flies. It shows that this variation in the LMNB2 gene might be involved in causing muscle weakness in broiler which also resulted in increasing the lightness of thigh muscle. The weakness of leg muscle in broiler is also consistent with the higher incidence of lameness in them.

5.5.2.5 Thigh muscle redness (T*a)

For thigh muscle redness (section 5.3.6), after using all the regression analyses (section 5.3.6), 3 SNP, rs15671752 in HRAS, rs14695299 in MTFR1, and rs13591700 in AKT1 gene were found most strongly associated (P=0.002) with T*a and were responsible for explaining 19% of residual variance for the trait. Several SNPs in the HRAS gene have been reported for their involvement in causing cancer and Castello syndrome in human (Wright *et al.* 2011; Burkitt-Wright *et al.* 2012; Maemoto *et al.* 2012) but, to date, no HRAS SNP in chicken is known to have association with any trait. However, the HRAS gene is involved in growth and proliferation of endothelial and haematopoietic cells (Meadows *et al.* 2001; Sykes & Kamps 2001). It was also up-regulated in broilers compared with layers in the gene expression experiment (Chapter 3 and 4). It seems likely that this variation was involved in increasing the blood supply to broiler thigh muscles in agreement with

the findings of Sandercock *et al.* (2009a) who observed that broiler thigh muscles had greater redness compared with layers.

However, no SNP in the AKT1 gene has been found to be associated with any trait in chicken. However, AKT1 gene is known to have a role in skeletal muscle growth and differentiation (Wilson & Rotwein 2007). Thus its association with muscle redness is consistent with the findings of Hu *et al.* (2012) who demonstrated that AKT1 is involved in enhancing the blood supply to muscles (Takahashi *et al.* 2002). A novel intronic mutation, rs14695299 in the MTFR1 gene, was also found associated with thigh muscle redness. As discussed in section 5.5.2.1 (B*L), Gu *et al.* (2009) have reported a role for the MTFR1 gene in rendering strength to skeletal muscles in horses; hence it might be involved in increasing the blood supply (haemoglobin) and ultimately redness of skeletal muscles and meat.

5.5.2.6 Thigh muscle yellowness (T*b)

In total 6 SNPs, rs14330679 in CALM, rs13595564 in CYP27A1, rs14604080 in HDAC4, rs14027066 in PLCG2, rs14416825 in SLC26A11 and rs14305263 in LMNB2 were found strongly associated ($P<0.001$) with this trait after running all the described regression analysis (section 5.3.6). These 6 significant SNPs were responsible for jointly explaining the 46% of the residual variance for thigh muscle yellowness.

As discussed under CK and B*L (section 5.5.1.1 and 5.5.2.1), it is speculated that the significant SNPs in CALM and HDAC4 genes might be involved in triggering cell death possibly through a raised intracellular Ca^{2+} level. They might be involved

in decreasing the yellowness of broiler muscle by increasing the rate of cell death and subsequent tissue damage. One novel synonymous mutation rs14416825 in the coding sequence of the SLC26A11 gene was significantly associated with T*b. This gene is involved in the transport of various anions like sulphate and chloride (Vincourt *et al.* 2003), which might be involved in giving yellow colour possibly by reacting with other cations, as discussed under the B*b (section 5.5.2.3).

Similarly, an intronic variation rs13595564 in the CYP27A1 gene was found significantly associated with T*b. CYP27A1 gene is known for its role in the conversion of cholesterol in the bile (Suh *et al.* 2012). Several mutations in this gene have been reported that are known to be associated with lipid storage disease in human. However, it could be hypothesised that this SNP might be involved in affecting the cholesterol/fat level in broiler thigh muscles, and had subsequently resulted in lower T*b. This hypothesis is further supported by data from Zhang *et al.* (2010) who studied the effects of dietary Acetyl-L-Carnitine (ALC) on meat quality in broilers and observed that addition of ALC resulted in a significant decrease in cholesterol and fat percentage and this decrease in fat% subsequently lowered thigh muscles yellowness (T*b). Alternatively, low fat contents in broiler thigh muscle could be linked to a synonymous mutation, rs14305263 in the LMNB2 gene in the present study, as the mutations in this gene have been reported to reduce the fat content from extremities and subcutaneous tissues. However, mutations in LMNB2 have also been reported to negatively affect the muscle strength and ability to walk (Munoz-Alarcon *et al.* 2007). This is also consistent with the performance of modern broilers that are characterised by a higher incidence of leg problems and lameness.

5.5.2.7 Initial pH (pHi)

In the case of pHi 5 SNPs, rs15305228 in CALM, rs13803200 in MTFR1, rs15031083 in the promoter of ENDOG, rs15126679 in PDCD6, and rs13535812 in PRKAG2 were significant (Table 5.3) and jointly explained 41% of the residual variance for pHi.

As discussed above, the CALM and PDCD6 genes are involved in causing cell death mediated through excessive uptake of extracellular Ca^{2+} (Rho *et al.* 2012). However, mutations in ENDOG have been reported to be associated with mitochondrial depletion and abnormal functions that leads to oxidative stress (McDermott-Roe *et al.* 2011; Zhang *et al.* 2011). Furthermore, the expression of ENDOG was greater in broiler controls compared with layers and the expression was further increased by heat-stress in broilers (chapter 4), suggesting that broilers are under greater oxidative stress compared with layers. Similarly, MTFR1 is also involved in anti-oxidant functions and the intronic SNP rs13803200 in the MTFR1gene was present very close to the coding sequence.

Similarly, mutations in the PRKAG2 gene have been reported to affect the glycogen reserve of cells and are associated with the incidence of type 2 diabetes in humans (Jablonski *et al.* 2010; Nouira *et al.* 2010) which is characterised by higher blood glucose levels. Additionally the SNPs present in CALM, ENDOG, MTFR1 and PDCD6 genes might be involved in causing oxidative stress and tissue damage that could further lead to an increase in blood glucose level. Hence, it could be hypothesised that synergistic combination of stress and raised blood glucose levels result in lowering the initial pH in broilers breast meat.

5.5.2.8 Ultimate pH (pHu)

For pHu, 7 SNPs were strongly associated and explaining 55% of the residual variance for this trait (Table 5.3). Out of these 7 SNPs, one intronic mutation, rs14645300 in the BCL10 gene, was associated with pHu. As discussed under the B*a (section 5.5.2.2), the BCL10 gene is involved in triggering the rate of cell death mediated by immune cells infiltration. However, one novel intronic mutation rs14695299 in the MTFR1 gene was significantly associated with pHu, as discussed in section 5.5.2.1 this gene is involved in muscle strength and athletic performance (Gu *et al.* 2009). Hence, it could be hypothesised that this gene might be involved in the storage of glycogen in skeletal muscle and thus involved in affecting pHu.

Similarly, one mutation rs15031083 in the promoter region of the ENDOG gene was significantly associated with pHu, similar to B*a and pHi as discussed in sections 5.5.2.2 and 5.5.2.7, this mutation could be involved in enhancing the expression of the gene and augmenting the degree of oxidative stress. One intronic variation rs14615869 in the CYP27A1 gene was significantly associated with pHu. As discussed under B*b (section 5.5.2.6) the CYP27A1 gene is involved in lipid storage diseases and can affect the concentrations of circulating triglyceride in broilers. Rohrer *et al.* (2012) have reported a mutation in PRKAG3 for its association with pH, colour, and water holding capacity of pork meat. PRKAG3 is located next to the CYP27A1 gene down-stream on chromosome 7 but on the reverse strand. It is also likely that mutations in the ENDOG gene have synergistic effects with the CYP27A1 gene because proteins encoded by both genes localise in mitochondria. Hence, damaged mitochondria would also affect the functions of CYP27A1.

One novel synonymous SNP, rs14946390, in the coding sequence of the GNB5 gene was significantly associated with pHu. Gerard and Gerard (1994) have reported that the protein of this gene along with the other members of heterotrimeric G family protein is involved in increasing the production of diacylglycerol which leads to production and accumulation of lipids and fat in the body. Similarly, a synonymous SNP rs14305263 in the LMNB2 gene was also associated with pHu. Consistent with our result, Hegele *et al.* (2006) have also discovered causative mutations in the LMNB2 gene responsible for the acquired partial lipodystrophy (APD) in human, characterised by the loss of subcutaneous fat around the trunk and extremities but accumulation of fat in head and thorax. However, affected individuals are highly prone to diabetes and hypertriglyceridemia. Hence both of these SNPs were involved in increasing the lipid contents in the body. In agreement with this Sirri *et al.* (2011) have reported that fast growing chicken had higher level of lipids and mono-unsaturated fatty acids and lower level of poly unsaturated fatty acid (PUFA) compared with slow and medium growing birds that might be the factor, partly, for the low pH of muscles of fast growing birds. But the association of these SNPs with meat pHu needs further investigation to identify their actual biological relationship with pHu.

Similarly, one novel SNP (rs15177275) in the intron of BCL2L1 gene was significant. The BCL2L1 gene is involved in anti-apoptotic functions in the body. In our previous experiments (Chapter 3 and 4) this gene was down-regulated in heat-stressed broiler (BH) compared with control broiler (BC), indicating greater rate of cell death in BH than BC. However, the biological association of this intronic SNP in the BCL2L1 gene with the ultimate pH merits further investigation.

Hence, out of these 7 SNPs, 3 of them were directly associated with cell death and tissue damage related effects. However, the other 4 SNPs were involved in raising the blood cholesterol level. Both of these effects, oxidative stress and hypercholesterolemia could lead to insulin resistance (type 2 diabetes) and ultimately higher blood glucose level that might be involved in decreasing the pHu in broilers.

5.6 Conclusion

1. A set of novel SNPs associated with effects on muscle and meat quality traits have been identified in chicken.
2. These SNPs are responsible for explaining a substantially large proportion of the phenotypic variation for these traits in different breeds of chicken.

Chapter 6 The role of low ambient temperature on productivity, muscle and meat quality in broiler chicken

6.1 Introduction

Genetic selection for weight-related traits in chicken has resulted in tremendous improvement in these traits, as a result of which modern broilers can achieve more than 2.5 kg body weight in 6 weeks or about 5-times more than the broilers available 50 years ago (Havenstein *et al.* 2003). These improvements in broiler traits are also considered to be associated with some detrimental effects on bird health, skeletal muscle integrity and metabolism, meat quality and welfare-related traits. It is increasingly recognised that genetic selection has resulted in a decrease in the ability of meat-type chicken to cope with different stressors (Mitchell & Sandercock 1995b; Sandercock & Mitchell 1999; Sandercock *et al.* 2009a).

In the results of our gene expression and bioinformatics studies described in chapters 3 and 4 it was observed that genes involved in inflammation, cell death, and other stress-related functions were up-regulated in broiler breast muscles compared with layer muscles from both treatments. These gene expression and bioinformatics results indicate that skeletal muscles in modern broilers are under stress-related damage and they also showed a substantial overlap of differentially expressed genes between control broilers and heat-stressed layers, and also in the significant canonical pathways and biological networks for these birds. This stress could be physiological or genetic in origin or a combination of both metabolic stress and genetic susceptibility to cope with these stressors.

The increase in the carcass weight of modern broilers is mainly due to the increase in relative size of pectoral muscles which in turn is due to the greater diameter of their myofibres. However, it is also recognised that due to the hypertrophy of myofibres

the muscle area to capillary ratio has been increased. Consequently, the birds are unable to properly get rid of their metabolic wastes and to take up sufficient oxygen, due to which meat-type chickens are under metabolic stress (MacRae *et al.* 2006; MacRae *et al.* 2007). This stress is further exacerbated during the challenge by ante-mortem stressors like catching, crating, shackling and transport-related heat stress. But heat-stress has more profound effects because it further reduces the ability of the bird to get rid of excessive metabolic heat, leads to the production of more metabolic waste by accelerating the metabolic rate, and leads to skeletal muscle damage, reduced meat quality and ultimately PSE-like meat (Debut *et al.* 2003; Owens *et al.* 2009; Ziober *et al.* 2010).

Furthermore, a large number of up- and down-regulated genes were found to be overlapping between control broiler and heat-stressed layer (Chapter 3). Hence, our gene expression and bioinformatics results led us to develop a hypothesis that breast muscles in broilers are in stress-related damage at conventional rearing temperatures. Therefore, it was decided to test this hypothesis by rearing the broilers at low ambient temperature and monitor its effect on meat quality. An outline of the experimental procedures described in this chapter is presented in Figure 6-1.

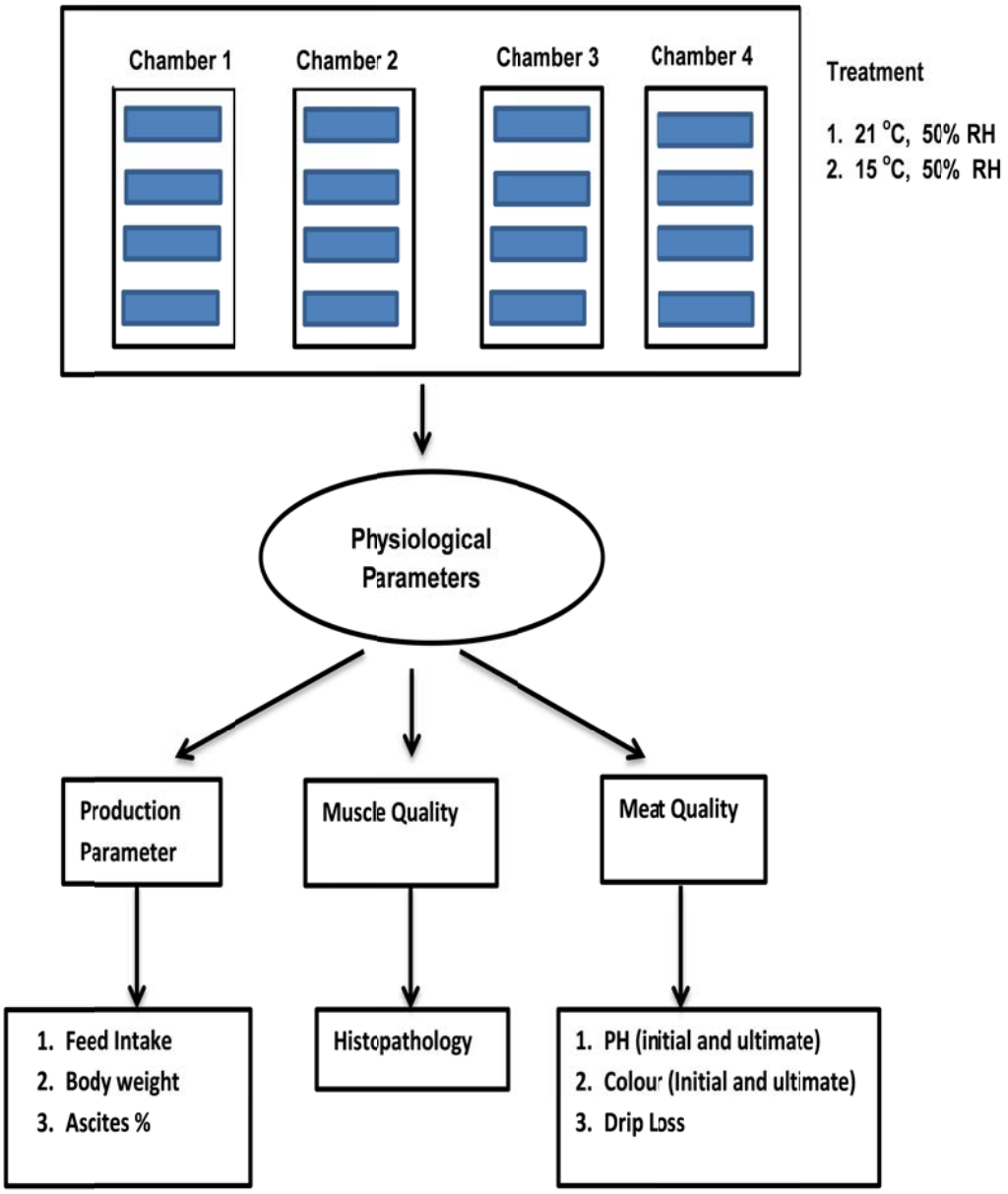
6.2 Objectives

This experiment was designed to evaluate the hypothesis that the muscle damage in broiler at conventional temperatures can be reduced by rearing them at a lower ambient temperature. So, the major objectives of the following experiment were

1) To confirm whether broilers are under metabolic stress that is physiological or genetic in origin or a combination of both.

2) To improve muscle and meat quality, and the welfare of meat-type chickens by developing management options to minimise metabolic stress.

Figure 6-1 Work flow diagram of Chapter 6 showing the experimental details and the study of physiological parameters including production parameters, muscle and meat quality in broiler chicken reared to conventional and low ambient temperature.



6.3 Materials and Methods

6.3.1 Animal Husbandry and Sampling

In total 100 day old broiler males chicks (Ross-308) were obtained from a commercial hatchery. The chicks were hatched from the eggs laid by hens that were 29 weeks old and were feather-sexed at hatch. The chicks were randomised to 4 climate chambers that were further subdivided into 4 pens. Each pen was comprised of approximately 2 m^2 area (1 x 2 m) and holding 6-7 birds. Wood shavings were used as litter and a 3-inch thick layer of litter was spread in each pen. During the first 2 days, the light to dark hour ratio was 22:2 and then it was reduced to 20:4 for all of the remaining period, until the end of the 6th week. During this period birds were given *ad-libitum* access to feed and water. During the first 3 weeks the birds were given broiler starter crumb and during the last 3 week they were offered broiler finisher pellets. Birds were reared under 2 climatic conditions, hot (conventionally normal) and cool, as outlined in the Table 6-1. The low temperature was based on criteria determined by Prof. R. Gous by simulation of a broiler growth model (personal communication). Treatments were randomly allocated to the 4 climate chambers. Data were collected for production parameters (body weight, feed intake, ascitic and mortality rate), muscle quality (histopathology) and meat quality (pH, colour and drip loss).

Table 6-1 Temperature and relative humidity (RH) requirements for conventional and low temperature treatments at different ages

Age	Conventional temperature		Low temperature	
Days	Temp (^{o}C)	RH (%)	Temp(^{o}C)	RH (%)
0	30	70	30	70
3	28	70	28	70
6	27	60	27	60
9	26	60	26	60
12	25	60	25	60
15	24	60	24	60
18	23	60	22	60
21	22	60	20.5	60
24	21	60	18.5	60
27	21	60	17	60
30	21	60	15	60
33	21	60	15	60
36	21	60	15	60
39	21	60	15	60
42	21	60	15	60

Temp = Temperature

RH = Relative humidity

On day 41, the rectal temperature was measured and a 2 ml blood sample was taken from every bird. For measuring the rectal temperature a thermistor probe (Model 612-849; RS Components Limited., Corby, Northants, UK) was inserted 5 cm into the rectum and maintained in position until the digital readout displayed a constant value. Blood samples (2 ml) were obtained by venepuncture of the brachial vein using a 5.0 ml syringe, fitted with a 25 guage, 5/8 inch needle. Each blood sample was transferred to a 2 ml blood collection tube containing 50 units Li-heparin anticoagulant and placed on ice. The samples were centrifuged at 1500g for 5 minutes. The plasma supernatant was pipette into duplicate plasma tubes and immediately frozen at -20^{o}C, pending analysis.

On day 42 the birds were euthanized using an intravenous injection of sodium pentabarbitone and weighed. The left breast fillet was used for measuring meat

quality parameters at 15 min and 24 hours after death. The right breast fillet was used to provide a tissue sample for histopathology.

6.3.2 Muscle quality

Muscle quality was monitored by studying the histopathology of breast muscles.

6.3.2.1 Histopathology

A slice of *Pectoralis major* muscle was cut and immediately fixed in 10% buffered neutral formalin (BNF). Approximately 2 cm muscle samples were taken from standardised regions of left *Pectoralis major* muscle. The muscle sections were stained with Haematoxylin and Eosin (H&E) by using the standard procedure for H & E staining. For histopathological examination, slides of both treatments were grouped into 4 categories; mild, moderate, high and severe on the basis of number of damaged and necrotic myofibres and the presence of inflammatory cells (mainly macrophages and heterophills).

6.3.3 Meat Quality

The following meat quality parameters were measured: pH, colour, and extruded water loss.

6.3.3.1 pH

Muscle samples (10 g) were collected form left breast muscle to determine pHi and pHu. Samples were stored in plastic bags at -80^0C until ready for analysis to prevent glycolysis. Semi-frozen breast muscle samples were homogenised (1:10 wt/vol) in

ice-chilled buffer (4°C) containing 5 mM sodium iodoacetate and 150 mMpotassium chloride (KCl) adjusted to pH 7.0 (Sandercock *et al.* 2009a). The pH was measured 15 min (pHi) and 24 hr (pHu) post-mortem from mixture of muscle homogenates by employing a combination pH electrodes (Model FC200 Hanna Instruments, Leighton Buzzard, UK) (Sandercock *et al.* 2009a).

6.3.3.2 Colour

The lightness (L*), redness (a*), and yellowness (b*) of the Pectoralis major muscle were evaluated using reflectance colorimetery (Minolta CR-300, CIELab, Minolta (UK) Ltd., Milton Keynes, UK). The colorimeter was calibrated using the standard white board supplied with the instrument (Minolta CR 400, Minolta GmbH) before measurements began. For each carcass, colour values were made at 15 min and 24 h postmortem (PM) respectively on the left breast muscle. Each of these values was an average of 2 measurements, one at the proximal/cranial side of the fillet and the other at the caudal/distal side (Berri *et al.* 2007). After killing the bird and removal of skin the carcass was allowed to stay at room temperature for the initial readings (L^*_i, a^*_i, and b^*_i) and the carcasses were stored overnight at 4^oC. After 24hr the ultimate colour values (L^*_u, a^*_u, and b^*_u) were recorded by the same procedure.

6.3.3.3 Extruded water loss

After 24 hours storage at 4°C a 1x1x1 cm cube of meat was cut from the breast fillet and was weighed before compressing. This cube was placed in between two Whatmann filter papers # 1 (with a diameter of 12.5cm) with two wooden discs each with a diameter of about 15 inch positioned below and above the cube and a piston was lowered onto the sample/wooden disc at a rate of 100 mm/min. A

maximum load of 400N was applied to the sample for 15 seconds. The cube was again weighed and the extruded water loss was calculated as

= [(Pre-weight – Post-weight)/Pre-weight)] x 100 (Petracci *et al.* 2012).

6.3.4 Experimental design

The experiment was 2 x 2 x 4 design, with 2 treatments and 2 chambers per treatment and 4 pens in each chamber. Treatment was used as fixed model and chamber/pen was used as blocking structure for all the variates (traits). Data were analysed by using general analysis of variance (ANOVA) in GenStat (www.vsni.co.uk/software/genstat). Data for ascites were analysed by using Chi-square test.

6.4 Results

Body weight and feed intake for both treatments were similar at the end of 6 weeks (Table 6-2). However, the number of ascitic birds from the conventional treatment was double that of the low temperature treatment.

Table 6-2 Body weight, Feed intake, Mortality (%) and Ascitic birds (%) in conventional and low temperature treatments

Trait	Conventional temperature	Low temperature	SED	Significance
Total no. of birds	50	50		
Body weight (g)	3559	3492	139	NS
Feed intake (g)	6050	6190	170	NS
Ascitic birds (%)	12	6	0.13	NS
Mortality (%)	6	6		NS

SED = SE of a difference between 2 category means.
N.S. = Non-significant

There were numerical improvements in most of meat quality parameters in the low temperature treatment compared with conventionally reared birds. However, a significant improvement in ultimate redness (a*u) was observed (Table 6-3).

Table 6-3 Meat quality parameters for conventional and low temperature treatments

Trait	Conventional temperature	Low temperature	SED	Significance
pHi	6.76	6.74	0.138	NS
L*i	58.33	56.99	0.791	NS
a*i	12.91	13.66	0.625	NS
b*i	8.59	7.56	0.501	NS
pHu	5.95	6.00	0.036	NS
L*u	54.20	52.35	0.703	NS
a*u	16.06	17.88	0.392	*
b*u	12.67	11.82	0.293	NS
Drip loss	24.68	24.19	0.210	NS

SED = SE of a difference between 2 category means.
*P< 0.05; N.S. = Non-significant
These data were collected from 40 birds of each treatment.

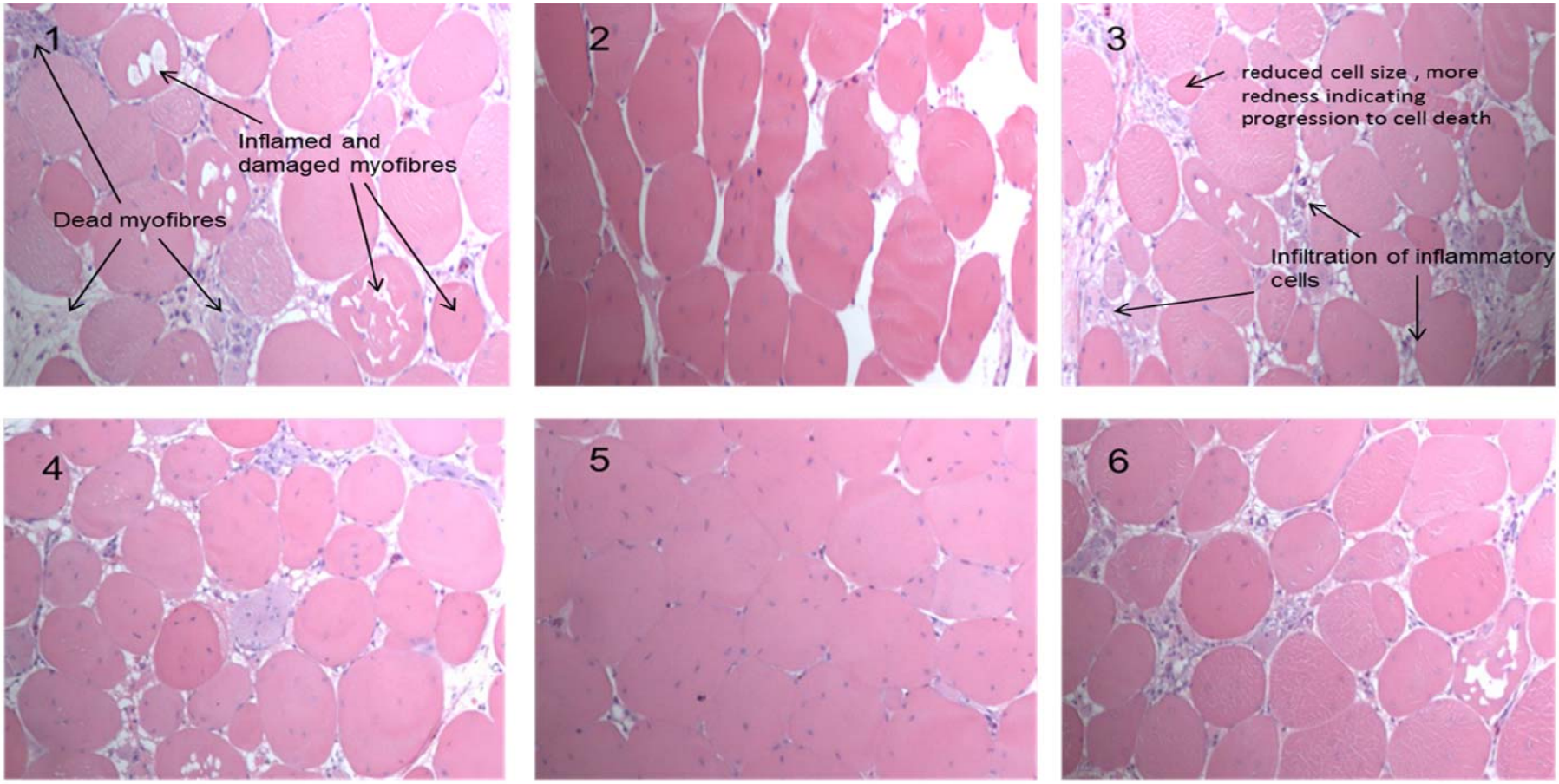
Histopathological examination revealed little difference in muscle quality of broilers reared at low compared with conventional temperatures (Table 6.4).

Table 6-4 Results of Histopathological examination of broiler breast muscles

Trait	Conventional temperature	Low temperature
Mild	2	4
Moderate	3	2
High	2	1
Severe	1	1

In total 16 birds (8/treatment) were used for histopathological examination of breast muscles.

Figure 6-2 Histopathological examination of pectoral muscles



Slide 1-3 showing histopathological results of pectoral muscle tissues in conventionally treated broiler and slide 4-6 showing results of pectoral muscles for low temperature broilers.

6.5 Discussion

The present study was conducted to evaluate the hypothesis that broiler breast muscles are under stress-related damage under conventional temperatures which underlies poor meat quality.

In the meat quality parameters, a significant (P<0.03) improvement in the ultimate redness (a^*_u) of breast muscles for the cool treatment was observed (Table 6-3). Similarly, though non-significant, a substantial decrease in the breast muscle lightness of low temperature birds was observed. Although this improvement in lightness was not statistically significant (P< 0.098), due to the limited number of chambers in this experiment, there was a reasonably big difference between the treatments means (Table 6-4). In the present study the mean lightness (L*) values for conventional and cool treatments were 54.20 and 52.35 respectively, showing that breast meat from cool treatment was comparatively darker. These results are also in agreement with the reports that lightness and drip loss are mainly controlled by the variations in pHu (Berri *et al.* 2001; Le Bihan-Duval *et al.* 2008b) because, although non-significant, some numerical (0.4) improvements in breast muscle pH of low temperature birds were observed.

The difference in histopathological examination in both treatments showed that there was some non-significant improvement that favoured the broilers reared at low temperature (Table 6-4) (Figure 6-2). Comparatively more damaged and necrotic myofibres were observed from the tissues of conventionally treated birds compared with low temperature (Figure 6-2). It was also observed that there was more infiltration of heterophils, macrophages, and monocytes in conventionally treated

birds (Figure 6-2). Consistent with our results, Aksit *et al.* (2006) reported that heat-stress increased the heterophil : lymphocyte ratio, an indicator of stress, in broilers. This higher activity of heterophils and macrophages in breast muscles of conventionally treated birds indicates that they were subject to stress-related damage and in turn associated with decreasing pH and increasing lightness (L*) of the breast muscles (Aksit *et al.* 2006).

The pathological changes observed in breast muscle of broilers were of poly-phasic nature, as it can be seen in the Figure 6-2 (3 slides in top row) the presence of some congested myofibres which start to decrease in width compared with other myofibres. Similarly the presence of greater numbers of inflammatory cells around these shrinking muscle fibres, and ultimate disappearance of these fibres from their place leaving a gap show that these changes did not occur suddenly but took a progressive course over a few days before cell death (Challa & Chan 2010; Geiger-Maor *et al.* 2012). The microscopic examination of muscle tissues revealed that affected myofibres were first changing into more red and then they were getting some irregular shapes with some angular edges and there was comparatively greater number of inflammatory cells around them. Finally, there was death of affected fibres and they were gradually engulfed by the macrophages. Some myofibres had nearly disappeared leaving a circular white area in their place with a large number of macrophages and heterophils.

Surprisingly, for the production traits, both groups had similar feed intakes and body weights. Because intensive genetic selection for growth-related traits has increased the appetite in broilers to its full extent (Richards 2003) it may not be affected by mild stressors (Mohammadrezaei & Toghyani 2011). When, in the 5th week, the

temperature was reduced to 15°C the birds started to huddle indicating that they were under some cold stress. Additionally, there was a strong draught in the chambers from the fans which may have resulted in decreasing the ambient temperature experienced by the birds as a "wind-chill" effect. This apparent cold stress in these birds may also explain the slightly lower weight gain and higher feed intake in these birds.

It was observed that birds in the conventional treatment were sitting and lying down on the floor mainly under the fans most of the time with their wings and legs outstretched, perhaps as an attempt to dissipate their body heat. Conversely the birds in the cool treatment chambers spent most of the time away from the fans. The difference in the body temperatures of both treatments was also non-significant but a numerical decrease (0.15°C) in the body temperature of low temperature birds was recorded. The reason for this difference in body temperature may be the presence of strong draught in the chambers which might have resulted in decreasing the temperature to some extent in conventionally treated chambers. Ultimately, this draught might have resulted in decreasing the difference in body temperature in both groups. In addition to the muscle damage in this study, we found that almost all the deaths during the 4-6 weeks of age were due to ascites and their number was the same in both treatments during the 6^{th} week, 3 birds in each treatment. During the measurement of meat quality traits 3 more birds in the conventional treatment were found ascitic (in total 6 ascitic birds were found in the conventional group and 3 in the low temperature group). The presence of ascitic birds in both groups might be due to insufficient supply of oxygen to skeletal muscles, most likely due to greater muscle size and ultimately increased muscle to capillary ratio (MacRae *et al.* 2006;

Berri *et al.* 2007). In agreement with this, some angiogenic pathways like VEGF signalling were also up-regulated, in our previous study (chapter 4) in control and heat-stressed broilers compared with layers. Consistent with this, Sandercock *et al.* (2006) have reported higher values of p_vCO_2 and lower blood pH_v compared with egg-type chickens at thermoneutral temperatures. In the present experiments about 10% of the birds were ascitic which is in agreement with the findings of De Smit et al. (2005) who reported that ascites could increase mortality rate up to 25% in commercial flocks that mainly occurred between 5 and 6 weeks of age.

6.6 Conclusion

Taken together, these results show that some improvements in muscle and meat quality could be made by simply rearing broilers at comparatively lower ambient temperatures. However, this may not be feasible or economical viable although it may result in reducing the production cost, by minimising the heating cost especially in temperate countries.

Although numerical improvements in most of the parameters were observed they were statistically non-significant showing that the experiment should be repeated. Nevertheless the relative lack of improvement confirms that muscle pathologies are not primarily because of the difficulty of dissipating heat but rather a consequence of metabolic changes leading to a hypoxia-like situation in broiler muscle in agreement with the bioinformatics results for chapters 2-5. Finally, on the basis of our results we conclude that meat quality traits are complex and it is very likely that our results (genetics and environment/management) will help to resolve the issue of poor

muscle and meat quality by eventually providing genetic markers for selection to improve muscle and meat quality.

Chapter 7 Summary and Conclusion

7.1 Introduction

The current project was conducted to identify the genes for muscle and meat quality traits in chicken. There is a growing incidence of muscle pathology and subsequently poor meat quality in broilers which can reach up to 40% within a flock depending on various factors like season, transportation, management and handling (Petracci *et al.* 2009). This poor quality meat, also known as PSE-type meat, is often characterised as meat having low pHu, higher lightness (L*), low redness and yellowness (a*, b*), poor water holding capacity, and higher cooking losses. Poor quality poultry meat and PSE-type meat is a cause of economic losses to processing plants and meat industry and the genetic basis of these issues are unknown.

7.2 Chapter 2

By exposing the broilers and layers to heat-stress, 32°C temperature and 75% RH, for 2 h a significant (P<0.001) rise in body temperature was observed, compared with control birds at 21°C and 50% RH. It was also found that the increase in the body temperature of broilers was more substantial (2.63°C) compared with layers (0.34°C), showing the greater susceptibility of broilers to heat-stress. The greater increase in body temperature in broilers may be due to their different anatomy, body size and mass, physiology and metabolism, compared with layers. Broilers and layers are types of chicken but they are the result of genetic selection for different quantitative traits (growth- and egg-related traits respectively) for more than half a century. It could be argued that the shape, size and anatomy of broilers bodies has made it difficult for them to dissipate heat. Alternatively, it is also possible that intensive genetic selection for different phenotypic traits has resulted in making

these two types of chicken quite different in terms of their response and susceptibility to ante-mortem stressors by the DNA sequence variations responsible for their respective production traits, although they both share the same genetic predecessor (traditional breeds and ultimately the red jungle fowl). In fact, the underlying cause for the different shape, size, physiology and metabolism of broiler is also their different genetics.

It was also discovered (for the first time) that broilers exhibited lower body temperature than layers in control conditions of this experiment, which has not been reported earlier, to our knowledge. It could be argued that layers were more active in their crates and that broiler could not move around due to their bigger size. However, the exact reason for this low temperature in not clear. Given that body temperature is related to the body metabolism of fast growing birds, broilers have greater feed intake and greater growth rate compared with layers and hence, their body temperature should not be lower than layers.

7.3 Chapter 3

Genome-wide expression studies were conducted by using the breast muscle RNA samples of broilers and layers, treated and sampled in the previous experiment (Chapter 2), discussed in section 7.2. A total of 2,213 differentially expressed genes were significant ($P<0.05$) for breed x treatment interaction. The gene ontology (GO) terms analysis for this gene set revealed that 567 (25.6%) transcripts had no Gene Symbol, and no GO terms against them for biological process/functions indicating that these genes are not yet characterised. However, from the remaining genes about 754 (34%) genes (having a Gene Symbol) were those for which there was no GO

term. It suggests that there are a large number of potential genes that are likely to be involved in the pathogenesis of heat-stress induced muscle damage in chicken that need further exploration. The remaining genes were apparently involved, directly or indirectly, in creating the pathology in breast muscles caused by heat-stress. However, it was also observed that a substantially large number of up and down-regulated genes in various comparisons were overlapping in BC and LH, suggesting that both of them were sharing the similar type of gene expression profile in their breast muscles. Alternatively, it could be argued that their breast muscles were suffering from, more or less, the same type of stress, on the basis of their expression pattern.

7.4 Chapter 4

The significant gene (2,213) set was analysed in BioLayout Express at 0.80 Pearson thresholds which resulted in the filtering out of 1,066 genes. Given that BioLayout Express only take into account the positive correlations; it could be asserted that these 1,066 genes were those that were not having a strong positive correlation with any of the other genes. The interesting clusters for the analysed gene set were selected for further subsequent investigation. It was observed that these clusters could be grouped into 6 different categories, on the basis of their clear expression pattern. The genes in the selected clusters were analysed in IPA, for each category separately, and interesting biological pathways and networks were selected. Similarly, the 1,066 genes filtered out by BioLayout Express were also analysed in IPA separately, and interesting pathways and networks were selected for them, to

select the most appropriate gene candidates, because we were not interested only in the genes that were positively correlated.

From the pathways and networks analyses of BioLayout analysed genes, it was discovered that genes involved in inflammation, stress response, oxidative stress, cell death, and tissue damage related functions were present in the Category I, IV, V, VI, and partly in category II. Out of these categories, pathways and networks from the 3 categories (I, IV, and VI) were up-regulated in BH compared with LC and LH. However, the expression levels of the genes present in Category I were even higher in BC compared with layers and they were further up-regulated after heat-stress. These pathways and networks of this category were all related to stress response, hypoxia, and inflammation. Whereas, in the case of layers, categories II and V were up-regulated and category V (with only 5 pathways) was the only category that was solely related to inflammation, cell death and tissue damage functions. In Category II some pathways and networks were related to inflammation and oxidative stress but, at the same time, others were related in the anti-apoptotic, cellular growth and development, protein synthesis and tissue repair related functions that might be a protective measure of the body to minimise the deleterious effects of heat-stress.

Category III had only 2 significant pathways (oestrogen receptor signalling and oxidative phosphorylation) that were up-regulated in LC but on response to heat-stress they were further up-regulated compared with broilers. The reason for the up-regulation of oestrogen receptor signalling pathway in layers might be that they are selected for egg and reproductive traits and have greater improvements in these traits compared with broilers. However, the up-regulation of this pathway in LH might be

a protective attempt of the body due to an anti-stress and anti-oxidant role of oestrogen. Hence, the further up-regulation of oestrogen-receptor signalling pathway in LH also explains the down-regulation of some stress, inflammation, and apoptotic pathways in LH compared with LC. This increased role of oestrogen to combat stress and its subsequent damaging effects of stress is also in agreement to the published literature reporting a decrease in egg production in layers on exposure to heat-stress. However, though the expression of oestrogen-related genes were up-regulated in layers on exposure to HS but a fall in the rate of egg production might be due to the greater role or activity of oestrogen in its anti-stress and anti-oxidant activity rather than its role in egg production. However, the further down-regulation of this pathway in BH suggests an involvement in the susceptibility of broilers to heat-stress, and this has not been reported earlier, to our knowledge.

The down-regulation of the oxidative phosphorylation pathway in BC compared with layers might be due to a hypoxia-like situation in broiler muscles. The further down-regulation of this pathway in BH might be due to the inability of the body to meet the increasing oxygen demand of the body to obtain nutrients and to eliminate metabolic waste to protect the cells. Alternatively, the down-regulation of this pathway in BC might be a protective measure of the body to produce less metabolic heat and was further down-regulated in broilers on exposure to thermal load, as a measure to minimise the effect of heat-stress.

The IPA analysis of BioLayout filtered genes revealed several pathways and networks that were related to oxidative stress, cell death, metabolism/diabetes, inflammatory and anti-angiogenic functions. They were up-regulated in broilers

compared with layers, and also in BH compared with BC. Interestingly, it was observed that though these genes were filtered out by BioLayout they were involved in oxidative stress and cell death related functions. The reason for which might be that all of these genes, filtered and un-filtered (analysed) by BioLayout were differentially expressed genes in response to heat-stress. Finally from the key positions of selected significant pathways and networks, genes were selected for SNP genotyping. In total, 24 candidate genes were selected and out of them 18 genes were from pathways and networks and the remaining 6 were selected on the basis of their GO terms.

These results show that broilers are not only under stress in control conditions compared with layers but are also more susceptible to heat-stress, that is further augmented to a greater extent, than in layers. These gene expression and bioinformatics results are also in agreement with the phenotypic data in which heat-stress resulted in a substantial increase (2.63°C) in the body temperature in broilers but only moderate (0.34°C) in layers compared with their respective controls.

On the basis of gene expression response from breast muscles, it was also concluded that broiler and layers were not only quiet different, in terms of their response to heat-stress, but they are nearly opposite to each other.

7.5 Chapter 5

All the SNPs reported in the current study are novel and none of them have been reported earlier for having association with any trait in chicken. For the muscle quality trait (CK), a synonymous mutation rs14604079 in the HDAC4 gene and

rs14216459 down-stream of the PDCD6 gene were strongly associated with CK and they jointly explained 15% of residual variance for the trait. Both of these genes are known to have well established roles in cell death and tissue damage. However, PDCD6 is also involved in inhibiting angiogenesis and in augmenting the cell death caused by other genes.

For breast muscle lightness (B*L) 7 SNPs were strongly associated and jointly explained 48% of residual variance for the trait. CALM and PDCD6IP are involved in causing cell death mediated by excessive uptake of extra-cellular Ca^{2+} and PLCG2 is involved in various inflammatory and immune disorders mediated by the excessive intake of Ca^{2+}. However, it could be argued that SNPs in the PDCD6IP and PLCG2 genes might have been involved in enhancing the function of these genes and causing cell death possibly through their associations with some other genes or SNPs not tested in the present study. The MTFR1 gene is involved in anti-oxidant functions and rs13803200 SNP was present at the junction of an exon and intron. Hence, it could be hypothesised that this mutation might have resulted in loss of the function in this gene, possibly by affecting gene splicing, and resulting in oxidative stress, muscle weakness and increased lightness.

In the case of breast muscle redness (B*a), 4 SNPs were significant and responsible for jointly explaining 28% of residual variance for this trait. The BCL10 gene is involved in angiogenesis-related functions and in enhancing the production of red blood cells and haemoglobin. It is speculated that the intronic mutation might have resulted in the decreased role for this gene possibly by interacting through some other gene. The exact mechanism by which this mutation affects B*a is not known.

The rs15126679 mutation in PDCD6 gene was very close to the exon and might be involved in splicing and an enhanced expression of this gene in broilers, and be involved in cell death and anti-angiogenic activities. CALM is involved in causing cell damage mediated by excessive uptake of extracellular Ca^{2+} and presence of a synonymous mutation rs10727941 might have resulted in greater tissue damage and increasing lightness. SNP in the promoter region of ENDOG might have resulted in reduction of its expression and, ultimately, a decrease in energy production and an increase in the degree of oxidative stress. With the exception of BCL10 all of these genes were up-regulated in broilers and may underlie broiler muscle damage whereas the down-regulation of BCL10 might have resulted in a decrease in B*a in broilers.

In the case of breast muscle yellowness (B*b) only 3 SNPs rs13535756, rs10726982 and rs14416824 were identified and they jointly explained 20% of residual variance for this trait. PRKAG2 gene had been reported to be involved in diabetes and muscle weakness caused by oxidative stress. Similarly, the ENDOG gene is also involved in mitochondrial depletion and enhancing oxidative stress, whereas SLC26A11 is involve in the transportation of anions like chloride and sulphate. It is speculated that these SNPs might have resulted in greater muscle damage by enhancing oxidative stress. But the role of anions and exact mechanism responsible for decreasing breast muscle yellowness is not known.

For the thigh muscle lightness (T*L), 8 SNPs jointly explained the 45% of residual variance. The SNPs found on CALM, MTFR1, MAP2K1, and PLCG2 and HDAC4 gene might be involved in affecting the thigh muscle lightness in a similar manner

(by excessive uptake of extracellular Ca2+, oxidative stress, and cell death) as they were for B*L. Mutations in LMNB2 have been reported to cause weakness of leg muscle, therefore, it is speculated that the synonymous mutation observed in the present study might also be associated with leg muscle weakness and subsequently increased muscle lightness. In agreement with this, modern broilers are unable to walk properly, and additionally have a higher incidence of leg problems.

In thigh muscle redness, 3 SNP explained 19% of residual variance for this trait. HRAS is involved in growth and proliferation of endothelial and haematopoietic cells (Meadows *et al.* 2001; Sykes & Kamps 2001) whereas AKT1 (up-regulated in layers compared with broilers) is also involve in increasing the blood supply to muscles (Takahashi *et al.* 2002). MTFR1 gene is known for its role in muscle strength and anti-oxidant activity and the mutation in this gene might have resulted in the impaired function of this gene in broilers that leads to lower redness of thigh muscles.

For the thigh muscle yellowness, in total 6 SNPs were jointly responsible for explaining the 46% of residual variance for the trait. Broilers exhibit significantly lower T*b compared with layers and traditional breeds (Sandercock *et al.* 2009a). SNPs in CALM, HDAC4, and PLCG2 might be involved in excessive Ca^{2+} uptake and cell damage whereas mutations in LMNB2 have also been reported to affect fat contents and muscle weakness. Similarly, the mutation in CYP27A1 is known to cause some lipid storage diseases. However, SLC26A11 is involved in transportation of anions but, to date, no mutation in this gene has been reported for its association with any trait. These results suggest that these mutations are involved in decreasing

yellowness by causing tissue damage and also by increasing the lipid contents of thigh muscles in broilers.

In the case of pHi 5 SNPs explained 41% of the residual variance for the trait. Mutations in PRKAG2 gene are involved in converting glycogen to glucose and hence raising the blood glucose level. Mutations in the promoter of ENDOG might have resulted in the impaired expression of this gene and hence leading to greater oxidative stress. Similarly the MTFR1 gene is involved in alleviation of oxidative stress and the SNP in this gene might have resulted in the decreased function of this gene. Similarly, SNPs in PDCD6 and CALM gene are involved in cell death and tissue damage by excessive uptake of Ca^{2+}. The data in the present study do not show whether any of these SNPs is causal in its effects or not and merit further investigation. But it is very likely these variations in DNA sequences are associated with stress like responses through their association with some other sequence variation and hence further promote the conversion of glycogen to glucose that may lead to the lower pHi in broilers.

For pHu 7 SNPs were found significantly jointly explained 55% of residual variance for this trait. MTFR1 and BCL2L1 genes are involved in imparting the functional strength to muscles by increasing the glycogen reserves of the body.

However, as discussed above, a mutation in the ENDOG had been reported to cause mitochondrial depletion, oxidative stress, lower oxidative phosphorylation, and hence greater blood glucose level. SNPs in BCL10 were associated with stress-related functions and tissue damage in the body. GNB5, LMNB2, and CYP27A1 were involved in augmenting the levels of saturated fatty acids and lipids. Finally,

higher levels of blood glucose due to stress (possibly mitochondrial depletion) and very likely insulin deficiency (section 5.6.2.8), along with the greater levels of lipids in broilers, might be involved in leading to lower ultimate pH that also increases the lightness of broiler meat and results in PSE-like meat.

7.6 Chapter 6

The hypothesis that broiler breast muscles are under stress-related damage at conventional rearing temperature was tested by rearing the broilers under lower ambient and at control temperatures. By rearing the broilers at lower temperature, a significant ($P<0.05$) improvement in breast muscle redness (a*) was observed. Statistically non-significant but substantial improvement in breast muscle lightness (L*) ($P<0.09$) and muscle quality from the histopathological examination were observed from low temperature reared birds. Similarly, non-significant numerical improvements in body temperature, pHi, pHu and drip loss were observed and this experiment should be repeated to confirm these observations.

Interestingly, it was also observed that feed intake and body weight for both treatments were similar. The reason for this might be that intensive genetic selection for growth-related traits has pushed the hunger up to such an extent that it is not affected by mild stressors. Additionally, the mortality % from the ascites in both treatments was also similar. However, the difference in the plasma level of acute phase protein (APP) in both treatments was non-significant. From the analysis of behavioural response, it was observed that the birds from the hot (conventional) treatment spent most of their time by sitting exactly under the fan that might reduce the difference in the observed parameters between the treatments. It was also

observed that there was strong draught within the chambers that might have resulted in some decrease in the internal temperature; which was beneficial for conventionally treated birds but harmful for those in the lower temperature, because, cold-stress is also known to cause muscle damage mediated by oxidative stress. It could be that the draught caused some stress on the low temperature treated birds and reduced the differences in the observed traits. However, it is suggested that perhaps further improvements in the observed traits could be achieved if it could be replicated by rearing the birds at 16-17°C and without and draught/wind chill effect.

These results shows that broiler breast muscles are under stress related damage at conventional rearing temperatures which was further increased under hot climatic conditions leading to poor quality meat (Bianchi *et al.* 2007). But, at the same time, the up-regulation of hypoxia related pathways in BC and BH compared with layers, and the presence of ascitic birds in the conventional treatment show that broilers also experience hypoxic-stress. This is likely to have a synergistic effect in combination with heat-stress and, therefore, further decrease broiler muscle and meat quality.

The method of rearing at the optimum (comparatively low) ambient temperatures may not be practical or economically feasible commercially but requires further investigation. It is unlikely that poor muscle and meat quality will be eliminated by this procedure and a primary genetic basis, as outlined in this thesis, is clearly important

7.7 Conclusion

Taken together, our results suggest that broilers are suffering from a metabolic syndrome characterised by higher blood glucose (due to insufficiency of insulin), hypoxic status of tissues, higher levels of cations (Ca^{2+}), and higher levels of lipids and cardiac myopathy (hypertrophy). Finally, this metabolic syndrome responsible underlies higher muscle pathology, poor muscle and meat quality and greater mortality due to higher incidence of ascites and stroke in modern broilers compared with layers and traditional breeds.

We have successfully identified a number of genes that underlie differences in muscle and meat quality that can now be taken forward for further proof and confirmation of an involvement in muscle function. Furthermore we have identified a number of SNP that may be associated or linked to genetic changes responsible for these relationships. Lastly, SNP may be examined for their role in selecting broilers for improved meat quality.

However, data in the present study do not provide enough evidence regarding the causal relationship between SNP and traits under study and, therefore, more investigation is recommended to confirm their association with muscle and meat quality traits.

7.8 Implications and Limitations

1. It is very likely that this SNP set would be incorporated into the available SNP chips for chicken.

2. It is hoped that this set of novel SNPs, explaining substantial phenotypic variance, would help poultry breeders to select the elite birds to produce the future generations of broilers with better muscle and meat quality attributes and help the poultry meat industry to reduce their losses due to poor muscle and meat quality.
3. Rearing broilers at comparatively low ambient temperature may provide some relief and improvement in meat quality, and help to reduce the heating cost in commercial broiler farming, especially in temperate countries. However, further research is needed to confirm this relationship.
4. These SNPs were validated previously and are currently available in the public domain and were genotyped in 34 breeds of chicken. However, it is recommended that they should be tested for association with muscle and meat quality traits in commercial pedigree selection populations.

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APPENDIXES

Appendix-1.1

Pilot Projects

Pilot projects were run with the following aims.

1. To check proper working (validation) of control environment chambers.
2. To develop experimental protocols and assays.
3. To satisfy the Home Office demands for the project licence and with the aim of getting a personal license.
4. To check the response of AIL birds (developed in Roslin Institute) to acute heat stress.

Pilot Experiment 1a

In this experiment, 24 six-week old AIL birds (developed and maintained at Roslin Institute), half male and half female, were taken. Birds were reared for 6 weeks and fed layer starter diet on ad-lib basis and given a photoperiod of 14 h light and 10 h darkness. These six week old birds were divided into two groups, each group contained 6 male and 6 female and one group was subjected to heat stress and other was treated as control. Two environment control chambers were used in this experiment, one for the heat stress (36^{o}C/75%RH) and the second for the control (21^{o}C/50%RH) treatments. There were 6 male and 6 female AIL birds in each chamber and they were placed on the floor littered wih wood shavings. Birds were placed in the chambers for 2 hours only. After completing the 2 hr treatment period the birds were taken out and their rectal temperatures were measured. For measuring the rectal temperature a thermistor probe was inserted 5 cm into the rectum and maintained in position until the digital readout displayed a constant value.

Blood samples (2 ml) were obtained by venepuncture of the brachial vein using a 2.0 ml syringe, fitted with a 25 gauge, 5/8 inch needle. Each blood sample was transferred to a 5 ml blood collection tube containing 50 units Li-heparin anti-

coagulant and placed on ice. The samples were centrifuged at 1500g for 5 minutes. The plasma supernatant was pipetted into plasma tubes and immediately frozen at -20°C, pending analysis. Birds were euthanized using an intravenous injection of sodium pentabarbitone. The birds were deemed dead when there was cessation of visible respiratory movements and then birds were weighed in the groups.

Pilot Experiment 1b

In this experiment, 24 nine-week old AIL birds (developed and maintained at Roslin Institute), half male and half female, were taken. Birds were from the same hatch of which 6 week old birds were used in the Pilot project A1. These nine week old birds were divided into two groups, each with 6 male and 6 female and one group was subjected to heat stress and other was treated as control. The rest of the procedures used in this experiment were the same as described above for Pilot 1a.

Pilot Experiment 2 (a,b)

In this experiment, 50 broiler chicks (Ross-308 broiler), half male and half female, were taken and reared in pens (started on 02-12-08 and ended on 16-01-2009). Birds were reared for 6 weeks and fed broiler starter and grower diet on ad-lib basis and with 16 hours light intervals and 8 hour darkness.

After six weeks, birds were divided into two groups each with 24 birds at 42 days of age. The first group was treated on day 1 and experiment was named as Pilot C1 and the second group on day 2 (Pilot C2). Sampling in each Pilot experiment was done by putting half of the birds in heat stress and the other half in cold/normal conditions. This procedure for the sampling was completed at 43 days of age.

Two chambers were used during each day, one for the heat stress (36°C/75%RH) and second for the control (21°C/50%RH) treatments. There were 6 male and 6 female broiler birds in each chamber and they were placed on the littered floor for 2 hours.

After completing the 2 hr treatment the birds were taken out and their rectal temperature was measured. For measuring the rectal temperature a thermistor probe

was inserted 5 cm into the rectum and maintained in position until the digital readout displayed a constant value.

The rest of the protocol used is the same as described above, in the case of Pilot 1a. The only difference in this trial is that after killing and weighing of birds, tissue samples (approximately 100 mg) for RNA extraction were taken from deep pectoral breast muscles of each bird.

STATISTICAL ANALYSIS

Pilot 1(a,b), was a 2 × 2 factorial design with 2 treatment (Control and Heat stress) and 2 sex (Female and Male). A similar design was used in Pilot 2 experiment.

Results and Discussion for the Pilot experiments

Pilot experiment 1a (birds at 6 weeks of age) and 1b (birds at 9 week of age)

Birds of AIL layers were subjected to Control and acute heat stress conditions at 6 weeks of age (pilot 1a) and at 9 weeks of age (pilot 1b). Mean environmental temperature and RH % for control were 21°C and 50% and for heat stressed were 32°C and 75% for. The data was analysed with in Genstat with ANOVA for testing sex and treatment as fixed effects as well as their interaction. The results for rectal temperature of pilot experiment 1a are presented in Table-1 and results body temperature and body weight of pilot experiment 1b are presented in Table-2 and Table-3.

It was observed that heat stressed condition (32°C and 75% RH) has significant (P<0.001) effect in increasing deep body temperature of AIL layers at 6 (Table 1) and 9 weeks of age (Table 2). At 6 weeks of age, an increase of 1.23°C was observed after heat exposure in AIL birds but at 9 week of age, a 0.7°C increase was recorded. A lower increase in the rectal body temperature of AIL birds at both ages suggests that these birds are more close to layer birds as layers are comparatively less sensitive to heat stress compared to broilers at the same age . This argument that AIL birds are more like layers is further confirmed by their low body weight at the same age as compared to broiler (Sandercock et al. 2009).

Treatments showed non-significant effects on body weight of birds at 9 weeks of age but there were significant associations / effects of sex (P<0.001) on body weight of birds at 9 weeks of age. Average body weight of male birds was 1541g that was significantly (P<0.001) higher than female body weight 1258 g.

Table 1: Mean body temperature of AIL layer at 6 week of age

Treatment	Body temp,°C	*P*-value
Control	41.708	<.001
Heat Stress	42.942	
S.E.D	0.18	

Table-2 Mean body Temperature of AIL layer at 9 week of age

Treatment	Body temp,°C	*P*-value
Control	41.92	<.001
Heat stress	42.62	
S.E.D	0.207	

Table-3 Mean Body weight of AIL layer at 9 weeks of age

Gender	Body weight, g	*P*-value
Female	1258	<.001
Male	1541	
S.E.D	79.8	

Pilot experiment 2(a & b) Ross-308 broiler

Birds of broiler (Ross-308) were subjected to Control and acute heat stress conditions at 6 weeks of age. Mean environmental temperature and RH % for control were 21°C and 50% and for heat stressed were 32°C and 75% RH. The data for body temperature and body weight were analysed within Genstat with ANOVA using a balanced design for testing sex and treatment as fixed effects as well as their interaction.

It was observed that there was a significant effect of Acute heat stress (32°C and 75% RH) on deep body temperature of birds (Table-4). Body temperature of broilers in the Control treatment was 41.33°C while in the High Temperature treatment it was 43.29°C. No effect of Sex and Sex X treatment interaction was observed in this case.

For body weight, male body weight (2319 g) was significantly (P<0.001) higher than female body weight (2084 g) (Table 5). Treatments and 'Treatment x Sex' interaction showed non-significant effects on body weight of broiler birds.

A greater increase in body temperature after exposure to acute thermal load indicates that broiler birds are more susceptible to heat stress as compared to AIL (Pilot 1 a, b) and layer birds (final experiment) at the same age. However, broiler birds are about three times heavier than layers and AIL birds at the same age which indicates that genetic selection for growth related traits in meat- type chicken have made the broiler birds more susceptible to heat stress, by limiting their thermo-regulatory responses on exposure to thermal challenge.

Table-4 Mean body temperature of broiler (Ross-308) at 6 weeks of age

Treatment	Body temp,°C	*P*-value
Control	41.44	<0.001
Heat Stress	43.27	
S.E.D	0.251	

Table 5 Mean Body weight of broiler (Ross-308) at 6 weeks of age

Gender	Body weight, g	*P*-value
Female	2084	<0.001
Male	2326	
S.E.D	66.0	

Table-1 Randomisation of treatment to chamber

Day	Chamber	Treatment	Order	Time on	Birds in	Birds out
1	1	C	3	0930	1015	1215
1	2	H	2	0845	0915	1115
1	3	H	1	0800	0830	1030
1	4	C	4	1015	1045	1245
2	1	C	2	0845	0915	1115
2	2	C	1	0800	0830	1030
2	3	H	3	0930	1015	1215
2	4	H	4	1015	1045	1245
3	1	H	3	0930	1015	1215
3	2	C	2	0845	0915	1115
3	3	H	1	0800	0830	1030
3	4	C	4	1015	1045	1245
4	1	H	1	0800	0830	1030
4	2	C	3	0930	1015	1215
4	3	C	2	1015	1045	1245
4	4	H	4	0845	0915	1115

Table-2 Randomisation of crates in chambers

Day	Chamber	Location (from door)				Pen
		1	2	3	4	
Monday	1	BM	BF	LF	LM	3
	2	LF	BF	LM	BM	7
	3	BM	LF	LM	BF	16
	4	LF	BF	BM	LM	17
Tuesday	1	LM	LF	BM	BF	2
	2	BM	BF	LM	LF	8
	3	BF	LM	BM	LF	11
	4	LF	LM	BM	BF	12
Wednesday	1	BF	LM	LF	BM	6
	2	LF	BM	LM	BF	9
	3	LM	BF	BM	LF	10
	4	LF	LM	BM	BF	15
Thursday	1	LM	BM	BF	LF	4
	2	BM	LM	LF	BF	5
	3	LM	BF	LF	BM	13
	4	LM	BM	BF	LF	14

Sexing layer chicks by PCR

Wing vein (brachial vein) of the birds was pierced by using 25 gauge needle and a drop of blood was collected by using the pasture pipette as a capillary. Blood was allowed to flow from pipette onto the CloneSaver Card in an even way, made sure by moving the pipette within the circle being filled. Bird No was recorded in the corresponding grid square and the card was air dried for few minutes.

Punch Preparation

From each circle, a piece of 1.2 mm was punched out using the Harris punch and put into the corresponding well of 96 well plate, used for the PCR. 150 µl of FTA Wash solution was added and allowed to incubate for 10 minutes at room temperature and then aspirated off solution to waste using a multi-channel pipette. This washing step was done twice (i.e. three times in total). Plate was allowed to incubate for 5 minutes at room temperature, with 150 µl of 1x TE solution in each well which was then aspirated off. This step was repeated, and finally all the liquid was aspirated off. Punches were allowed to air dry completely overnight, and then used in PCR, as DNA samples.

Table-3 PCR Solution Preparation for sexing chicks

Reagents	Per Sample	1 x 96 well plate
W3 primer (10pm/ µl)	0.4	40
W5 primer (10pm/ µl)	0.4 µl	40µl
R1 primer (10pm/ µl)	0.5 µl	50 µl
R2 primer (10pm/ µl)	0.5 µl	50 µl
10 x NTP	1.5 µl	150 µl
10 x Buffer (w/o Mg)	1.5 µl	150 µl
Mg2+ (2mM final)	1.2 µl	120 µl
5 x BB/Sucrose*	3.0 µl	300 µl

DMSO	0.75 µl	75 µl
Taq	0.075 µl	7.5 µl
H2O	5.175 µl	517.5 µl
Purified FTA Punch		

i.e. Total per well = 15 µl

*5 x BB/Sucrose = 16.5% (w/v) Sucrose + Bromophenol Blue.

Taq and corresponding buffer and Mg2+ from Abgene.

Primer Sequence

W3 5' GAA ATG AAT TAT TTT CTG GCG AC 3'

W5 5' CCC AAA TAT AAC ACG CTT CAC T 3'

R1 5' AGC TCT TTC TCG ATT CCG TG 3'

R2 5' GGG TAG ACA CAA GCT GAG CC 3'

PCR Conditions

(i) 94^{o}C 2 minutes

(ii) 94^{o}C 10 seconds

(iii)54^{o}C 15 seconds

(iv)72^{o}C 20 seconds

repeat steps (ii)– (iv) (x 30 in total)

Final extension at 72^{o}C for 5 minutes and then finish.

PCR products were loaded directly onto gel.

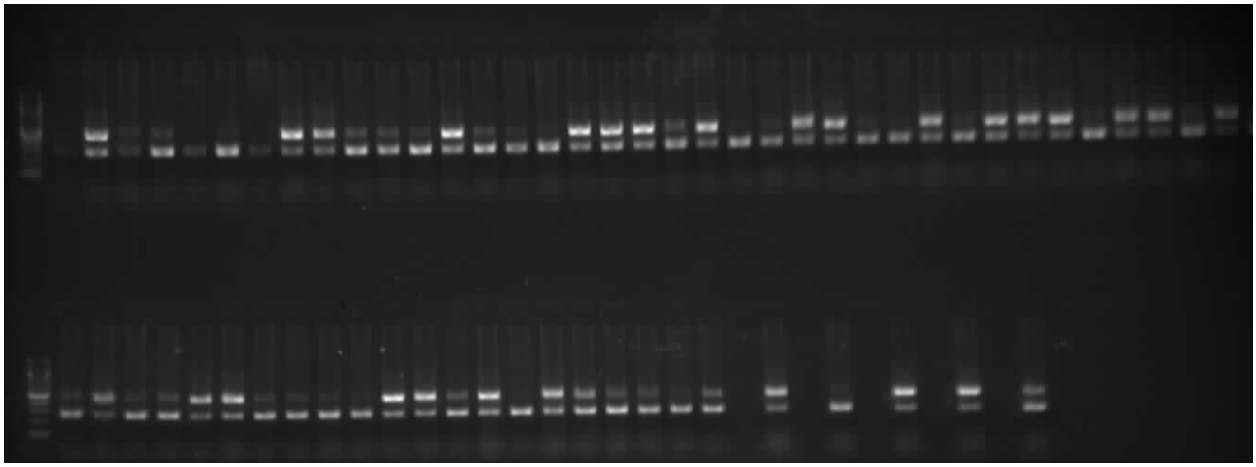
Gel preparation

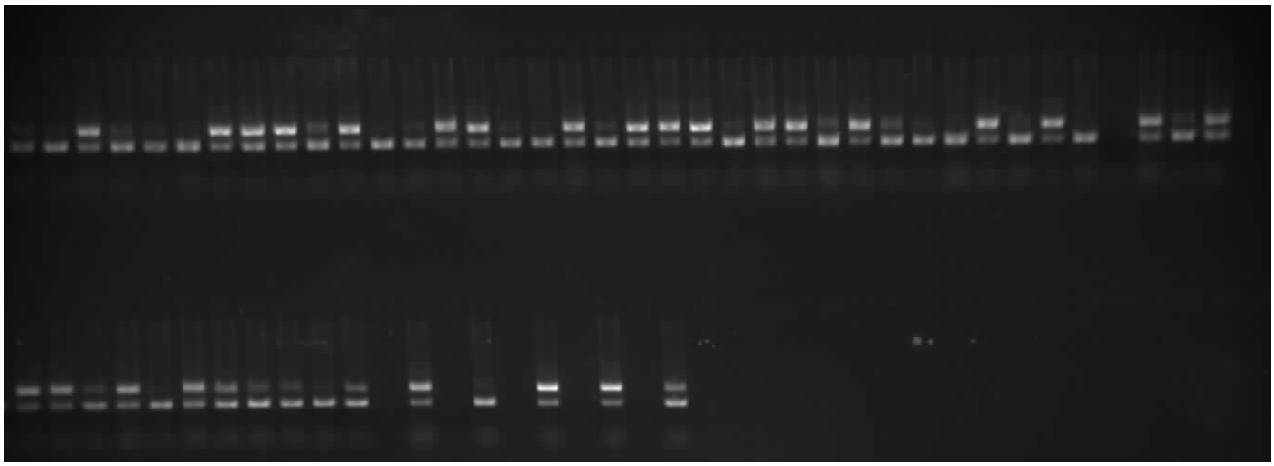
The gel was prepared by dissolving 2% Agarose in TAE buffer, solution was heated in microwave until it became transparent. After cooling the solution 2 µl of ethidium bromide per 100 ml of Agarose was added and mixed. Then gel was poured into a casting bench, with appropriate combs in it, and allowed to solidify. After that gel was placed into the tank containing TAE buffer and combs were removed. Finally, PCR products were loaded into the wells, in the gel, and gel was run under 100v for

20 minutes.

Images of the gels are presented in the following two figures.

Images of DNA after sexing PCR (Left to Right)





(Double band is showing male bird and single band is showing female bird)

RNA Extraction Protocol

For RNA extraction, samples were randomised prior to extraction and so 8 of randomised samples were used in one extraction. RNA was extracted by using the following protocol.

Homogenisation

100 mg of frozen (at -80°C) breast tissue samples were placed in the beaded tubes, on ice, and then 1ml of Tri-reagent was added in each tube and after ensuring the lids are on lightly samples were placed in the FastPrep FP 120 for agitation, at the speed of 6m/s for 40 seconds.

Phase Separation

After incubating, the tubes for 5 minutes at room temperature, 0.2ml BCP was added in each tube followed by vigorous manual shaking for 15 seconds. The tubes were then set in the centrifuge machine at 12000 x g for 15 minutes at room temperature, after incubation for 3 minutes at 15 - 30ºC. Then the upper aqueous phase was added into new RNase free tubes, by using P 200.

RNA precipitation

500 µl of isopropyl alcohol was added into each tube and then tubes were incubated for 10 minutes at 2-8°C (on ice). Tubes were centrifuged at 15000 x g for 30 minutes at room temperature at the end of centrifugation RNA precipitate to form a pellet on the bottom of the tube.

RNA Wash

Supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol. After vortexing the samples were again subjected to centrifugation at the speed of 11000 x g for 30 minutes at room temperature. After washing twice, the supernatant was discarded and RNA pellet was air dried for 10 minutes.

Redissolving the RNA

Finally the pellet was resuspended in 50 µl of RNase free water and tube was put at room temperature for 10 minutes and at the end after labelling with new labels, tubes were stored at -80°C.

ARK-Genomics Quality Control Protocol

TITLE: **Affymetrix Fragmentation, Hybridisation and Staining of 3' IVT Express Labelled arrays**

SCOPE: This SOP should be used when using the Affymetrix 3' IVT Express labelling kit which replaced the one and two cycle labelling kits in 2008. Any projects requiring data comparisons with samples labelled using the one or two cycle kits should NOT use this labelling method but stick to the older methods.

Purpose

This describes the assay procedures recommended for eukaryotic target labelling in expression analysis using Affymetrix GeneChip 3' brand probe arrays. Projects requiring comparison with samples labelled using the one or two cycle amplification methods should not use this method but remain with the older methods. All new projects should use this labelling protocol which requires significantly less starting material.

Equipment/Reagents

GeneChip 3' IVT Express Kit – 10 reactions (Affymetrix - P/N 901228), 30 reactions (Affymetrix – P/N 901229)

NB If planning on using the kit for Human, Mouse or Rat GeneChips there is an additional cost saving by buying a bundle of labelling kit and the appropriate number of arrays. Check the Affymetrix website for details

SOP IGF114.00 - Numbering System used for Affymetrix GeneChip arrays in the ARK Genomics laboratory.

SOP IGF152.00 – 3' IVT Express Labelling

SOP IGF141.00 – Sample Entry for Affymetrix Experiments using Command Console.

Principle

The 3' IVT Express kit improves on the Affymetrix One and Two Cycle amplification kits with lower input total RNA requirements, streamlined workflow using Mastermix and magnetic bead aRNA purification for high recovery and ease of use. The biotinylated aRNA targets produced are, fragmented prior to hybridisation to Affymetrix GeneChip 3' Expression arrays. After hybridisation the arrays are washed, stained and scanned to visualise the results.

Procedure

Fragmentation

Assemble the aRNA fragmentation mixture according to the table below. Refer to the specific probe array package insert for information on the array format.

Component	**49/64 Format**	**100 Format**	**169/400 Format**
aRNA	15ug (1 to 32ul)	12ug (1 to 23.6ul)	7.5ug (1 to 16ul)
5xFragmentation Buffer	8ul	6.4ul	4ul
Nuclease free water	Up to 40ul	Up to 30ul	Up to 20ul
Total	**40ul**	**30ul**	**20ul**

4.1.1 Run the XPRSF protocol on the Dyad PCR machine. This incubates the fragmentation reaction at 94C for 35 minutes before holding at 4C. Store at -80C or place on ice and continue with setting up the hybridisations.

Hybridisations of aRNA to Affymetrix 3' GeneChips

** It is imperative that frozen stocks of 20x GeneChip Eukaryotic Hybridisation Controls are heated to 65C for 5 minutes to completely resuspend the aRNA before aliquating **

4.2.1 Remove the arrays from the fridge and allow them to equilibrate to room temperature prior to use. If the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.

4.2.2 Remove the arrays from their packets and number them according to the numbering system described in SOP IGF 114.00. Enter the samples into Command Console as described in SOP IGF 141.00.

4.2.3 Please refer to the tables below for the necessary amount of aRNA required for the appropriate array format. If you are unsure of your array format consult the insert sheet which is included in the box with the arrays.

Array	Volume
49 Format (Standard)	200ul
64 Format	200ul
100 Format (Midi)	130ul
169 Format (Mini)	80ul
400 Format (Micro)	80ul

4.2.4 Prepare a mastermix for the hybridisation buffer (minus the fragmented aRNA) according to the table above. Allow extra volume for pipetting errors.

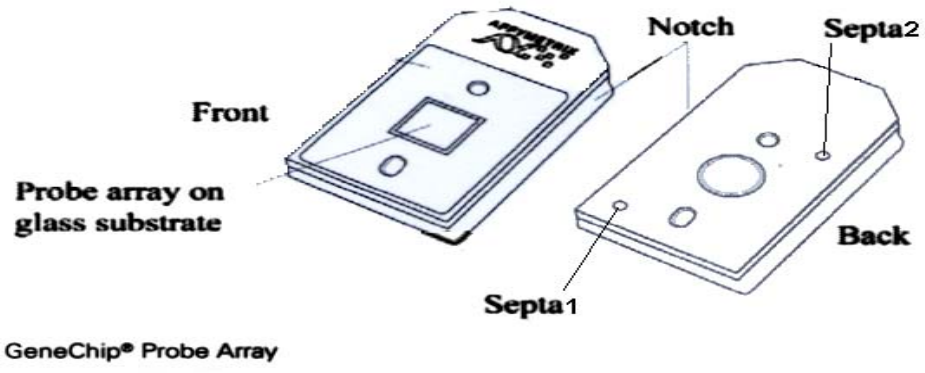
4.2.5 Pipette the appropriate volume of hybridisation buffer mastermix into individual screw capped microfuge tubes. To these tubes add the appropriate amount of fragmented aRNA. Mix by vortexing and centrifuge briefly to return the contents of the tube to the bottom.

4.2.6 Once equilibrated, wet the probe arrays by filling with 1x Hybridisation Buffer. The appropriate fill volume can be found in the first table above. Place a P10 tip in Septa 1 (see figure below) to provide venting of air from the hybridisation chamber. Then pipette the 1x hybridisation buffer in through Septa 2, tilt the chip at an angle so that the Septa 1 is highest and Septa 2 is directly beneath it. Be careful not to waggle the tip while inserted into the septa since this will increase the chances of leaks during the hybridisation. Once full, tap the chip on all sides to ensure small air bubbles do not prevent the array to be properly wetted. Place the arrays in racks in

the Hybridisation Oven at 45ºC for at least 10 minutes while the hybridisation cocktails are prepared.

Hybridisation Cocktail for Single Probe Array

Component	Array Format			Final Dilution
	49/64	**100**	**169/400**	
Fragmented aRNA	12.5ug (33.3ul)	10ug (26.7ul)	5ug (13.3ul)	0.05 ug/ul
Control Oligo B2 (3nM)	4.2ul	3.3ul	1.7ul	50pM
20x Hyb Controls (bioB, bioC, bioD, cre)	12.5ul	10ul	5ul	1.5, 5, 25 & 100pM respectively
2x Hyb Mix	125ul	100ul	50ul	1x
DMSO	25ul	20ul	10ul	10%
Herring Sperm DNA (10mg/ml)	2.5ul	2ul	1ul	0.1mg/ml
BSA (50mg/ml)	2.5ul	2ul	1ul	0.5mg/ml
Water	45ul	36ul	18ul	
Total	**250ul**	**200ul**	**100ul**	
Master mix Vol to add	**216.7ul**	**173.3ul**	**86.7ul**	



4.3.7 Heat the tubes containing the hybridisation cocktails at 99C for 5 minutes in a heated block.

4.2.8. Transfer the hybridisation cocktails to the 45C hybridisation oven for 5 minutes, placing the tubes on the bottom of the oven.

4.3.9. After incubation for 5 minutes, spin the hybridisation cocktails at maximum speed in a centrifuge for 5 minutes. This will remove any insoluble material from the hybridisation mixture.

4.3.10. The probe arrays will now have been incubated for over 10 minutes in the hybridisation oven. Remove the buffer from the probe array cartridge and fill the appropriate volume of hybridisation cocktail (see the table above). Avoid any insoluble material at the bottom of the tube.

4.3.11. Place the probe arrays in the hybridisation oven, set to 45C. Avoid stress to the motor by loading the arrays in a balanced configuration around the axis. Rotate at 60rpm and hybridise for 16 hours.

Washing and Staining the GeneChip Arrays

4.4.1 After 16 hours of hybridisation, remove the hybridisation cocktail to a labelled microfuge tube. Fill the probe array with the appropriate volume of Wash Buffer A, as detailed in the first table in 4.2.3. If necessary the probe array can be stored at 4°C until required, equilibrate the probe array to room temperature before washing and staining.

N.B. The hybridisation cocktails can be stored at -80C and can be reused if required.

4.4.2 Log in to the Affymetrix computer and start Affymetrix Launcher, this starts the Command Console software. Double click AGCC Fluidics Control.

4.4.3 In the Step 2 box, ensure the List All Protocols radio button is checked. Choose Prime_450 from the drop down list of protocols. If all modules are to be used then click the Check/Uncheck All Stations and Modules buttons at the top of the screen, else choose the individual modules which you wish to use. Check in IGF Visitors\AFFY\Affy Chip Useage to see which module was used last and start with the next one in sequence.

4.4.4 Ensure that bottles of Wash Buffer A, Wash Buffer B, water and waste are in position at the side of the Fluidics Stations and that the waste bottle is empty.

4.4.4 In Step 3 box in the AGCC Fluidics Control window, click the Copy to Selected Modules button. Click Run All at the top right of the window and follow the instructions on the fluidics stations themselves.

4.4.5 Prepare the staining reagents according to the tables below. NOTE: Streptavidin Phycoerythrin (SAPE) should be stored in the dark at 4°C, either foil wrapped or in an amber tube. Remove the SAPE from the fridge and tap the tube to mix well before preparing the stain solution. Make up a mastermix for all the chips to be stained on the day, allowing a little extra for pipetting errors. Only eight chips can be processed at one time on the Fluidics Stations, leave the remaining SAPE and Antibody mixes on ice in the dark until ready to use. Do not freeze the SAPE. Always prepare the SAPE stain solution fresh on the day of use.

SAPE Solution Mix – per chip.

Components	Volume	Final Concentration
2x Stain Buffer	600ul	1x
50mg/ml BSA	48ul	2mg/ml
1mg/ml SAPE	12ul	10ug/ml
Water	540ul	
Total Volume	**1200ul**	

Antibody Solution Mix – per Chip

Components	Volume	Final Concentration
2x Stain Buffer	300ul	1x
50mg/ml BSA	24ul	2mg/ml
10mg/ml Goat IgG Stock	6ul	0.1mg/ml
0.5mg/ml biotinylated antibody	3.6ul	3ug/ml
Water	266.4ul	
Total Volume	**600ul**	

4.4.6. Mix the reagents for the SAPE solution well and place into two 1.5ml microfuge tubes containing 600ul each. These are used for positions 1 and 3 on the fluidics stations.

4.4.7. Mix the reagents for the antibody solution well and place 600ul in a 1.5ml microfuge tube. This is used in position 2 of the fluidics stations.

4.4.8 Rack up the tubes containing SAPE and Antibody Solutions for one round of use on the Fluidics Station (maximum eight chips) and transfer, along with the chips themselves, to the Fluidics Station.

4.5.3 Transfer 30ul of IVT Master Mix to each (30ul) double-stranded cDNA sample. Mix thoroughly by gentle vortexing and centrifuge briefly to return the contents of the tube/plate to the bottom.

4.5.4 Once assembled place in the Dyad PCR block and run one of the two protocols listed in the table below, depending on the amount of input Total RNA in the reaction.

RNA Amount	IVT Incubation Time	PCR Program
50 – 250ng	16 hours	IVT16
500ng	4 hours	IVT4

****NOTE: Optimal RNA input amount and IVT incubation time are sample type dependant and should be determined empirically, It is recommended to keep input amount and IVT incubation time consistent within a given experiment****

4.5.5 Once the incubation is complete the PCR programs will hold at 4°C. Place the cRNA on ice briefly before proceeding with cRNA purification or freeze immediately at -20°C for overnight storage.

*****STOPPING POINT IF REQUIRED*****

cRNA Purification

Components of the GeneChip IVT Express Kit, box 1 are used for this step.

After synthesis, the cRNA is purified to remove enzymes, salts and unincorporated nucleotides. Photos of the cRNA purification process can be found at the end of this section.

4.6.1 Aliquot the appropriate amount of the aRNA elution solution (50ul per samples plus ~10% excess)

4.6.2 At room temperature, assemble the aRNA Binding Mix in a nuclease free tube for all the samples in the experiment, plus an extra half reaction to cover pipetting error, following the instructions in the table below.

Component	Amount
RNA Binding Beads*	10ul
aRNA Binding Buffer Concentrate	50ul

* Mix the RNA Binding Beads by vortexing before dispensing.

4.6.3 Add 60ul aRNA Binding Mix to each sample and transfer to a well of the U Bottom plate supplied with the cleanup kit, mix by pipetting up and down several times. *****Ensure you have a record of which sample went in which well of the plate.*****

4.6.4 Add 120ul of 100% ethanol to each sample, mix by pipetting up and down several times.

4.6.5 Transfer the plate to the Heidolph Titramax 1000 Shaker in ARK Genomics big lab. Set the shaker speed to 450rpm and shake for 2 minutes. The aRNA in the sample will bind to the RNA Binding Beads during this incubation.

4.6.5 Move the plate to the magnetic stand (QIAGEN Biorobot Magattract stand) and capture the magnetic beads for 7 minutes. When capture is complete, the mixture becomes transparent and the RNA Binding beads will form pellets against the magnets in the magnetic stand. See pictures at the end of this document.

4.6.6. Carefully aspirate and discard the supernatant without disturbing the magnetic beads; remove the plate from the magnetic stand.

****** ENSURE THE ETHANOL HAS BEEN ADDED TO THE BOTTLE OF aRNA WASH SOLUTION CONCENTRATE BEFORE USING IT******

4.6.7 Add 100ul aRNA Wash solution (check that ethanol added to bottle) to each sample and transfer to the Illumina shaker in Lab620. Shake at 800rpm for one minute.

****NOTE: the RNA Binding Beads may not fully disperse during this step; this is expected and will not affect RNA purity or yield. See pictures at the end of this document*****

4.6.8 Move plate to the magnetic stand and capture the magnetic beads for 7 minutes.

4.6.9 Carefully aspirate and discard the supernatant without disturbing the RNA Binding Beads and remove the plate from the magnetic stand.

4.6.10 Repeat steps 4.6.7 to 4.6.9 to wash a second time with 100ul of aRNA Wash Solution.

4.6.11 Move the plate to the Illumina shaker and shake the plate at 1100rpm for 1 minute to evaporate residual ethanol from the beads.

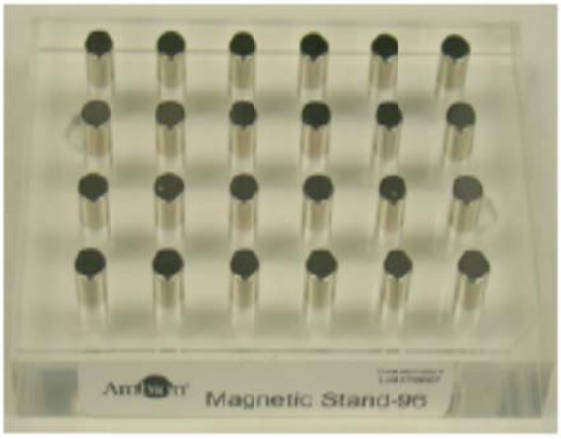
4.6.12 Elute the purified aRNA from the RNA Binding Beads by adding 50ul preheated (55°C) aRNA Elution Solution to each sample. Transfer the plate to the Illumina shaker.

4.6.13 Shake the plate for 3 minutes at 1100rpm. Then check to make sure the RNA Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed – it is recommended to switch the pulse option on during the second shake if there has been a problem with the bead dispersal.

4.6.14 Once the beads are fully dispersed transfer the plate to the magnetic stand and capture the RNA Binding Beads for 7 minutes.

4.6.15 Transfer the supernatant, which contains the eluted aRNA, to a nuclease-free microfuge tube. Store at -80°C or place on ice and proceed with quantitation, QC and fragmentation (SOP IGF153.00).

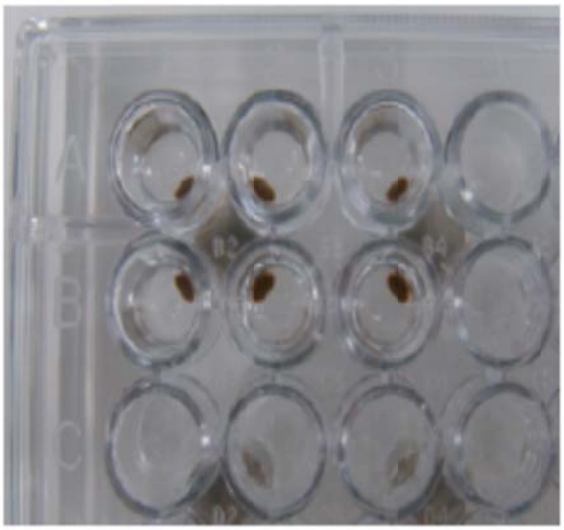
Pictures for the Magnetic Bead Clean up Stages



Magnetic Stand

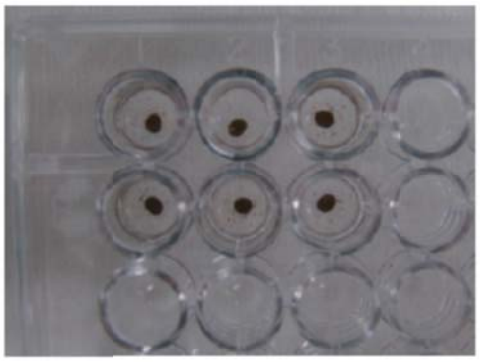
aRNA Binding Step

After addition of ethanol and mixing



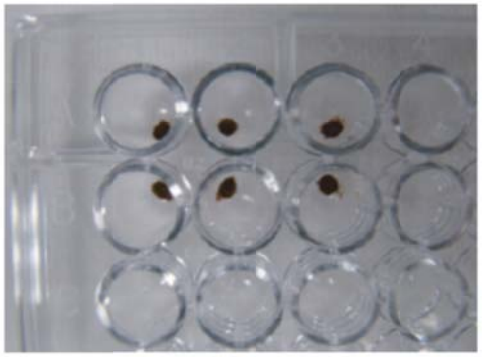
RNA Binding Beads Capture

After 5 minutes on magnetic stand



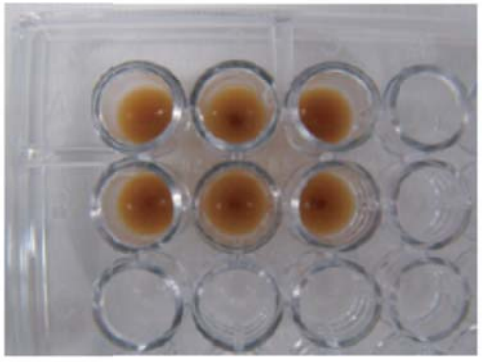
Bead Washing

After second wash and 1 minute shake



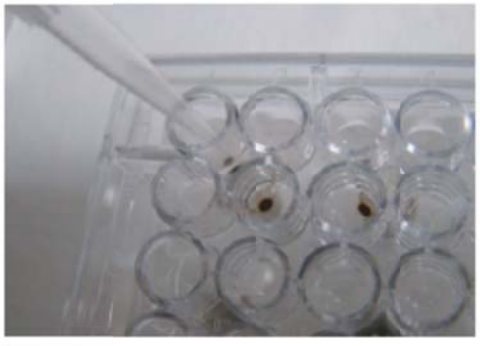
Removal of Ethanol

Dry beads following 1 minute shake



aRNA Elution

Dispersed beads following 3 minute shake



Elution Step

Recovery of purified aRNA following bead capture

Experimental design of Microarray

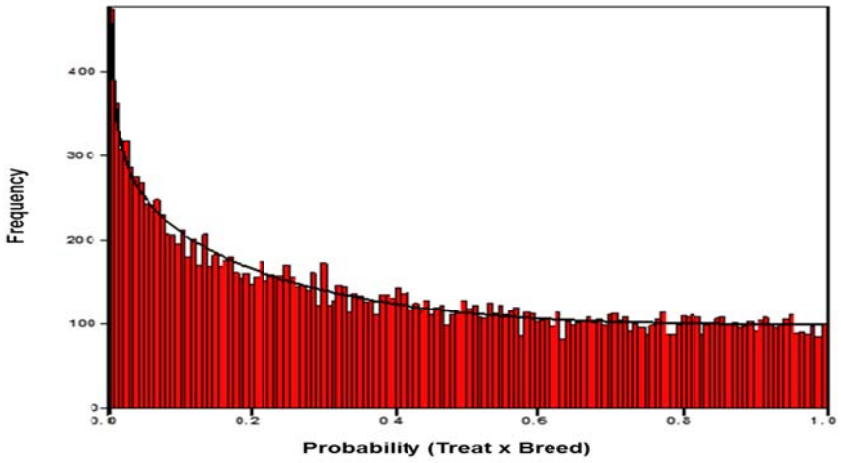
ST!	Filename	Treat!	Breed!	Day!	Chamber!
BH	129	Heat-stressed	B	1	3
BH	130	Heat-stressed	B	1	2
BH	131	Heat-stressed	B	2	3
BH	132	Heat-stressed	B	2	4
BH	133	Heat-stressed	B	3	3
BH	134	Heat-stressed	B	3	1
BH	135	Heat-stressed	B	4	1
BH	136	Heat-stressed	B	4	4
BC	137	Control	B	1	1
BC	138	Control	B	1	4
BC	139	Control	B	2	2
BC	140	Control	B	2	1
BC	141	Control	B	3	2
BC	142	Control	B	3	4
BC	143	Control	B	4	3
BC	144	Control	B	4	2
LH	145	Heat-stressed	L	1	3
LH	146	Heat-stressed	L	1	2
LC	147	Control	L	1	1
LC	148	Control	L	1	4
LC	149	Control	L	2	2
LC	150	Control	L	2	1
LH	151	Heat-stressed	L	2	3
LH	152	Heat-stressed	L	2	4
LH	153	Heat-stressed	L	3	3
LC	154	Control	L	3	2
LH	155	Heat-stressed	L	3	1
LC	156	Control	L	3	4
LH	157	Heat-stressed	L	4	1
LC	158	Control	L	4	3
LC	159	Control	L	4	2
LH	160	Heat-stressed	L	4	4

BH = Broiler Heat-stressed
BC = Broiler control
LH = Layer Heat-stressed
LC = Layer control

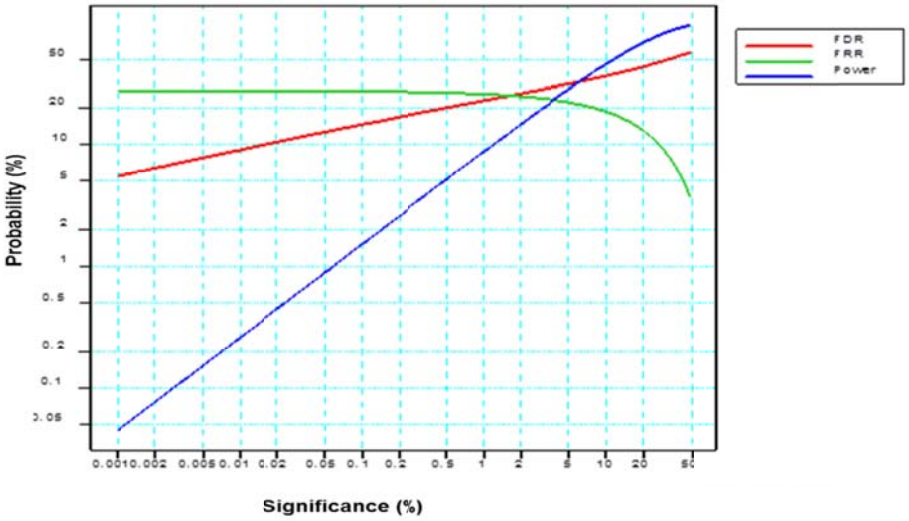
Microarray QC Results (Broiler slides)

A microarray is a sequence of dots of DNA, protein, or tissue arranged on an array for easy simultaneous analysis. The DNA microarray chip plays an integral role in gene expression profiling.

Graphs for the FDR for the analysis on the breed x treatment basis



Probability (Breed x Treat) Inference rates on log scale



Concentration of RNA samples (used in Microarray experiment)

Heat stressed male	RNA Conc.	Pools	Control male	RNA Conc.	Pools
344 121	2247	344_129	344 034	2288	344_137
344 105	1536		344 065	2534	
344 017	3168	344_130	344 074	63	344_138
344 009	2829		344 082	3395	
344 036	1367	344_131	344 043	1531	344_139
344 075	947		344 099	2425	
344 068	2396	344_132	344 051	2772	344_140
344 116	1755		344 003	2365	
344 093	2220	344_133	344 062	2559	344_141
344 085	2631		344 029	1516	
344 006	1443	344_134	344 126	3100	344_142
344 102	890		344 022	3146	
344 063	2309	344_135	344 016	2441	344_143
344 031	1695		344 112	2124	
344 055	2112	344_136	344 120	2273	344_144
344_048	2014		344_096	513	

Data Filtration

32 slides were converted to expressions in four different batches because GenStat was unable to convert the 32 Cel files (Affymetrix chicken array, 38.5K) into expression files in a single go, on a 32-bit computer. So 4 spreadsheets each having 8 slides were developed, 4 broiler slides (2 heat-stressed and 2 control) and four layer slides (2 heat-stressed and 2 control), on the basis of days i.e. birds treated on day 1 was included in spreadsheet one and birds treated on day 2 were included in second spreadsheet and so on for 3rd and 4th day treated birds. Each of the 4 spreadsheets was split into 8 sub-sheets on the basis of slides individually. Each subsequent sub-sheet had 4 (Slide number, Probe ID, Expression and SE) and 38,535 rows, one for each probe. Each of these sub-sheets (one for each slide) was saved in Excel format. Ultimately, data from all of these 32 files were copied, one by one, and pasted to develop a single file containing expressions and SE data for each slide.

In this Microsoft Excel file, the Probe IDs of all of the 38,535 probes were pasted in column 1, in the same format as returned by GenStat, and their respective expression on each slides were pasted in next columns in left to right direction, expression and SE of 129 slide, in extreme left, next to Probe Ids and then next two columns for the data of 130 slide and so on until the last slide, 160. So in this way, new excel file had 38,535 rows and 65 columns, one for Probe Ids and two columns for each slide, as mentioned above. In 66th column highest value 'Max value' for each row (R *et al.*) was calculated by using the formula for 'Max', provided in Microsoft excel. Hence, data were filtered on the basis of 'Max' values. The rows (Probes) having their Max values less than or equal to 1 were filtered which resulted in the reduction of probes (rows) from 38,535 to 19,038 for each slide. The filtered excel sheet was used to create a new filtered spreadsheet, having 4 columns, Slide number, Probe Ids, Expression and SE. Data for the each filtered slide were pasted in ascending order i.e. 129 at the top and 160 at the end. Finally this spreadsheet was saved in M. Excel and imported into GenStat, and columns Slide number and Probe Ids were converted to 'Factor'.

Appendix 4-1

Pathway Analysis of Category I

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	4.65E00	7.84E-02
fMLP Signaling in Neutrophils	4.24E00	6.25E-02
Clathrin-mediated Endocytosis Signaling	3.51E00	5.23E-02
Ephrin Receptor Signaling	3.28E00	4.52E-02
Regulation of Actin-based Motility by Rho	3.07E00	6.59E-02
Integrin Signaling	2.96E00	4.31E-02
Actin Cytoskeleton Signaling	2.87E00	3.78E-02
Cdc42 Signaling	2.58E00	3.45E-02
Rac Signaling	2.58E00	4.88E-02
RhoA Signaling	2.58E00	5.36E-02
Axonal Guidance Signaling	2.4E00	2.78E-02
Germ Cell-Sertoli Cell Junction Signaling	2.31E00	4.19E-02
CCR5 Signaling in Macrophages	2.17E00	4.26E-02
G Protein Signaling Mediated by Tubby	2.16E00	7.32E-02
FAK Signaling	2.05E00	4.9E-02
CD28 Signaling in T Helper Cells	1.97E00	3.79E-02
Agrin Interactions at Neuromuscular Junction	1.79E00	5.8E-02
CCR3 Signaling in Eosinophils	1.73E00	3.94E-02
Mechanisms of Viral Exit from Host Cells	1.71E00	6.67E-02
α-Adrenergic Signaling	1.64E00	3.81E-02
Crosstalk between Dendritic Cells and Natural Killer Cells	1.64E00	3.09E-02
NRF2-mediated Oxidative Stress Response	1.55E00	3.11E-02
Virus Entry via Endocytic Pathways	1.51E00	4E-02
Breast Cancer Regulation by Stathmin1	1.51E00	2.88E-02
Cholecystokinin/Gastrin-mediated Signaling	1.49E00	3.77E-02
VEGF Signaling	1.49E00	4.04E-02
Leukocyte Extravasation Signaling	1.48E00	3.02E-02
Role of MAPK Signaling in the Pathogenesis of Influenza	1.43E00	4.62E-02
Selenoamino Acid Metabolism	1.34E00	2.94E-02

Chemokine Signaling	1.33E00	4.05E-02
Cellular Effects of Sildenafil (Viagra)	1.32E00	2.65E-02
Caveolar-mediated Endocytosis Signaling	1.3E00	3.53E-02
Corticotropin Releasing Hormone Signaling	1.3E00	2.99E-02

Appendix 4-2

Pathway Analysis of Category II

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Ceramide Signaling	3.15E00	4.6E-02
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	2.89E00	4.05E-02
Regulation of eIF4 and p70S6K Signaling	2.61E00	3.03E-02
p70S6K Signaling	2.45E00	3.08E-02
Non-Small Cell Lung Cancer Signaling	2.34E00	3.8E-02
Renal Cell Carcinoma Signaling	2.13E00	4.05E-02
RANK Signaling in Osteoclasts	2.07E00	3.16E-02
EIF2 Signaling	2.01E00	2.97E-02
HMGB1 Signaling	1.92E00	3E-02
Chronic Myeloid Leukemia Signaling	1.92E00	2.86E-02
Glioma Signaling	1.91E00	2.68E-02
Role of PKR in Interferon Induction and Antiviral Response	1.82E00	4.35E-02
Sphingosine-1-phosphate Signaling	1.76E00	2.52E-02
PI3K/AKT Signaling	1.76E00	2.14E-02
P2Y Purigenic Receptor Signaling Pathway	1.71E00	2.22E-02
Melanoma Signaling	1.7E00	4.35E-02
Synaptic Long Term Depression	1.59E00	2.05E-02
Relaxin Signaling	1.59E00	1.9E-02
IL-2 Signaling	1.56E00	3.45E-02
CNTF Signaling	1.54E00	3.64E-02
Molecular Mechanisms of Cancer	1.54E00	1.33E-02
IL-15 Signaling	1.48E00	2.94E-02
Endometrial Cancer Signaling	1.48E00	3.51E-02
Role of MAPK Signaling in the Pathogenesis of Influenza	1.48E00	3.08E-02
Role of NFAT in Regulation of the Immune Response	1.46E00	1.5E-02
IL-17A Signaling in Airway Cells	1.44E00	2.78E-02
Chemokine Signaling	1.4E00	2.7E-02
Melatonin Signaling	1.39E00	2.6E-02
GM-CSF Signaling	1.39E00	2.99E-02
Glioblastoma Multiforme Signaling	1.38E00	1.83E-02

JAK/Stat Signaling	1.37E00	3.12E-02
Erythropoietin Signaling	1.35E00	2.56E-02
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.35E00	1.6E-02
Neurotrophin/TRK Signaling	1.34E00	2.6E-02
CXCR4 Signaling	1.31E00	1.78E-02
FLT3 Signaling in Hematopoietic Progenitor Cells	1.3E00	2.7E-02
Small Cell Lung Cancer Signaling	1.3E00	2.25E-02
IL-3 Signaling	1.29E00	2.7E-02
IL-17 Signaling	1.29E00	2.7E-02
Acute Myeloid Leukemia Signaling	1.29E00	2.44E-02

Appendix 4-3

Pathway Analysis of Category III

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Estrogen Receptor Signaling	1.42E00	1.47E-02
Inositol Phosphate Metabolism	1.37E00	1.12E-02
Oxidative Phosphorylation	1.33E00	1.26E-02
Glycosphingolipid Biosynthesis - Globoseries	1.28E00	2.56E-02
Pentose Phosphate Pathway	1.13E00	1.22E-02
Docosahexaenoic Acid (DHA) Signaling	1.04E00	2.04E-02
Galactose Metabolism	9.76E-01	9.43E-03
TNFR1 Signaling	9.59E-01	1.89E-02
Colorectal Cancer Metastasis Signaling	9.47E-01	7.78E-03
Assembly of RNA Polymerase II Complex	9.42E-01	1.79E-02
CD27 Signaling in Lymphocytes	9.34E-01	1.75E-02
Semaphorin Signaling in Neurons	9.26E-01	1.92E-02
Lymphotoxin β Receptor Signaling	9.1E-01	1.64E-02
Protein Ubiquitination Pathway	8.81E-01	7.3E-03
Glutathione Metabolism	8.81E-01	1.11E-02
Glucocorticoid Receptor Signaling	8.79E-01	6.78E-03
Purine Metabolism	8.71E-01	5.12E-03
Induction of Apoptosis by HIV1	8.68E-01	1.52E-02
IL-15 Signaling	8.54E-01	1.47E-02
GM-CSF Signaling	8.54E-01	1.49E-02
Angiopoietin Signaling	8.35E-01	1.35E-02
Agrin Interactions at Neuromuscular Junction	8.17E-01	1.45E-02
Ubiquinone Biosynthesis	8.17E-01	8.93E-03
Renal Cell Carcinoma Signaling	8.06E-01	1.35E-02
Starch and Sucrose Metabolism	8.06E-01	5.92E-03
Small Cell Lung Cancer Signaling	8E-01	1.12E-02
OX40 Signaling Pathway	7.48E-01	1.11E-02
Regulation of Actin-based Motility by Rho	7.34E-01	1.1E-02
FAK Signaling	7.2E-01	9.8E-03
VEGF Signaling	7.2E-01	1.01E-02

PAK Signaling	7.11E-01	9.35E-03
Apoptosis Signaling	7.11E-01	1.04E-02
Glycolysis/Gluconeogenesis	7.07E-01	7.46E-03
Molecular Mechanisms of Cancer	7.05E-01	5.31E-03
p53 Signaling	6.94E-01	1.04E-02
Chronic Myeloid Leukemia Signaling	6.9E-01	9.52E-03
Amyotrophic Lateral Sclerosis Signaling	6.7E-01	8.4E-03
Nicotinate and Nicotinamide Metabolism	6.7E-01	7.41E-03
Natural Killer Cell Signaling	6.54E-01	9.09E-03
Rac Signaling	6.51E-01	8.13E-03
Pancreatic Adenocarcinoma Signaling	6.43E-01	8.4E-03
Renin-Angiotensin Signaling	6.4E-01	8.06E-03
PTEN Signaling	6.36E-01	8.06E-03
Role of Tissue Factor in Cancer	6.33E-01	8.77E-03
Metabolism of Xenobiotics by Cytochrome P450	6.12E-01	5.08E-03
CCR3 Signaling in Eosinophils	6.12E-01	7.87E-03
PI3K/AKT Signaling	6E-01	7.14E-03
GNRH Signaling	5.87E-01	6.9E-03
Mitochondrial Dysfunction	5.55E-01	5.71E-03
B Cell Receptor Signaling	5.29E-01	6.41E-03
CXCR4 Signaling	5.07E-01	5.92E-03
Cdc42 Signaling	4.98E-01	5.75E-03
Germ Cell-Sertoli Cell Junction Signaling	4.95E-01	5.99E-03
Wnt/β-catenin Signaling	4.69E-01	5.75E-03
Ephrin Receptor Signaling	4.65E-01	5.03E-03
IL-8 Signaling	4.63E-01	5.18E-03
ERK/MAPK Signaling	4.42E-01	4.9E-03
Integrin Signaling	4.17E-01	4.78E-03
Actin Cytoskeleton Signaling	3.92E-01	4.2E-03
Huntington's Disease Signaling	3.84E-01	4.2E-03
Axonal Guidance Signaling	2.1E-01	2.31E-03

Appendix 4-4

Pathway Analysis of Category IV

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Melatonin Signaling	3.3E00	2.6E-02
Leptin Signaling in Obesity	3.25E00	2.44E-02
Melanocyte Development and Pigmentation Signaling	3.13E00	2.2E-02
α-Adrenergic Signaling	3.1E00	1.9E-02
G Beta Gamma Signaling	3.08E00	1.71E-02
Glioma Signaling	3.03E00	1.79E-02
Neuropathic Pain Signaling In Dorsal Horn Neurons	2.98E00	1.85E-02
Renin-Angiotensin Signaling	2.92E00	1.61E-02
Corticotropin Releasing Hormone Signaling	2.91E00	1.49E-02
P2Y Purigenic Receptor Signaling Pathway	2.85E00	1.48E-02
Cellular Effects of Sildenafil (Viagra)	2.75E00	1.32E-02
Ovarian Cancer Signaling	2.74E00	1.41E-02

Appendix 4-5

Pathway Analysis of Category V

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Glycerophospholipid Metabolism	2.78E00	1.12E-02
MIF-mediated Glucocorticoid Regulation	1.82E00	2.38E-02
MIF Regulation of Innate Immunity	1.7E00	2E-02
Linoleic Acid Metabolism	1.68E00	9.43E-03
Eicosanoid Signaling	1.62E00	1.32E-02
Role of MAPK Signaling in the Pathogenesis of Influenza	1.49E00	1.54E-02
Atherosclerosis Signaling	1.46E00	9.35E-03
Arachidonic Acid Metabolism	1.45E00	4.93E-03
Phospholipid Degradation	1.41E00	1.08E-02
p38 MAPK Signaling	1.27E00	9.43E-03
Fc Epsilon RI Signaling	1.25E00	9.01E-03

Pathway Analysis of Category VI

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Purine Metabolism	2.76E00	7.67E-03
Assembly of RNA Polymerase I Complex	2.08E00	7.69E-02
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	1.65E00	3.7E-02
Interferon Signaling	1.51E00	2.78E-02
TNFR1 Signaling	1.36E00	1.89E-02
Chondroitin Sulfate Biosynthesis	1.35E00	1.56E-02
Phototransduction Pathway	1.32E00	1.54E-02

Pathway Analysis of Biolayout Filtered genes

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Granzyme B Signaling	3.68E00	3.12E-01
Tumoricidal Function of Hepatic Natural Killer Cells	2.95E00	2.08E-01
Toll-like Receptor Signaling	2.65E00	1.27E-01
Type I Diabetes Mellitus Signaling	2.35E00	8.26E-02
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.34E00	5.81E-02
Neurotrophin/TRK Signaling	2.21E00	1.04E-01
Parkinson's Signaling	2.17E00	2.22E-01
Regulation of eIF4 and p70S6K Signaling	2.17E00	7.26E-02
Apoptosis Signaling	2.13E00	9.38E-02
Role of MAPK Signaling in the Pathogenesis of Influenza	2.04E00	1.06E-01
PI3K/AKT Signaling	1.88E00	7.14E-02
Retinoic acid Mediated Apoptosis Signaling	1.84E00	7.35E-02
Role of PKR in Interferon Induction and Antiviral Response	1.77E00	1.09E-01
Lymphotoxin β Receptor Signaling	1.76E00	9.84E-02
IL-2 Signaling	1.76E00	1.03E-01
Erythropoietin Signaling	1.73E00	8.97E-02
Non-Small Cell Lung Cancer Signaling	1.73E00	8.86E-02
AMPK Signaling	1.65E00	6.55E-02
Inhibition of Angiogenesis by TSP1	1.64E00	1.28E-01
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.6E00	7.87E-02
PI3K Signaling in B Lymphocytes	1.58E00	6.99E-02
Allograft Rejection Signaling	1.55E00	3.12E-02
Endometrial Cancer Signaling	1.55E00	1.05E-01
IL-1 Signaling	1.54E00	7.48E-02
Insulin Receptor Signaling	1.46E00	7.14E-02
PTEN Signaling	1.44E00	7.26E-02
CD27 Signaling in Lymphocytes	1.42E00	8.77E-02
Wnt/β-catenin Signaling	1.38E00	6.9E-02

Myc Mediated Apoptosis Signaling	1.38E00	9.84E-02
PKCθ Signaling in T Lymphocytes	1.34E00	5.63E-02
ERK5 Signaling	1.34E00	9.38E-02
Nur77 Signaling in T Lymphocytes	1.29E00	6.35E-02

Selected Network from category I cluster analysis

ID	Genes in Network	Score	Focus Genes	Top Functions
1	14-3-3, **ABLIM2, AKAP13**, Arp2/3, **ARPC4**, **ARPC1A, ARPC1B, BAK1, BARX2,** Calcineurin protein(s), **CALD1, CAMK4,** CaMKII, Caspase, **CHRNG, CIB1,** Cytochrome c, **DMD,** F Actin, **LONP2, LSP1, LTBP1,** MEF2, **MYOG, NCAM1,** Nfat (family), NFkB (complex), **PDCD6IP, PLEKHO1, SCLY, SH3GL1, SH3KBP1, SRP72, ST8SIA4, TNNI1**	42	25	Genetic Disorder, Skeletal and Muscular Disorders
4	**ACTR3,** ADCY, **AHR,** Akt, **AQP1, ARL6IP5,** Ck2, E2f, **FKBP3,** FSH, hCG, Histone h3, Histone h4, **HSF2,** Hsp70, Hsp90, **HSPG2,** IL1, Insulin, Lh, **NAP1L1, NCL, NUCB2,** P38 MAPK, **PHLDA2, PMEPA1, PTMA,** Rb, RNA polymerase II, **SMARCA5, SPATS2L, STMN1,** Tgf beta, **THBS2,** Vegf	27	17	Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders
6	**AP2M1,** APP, ARID1A, **AS3MT, COL12A1,** CTNNA2, **CTNNA3,** CTNNB1, DAB2, DSTN, EGFR, **FKBP3, GDI2, GNMT,** GRB2, Grb2-Shc1-Sos, **GSR,** H3F3C, IGF2BP1, LDB2, **LMO4,** Lrrfip1, MYC, NR3C1, **OAZ1,** POLR1B, **PXDN,** RPL41, **SARDH, SERPINI1,** SIM1, SMARCD1, SMARCD2, SNIP1, **TRIM45**	20	14	Hair and Skin Development and Function, Cell Morphology
7	ACTN4, **ALDH6A1, C12orf51,** CCAR1, CDT1, CHAF1B, CLIC4, Cofilin, CXCR1, **EGFL6, EPHA3, GLIPR1,** Grb2-Shc1-Sos, Histone H1,IL-2R, KPNA1, KRT10, **LRWD1, NEU2, PDCD6IP, PDLIM4,** PHACTR1, PP1 protein complex	19	13	Cell Death, Cell Cycle

	group, **PPP1R14C, PTBP1,** PTEN, **SEL1L,** SHC1, SPTAN1, SRC, SURF4, TP53, TUBB4, YWHAZ, ZFP36L1			
9	ADRB, **AKR1D1, ARCN1,** ARRDC1, ARRDC2, ARRDC3, ASB2, ATG7, AVPR2, FLCN, FNIP1, GMPPA, GNB2L1, **IPO5,** MAP1LC3A, **MPP6, PDCD6IP,** PLEC, RORA, **SEC61A1,** SERPINH1, **SLC25A24, SLC41A2, SLC9A6, TCEB1,** TGFB1, TLR5, TOM1, TSC22D1, **UBB,** Ubb, Ubiquitin, **WDFY3,** ZC3H12A, ZNF768	18	12	Cell Morphology, Infection Mechanism, Neurological Disease

Bold Overlapping genes from submitted list.
Bold Red Genes, from submitted list, common in two or more networks

Selected Network from the analysis of category II genes

ID	Genes in Network	Score	Focus Genes	Top Functions
1	**AKT1,** Akt, **AQP3, CDK6, COPS2, CUL2, CUL3, DBT, DHX15,** ERK1/2, FSH, G protein alphai, **GNAI1, GPS1, HBP1,** Hsp90, IKK (complex), Jnk, **KIF5B,** Lh, **MAP2K1,** Mapk, **MFN2, MTF1,** NFkB (complex), P38 MAPK, Pdgf(complex), **PGAM5, PTP4A1,** Ras, **S1PR1, TAB2, TRIP11,** Vegf, **ZFAND5**	45	21	Cell Cycle, Cardiovascular System Development and Function
3	**AGL,** CXCR4, DNTT, EIF2C3, **EIF2C4, EPDR1,** GOLGA2, GRB2, **HEATR3,** IRF8, MAX, MYC, NCOR1, NHLRC1, NR0B1, **NR1D2,** NR2F1, PSD, **PTPN4, RNF10, RNF114,** SCT, **SENP6,** SP1, SRY, TNRC6B, UBC, UBQLN4, UIMC1, **USP16, USP9X, WDR44, XPO7, ZDHHC3, ZFR**	28	14	Gene Expression, Cellular Development
4	**AFG3L2,** ALPP/ALPPL2, Ampa Receptor, **ATG16L1,** ATM, **CCDC6, CNOT1,** DAPK1, DAXX, ERBB2, H19, IKBKG, JUN, KIAA0368, MAPK6, **MTHFR,** MUC4, **NPTN, NR2C2,** NUAK1, NUP210, PAK3, **PPP2R2A, PSME4,** RAD23B, RNF7, SHMT1, TGFB1, TNFRSF10A, TNFRSF10B, TNFRSF1A, TOP1, **TRPC1, USP34, USP9X**	23	12	Cell Death, Inflammatory Response, Cancer
5	AXL, BIRC5, BRPF1, CCNE1, CFLAR, DYRK1A, ERK,GTP, Histoneh3, HSPD1, ING5, ITGAL, **KDM3A,** LRP, MAP3K10, MAPK6, MUC4, **MYST3,** NCOA2, NF1,	10	6	Skeletal and Muscular System Development and Function,

	NF2, NFE2L2, PI3K (complex), **PIGK,** PTPRF, **RAB21,** RAB1A, RABGEF1, Raf, **RANBP9,** S100A7, **S1PR1,** Sos, TNFSF10, ULBP2	Cancer

Bold	Overlapping genes from submitted list.
Bold Red	Genes, from submitted list, common in two or more networks

Selected Network from the analysis of category III genes

ID	Genes in Network	Score	Focus Genes	Top Functions and Diseases
1	**ASB15,** ASB3/GPR75-ASB3, **BCCIP,** BRIP1, CNKSR2, EXO1, **FAM82A2,** HNF4A, **IPO8, KIAA0415,** MOV10, **NDUFS1,** PAK1, **PAK3,** PCBD1, **PGM1, PMS1,** POLR1C, POLRMT, PRPF31, **RBM15,** RNF40, **RPRD1B, SLC25A32, SNX27,** SRP19, **TFB2M,** TUT1, UBA5, **USP5,** USP13, USP15, USP46, USP9Y, YWHAB	33	15	Cell Morphology, Genetic Disorder
2	Akt, ATP5A1, ATP5B, **ATPAF1, BCL2L1, C16orf70, C2orf56,** Calcineurin protein(s), **CMYA5,** CNKSR2, **DIDO1,** DLG4, dopamine, GFI1B, GRIN2B, GRIN2C, HTT, iron, KIF17, **MED9, MED31, MON1A, NDUFS1,** NDUFS3, NDUFS4, NDUFS5, NDUFV2, PAK1, **RG9MTD1,** RPA2, SEC24B, testosterone, TLN1, **TPMT, TTLL11**	29	14	Cell Death, Energy Production

Bold Overlapping genes from submitted list
Bold Red Genes, from submitted list, common in two or more networks

Appendix 4-11

Selected Network from IPA analysis of BioLayout filtered genes

ID	Genes in Network	Score	Focus Genes	Top Functions
4	Akt, **ARFGAP1, ATE1, BNIP2, CDON, CLK2, CLK3, DAB2IP,** Fcer1, Gm-csf, GTPASE, **GUSB,** Ifn, IFN alpha/beta, Ikk(family), **INPP5E, IRAK2, IRAK4,** IRAK, **IRF5, Lrrfip1, MRC1, MYD88,** NFkB (family), **RAB3GAP2, RGS5, RGS14, RNF219, RPTOR, SH2B2, SNX13, SOCS1, TIRAP,** Tlr, **TTC4**	35	25	Inflammatory Response, Organismal Injury and Abnormalities
7	20s proteasome, 26s Proteasome, Alpha tubulin, **ARRDC1, ASB2, CDKL2,** Clathrin, Cyclin B, **DNM1L,** Dynamin, **EIF3E, EPS15, FOXO3,** Gi-coupled receptor, **HIRA,** Ikb, **ITSN2,** Mapk, **NFKBIA, NFU1,** Proteasome PA700/20s, **PSMA1, PSMB3, PSMB7, PSMD1, RAB8B, RAD18, REV1, SH3BP4, SMARCE1,** STAT5a/b, **TP53BP1, TXNRD2,** Ubiquitin, **USP8**	31	23	Cell Cycle, Cellular Assembly and Organization
17	60S ribosomal subunit, **ADAMTS13,** Ca2+, CAD, Calcineurin A, **CEP57, CORO1C, COX6A1,** DUSP1, **Egln1,** EIF4A1, F2,FGA, **FKBP7, LAMP1, LMNB2,** Mlc, MYC, MYO1B, Myosin, NME2, PHLDA3, POLR2A, **PPID,** PPIF, RPL13, **RPL38, RPS12, SLC25A19, SRP14, STIM2,** TMSB4, **TRAK1,** Tropomyosin, **XPO5**	16	16	Skeletal and Muscular System Development and Function
24	AEBP1, **COX15, COX6A1,** CREBBP, CUL5, Cytochrome c oxidase, **EPHX2,** Ferritin, **FNBP4,** HTT, MED31, MT-CO3, MYOD1, NFATC4,	13	13	Tissue damage, Energy Production

	NFKB1, NfkB1-RelA, **ORAI2,** PDE8A, PIAS4, **PKHD1,** PLIN4, PNPT1, PPARG, PRMT2, **PTGR2, SCN4B, SRGAP1,** TAB1, **TMCC2, TMED8, TMEM66, TPR,** Tsc22d3, UCP1, ZFP36			
25	**AMDHD2,** ARAF, **BSPRY,** CHAF1A, **EARS2,** EIF4G1, **ENDOV, EPRS,** ETS1, **FAN1,** glutamate-tRNA ligase, HDAC9, IARS, **LARP1, LCLAT1,** MET, miR-155 (human, mouse), MITF, NFKB2, **NHEJ1,** POLR2D, POU2F1, QARS, **QDPR,** RAI14, RPS6KA1, **SH3BP4,** TCF7L2, TFE3, TH1L, **TSR1,** XRCC5, YWHAZ	13	12	Developmental Disorder, Genetic Disorder

Bold	Overlapping genes from submitted list
Bold Red	Genes, from submitted list, common in two or more networks

Appendix 4-12

Merge Network 1, 7, & 9 (Category I)