TRANSCRIPTOMIC ANALYSIS OF HYPOTHALAMIC RESPONSES TO HEAT STRESS IN MODERN AND LEGACY CHICKEN LINES

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

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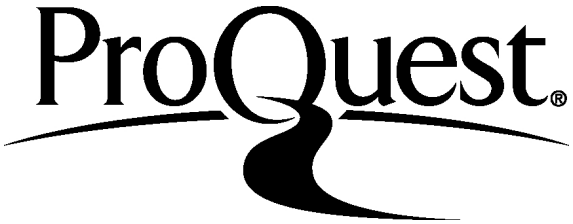

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ABSTRACT

Modern commercial broilers have been intensively selected for high growth rate, feed efficiency and meat yield. This enhanced genetic selection, at the expense of the functional efficiency of other physiological systems, has reduced their ability to withstand high temperatures compared with the legacy lines. Broilers reared under high temperatures have lower growth rates and feed efficiency compared to those raised at optimal temperatures. Maintenance of thermoregulation, feeding behavior and energy homeostasis is mainly controlled by the hypothalamus. Based on these factors, we hypothesize that heat stress may differentially impact the hypothalamic gene expression patterns in modern and legacy broiler lines. To explore this hypothesis, RNA-seq was used to study the hypothalamic transcriptomic responses to heat stress in modern and legacy broiler lines and identify the differentially expressed genes at day 28 and 42 post hatch. Chickens in the heat stress group were subjected to a temperature of 41°C for 10hrs daily, from day 21 post hatch, while the controls were maintained at 25°C. The body weights of the heat stress chickens were lower compared to the controls in both the lines, however the difference is only statistically significant in Ross 708 line, at both the time points. Among the 13,642 genes analyzed, 187 and 182 genes were differentially expressed in response to heat stress in Ross 708 and Illinois line, respectively, at day 28 post hatch. These included, genes encoding rate-limiting enzymes that are primarily involved in regulating feed intake. The transcriptional profiles, at day 42-post hatch, identified 91 genes in Ross 708 and 393 genes in Illinois lines that are differentially expressed. These genes were predominantly involved in immune regulation. However, genes associated with feed intake regulation were also differentially expressed in Illinois

line. This study suggests that hypothalamic regulation of feed intake during heat stress may have affected the body weights in Ross and Illinois lines. This study provides a broader understanding of the mechanisms underlying heat stress response in the hypothalamus of modern and legacy chicken lines.

Chapter 1

GENERAL INTRODUCTION

Body homeostasis can be achieved by maintaining a stable internal environment in response to external variations. Environmental stressors often challenge this homeostasis (1). Modern broilers are highly susceptible to temperature related environmental challenges, especially heat stress (2-4). Heat stress is one of the important issues affecting the broiler chicken production in the United States as well as in the other countries of the world due to global climatic change (5). An economic study conducted in the United States by St. Pierre *et al.* (2003), based on decreased performance, reproduction and increased mortality due to heat stress, estimated a total economic loss of $51.8 million annually in broiler production (6). According to Intergovernmental Panel on Climate Change (IPCC, 2014), the global average surface temperatures by the end of 21st century is likely to increase by 1.0 to 3.7°C relative to the temperatures in 1986-2005 period (7). These predictions suggest that the negative effects of heat stress will become more apparent in the future (8). The bird's physiology is changed by heat stress, which places stress on many systems and thereby causes decreased performance and increased mortality (9). Moreover, commercial broilers, which are selected for rapid growth and high feed efficiency, produce more heat because of high metabolic activity and thus become more susceptible to heat stress (2-4, 10, 11).

Chickens are homoeothermic and maintain their body temperatures within a narrow thermoneutral zone (9, 12). Thermoneutral zone is a range of ambient temperatures where the bird does not expend energy to gain or lose heat (13). Exposure to

severe environmental conditions (hot or cold) that results in extreme changes in the body temperatures could lead to a cascade of irreversible thermoregulatory events that can be lethal to the birds (9). When the external temperature rises above thermoneutral zone, body temperature increases and heat-loss mechanisms are activated to dissipate excess body heat. Similarly, when external temperature drops below thermal neutral zone body temperatures decrease and thermogenic mechanisms are activated (14, 15). It has been proposed that central nervous system regulates the body temperature by controlling these heat-loss and heat gain mechanisms and this regulatory mechanism is mainly centered in the diencephalon (15). Kanematsu *et al.* have shown this by placing electrolytic lesions in the hypothalamus of chickens (16). Several researchers have proposed that hypothalamus plays a major role in thermoregulation of birds (17-20). Hypothalamus coordinates inputs from the brain, spinal cord, peripheral and deep body thermoreceptors and elicits various thermoregulatory responses, thereby maintaining a relatively constant core body temperature (20). In addition, several releasing hormones are also produced by the hypothalamus that affects the overall body homeostasis (21).

As commercial broiler lines that are selected for high growth rate and body weight are more susceptible to heat-stress inducing conditions compared to the legacy lines and as hypothalamus plays an important role in thermoregulation, this thesis focuses on comparing the hypothalamic transcriptome profiles between chickens under heat stress conditions and controls within legacy and commercial broiler lines so that important genes involved in heat stress regulation can be identified.

Chapter 2

LITERATURE REVIEW

2.1 Anatomy of Avian Hypothalamus

During early embryogenesis the primitive forebrain is divided into two subdivisions: the telencephalon and the diencephalon (22). The avian hypothalamus develops from the ventral part of the diencephalon (23, 24). The hypothalamus is located on either side of the third ventricle, below the thalamus and dorsal to the optic chiasma and pituitary gland. It consists of several well-defined nuclei and is traversed by several major neuronal fiber tracts. Based on the location of fiber tracts and nuclei, the hypothalamus is divided into three major regions: preoptic (anterior), tuberal (middle) and mammillary (posterior) hypothalamic regions. Two areas and nineteen nuclei were identified within these regions (23). Of these nuclei, ventromedial hypothalamic nuclei (VMN), the infundibular nuclei (IN), the paraventricular nuclei (PVN) and the lateral hypothalamic area (LHA) are mainly responsible for feed intake and body weight regulation (25). The suprachaismatic nucleus regulates the circadian rhythms (24, 26) and the preoptic area of the anterior hypothalamus (PO/AH) is mainly involved in thermoregulation (19, 20) (fig 1). The hypothalamic hormones are mainly produced by the neurons present in the magnocellular and parvocellular systems in the hypothalamus (27).

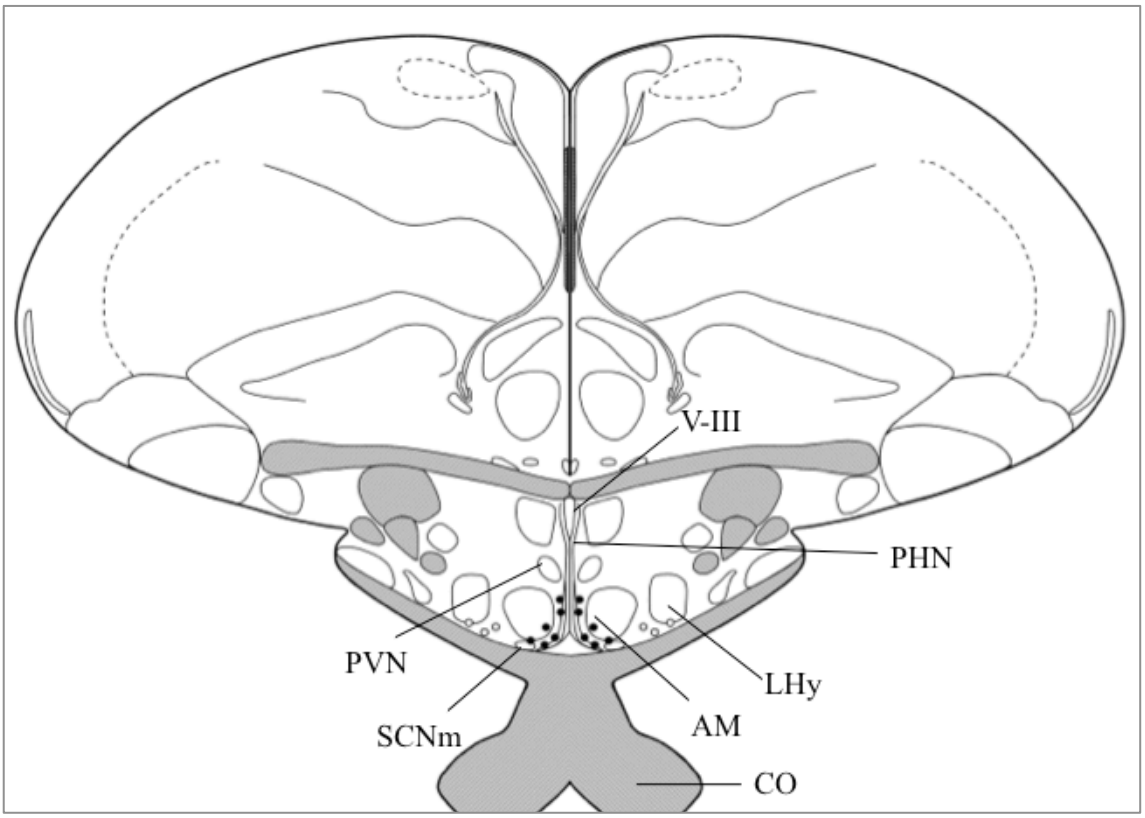


Figure 1: Coronol rostrocaudal section showing the anatomic locations of the hypothalamic nuclei of the broiler chickens. PVN- Nucleus Paraventricularis Magnocellularis; PHN- Nucleus Perventricularis Hypothalami, AM- Nucleus Anterior Medialis Hypothalami, LHy- Regio Lateralis Hypothalami, SCNm- Nucleus Suprachiasmaticus pars medialis, V III- Ventricularis tertius, CO- Chiasmia Opticum. *Image adapted from the chicken atlas for nomenclature forum by Dr. Wayne Kuenzel. (http://avianbrain.org/nomen/Chicken_Atlas.html).*

2.2 Ontogeny of Hypothalamic Thermoregulation in Birds

Afferent temperature signals from various areas of the body are integrated by thermosensitive neurons present in the PO/AH. These signals elicit appropriate thermoregulatory responses by controlling behavioral, physiological and endocrinal responses to maintain constant body core temperatures (20, 28). The development of

brain and body temperature regulation is completed at 10 days post hatch in broiler chicks (29). Prior to 10-day post hatch, low body temperatures are maintained compared to the adults and as age increases body and brain temperatures increases (29). This change is attributed to the shift in the ratio between cold and warm sensitive neurons in the PO/AH (29, 30). Between the last week of prenatal incubation and fifth day post hatch, there is an increase in cold sensitive neurons by 20-30% and decrease in hot sensitive neurons by 12.5-5%. However, between fifth and tenth day post-hatch the proportion of cold sensitive neurons decreases to 14% and the proportion of hot sensitive neurons increases to 15% in the PO/AH (29, 30).

2.3 Neuroendocrine Secretory System of the Avian Hypothalamus

The hypothalamus connects the nervous system with the endocrine system through the pituitary gland and is mainly responsible for hormone production (21). The neurons present in the magnocellular and parvocellular hypothalamic systems produce hypothalamic hormones that are involved in regulating the functions of the pituitary gland (27).

In chickens, Arginine Vasotocin (AVT) and Mesotocin (MT), homologs of mammalian Arginine Vasopressin (AVP) and oxytocin (OXT), are secreted from the neurons in the magnocellular region (27, 31, 32). These secretions directly reach the neurohypophysis of the pituitary gland where they are stored prior to the release into the systemic circulation (33). OXT and AVP genes encode precursor proteins of the MT and AVT respectively (33). AVT is necessary for maintaining osmoregulation and acts as an anti-diuretic hormone in birds (9, 33, 34). AVT and MT together play an important role in oviposition in laying hens with AVT being more potent (33, 35). Recently, it was reported that MT is associated with brooding behavior in Thai chickens (36).

Intracerebroventricular (ICV) injections of AVT inhibited feeding behavior, increased rectal temperatures and plasma corticosterone concentrations in chickens (37).

Several neuropeptides are secreted by the peptidergic and aminergic neurons in the parvocellular region. Some of these neuropeptides act as neurohormones or releasing factors, which control the production and the release of hormones from the anterior pituitary gland (27). In response to these releasing factors, the pituitary gland produces tropic factors and relays information to endocrine targets (38). Parvocellular neurohormones identified in chicken hypothalamus include: Corticotropin Releasing hormone (CRH) (39), Gonadotropin releasing hormones (GnRH-I and GnRH-II) (40, 41), Gonadotropin Inhibiting Hormone (GnIH) (42), Growth Hormone Releasing Hormone (GHRH) (43), Somatostatin (SS) (44), Vasoactive intestinal polypeptide (VIP) (45) and Thyrotropin releasing hormone (TRH) (46). CRH and TRH released from hypothalamus not only affected the corresponding hormone release from the pituitary but also play an important role in regulating physiological responses to stress (9).

Neurosecretory cell bodies that release CRH and AVT regulate the secretion of Adrenocortico hormone (ACTH) from pituitary corticotrophs thereby stimulating adrenal cortical cells to release corticosteroids (33, 47). Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) secretion from pituitary gonadotrophs are positively regulated by GnRH-I and GnRH-II and negatively by GnIH, and thus controls gonads. VIP promotes prolactin (PRL) release from pituitary lactotrophs. Pituitary somatotrophs that secrete Growth hormone (GH) are stimulated by GHRH and TRH and inhibited by SS. TRH and CRH are potent secretagogues of Thyroid-stimulating hormone (TSH) release from pituitary thyrotrophs, which further stimulates thyroid glands to release thyroid hormones (33) (table 1). These hypothalamo-hypophyseal hormones are involved in the regulation of various important physiological processes like metabolic homeostasis,

growth, reproduction, immunity, defensive reactions such as fear or rage, autonomic system and circadian rhythms (21).

Table 1: Hypothalamic hormones and their effects on the target pituitary hormones

HYPOTHALAMIC HORMONES	TARGET PUITARY HORMONES	EFFECTS + Stimulatory effect − Inhibitory effect
CRH	ACTH	+
GnRH-I and GnRH-II	FSH, LH	+
GnIH	FSH, LH	−
GHRH and TRH	GH	+
SS	GH	−
TRH, CRH	TSH	+
VIP	PRL	+

2.4 Hypothalamic Control of Energy Homeostasis:

In mammals as well as in birds, the hypothalamus plays an important role in regulation of energy homeostasis (25, 48). For example, lesions in medial hypothalamus increase the feed intake whereas lesions in the LHA decrease the feed intake (25). Neuropeptides present in the hypothalamus ensure energy balance by maintaining crosstalk between hypothalamus and gastrointestinal tract (49). Two populations of orexigenic and anorexigenic neuropeptides have been identified in the chicken hypothalamus that regulate feed intake (50-52). Neuropeptide Y (NPY) and agouti-related peptides (AgRP) are orexigenic (50) whereas pro-opiomelaocortin (POMC), CRH, Cholecystokinin (CCK) and cocaine and amphetamine-regulated transcript (CART) are anorexigenic (51-53). GnIH was identified as an orexigenic peptide in the chicken brain (54). Neuromedin U (NMU) is another neuropeptide abundantly expressed

in the hypothalamus (55) and ICV administration of NMU in chickens suppressed feed intake (56).

2.5 Concepts of Stress and Homeostasis

Stress is a biological response of an organism to adverse stimuli called stressors, which disturb homeostasis (1). Stressors include all possible environmental conditions in which the bird lives that have negative impact on bird health and welfare (57, 58) Extrinsic and intrinsic stressors cause environmental stress. Extrinsic stressors consist of abiotic (temperature, climatic factors and chemical components) and biotic (competition, predation and parasitism) factors while intrinsic stressors include inbreeding and changes in genetic architecture, which cause genetic stress. These stressors can often act synergistically (59) and affect the hormonal control of the metabolism, reproduction, growth and immunity (60).

Success in coping with the stress depends on the type, intensity and severity of the stressors along with the individual's ability to physiologically respond (57, 61). Maintenance of homeostasis by counteracting aversive stimuli requires the activation of regulatory processes, which are collectively called stress responses (57). Siegel (1980) classified the stress responses as specific and nonspecific (57). Specific responses are usually short term and elicited by a particular stimulus (57, 62). For example, sudden rise in environmental temperature causes panting, dilation of surface blood vessels and rearrangement of feathers to reduce insulation in birds (63). In specific response, animals try to combat the stressor (62). In contrast, nonspecific responses are long term and lead to adaptation rather than combating the stressor (57, 62). Non-specific responses occur regardless of the stressors (heat, cold or hypoxia) and the birds respond to them in a generalized manner (64).

The stress response that is activated when an animal first encounters the stressor is sympathetic-adrenomedullary (SAM) system, which causes the release of catecholamines from the adrenal medulla (57). This is termed as “fight or flight” response (65). Failure to combat the stressor by SAM activates hypothalamic-pituitary-adrenal cortical (HPA) system (57, 62). Activation of this system causes the release of CRH from hypothalamus, which in turn stimulates ACTH release from pituitary gland. ACTH secretion causes the release of corticosteroids (9, 33). An elevated level of corticosteroids in circulation causes altered glucose, lipid and mineral metabolism, along with inhibition of immune system functions (62, 63). Broiler performance and carcass characteristics are deteriorated due to increased blood corticosteroid levels (62).

2.6 Heat Stress

Heat stress is a condition that results when an animal experience a negative balance between net energy released into its surrounding environment and the amount of heat energy it produces (66). Heat stress can be expressed as ‘acute’ and ‘chronic’. Acute heat stress occurs when an animal is exposed to short, sudden periods of high temperature whereas chronic heat stress occurs when exposed to long periods of high temperature (67).

Chickens are homoeotherms and thus maintain their core body temperatures within a narrow range of around 40.5-41.5°C (9, 12). The optimum environmental temperature for performance is between 18-22°C in broilers and 19-22°C in layers (68). Any deviation from these temperatures can affect the performance. The upper critical temperature is a temperature at which the body temperature increases until it reaches a stage at which the bird expires (9). The upper critical body temperature is estimated to be between 29-32°C (69, 70) and is usually dependent upon relative humidity (RH) (12, 71-

73). At high temperatures heat production mechanisms decrease and heat dissipative mechanisms increase (12, 74, 75). As chickens lack sweat glands, they depend on evaporative cooling (panting) as a body temperature regulatory mechanism (12, 73-75). Increase in RH decreases the amount of heat dissipated by evaporative cooling, resulting in respiratory alkalosis, electrolyte imbalance and hyperthermia (9, 12, 72, 73, 76).

Intensive genetic selection in modern broilers increased growth rate under optimal temperatures (77). In broilers decrease in body weight by 23% and feed efficiency by 15% was observed, from 4wk to 7wk period, when reared under high temperatures compared to the controls raised at optimal temperatures (77). Genetic selection has favored high growth rate and muscle mass at the expense of the functional efficiency of cardiovascular and respiratory systems (11), which led to low capability to achieve energy and body water balance and increased sensitivity to heat stress conditions (3, 77-80). Faster growing broilers produce more internal heat (81-83) and due to the absence of sweat glands they have greater difficulty in dissipating heat by conduction and convection at high temperatures (73, 84). This leads to increase in their body temperatures and reduced performance (83). Broilers are more sensitive to heat stress compared to layers (85). Increased size and insulation in older broilers, which reduces their ability to dissipate heat, makes them more susceptible to heat stress compared to younger birds (85).

2.7 Hypothalamic Responses to Heat Stress

The hypothalamus maintains body homeostasis by producing several releasing hormones that serve as an important link in the flow of information among different cells and tissues in an animal to initiate heat stress responses (9). The extent to which these responses are mediated depends upon the severity of heat stress (9).

AVT, secreted from the hypothalamus, is the principal antidiuretic hormone in non-mammalian vertebrates (9, 33, 34). However, AVT functions in heat dissipation, independent of its role in osmoregulation (9, 86). During acute heat stress, plasma concentrations of AVT are increased without any change in plasma osmolality (9, 86). MT reduces the circulating levels of aldosterone and also influences the blood flow to some organs (33). Circulating levels of MT are decreased during heat stress (86) and this can be attributed to increased levels of AVT, as increased AVT levels suppress MT release (9, 87).

Heat stress stimulates the activation of HPA axis, which results in release of corticosterone from adrenal glands (9). The main upstream regulator of corticosterone release is CRH, secreted from the hypothalamus, which in turn stimulates ACTH release from anterior pituitary (9, 33). The adrenal cortex increases the production and release of adrenal hormones, corticosterone and aldosterone, in response to ACTH stimulation (9, 33). High levels of plasma corticosterone are maintained for short periods to cope with acute heat stress and combat initial hyperthermia (9). Increased levels of corticosterone results in regression of lymphoid tissues, increased heterophil-to-neutrophil ratio and impaired function of immune system (62). Also, live performance of the bird is impaired due to corticosteroid-induced gluconeogenesis (62). Increased levels of plasma aldosterone together with increased plasma AVT concentrations facilitate thermoregulation by increasing renal absorption of water and promoting evaporative cooling (9). Chronic exposure to high temperatures reduces plasma corticosteroid levels (9). Heat stress can disrupt the normal status of reproductive hormones at the level of hypothalamus and can affect the reproductive function of poultry (9).

TRH mediates the release of thyroid hormones, triiodothyronine (T3) and thyroxine (T4), and regulates body temperature and metabolic activity (9, 33, 88). In

chickens increased temperature decreases the secretion of thyroid hormones and heat tolerance is increased as the function of the thyroid is reduced (9, 88). Previous studies reported that plasma T3 levels decreased consistently on prolonged exposure to high temperatures (89) whereas, the plasma T4 levels were inconsistent at high temperatures with studies reporting decrease (90), increase (91) or no change (92, 93).

2.8 Behavioral, Physiological and Molecular Responses to Heat Stress

Birds alter their behavioral, physiological and molecular homeostasis at high temperatures to maintain thermoregulation (9, 94). Feed consumption is reduced and water intake is increased when birds are subjected to high temperatures (3, 9, 80). Feed intake of heat stressed birds is reduced in an attempt to reduce metabolic heat production and thus overall heat load (73, 89). Panting is one of the visible responses of poultry when exposed to heat stress (9, 76). Panting reduces the partial pressure of carbon dioxide and bicarbonate, causing lowered concentration of hydrogen ions and rise in plasma pH, a condition referred to as respiratory alkalosis (9, 76). The feed conversion ratio is increased and the weight gain and the quality of meat are reduced in broilers when exposed to high ambient temperatures (9, 95, 96). Heat stress is also associated with decrease in blood pressure and peripheral resistance and increase in cardiac output (9). The large increase in cardiac output in birds indicates the high demand placed on cardiovascular system during heat stress to dissipate heat (9). Heat stress also disturbs the osmotic balance between the intracellular and extracellular fluids (9).

The structure and physiology of the cells is altered when exposed to high temperatures affecting transcription, RNA processing, translation, oxidative metabolism, membrane structure and function (94, 97, 98). Heat stress increases oxidative stress and disturbs the balance between the reactive oxygen species (ROS) and antioxidant systems

in the broilers (99-101). Change in redox balance due to heat stress impairs breast muscle membrane integrity and leads to pale, exudative meat characteristics in chickens (95). Exposing 14 day old chicks to high ambient temperatures of 35°C for 48hrs increased the oxidative damage of diencephalon (thalamus and hypothalamus) and altered the free amino acid concentrations in the diencephalon and the plasma (94). The altered free amino acid concentrations may contribute to physiological, behavioral and thermoregulatory responses to heat stress (94).

2.9 Heat Shock Proteins

Heat Shock Proteins (HSPs) were first discovered in 1962 (102). These proteins are evolutionarily conserved with respect to both structure and function and are present in all organisms from bacteria and yeast to humans. Expression of these proteins is induced by heat shock and other stressors including oxygen starvation, nutrient deprivation or presence of oxygen radicals (9, 103, 104). HSPs have strong cytoprotective effects and behave as molecular chaperones by binding to other cellular proteins, assisting in intracellular transport and folding, preventing protein denaturation and aggregation of denatured proteins, repairing damaged cellular proteins, and protecting the genome (103, 105-109). HSPs are grouped into various families based on their molecular weights (103) including HSP 60, 70, 90, 110/104, and small HSPs ranging from 16 to 40kDa (103, 110). HSPs are broadly classified into constitutive HSPs, which are present prior to stress exposure and are enhanced by heat treatment and inducible HSPs, which become detectable following stress (104, 110). HSPs are regulated by Heat Shock Transcription factor (HSF) (109) and in avian cells HSP transcription is mediated by HSF1 and HSF3 (111, 112).

Among all the families, HSP70 is the most prominent heat induced protein and has strong cytoprotective effects (110, 113). Several studies have shown that elevated levels of HSP70 following heat shock treatment correlated with the increased thermotolerance (114, 115). HSP 90 regulates the responses to steroid hormones (103, 104) by binding the steroid hormone receptors and rendering them inactive. When steroid hormones are present, HSP90 is displaced, leading to receptor activation (104).

2.10 Embryonic Thermal Conditioning

Several theories have been proposed to sustain thermotolerance and to avoid deleterious consequences of heat stress, of which, one is epigenetic thermal adaptation (29, 116-118). Epigenetic thermal adaptation is based on the assumption that environmental factors, especially ambient temperatures, strongly influence the determination of the 'set point' during critical developmental phases for physiological control systems (designated as 'determination rule') (119). This adaptation can be described as an innate, but non-genetic change, affecting the gene expression patterns during the critical developmental phases (120, 121). It was previously reported that post hatch capacity to adapt to hot environments have been improved by exposing the embryos to high temperatures during incubation period (29, 122, 123). The critical phase, the temperature level and the duration of exposure are the three important parameters that are considered in the approach of embryonic thermal manipulation (TM) (110).

Determination of critical phase of embryogenesis is based on the hypothesis that application of TM during the development/maturation of hypothalamo-hypophyseal-thyroid axis (thermoregulation) and HPA axis (stress response) alters the 'set point' for the acquisition of thermotolerance(116, 121, 124, 125). Recent studies have demonstrated that when intermittent TM of 39.5°C and 65% RH for 12h/d was applied during critical

phase of embryogenesis i.e., between embryonic (E) days E7 and E16, coinciding with the maturation of thyroid and adrenal axis yield improved thermotolerance during post hatch heat stress (116, 124). There was a significant decrease in body temperatures and plasma T3 and T4 concentrations of the hatched chicks(110, 116). This intermittent heat treatment did not have a negative effect on hatchability, chick quality and performance(116, 121). Adopting E7 to E16 as the critical phase in TM improved the thermotolerance of 35d old broilers exposed to 35°C for 4hrs, characterized by lowered levels of plasma corticosterone and reduced mortality (124). Birds which were subjected to TM during embryogenesis (E 12-18) showed significant increase in mRNA levels of HSP90, HSP60 and HSF1 in the muscle, brain and heart tissues, when subjected to thermal challenge at 43°C for 6hrs at day 10 and 28 post hatch (126).

RRAS3, Brain derived neurotropic factor (BDNF) and 14-3-3ε, affects the TM of PO/AH in chickens. RRAS3 belongs to Ras subfamily of small GTP-binding proteins and is mainly expressed in brain (127). It plays an important role in cell proliferation, differentiation and modulation of synaptic functions (110). BDNF activates phosphothyrosine-binding site by binding to Trk-B receptor and initiates signaling via the Ras pathway, which results in gene transduction responsible for neuronal growth and maintenance (110, 128). 14-3-3ε is a chaperone, which plays an important role in phosphorylation and determination of cellular localization in the establishment of thermal control (129). Significant increase in RRAS3 (127), BDNF (128) and 14-3-3ε (129) were detected in chick PO/AH, during TM.

2.11 Hypothalamic Gene Expression in Heat Stressed Chickens

Recently, different researchers have studied the gene expression patterns in hypothalamus under varied heat stress conditions in poultry. High temperatures and

humidity of 31± 1.5°C and 82.0±2.2% respectively, in layer chickens, affected the gene expression of feed intake regulatory peptides including an increase in mRNA levels of ghrelin and CART and decreased mRNA levels of cholecystokinin in the hypothalamus (130). Feed reduction due to heat stress increased the mRNA levels of GnIH in the PVN of the hypothalamus (131). Significant expression of heat shock proteins HSP90 (HSPCB) and HSP40 (DNAJC13) was identified in the hypothalamus, when 56d old female broilers were subjected to acute heat stress at 34°C for 24hrs. In this study, they have also identified differential expression of genes related to hormones, meat quality and growth (PTGS2, FIGF, ACTC1, MYH11, BMP3, and TH) in the hypothalamus (132). Exposing chickens to high ambient temperatures of 37.5°C or 40°C attenuated the number of newborn cells in chicken hypothalamus. These results were predicted to be mediated by the regulation of RELN by miR138 (133).

2.12 Illinois and Ross 708 lines

Domestication of chickens was started initially in Asia about 7000 to 10000 years ago from jungle fowl (134-136). With the commercialization of poultry rearing in the early 20th century, domesticated chickens were subjected to intensive genetic selection for both meat (broiler) and egg (layer) production traits. By the mid 20th century the poultry industry largely focused on breeding chickens for meat consumption (10, 11, 137-139). To improve production, commercial broilers have been selected genetically for rapid growth, high meat yields and high feed efficiency (10, 11, 89, 137). With this genetic selection, the age at which the broilers reached market weight was reduced from 16wks in 1950 to 6 to 7wks by 1990 (137, 140, 141). Similarly, the feed conversion ratio (FCR) was also reduced from 2.34 in 1957 to 1.63 in 2001 (10). This intensive selection provides an excellent platform to understand the effect of human selection on gene

expression patterns and growth properties of broiler chickens. This can be achieved by comparing the gene expression patterns and morphometric differences between commercial broilers and legacy lines (10, 11, 138, 142).

The present study uses the legacy lines that are maintained at the University of Illinois (referred to as Illinois lines). These legacy lines are the progeny of New Hampshire males crossed with females carrying Columbian feather pattern (142). These birds represent the birds used in the poultry industry in the early 1950s. The commercial birds used are the modern Ross 708 line, provided by the local hatchery (142). In the study performed by our lab previously, morphological properties between the Ross 708 and Illinois were compared. By 35day post hatch, the average body weights of Ross and Illinois were 1800g and 1046g respectively. These body weights represented a growth rate of 59g/d in Ross and 32g/d in Illinois birds with Ross birds growing 1.8 times faster than Illinois birds. When breast muscle mass is compared, it constituted 18% of the body mass in the Ross whereas it was only 9% in Illinois birds. The growth rate of breast muscle in Ross birds is 3.8 times faster than that of Illinois. These results indicated that genetic selection increased the growth rates in modern broilers particularly in the breast muscle mass (142). Comparison of gene expression changes in breast muscle tissues of Ross and Illinois birds has identified differential regulation of genes involved in myogenic growth and differentiation (143).

The present study focuses on comparing the hypothalamic transcriptome profiles between chickens subjected to heat stress conditions and controls within Illinois and Ross 708 lines so that important genes involved in heat stress regulation can be identified.

Chapter 3

HYPOTHESIS AND OBJECTIVES

Heat stress is one of the important issues affecting the broiler chicken production. Broiler chickens that are selected for rapid growth rate and high body weights are more susceptible to high ambient temperatures compared to the lines with lower growth rate and body weights. The hypothalamus plays an important role in maintaining thermoregulation and overall energy homeostasis. On the basis of these factors, we hypothesized that comparing the post-hatch hypothalamic gene expression profiles between chickens subjected to heat stress conditions and controls within modern and legacy lines, may lead to identification of genes important for heat stress regulation. For this, we used modern commercial broiler line, Ross 708, and a legacy line, which has the properties of meat birds unselected since 1950s, referred to as Illinois. Illinois line has lower growth rate and body weight compared to the Ross 708 line. This study consists of following three objectives:

1) Study the effect of heat stress conditions on growth rates in Ross and Illinois lines
2) Identify the genes important for heat stress response by comparing the hypothalamic gene expression profiles within modern and legacy chicken lines using the transcriptomic data.
3) Validate the results for selected genes from objective two using Quantitative reverse transcription Polymerase chain Reaction (qRT-PCR)

Chapter 4

MATERIALS AND METHODS

4.1 Animal Rearing, Experimental Set-up and Tissue Collection

Illinois and Ross 708 eggs were obtained from University of Illinois and Mountaire farms in Delaware respectively. After hatch, the female chicks were culled to minimize the gender effects. Chickens were equally divided and raised in two different houses on the University of Delaware farm. Chickens in one house are subjected to Heat Stress temperatures while those in other house were maintained at normal temperatures Ross 708 and Illinois birds subjected to same treatment were raised in same house. The birds were provided with *ad libitum* access to feed and water.

At hatch, chickens in both the houses were maintained at 37°C and the temperatures were gradually brought down to 25°C by 21 day (D) post hatch, with about 4°C reduction in temperature per week. After D21, the birds in Heat Stress (HS) group were subjected to a temperature of 41°C for 10hrs daily, mimicking heat wave conditions, and the remaining time they were maintained at 25°C until D42 post hatch. Control (C) birds were maintained at a temperature of 25°C throughout the study.

The birds were euthanized by cervical dislocation, the body weights of Ross and Illinois chickens were taken and the hypothalamic tissue samples were collected at D28 and D42 post hatch. The samples of the D28 chickens used in this study were from the trail conducted in the fall-2013 while the D42 samples were from fall-2012 trial The samples were flash frozen in liquid nitrogen and then stored at -80°C until total RNA isolation.

4.2 Total RNA isolation

Total RNA was isolated using Qiagen RNeasy Mini Kit as per the manufacturer's instructions. Approximately 30mg of Hypothalamic tissue was used for RNA isolation. The number of samples used for RNA isolation at each time point and for each line is summarized in Table2. Nanodrop ND-100 spectrophotometer was used to assess the concentration of RNA and the overall RNA quality was assessed using Agilent 2100 BioAnalyzer. All the samples have the RNA Integrity Number (RIN) of 8 or more, are used for the construction of RNA-seq libraries.

Table 2: Total number of HS and C hypothalamic samples used for RNA extraction at D28 and D42 time points for Ross and Illinois lines

DAY	LINE	NUMBER OF HEAT-STRESS SAMPLES	NUMBER OF CONTROL SAMPLES	TOTAL NUMBER OF SAMPLES
28	ROSS	8	7	15
28	ILLINOIS	5	6	11
42	ROSS	7	7	14
42	ILLINOIS	5	5	10

4.3 cDNA Synthesis and RNA-seq Library Preparation

For each sample, from 4µg of total RNA, mRNA was purified using magnetic oligo-dt beads. From these purified mRNA samples, cDNA synthesis followed by transcriptomic library construction was performed using Illumina TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) as per the manufacturers instructions.

4.4 Sequencing and Alignment

HiSeq 2000 Sequencing System (Illumina) at the Delaware Biotechnology Institute's Sequencing and Genotyping Centre (Newark, DE) was used to sequence the prepared libraries. All libraries were sequenced at a depth of ~30 million reads per library. Sequence reads were mapped against the Galgal4 version (2011) of the chicken reference genome sequence using Tuxedo suite and the transcript levels were quantified in reads per kilobase of gene per million mapped reads (RPKM).

4.5 Differential Gene Expression Analysis of the Transcriptomic Data

Transcriptional data analysis was performed using JMP software (JMP Pro 11). Genes with mean RPKM value greater than 0.1 were considered as this threshold corresponds to the results when assayed by qRT-PCR. Log_2 transformation of the ratio between the means of the RPKM values of HS and C chickens were determined at D28 and at D42 for both Ross 708 and Illinois birds. Genes with $\log_2$ values ≥ 1 and ≤ -1 were considered for further analysis, as they show more than two fold changes in expression. On these genes, paired t-test was performed and genes with p-value ≤ 0.05 are identified as differentially expressed. Gene ontology (GO) analysis of these differentially expressed genes is done using Panther (144) and pathway analysis is performed using KEGG (145), and PathRings. PathRings use the information available for human orthologous of chicken proteins in the Reactome database to visualize and identify the biological pathways, genes encoding the rate-limiting enzymes as well as cross talking pathways. This tool uses the Fischer Exact test to identify the significantly enriched pathways (146).

4.6 Body Weight Analysis

The body weights (BW) between HS and the C chickens at D28 and D42 were analyzed using one-way ANOVA for both the lines.

4.7 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

To confirm the results obtained from Illumina sequencing, probe-based qPCR was performed on nine selected genes including genes that encode rate-limiting enzymes, neuropeptides and hormones. From the previously isolated hypothalamic RNA samples, complementary DNA (cDNA) was synthesized using the Superscript II first strand synthesis kit (Invitrogen). The quantity and the quality of the cDNA were assessed using Qubit 2.0 Flurometer (Life Technologies). PCR-specific primers and probes for the target genes were designed using Primer Express 3.0 software (Life Technologies). BLAT (147) was used to check for specific mapping of the primers and probes to the targeted genes in the chicken genome. To construct standard curves for quantification, custom oligonucleotides were designed for each primer-probe set. The sequence of custom standards consisted of forward primer, probe and reverse complement of the reverse primer each separated by a single nucleotide. Probe qPCR was performed using QuantiTect probe qPCR kit (Qiagen) and 7500 Fast Real Time PCR System (Applied Biosystems). For statistical analysis, two-sample t-test was performed to identify the significance. Primers and probes used in qPCR were summarized in Table 3.

Table 3: Probe and the primer sequences used for qRT-PCR

Gene	Primers and Probes	Gene Accession number
AVP	For- TCCGGGCACACTCAGCAT Rev-ATGTAGCAGGCGGAGGACAA Probe-AGAGCCTTCGCTGCCCCTCTCCTT	NM_205185.2
LPL	For- TGGTCCCACCTTTGAGTATGC Rev-TGCAGGACATCCACAAAGTCA Probe-ATGCCCCTATCCGCCTCTCCCC	NM_205282.1
PPP1R9A	For- GCCAGGTTAACAATAACAACAACATATT Rev- TCCAGACAGGAGACTTGCTTAGTATC Probe- TTGGAGAAGTTTCTAGAGGAG	XM_418668.4
PTGS1	For-GCTGCGGCACAAATTTGAA Rev-TGAAGAACTGGTGGGTGAAATG Probe-CACCAACTTGATGTTTGCCTTCTTCGCT	XM_425326.4
SREBF1	For- CTTTGTCTTCCTCTGCCTCTCTTT Rev- CAGCCATGATGCTTCTTCCA Probe- CTCCGTGGCTCCAGTGCCCC	NM_204126.1
SS2	For-GCCAGCCTGGTGTCTGTTCT Rev-TCTGTAACGCGAGTCTCTCTTCTC Probe-CGTGAGAGCCACTGCGCTGCC	NM_204455.1
TRH	For-GCAGAAAATCACAATGCCATCTAT Rev-GTTGAGGCAAACACCAGACAAG Probe-AGCTGCCAGTGCTACTCCTTTGCCTGA	AJ703806.2
NMU	For-TGCGCGTCTGCAAAGGT Rev-TCTCCTTCCACAGCTGCAGTT Probe-CCCGATGCCGTCCCAAGCG	NM_001277921.1
PPARG	For- CACTGCAGGAACAGAACAAAGAA Rev- TCCACAGAGCGAAACTGACATC Probe- CAATTCGCATTTTCCA	NM_001004600.1

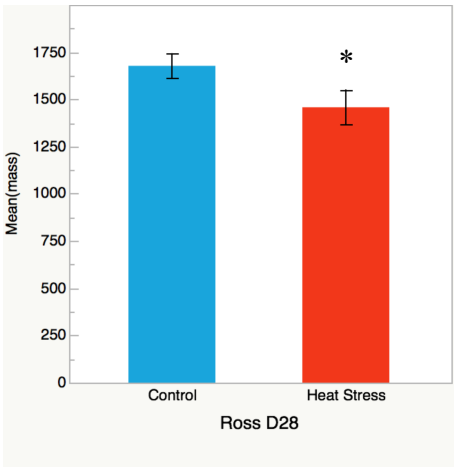
Chapter 5

RESULTS AND DISCUSSION

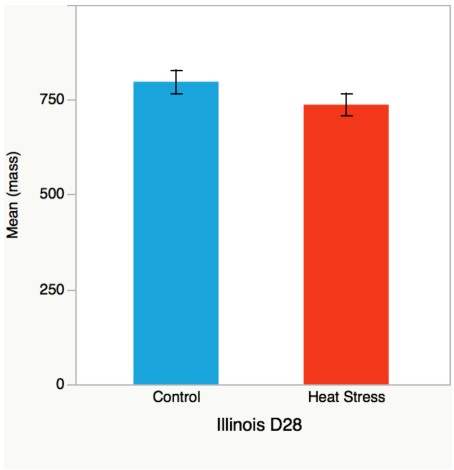
5.1 Analysis of Body Weights

The body weights of the HS and C birds in both the lines were compared at D28 and D42. At D28 and D42, the mean body weights of HS birds in both Ross 708 and Illinois lines were lower than the control birds. However, only the body weights of HS chickens in Ross 708 were significantly lower compared to the controls at both the time points (fig 2)

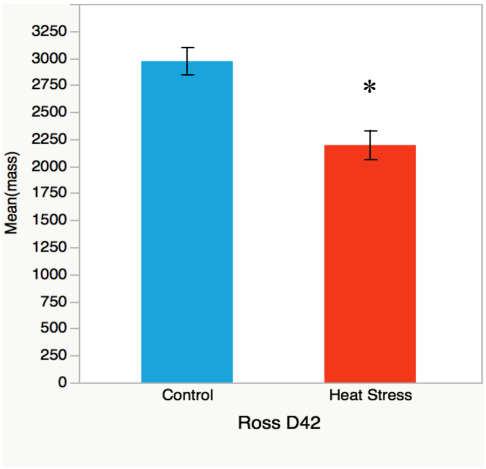
a



b



c



d

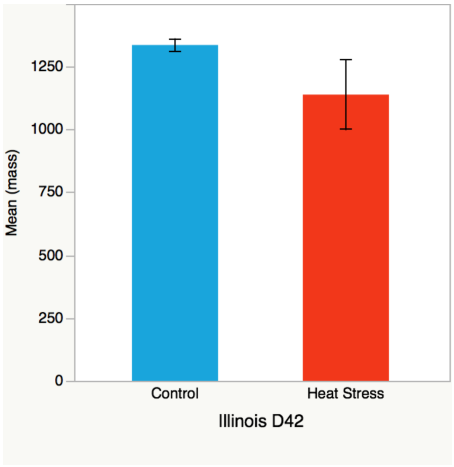


Figure 2: Comparison of body weights between Heat Stress and Control chickens in Ross 708 and Illinois lines (a-d). Bar graphs represent the mean value of body weights (in grams) for each condition. Error bars represent 1SEM. Ross D28 HS n = 12, C n = 11, Illinois D28 HS n = 12, C n = 10, Ross D42 HS n = 12, C n = 12, Illinois D42 HS n = 5, C n= 6. * indicates significant at $p < 0.05$.

5.2 Differential Expression of the Hypothalamic Genes

To identify the gene expression changes in the hypothalamus in response to heat stress, a total of 13,642 genes were analyzed from both Ross 708 and Illinois birds. RPKM values of all the genes for HS and C birds in Ross 708 and Illinois were compared and figure 3 shows the relationship between the genes at each time point. Of these, the number of genes that have RPKM > 0.1 at each time point in both the lines were summarized in Table 4.

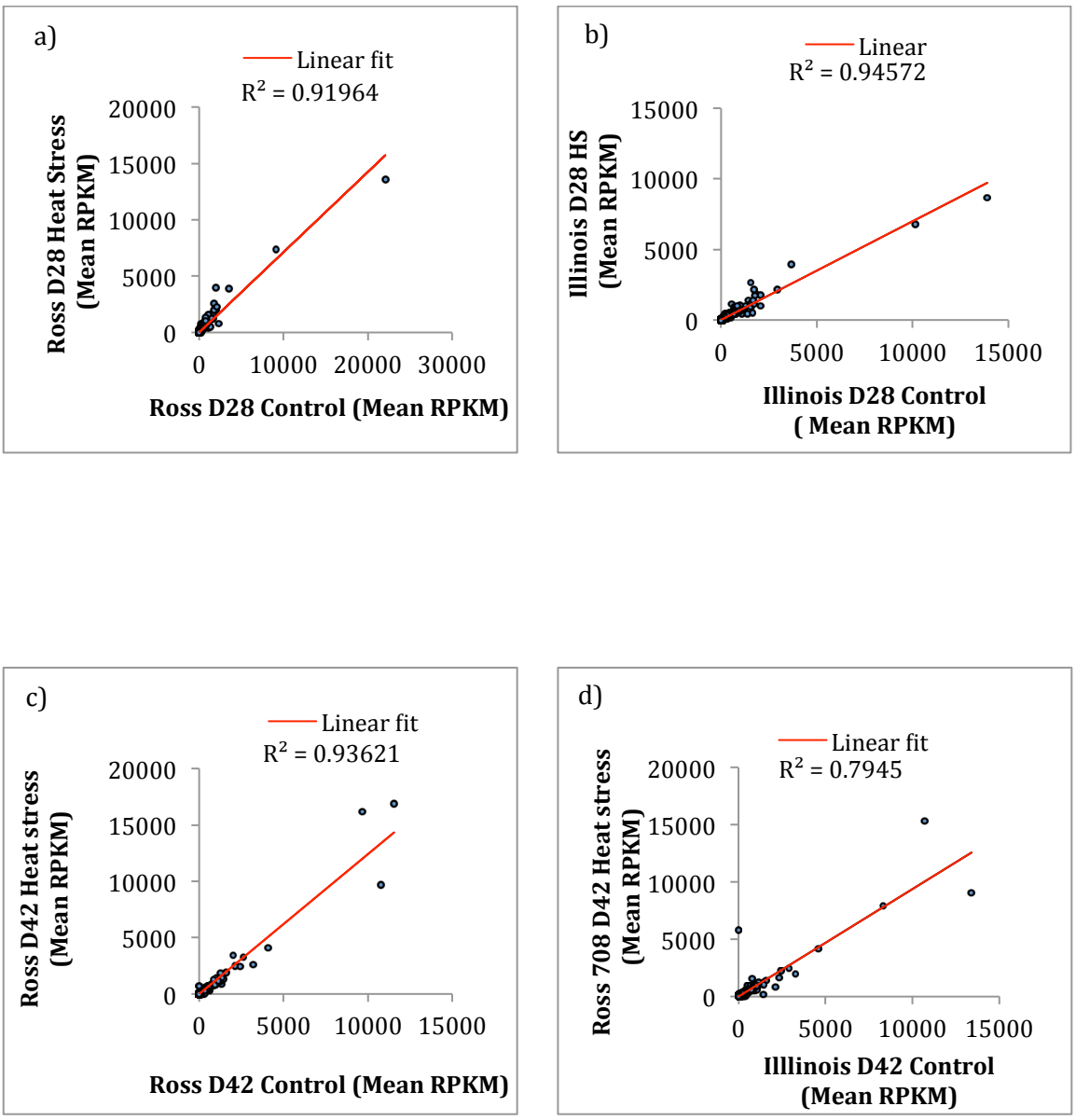


Figure 3: Scatter plot comparison of expression level (RPKM) of all the genes between Heat Stress vs. Control chickens for Ross and Illinois lines at D28 and D42 post hatch (a-d). The expression levels of all the 13,642 genes were compared. R^2 and the line for best fit were included.

Table 4: Total number of genes with RPKM > 0.1 at D28 and D42 in both the lines.

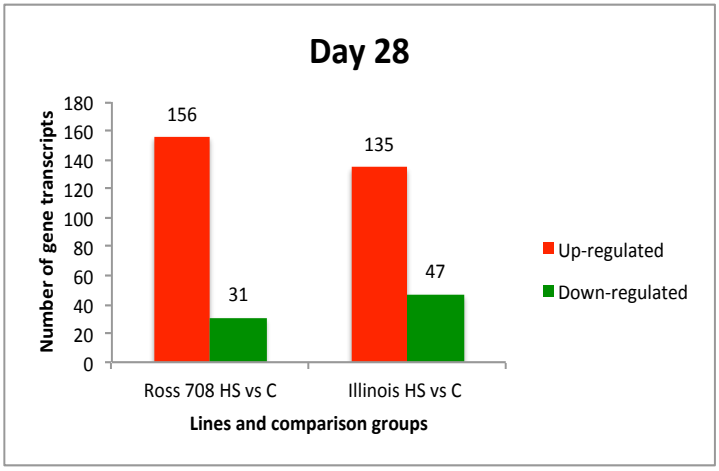
Lines	D28	D42
Ross 708	12,077	12,101
Illinois	12,043	12,113

Gene expression profiles were compared between HS vs. C groups at D28 and D42 for both the lines. The total number of differentially enriched genes for HS vs. C in both the lines at D28 and D42 were summarized in Table 5. Fig 4 shows the number of gene transcripts that were up- and down-regulated in HS vs. C at D28 and D42 for both the lines.

Table 5: Total number of differentially enriched genes for HS at D28 and D42.

Lines	Day 28	Day 42
Ross 708	187	91
Illinois	182	393

a.



b.

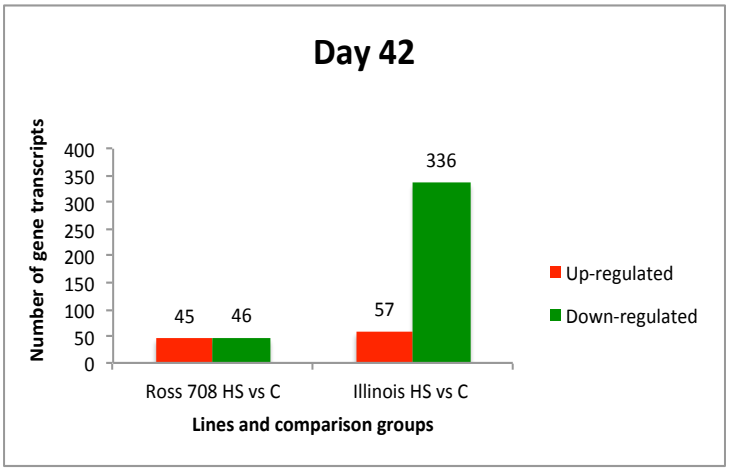
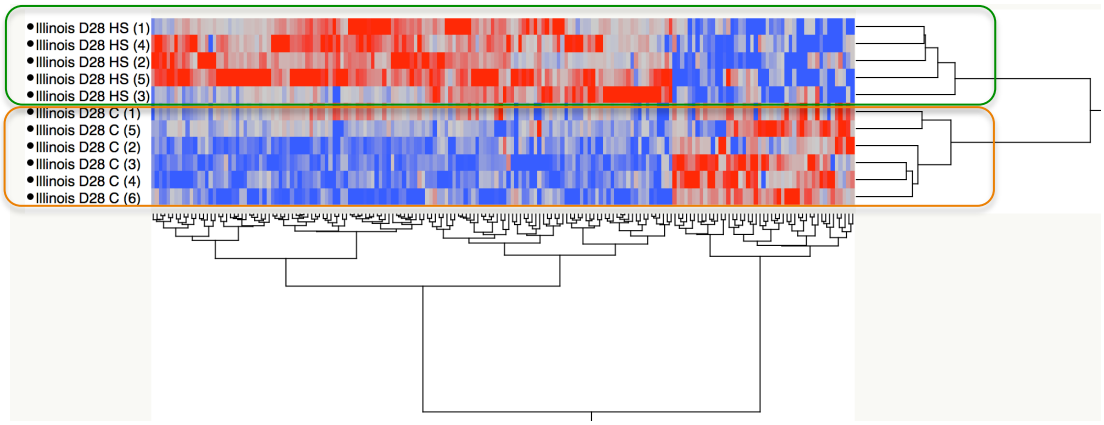


Figure 4: Total number of differentially expressed genes at D28 (a) and D42 (b) in Ross 708 and Illinois line. Bar graphs represent the total number of differentially expressed genes in each condition. Fold change ≥ 2, HS Heat stress, C control.

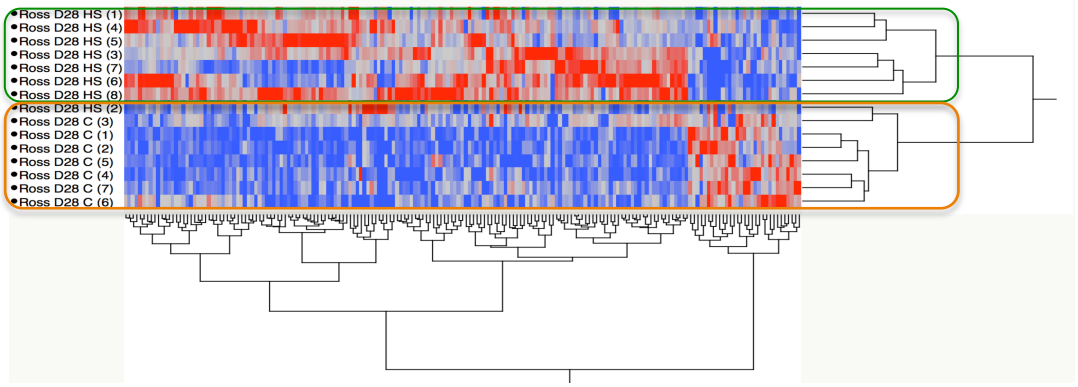
5.3 Hierarchical Clustering of the Differentially Expressed Genes

Hierarchical clustering of the differentially enriched genes was performed for HS vs. C in Ross 708 and Illinois lines at both the time points to study the expression pattern. Heat maps indicated that heat stress and control groups were different from each other in both the lines at D28 (fig 5a-b) and D42 (fig 5c-d).

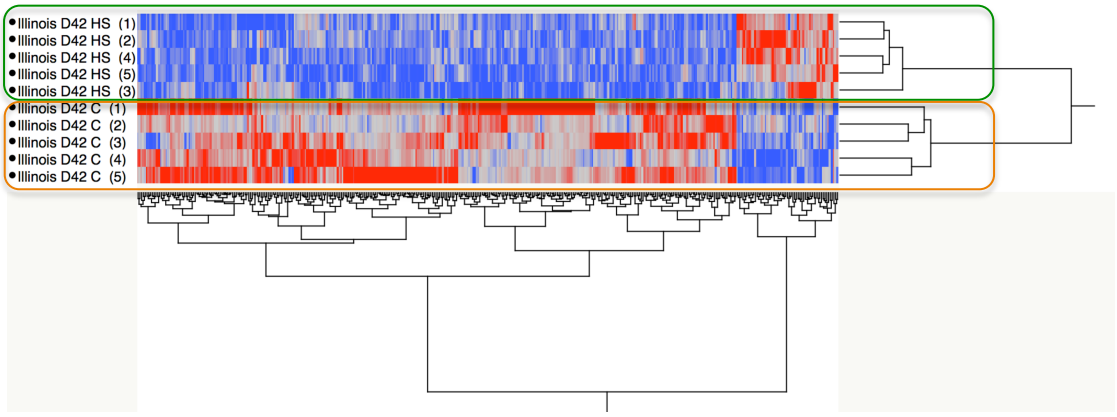
a)


Illinois D28 HS (1)
Illinois D28 HS (4)
Illinois D28 HS (2)
Illinois D28 HS (5)
Illinois D28 HS (3)
Illinois D28 C (1)
Illinois D28 C (5)
Illinois D28 C (2)
Illinois D28 C (3)
Illinois D28 C (4)
Illinois D28 C (6)

b)


Ross D28 HS (1)
Ross D28 HS (4)
Ross D28 HS (5)
Ross D28 HS (3)
Ross D28 HS (7)
Ross D28 HS (6)
Ross D28 HS (8)
Ross D28 HS (2)
Ross D28 C (3)
Ross D28 C (1)
Ross D28 C (2)
Ross D28 C (5)
Ross D28 C (4)
Ross D28 C (7)
Ross D28 C (6)

c)


Illinois D42 HS (1)
Illinois D42 HS (2)
Illinois D42 HS (4)
Illinois D42 HS (5)
Illinois D42 HS (3)
Illinois D42 C (1)
Illinois D42 C (2)
Illinois D42 C (3)
Illinois D42 C (4)
Illinois D42 C (5)

d)

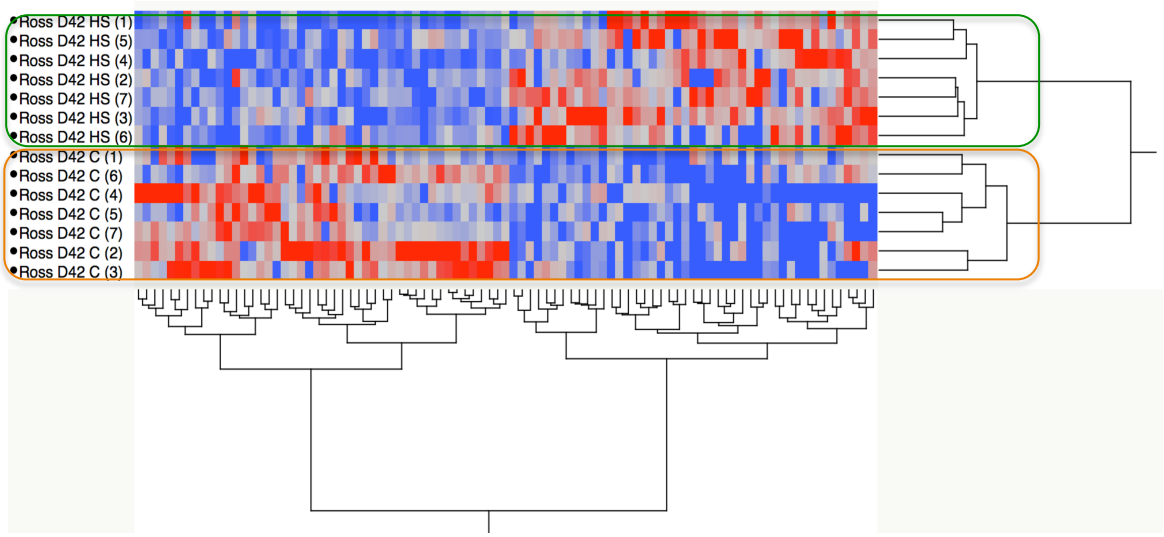
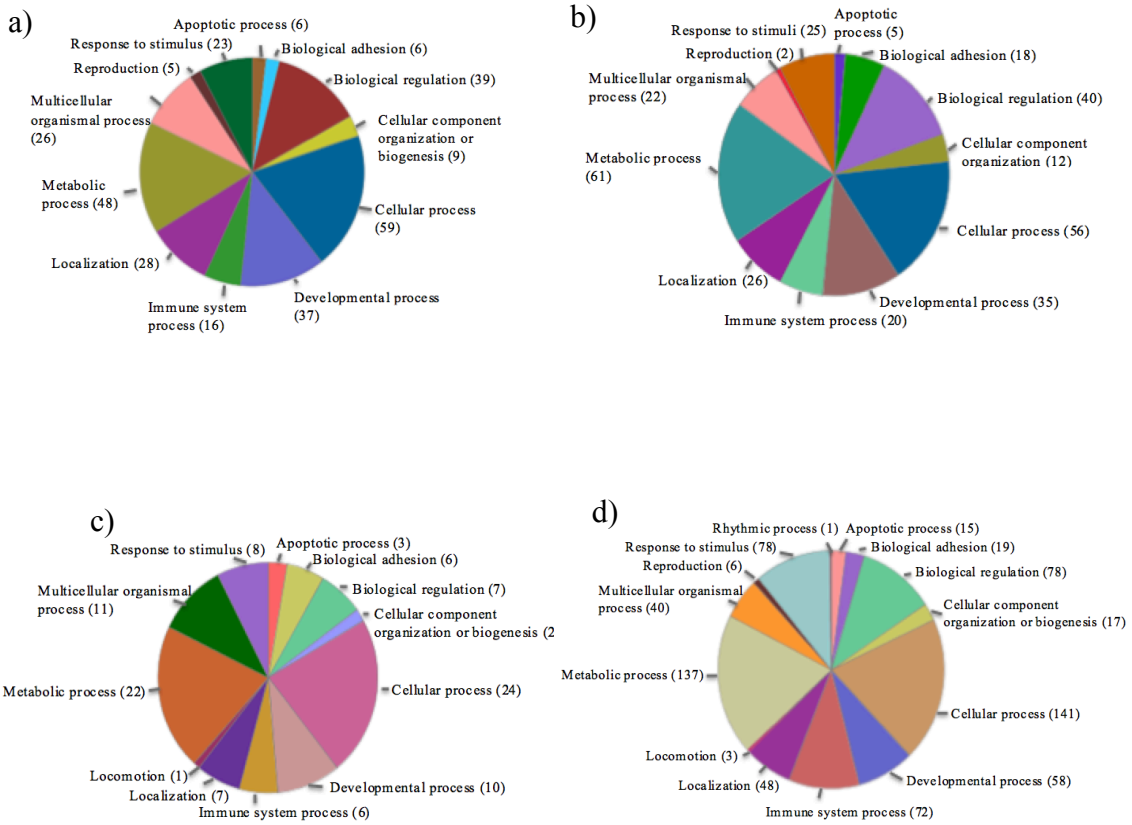


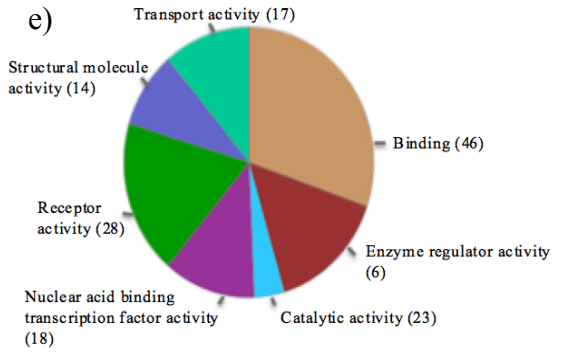
Figure 5: Hierarchical clustering of significantly enriched genes. Hierarchical cluster was performed on the differentially enriched genes at D28 and D42 for Ross 708 and Illinois-a) Illinois D28 b) Ross 708 D28 c) Illinois D42 d) Ross 708 D42. Dendrograms are in a distance scale.

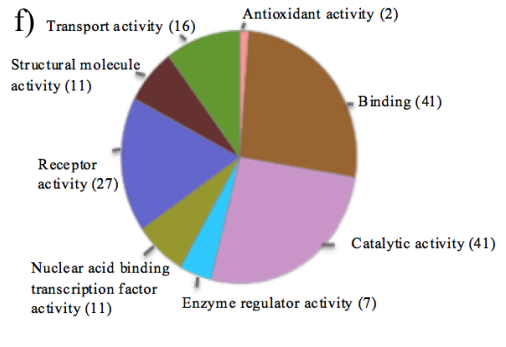
5.4 Gene Ontology (GO) Analysis of Differentially Expressed Genes

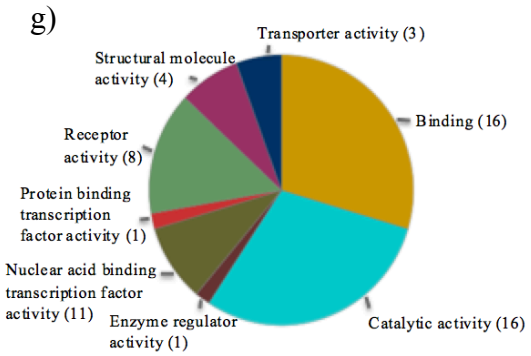
GO analysis is performed to evaluate the function of differentially expressed genes. Important biological process that is unique for Ross and Illinois at D28 and D42 (fig 6a-d) includes metabolic process, immune system process, cellular process, developmental process, biological adhesion and biological regulation. Several important molecular functions that were uniquely affected are nucleic acid binding transcription factor activity, receptor activity, catalytic activity, enzyme regulator activity, structural

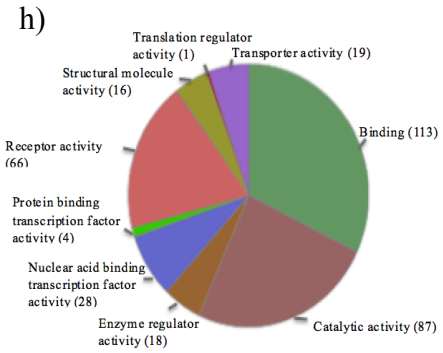
molecule activity and binding (fig 6e-f). Finally, cellular components including extracellular region and cell part were observed in both the lines at D28 and D42(fig 6i-l).

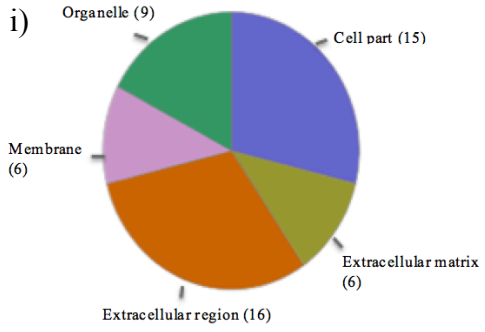


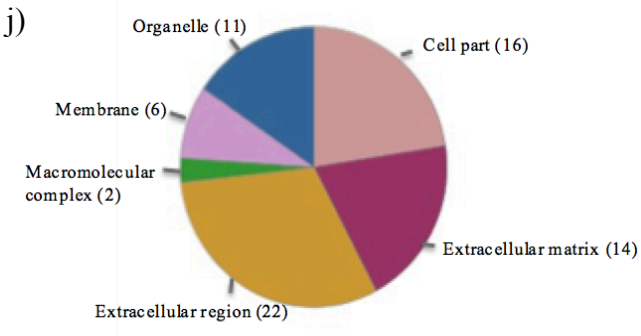

e)
Transport activity (17)
Structural molecule activity (14)
Binding (46)
Receptor activity (28)
Enzyme regulator activity (6)
Nuclear acid binding transcription factor activity (18)
Catalytic activity (23)


f)
Transport activity (16)
Antioxidant activity (2)
Structural molecule activity (11)
Binding (41)
Receptor activity (27)
Catalytic activity (41)
Nuclear acid binding transcription factor activity (11)
Enzyme regulator activity (7)


g)
Transporter activity (3)
Structural molecule activity (4)
Binding (16)
Receptor activity (8)
Protein binding transcription factor activity (1)
Nuclear acid binding transcription factor activity (11)
Enzyme regulator activity (1)
Catalytic activity (16)


h)
Translation regulator activity (1)
Transporter activity (19)
Structural molecule activity (16)
Binding (113)
Receptor activity (66)
Protein binding transcription factor activity (4)
Nuclear acid binding transcription factor activity (28)
Enzyme regulator activity (18)
Catalytic activity (87)


i)
Organelle (9)
Cell part (15)
Membrane (6)
Extracellular matrix (6)
Extracellular region (16)


j)
Organelle (11)
Cell part (16)
Membrane (6)
Macromolecular complex (2)
Extracellular matrix (14)
Extracellular region (22)

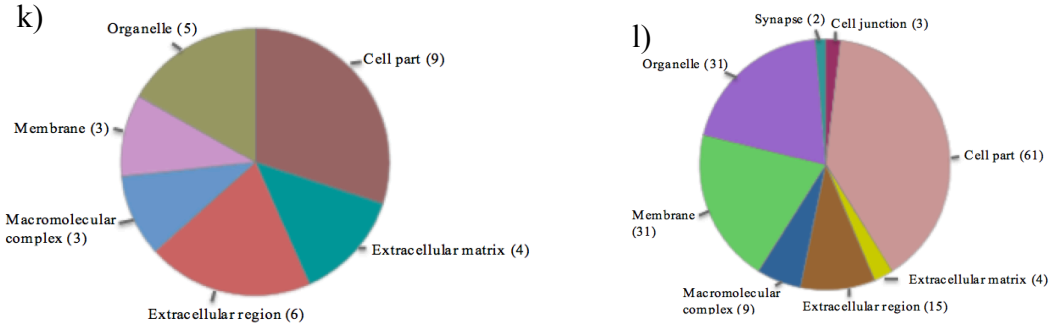
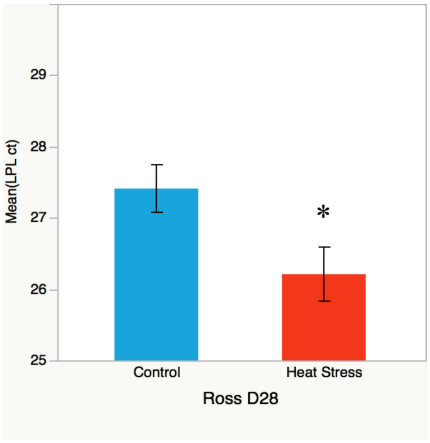


Figure 6: Gene ontology terms identified for Ross 708 and Illinois lines at D28 and D42 showing biological process (a-d), molecular function (e-h) and cellular component (i-l). Chart labels are GO terms followed by number of genes associated with the GO term

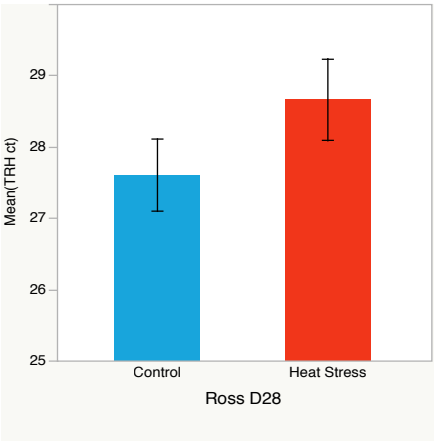
5.5 Validation of Probe-based qRT-PCR Results

Probe-based PCR was performed on nine selected genes that included genes encoding rate-limiting enzymes from both Ross 708 and Illinois line. Among the nine tested genes, five differentially expressed genes were selected from D28 of Ross 708 line (AVP, LPL, NMU, SREBF1, and TRH); three from D28 of Illinois line (PTGS1, PPARG, and PPP1R9A); one from D42 Illinois (SS2). The qRT-PCR results showed the same expression pattern for the eight selected genes out of nine although the fold change differed for the two methods (fig 7, 8). For PPARG, we were unable to validate the expression pattern with the primer-probe sets that we designed.

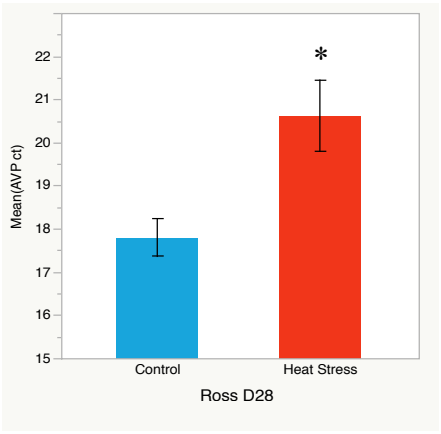
a


Mean(LPL ct)
29
28
27
26
25
*
Control
Heat Stress
Ross D28

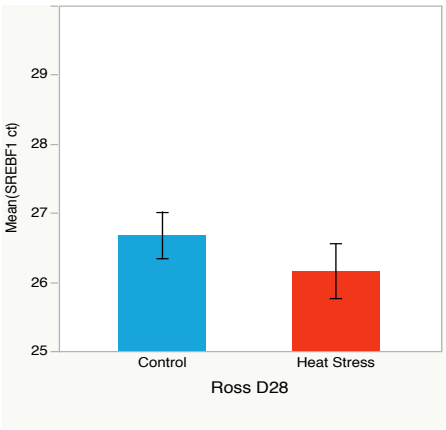
b


Mean(TRH ct)
29
28
27
26
25
Control
Heat Stress
Ross D28

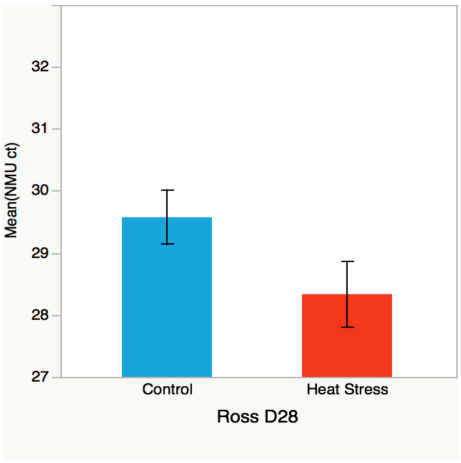
c


Mean(AVP ct)
22
21
20
19
18
17
16
15
*
Control
Heat Stress
Ross D28

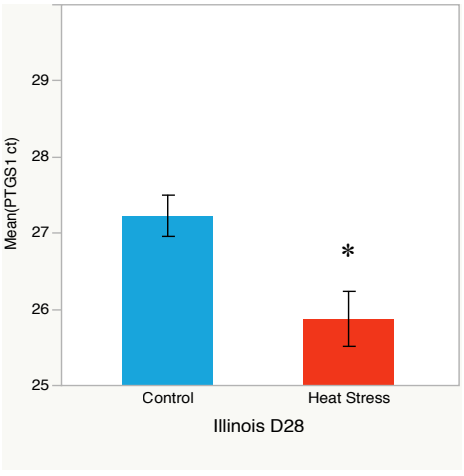
d


Mean(SREBF1 ct)
29
28
27
26
25
Control
Heat Stress
Ross D28

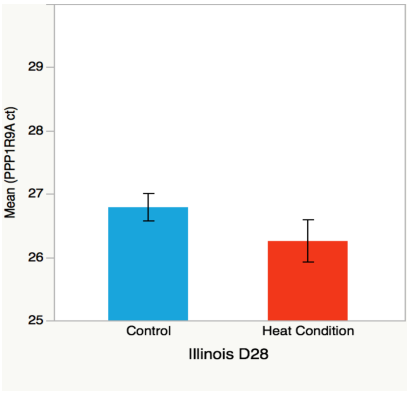
e


Mean(NMU ct)
32
31
30
29
28
27
Control
Heat Stress
Ross D28

f


Mean(PTGS1 ct)
29
28
27
26
25
*
Control
Heat Stress
Illinois D28

g



h

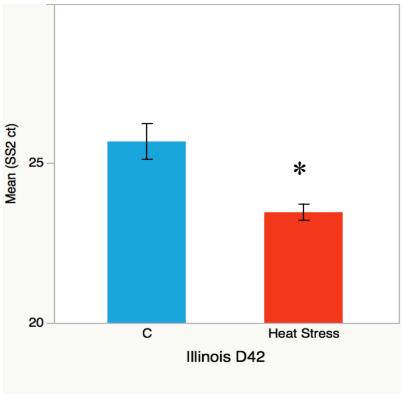


Figure 7: Probe-bases qPCR verification of differentially enriched genes between heat stress and Control hypothalami samples at D28 and D42. Bars represent mean ct-value for each condition of a given gene. Errors bars represent 1 SEM. Ross 708 D28 HS n = 7 C n = 7; Illinois D28 HS n = 5 C n = 6; Illinois D42 HS n = 5 C n = 5. * indicates significance at $p < 0.05$

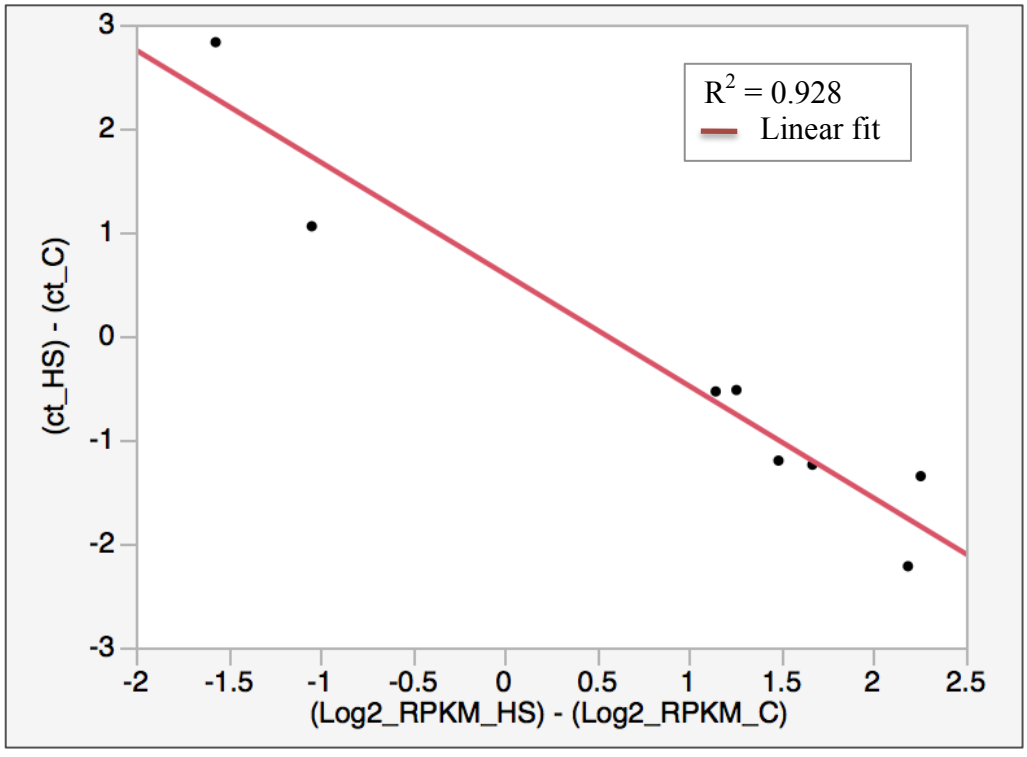


Figure 8: Correlation of the relative expression of selected genes determined by qRT-PCR and RNA-seq (RPKM)

5.6 Pathway Analysis of the Differentially Expressed Genes at D28 Post Hatch

The differentially enriched genes, which have a fold change of ≥ 2, were subjected to pathway analysis using PathRings and the significant pathways were identified which have p ≤ 0.05. Metabolism of lipids and lipoproteins, thyroxine biosynthesis, signaling by G-protein coupled receptors are the significant pathways that are differentially regulated in Ross 708 line at D28 (fig 8). Arachidonic acid metabolism, nucleotide metabolism and signaling by G-protein coupled receptors are the significant pathways that were differentially expressed in Illinois line at D28 (fig 9). The list of the genes that were differentially expressed in the above pathways for Ross 708 and Illinois at D28 were summarized in the Appendix A.

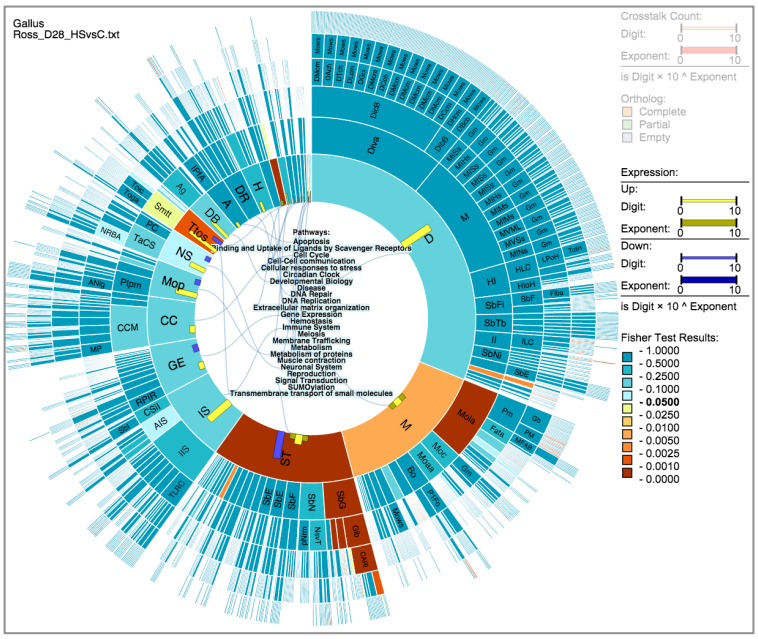


Figure 9: Pathway analysis of differentially expressed genes in Ross 708 at D28 using PathRings.

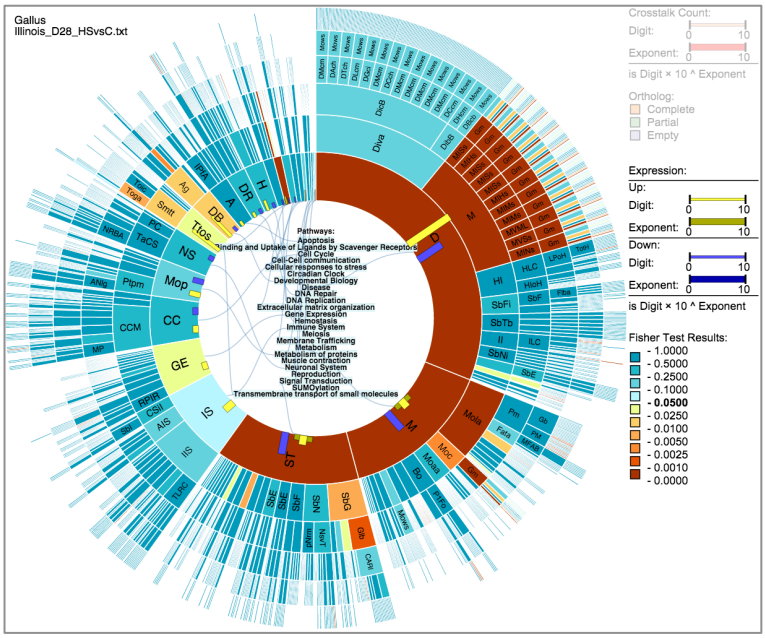


Figure 10: Pathway analysis of the differentially expressed genes in Illinois at D28 using PathRings.

5.6.1 Differentially Expressed Genes in Ross 708 at D28

Two genes encoding rate-limiting enzymes, lipoprotein lipase (*LPL*) and sterol regulatory element binding transcription factor 1 (*SREBF1*), were significantly up-regulated by heat stress in Ross 708 at D28. LPL encodes the rate-limiting enzyme that hydrolyzes the triglyceride (TG) core of the TG-rich lipoproteins, chylomicrons and very-low density lipoproteins (VLDLs) and produces chylomicron remnants and intermediate density lipoproteins (148). It was reported that LPL is also synthesized and expressed in the CNS neurons (149, 150). LPL regulates the metabolism of TG- rich glycoprotein and cellular uptake of n-3 polyunsaturated fatty acids (PUFA), thereby providing signals to the hypothalamus in regulating the expression of orexigenic neuropeptides (AgRP/NPY) and thus affecting feed intake(150). Recent studies in mice indicated that homozygous (NEXLPL -/-) as well as heterogeneous (NEXLPL +/-) knock out of LPL increased the

body weights of these mice, which is attributed to LPL specific decrease in PUFA levels and increased gene expression of orexigenic neuropeptides in the hypothalamus (148, 150). Based on this, we hypothesize that increased LPL in the hypothalamus may lead to increased uptake of PUFA leading to enhanced brain lipid sensing thereby decreasing the expression of orexigenic neuropeptides and feed intake (fig 10).

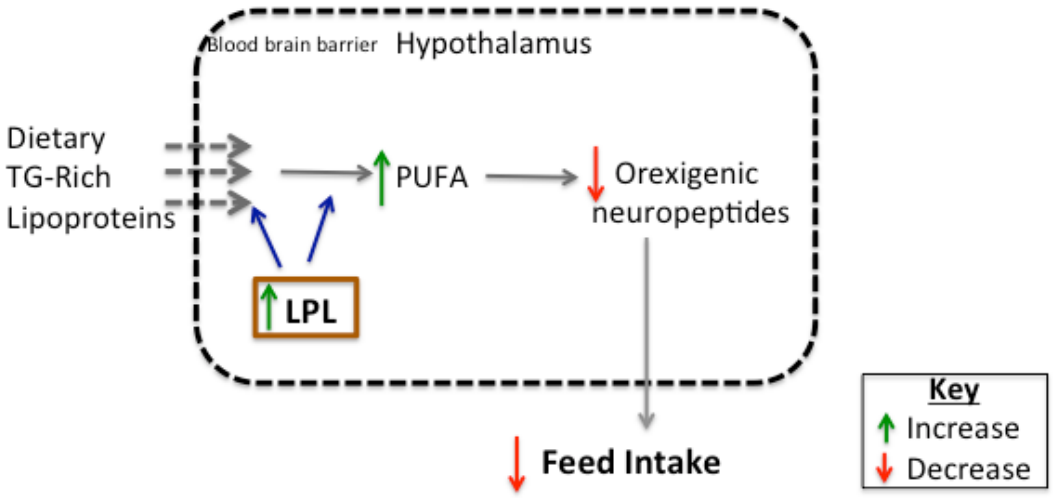


Figure 11: Hypothetical mechanism of regulation of feed intake due to increased LPL mRNA levels in hypothalamus during heat stress in Ross 708 chickens. At D28, significant up-regulation of LPL transcript levels is observed in Ross 708 line during HS. Figure modified from Wang et al. 2011, Cell metabolism (150).

SREBF1 is a membrane bound transcription factor, which belongs to a family of sterol regulatory element binding proteins(151). It controls the expression of genes including acetyl co-A carboxylase *(ACC)* and fatty acid synthase *(FAS)*, which are mainly involved in regulating fatty acid homeostasis(151, 152). Studies have shown the presence of mRNA of SREBF1 in lipogenic as well as in non-lipogenic tissues (brain,

kidney, muscle) of chickens (153). Furthermore, we have also seen significant up regulation of acetyl-CoA carboxylase beta *(ACACB)*, an isoform of ACC, in the hypothalamus of Ross 708 line at D28. ACACB catalyzes the conversion of acetyl co-A to malonyl co-A in the fatty acid metabolism (154-156). High hypothalamic concentrations of malonyl co-A inhibits the activity of carnitine palmitoyl co-A transferase-1 *(CPT1)*, a transport protein located in the inner mitochondrial membrane that regulates the uptake of long chain fatty acids into the mitochondria to undergo β-oxidation. Inhibition of CPT1 by increased malonyl co-A thereby increases the accumulation of long chain acyl co-A in the cytosol (154-156). Increase in cellular levels of malonyl co-A and long chain acyl co-A causes increased expression of anorexigenic neuropeptides (POMC) and decreased expression of orexigenic neuropeptides in the hypothalamus (154, 156).

In chickens as well as in mammals, hypothalamic regulation of feed intake is mediated by adenosine monophosphate activated protein kinase *(AMPK)* pathway and the housekeeping enzymes involved in this pathway include AMPK, ACC, FAS and malonyl co-A decarboxylase *(MCD)* (155-157). At the hypothalamic level, AMPK plays a key role in regulation of appetite control (158). A variety of conditions that include heat shock, insulin, ghrelin, leptin, and fasting/re-feeding cycles can affect the AMPK activity in the hypothalamus (154, 158). In the absence of AMPK activity, ACC is not phosphorylated and remains active. Active ACC increases the cellular malonyl co-A levels leading to the inhibition of CPT 1 and fatty acid oxidation, which ultimately results in decreased feed intake (159). Multiple lines of evidence suggest that AMPK phosphorylates and decrease the expression of SREBF1(160-162). In addition, loss of AMPK activity leads to increased levels of SREBF1 in the hypothalamus (162).

Based on these observations, it is hypothesized that, in Ross 708 chickens, heat stress increases the mRNA levels of SREBF1 and ACACB in hypothalamus, which leads to increased malonyl co-A levels and subsequent decrease in feed intake and body weights (fig 11). Phenotypically, these results correlate when body weights were compared between the HS and C chickens. There is a significant decrease in bodyweights in HS chickens compared to the controls at D28 in Ross 708 birds (fig 2a).

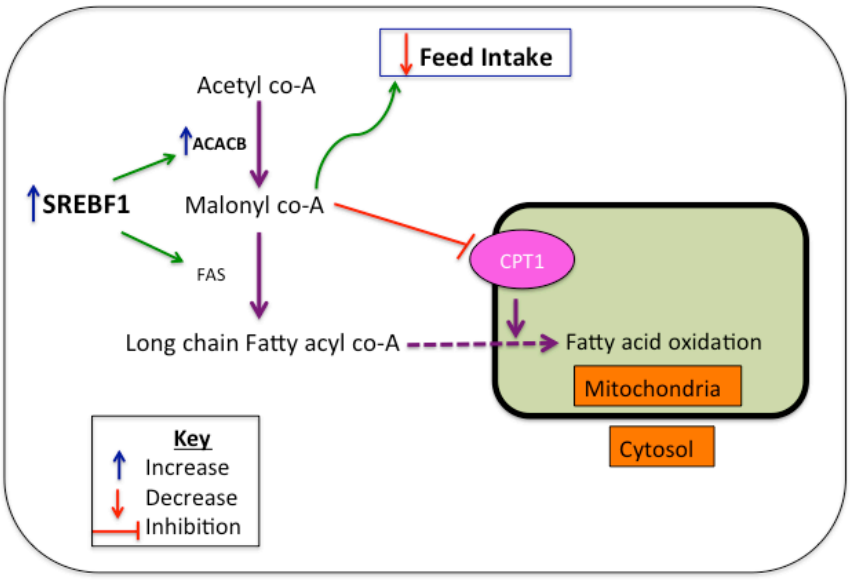


Figure 12: Probable mechanism of regulation of SREBF1 and ACACB on hypothalamic feed intake during heat stress in Ross 708 chickens at D28. Significant up-regulation of mRNA levels of SREBF1 and ACACB were observed in Ross 708 line during Heat stress at D28. Figure modified from Lopez *et al*. (2007) (155)

D28 Ross samples showed enrichment of two gene transcripts in the hypothalamus during heat stress involved in thyroxine (T4) biosynthesis that includes thyroid stimulating hormone beta *(TSHB)* and its binding partner glycoprotein hormones, alpha polypeptide *(CGA)*. Both these genes were thought to be restricted to the pituitary

gland (163), however their gene expression was also observed in the hypothalami of mammals (164) as well as in chickens (165, 166). Biological activity of TSH requires non-covalent binding of TSHB and CGA (163). TSH stimulates the release of T4 from the thyroids (33). Therefore, increased transcript levels of TSHB and CGA likely increases the circulating levels of T4 hormones in Ross chickens during heat stress at D28. Previous studies indicate that plasma T4 levels were inconsistent in response to heat stress with studies reporting decrease (90), increase (91) or no change (92, 93). Conversely in our study, down-regulation of TRH, which is a potential secretagogue of TSH and GH release from pituitary gland (33), is observed during heat stress. It was reported that thyroid hormones can negatively feedback TRH release from the hypothalamus (167). Probably increase T4 levels may be responsible for down regulation of TRH in the hypothalamus of Ross heat stress chickens.

The neuropeptide, neuromedin U *(NMU)* is significantly enriched in HS at D28 in Ross chickens. NMU is a brain-gut neuropeptide in chickens. In both layers and broilers, ICV administration of NMU suppressed feed intake (168, 169). In broilers, CRH is proposed to act as a downstream regulator of anorexigenic action of NMU(168).

Another gene, 5-hydroxytryptamine (serotonin) receptor 1E *(HTR1E)* that is involved in regulation of feed intake, is significantly enriched during heat stress in the hypothalamus of Ross 708 chickens. Of the fourteen 5-hydroxytryptamine (serotonin) (5-HT) receptors that are expressed in the hypothalamus, only HTR1E is differentially expressed in our study. 5-HT is one of the abundant neurotransmitter distributed throughout the brain as well as in several peripheral organs (170). In birds, 5-HT receptors identified in the hypothalamus regulate feeding behavior (171, 172). Studies indicate that 5-HT receptors induce inhibition of feed intake in chickens (172).

Significant down-regulation of gene transcripts, arginine vasotocin *(AVT)* and mesotocin *(MT)*, were observed in heat stress in Ross 708 line. AVT is a principle antidiuretic hormone and also functions in heat dissipation. MT gene encodes the precursor protein that is processed to produce mesotocin. During acute heat stress plasma AVT concentrations increased without change in plasma osmolality (9, 86) while circulating levels of MT are reduced during heat stress (86). However, in our study at D28, where the chickens were subjected to a chronic heat stress for seven days, significant down-regulation of both AVT and MT were observed. This may be an acclimatization response on the part of Ross chickens to chronic heat stress.

Ross D28 hypothalamus also showed enrichment of Bradykinin receptor B1 *(BDKRB1)* with a fold change of 29.4. BDKRB1 gene encodes Bradykinin receptor B1 that belongs to a family of G-protein coupled receptor. B1 receptors are rapidly up regulated following inflammatory stimuli (173, 174). BDKRB1 binding leads to increase in intracellular Ca^{2+} and results in an inflammatory response (175). It has been reported that the up-regulation of BDKRB1 during heat stress might promote an inflammatory response (176).

5.6.2 Differentially Expressed Genes in Illinois at D28

Two genes that are involved in arachidonic acid metabolism are up-regulated in hypothalami of Illinois chickens at D28: Prostaglandin-endoperoxidase synthase 1 *(PTGS1)* and prostaglandin D2 synthase *(PTGDS)*. PTGS1 encodes a rate-limiting enzyme that is involved in the biosynthesis of prostaglandins. The enzyme encoded by PTGS1 catalyzes the conversion of arachidonic acid to prostaglandin H2 *(PTGH2)*, which is a precursor of all prostaglandins, prostacyclins and thromboxanes. PTGS1 is constitutively present in all tissues and maintains tissue homeostasis by regulating basal

prostanoid levels (177). PTGDS, which is synthesized and secreted by the cells of the central nervous system, catalyzes the conversion of PTGH2 to prostaglandin D2 *(PGD2)* (178, 179). Furthermore, significant enrichment of peroxisome proliferator-activated receptor gamma *(PPARG)* is also observed in hypothalami of Illinois D28 chickens. PPARG gene encodes a member of nuclear receptor family of ligand inducible transcription factors (180). The dehydration product of PGD2, 15-deoxy-Δ12, 14-PGJ2 *(PGJ2)*, is a natural ligand for PPARG (177). Binding of the ligands to the PPARG induces a conformational change in PPARG, which then recruits different cofactors and modulates the expression of target genes (180). In mammals, it has been reported that activation of PPARG in the brain leads to increase in food intake and decrease in inflammation (180, 181). PTGS1 and PTGDS in hypothalamus of Illinois birds during heat stress may lead to increased production of PGD2, which subsequently dehydrates to PGJ2 and binds to PPARG. Binding of PGJ2 to PPARG subsequently may result in reduced inflammation and increased feed intake in Illinois chickens (fig 12).

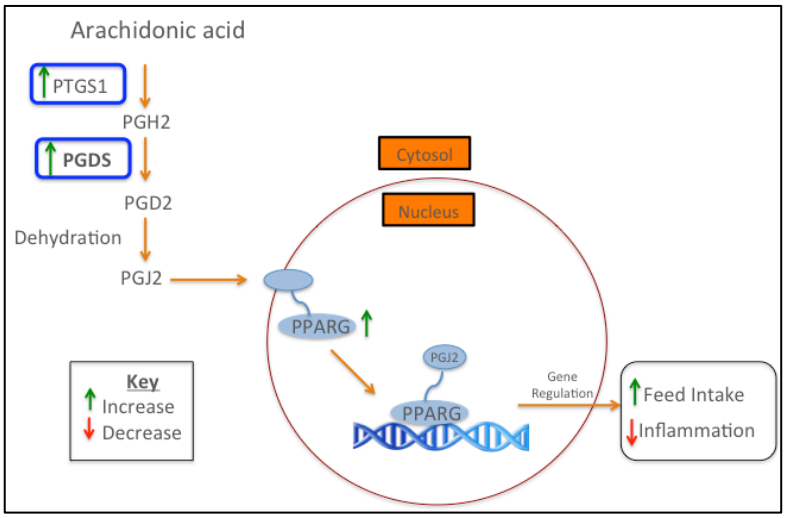


Figure 13: Probable mechanism of action of increased transcript levels of PTGS1, PGDS and PAPRG in the hypothalamus during heat stress in Illinois line at D28.

Illinois D28 hypothalamus also showed significant enrichment of carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase *(CAD)* gene. CAD gene encodes a multifunctional rate-limiting enzyme that catalyzes the initial three steps in *de novo* pyrimidine biosynthesis. It has been reported that direct stimulation of CAD provides the building block for the synthesis of DNA and RNA and subsequent cell growth (182-184). Increased gene expression of CAD in response to heat stress in the hypothalamus of Illinois birds may reflect increased cell proliferation and adaptation to stress.

The neuropeptide neuromedin B *(NMB)* is down-regulated significantly in the hypothalamus of Illinois birds at D28. In chickens, NMB was isolated as a homologue of bombesin-like peptides of mammals (185, 186). Bombesin, which is a 14-amino acid

peptide, is associated with anorexigenic effects in mammals as well as in chickens (185-188). ICV and peripheral injection of NMB is also associated with suppressed feed intake in chickens (186, 189) and its receptors are abundantly found in the brain (190).

Another hypothalamic gene, ghrelin/obestatin prepropeptide *(GHRL)* that encodes ghrelin and obestatin is down regulated in Illinois birds at D28 during heat stress. In birds, little is known about the function of obestatin and the actual mature peptide of obestatin is yet to be identified (191). In chickens, GHRL has an anorexigenic effect (192-194) as opposed to mammals where it stimulates feed intake (195). mRNA expression of ghrelin and its receptors are found in the hypothalamus of chickens (192). The anorexigenic effect of ghrelin is mediated by secretion of corticotropin releasing factor (CRF) (194). Recent studies have indicated that ghrelin stimulates the release and synthesis of 5-HT, thereby stimulating the secretion of CRF and resulting in reduced feed intake (172). In the present study, decreased mRNA levels of NMB and GHRL in Illinois chickens during heat stress may suggest that there is less feed intake suppression.

5.7 Pathway Analysis of the Differentially Expressed Genes at D42 Post Hatch

The differentially enriched genes, which have a fold change of ≥ 2, were subjected to pathway analysis using PathRings and the significant pathways were identified which have $p \leq 0.05$. Adaptive immune system is the significant pathway differentially regulated in the hypothalamus of Ross 708 line (fig 13). Arachidonic acid metabolism, integration of energy metabolism and immune system are the important pathways that are differentially regulated in Illinois D42 (fig 14). The list of the genes that were differentially expressed in the above pathways for Ross 708 and Illinois at D42 were summarized in the Appendix A.

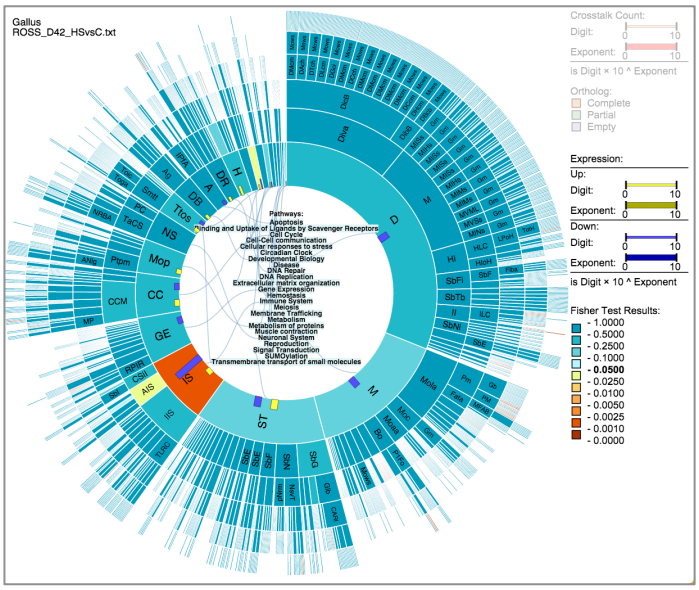


Figure 14: Pathway analysis of the differentially expressed genes in Ross at D42 using PathRings.

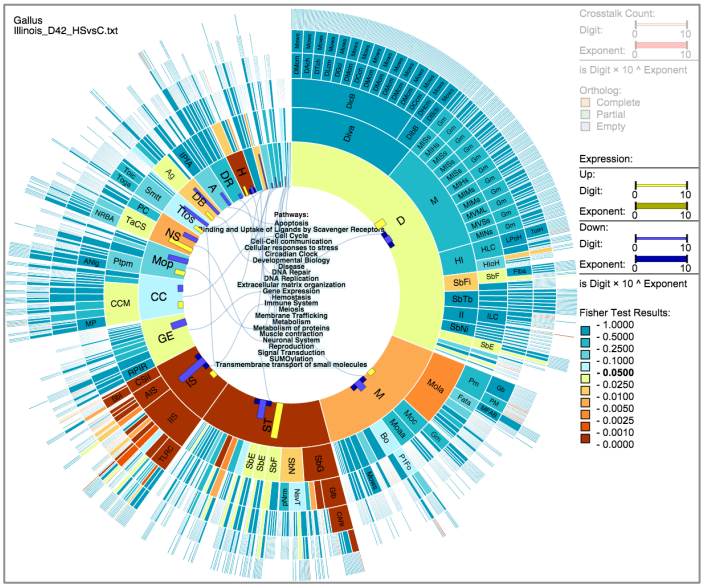


Figure 15: Pathway analysis of the differentially expressed genes in Illinois at D42 using PathRings.

5.7.1 Differentially Expressed Genes in Ross 708 at D42

Neutrophil cytosolic factor -1 *(NCF1)*, Inducible T-cell co-stimulator *(ICOS),* IL-2 inducible T-cell kinase *(ITK)* and Fyn binding protein *(FYB)* are the differentially expressed genes that are involved in adaptive immune system pathway. NCF1, ICOS, ITK and FYB are significantly down regulated in Ross 708 at D42. ICOS belongs to CD-28 gene family of co-stimulatory molecules. ICOS is up regulated following T-cell activation and promotes T-cell survival, proliferation and memory (196). ITK and FYB are involved in T-cell receptor (TCR) signaling. Both FYB and ITK act an important link between TCR stimulation and integrin activation (197, 198). NCF1 gene encodes a cytosolic co-factor p47-phox, which is an essential component of the NADPH-oxidase enzyme complex. In phagocytic cells NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen leading to oxidative burst (199). Down regulation of these genes may suggest immune suppression due to chronic heat stress.

5.7.2 Differentially Expressed Genes in Illinois at D42

Two genes involved in integration of energy metabolism were significantly down regulated in the hypothalamus of D42 Illinois chickens: protein kinase, AMP-activated, beta 2 non-catalytic subunit *(PRKAB2)* and adenylate cyclase 7 *(ADCY7)*. PRKAB2 encodes the regulatory beta 2 subunit of the AMPK. AMPK is a heterotrimeric enzyme complex consisting of one catalytic (alpha) and two regulatory subunits (beta and gamma) (157). The beta subunit of AMPK provides linkage between alpha and gamma subunits and is considered a molecular scaffold (200). All the three subunits are necessary for the formation of active AMPK enzyme complex(201). In the absence of AMPK, ACC is not phosphorylated and remains active. Active ACC increases the cellular malonyl co-A levels leading to the inhibition of CPT 1 and fatty acid oxidation, which ultimately results in decreased feed intake (159). Down regulation of PRKAB2,

therefore, may lead to low levels of AMPK enzyme resulting in subsequent decrease in feed intake. Furthermore, we have seen significant down regulation of ADCY7 gene, which encodes adenylate cyclase 7. ADCY7 is a membrane-bound protein that catalyzes the synthesis of cyclic adenosine monophosphate (cAMP) from ATP (202). In rats, injection of cAMP analogs or adenylyl cyclase activator into perifornical and lateral hypothalamus increased feed intake (203, 204). In our study, down regulation of ADCY7 may lead to decreased feed intake.

Illinois D42 hypothalamus also showed significant down regulation of aldehyde dehydrogenase 1 family, member A1 *(ALDH1A1)*. ALDH1A1 encodes an enzyme that mainly functions in the dopamine metabolism and also acts as the rate-limiting enzyme regulating the conversion of retinaldehyde to retinoic acid (205-208). In mice, absence of ALDH1A1 gene increased extracellular concentrations of dopamine (205). In our study, decreased ALDH1A1 gene expression may lead to reduce dopamine metabolism. In chickens, dopamine has an anorexigenic effect on feed intake (187). Therefore, increased dopamine concentrations may lead to decreased feed intake in heat stressed Illinois chickens at D42.

Conversely, there is also significant down-regulation of mRNA level of POMC. POMC, a precursor of anorexigenic neuropeptides ACTH and alpha melanocyte-stimulating hormone *(αMSH)*, suppresses feed intake in chickens as well as in mammals (51, 209-211). Down regulation of POMC during heat stress in D42 Illinois may indicate decreased anorexigenic effect.

Down regulation of PRKAB2, ADCY7 and ALDH1A1 in the hypothalamus of Illinois chickens suggests that there is suppression of feed intake during heat stress. However, POMC is also down regulated, which may lead to increased feed intake. These

results suggest that in chronic heat stress conditions Illinois birds are becoming acclimatized to heat stress.

In addition to genes regulating feed intake, there is a significant down regulation of genes that are involved in adaptive, innate and cytokine signaling in immune system in Illinois at D42. The important genes down regulated in adaptive immune system include TAP 1, TAP 2, TAPBP, CD8A, CD8B, CD4, ITK, LCK, ICOS; innate immune system include NFKB, TLR3, NLRC5, C3, C1R, C1S and cytokine signaling in immune system include IRF-1, IRF-4, IRF-5, IRF-7, SOCS1, SOCS3, IL7R, STAT1, MX1, OASL (fig 16). These results suggest probable immune suppression in D42 Illinois chickens. Chronic heat stress results in suppressed or dysregulated immune responses (212). In chickens heat stress resulted in increased heterophil: lymphocyte ratio, with increased circulating numbers of heterophil and decreased lymphocyte numbers (213, 214).

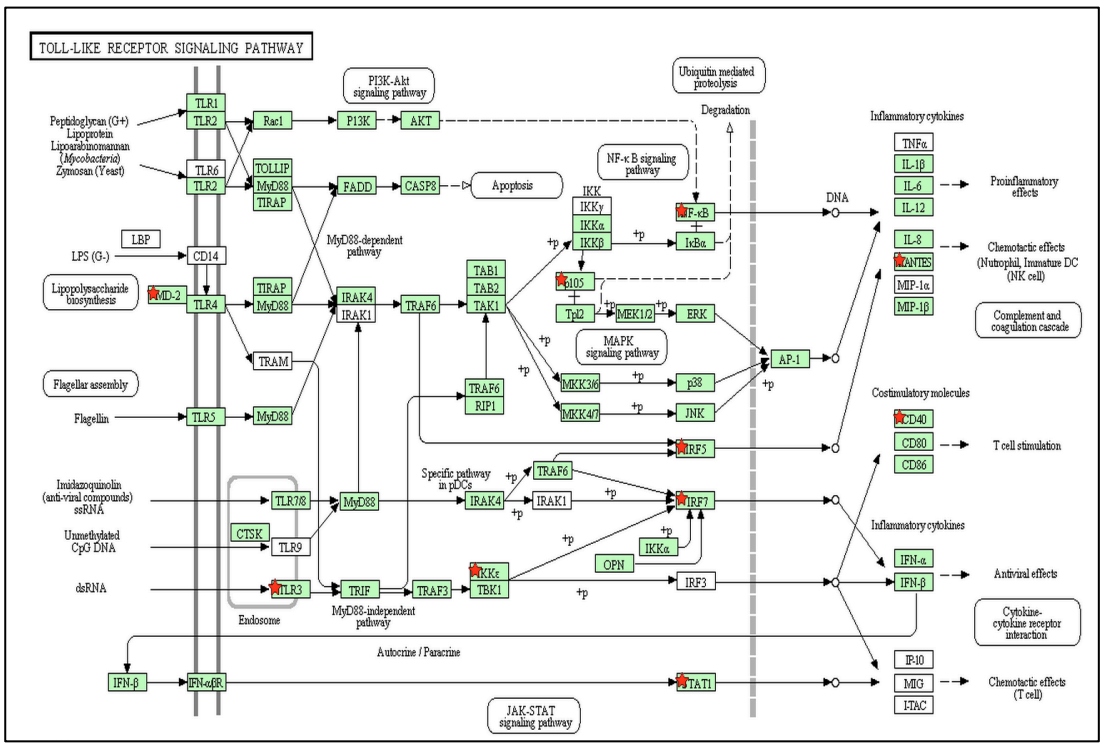


Figure 16: Pathway analysis of the differentially expressed genes at Illinois D28 using KEGG. Toll like receptor signaling pathway is an important component of innate immune system. Red stars indicate differentially expressed genes in this pathway.

5.8 Summary

Modern commercial broilers that are intensively selected for high growth rate and feed efficiency produce more heat because of high metabolic activity and are susceptible to increased temperatures (2-4, 10, 11). Exposing chickens to high ambient temperatures leads to decreased feed intake, growth rate, feed efficiency and survivability (4, 9, 94). Decrease in feed intake is suggested to be responsible for negative effects of heat stress on growth rate and productivity (94, 215, 216).

At D28, in Ross line genes encoding LPL, SREBF1, ACACB, NMU, and HTR1E, which play a major role in appetite suppression, were up regulated. Significant down regulation of the genes that encode hypothalamic hormones (TSH, AVP, OXT) were also observed. In Illinois line, differential expression of GHRL, NMB, PTGS1, PGDS and PPARG were observed, leading to reduce anorexigenic effects. This down regulation is probably due to the effect of chronic exposure to heat stress. Based on these results at D28, we conclude that, changes in hypothalamic gene expression patterns during heat stress mainly played an important role in feed intake regulation.

At D42, in both the Ross 708 and Illinois lines predominantly down regulation of immune related genes was present. The observed down regulation of immune genes was more prominent in Illinois line. In addition, differential expression of genes including PRKAB2, ADCY7, ALDH1A1 and POMC, possibly resulting in decreased feed intake, was observed in Illinois chickens. These results suggest that chronic heat stress is leading to immunosuppression predominantly in Illinois chickens.

Chapter 6

CONCLUSIONS

In the present study, we have compared the gene expression patterns between the heat stress and the control chickens in the hypothalamus of Ross 708 and Illinois lines at day 28 and 42 post hatch. Ross 708 represents the modern commercial lines and Illinois represents the legacy lines that grow with the properties of the meat birds unselected since 1950s. Illinois line has lower growth rate and body weight compared to the Ross 708 line and is expected to have high capacity to withstand heat stress temperatures. In this study, the body weights of the heat stress chickens were low in both Ross 708 and Illinois line at D28 and D42. However, the body weights were significantly lower only in Ross 708 line. This suggests that Ross line is more susceptible to heat stress conditions.

In transcriptomic analysis of the hypothalamus at D28 in Ross and Illinois lines, differential expression of several genes that are involved in regulating feed intake were identified. In Ross line, gene expression data is consistent with a net decrease in feed intake. These results were expected, and explain the significant decrease in body weights in Ross line at D28. In Illinois line gene expression data indicated that there is net increase in feed intake. The results were not consistent with the expected hypothesis, where the body weights were decreased in Illinois line during heat stress. The gene expression data at D28 may probably indicate that Illinois chickens are acclimatizing faster than Ross chickens in response to chronic heat stress.

At D42, no significant differences were observed in the expression of genes regulating feed intake in both the lines, suggesting that Ross birds are also getting

acclimatized to chronic heat stress. Several genes that are involved in immune responses were down regulated in the in the hypothalamus of heat stressed birds in both the lines. These effects were more prominent in the Illinois line. These results may probably suggest that suppression of immune response is a mechanism of acclimatization in response to chronic heat stress.

Future work aims at

1) Confirming the observed gene expression changes at protein level.
2) Studying the gene expression patterns at earlier time points.
3) Perform qRT-PCR on samples from 2013 trial to confirm the expression of immune genes.
4) Confirm the observed immune gene expression changes in the hypothalamus, whether it is due to infiltrating immune cells or by hypothalamic neuronal cells.

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Appendix A

LIST OF THE DIFFERENTIALLY EXPRESSED GENES INVOLVED IN THE PATHWAYS

Significant Pathways in Ross 708 at D28

1. Metabolism

A. Metabolism of lipids and lipoproteins
- 1) Lipid digestion mobilization and transport
  - HSPG2- heparan sulfate proteoglycan 2
  - LPL- Lipoprotein lipase
- 2) Metabolism of steroid hormones and vitamin D
  - HSD11B1- hydroxysteroid (11-beta) dehydrogenase 1-like
  - CGA- glycoprotein hormones, alpha polypeptide
- 3) Arachidonic acid metabolism
  - PTGDS- prostaglandin D2 synthase 21kDa (brain)
  - DPEP1- dipeptidase 1 (renal)
- 4) Regulation of Cholesterol Biosynthesis by SREBP
  - SREBF1- sterol regulatory element binding transcription factor 1
  - ACACB- acetyl-CoA carboxylase beta

B. Metabolism of amino acids and derivatives
- 1) Thyroxine biosynthesis
  - CGA- glycoprotein hormones, alpha polypeptide
  - TSHB- thyroid stimulating hormone, beta

2. Signal Transduction

A. Signaling by G-protein coupled receptor

a) GPCR ligand binding
- BDKRB1- bradykinin receptor B1
- NMU- neuromedin u
- HTR1E- 5-hydroxytryptamine (serotonin) receptor 1E
- CGA- glycoprotein hormones, alpha polypeptide
- TSHB- thyroid stimulating hormone, beta
- RGR- retinal G protein coupled receptor
- GNG11- guanine nucleotide binding protein (G protein), gamma 11
- IHH- Indian hedgehog
- FZD4- frizzled family receptor 4

AVP- arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)
OXT- oxytocin receptor
TRH- thyrotropin-releasing hormone receptor
PTGFR- prostaglandin F2 receptor negative regulator
GPR68- G protein-coupled receptor 68

b) GPCR downstream signaling

GNG1- guanine nucleotide binding protein (G protein), gamma 11
BDKRB1- bradykinin receptor B1
CGA- glycoprotein hormones, alpha polypeptide
TSHB- thyroid stimulating hormone, beta HTR1E
NMU-neuromedin u
RGR- retinal G protein coupled receptor
RGS11-regulator of G-protein signaling 11
AVP- arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)
OXT- oxytocin receptor
PTGFR- prostaglandin F2 receptor negative regulator
TRH-thyrotropin-releasing hormone receptor
GPR68- G protein-coupled receptor 68

c) Gastrin-CREB signaling pathway via PKC and MAPK

NMU-neuromedin u
GNG11- guanine nucleotide binding protein (G protein), gamma 11
BDKRB1- bradykinin receptor B1
PTGFR- prostaglandin F2 receptor negative regulator
TRH-thyrotropin-releasing hormone receptor
GPR68- G protein-coupled receptor 68
AVP- arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)
OXT- oxytocin receptor

3. Transmembrane transport of small molecules

A.SLC-mediated transmembrane transport

SLC39A8-solute carrier family 39 (zinc transporter), member 8
SLC4A2- solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)
SLCO1B1- solute carrier organic anion transporter family, member 1B1
AVP- arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)

B. Aquaporin-mediated transport

GNG11- guanine nucleotide binding protein (G protein), gamma 11
AVP- arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)

C. Ion Channel transport

ATP1A2- ATPase, Na+/K+ transporting, alpha 2 polypeptide
GLRA4- glycine receptor, alpha 4
RYR3- ryanodine receptor 3

4. Extracellular matrix organization

A. Collagen formation
COL5A1- collagen, type V, alpha 1
COL13A1- collagen, type XIII, alpha 1
COL2A1- collagen, type II, alpha 1

B. Degradation of the extracellular matrix
COL13A1- collagen, type XIII, alpha 1
CTSK- cathepsin K
HSPG2- heparan sulfate proteoglycan 2

C. Elastic fibre formation
ELN- elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)
BMP4- bone morphogenetic protein 4
LTBP2- latent transforming growth factor beta binding protein 2

D. Laminin interactions
HSPG2-heparan sulfate proteoglycan 2
LAMA2- laminin, alpha 2

E. Non-integrin membrane-ECM interactions
HSPG2- heparan sulfate proteoglycan 2
LAMA2- laminin, alpha 2

E. ECM proteoglycans
TNXB- tenascin XB
LAMA2- laminin, alpha 2

F. Integrin cell surface interactions
HSPG2- heparan sulfate proteoglycan 2
COL13A1- collagen, type XIII, alpha 1

Significant Pathways in Illinois at D28

1. Disease

A. Mucopolysaccharidoses
LUM- lumican
OGN- osteoglycin
PRELP- proline/arginine-rich end leucine-rich repeat protein
CSPG4- chondroitin sulfate proteoglycan 4
DCN- decorin
GLB1- galactosidase, beta 1

2. Metabolism

A. Metabolism of lipids and lipoproteins
a) Arachidonic acid metabolism

PTGDS- prostaglandin D2 synthase 21kDa (brain)
PTGS1- prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
DPEP1- dipeptidase 1 (renal)

b) Fatty acid triacylglycerol and ketone body metabolism
PPARG- peroxisome proliferator-activated receptor gamma
TECRL- trans-2,3-enoyl-CoA reductase-like
ACACB- acetyl-CoA carboxylase beta

c) Sphingolipid metabolism
ARSI- arylsulfatase family, member I
DEGS2- degenerative spermatocyte homolog 2, lipid desaturase (Drosophila)
GLB1- galactosidase, beta 1

B. Metabolism of carbohydrates

Glycosaminoglycan metabolism
LUM- lumican
OGN- osteoglycin
PRELP- proline/arginine-rich end leucine-rich repeat protein
DCN-decorin
GLB1- galactosidase, beta 1
CSPG4- chondroitin sulfate proteoglycan 4

3. Signal transduction

A. Signaling by GPCR

a) Gastrin-CREB signaling pathway via PKC and MAPK
CASR- calcium-sensing receptor
GHRL- ghrelin/obestatin prepropeptide
NMB- neuromedin B

b) GPCR downstream signaling
CASR- calcium-sensing receptor
TAS2R7- taste receptor, type 2, member 7
OPN1LW- opsin 1 (cone pigments), long-wave-sensitive
VIPR2- vasoactive intestinal peptide receptor 2
NMB- neuromedin B
GHRL- ghrelin/obestatin prepropeptide

c) GPCR ligand binding
OPN1LW- opsin 1 (cone pigments), long-wave-sensitive
WNT6- wingless-type MMTV integration site family, member 6
FZD4- frizzled family receptor 4
TAS2R7- taste receptor, type 2, member 7
NMB- neuromedin B
GHRL- ghrelin/obestatin prepropeptide

B. Visual transduction

STRA6- stimulated by retinoic acid gene 6 homolog (mouse)

OPN1LW- opsin 1 (cone pigments), long-wave-sensitive
RBP2- retinol binding protein 2, cellular

C. Signaling by PDGF
PDGFRB-platelet-derived growth factor receptor, beta polypeptide
COL9A3- collagen, type IX, alpha 3
COL6A2- collagen, type VI, alpha 2
COL6A1- collagen, type VI, alpha 1
COL6A3- collagen, type VI, alpha 3

4. Extracellular matrix organization

A. Integrin cell surface interactions
ITGA8- integrin, alpha 8
LUM- lumican
COL9A3- collagen, type IX, alpha 3

B. Collagen formation
COL5A1- collagen, type V, alpha 1
COL15A1- collagen, type XV, alpha 1
COL1A2- collagen, type I, alpha 2
COL6A2- collagen, type VI, alpha 2
COL9A3- collagen, type IX, alpha 3
COL6A3- collagen, type VI, alpha 3
COL4A6- collagen, type IV, alpha 6
LOX- lysyl oxidase

C. Elastic fibre formation
FBLN2- fibulin 2
ITGA8- integrin, alpha 8
LTBP2- latent transforming growth factor beta binding protein 2
LTBP1- latent transforming growth factor beta binding protein 1
LOX- lysyl oxidase

5. Transmembrane transport of small molecules

A. SLC-mediated transmembrane transport
SLC47A1- solute carrier family 47, member 1
SLC47A2- solute carrier family 47, member 2
SLC30A8- solute carrier family 30, member 8
SLC39A8- solute carrier family 39, member 8
ABCC4- ATP-binding cassette, sub-family C (CFTR/MRP), member 4
SLC9A2- solute carrier family 9 (sodium/hydrogen exchanger), member 2

Significant Pathways in Ross 708 at D42

1. Immune system

a) Adaptive Immune system
NCF1- neutrophil cytosolic factor 1
ICOS- inducible T-cell co-stimulator
ITK- IL2-inducible T-cell kinase

FYB- nuclear transcription factor Y, beta

Significant Pathways in Illinois at D42

1. Metabolism

A. Metabolism of Lipids and Lipoproteins

a) Arachidonic acid metabolism

HPGDS- hematopoietic prostaglandin D synthase

TBXAS1- thromboxane A synthase 1 (platelet)

b) Lipid digestion, mobilization and transport

SDC1- syndecan 1

ABCG5- ATP-binding cassette, sub-family G (WHITE), member 5

B. Biological oxidations

POMC- proopiomelanocortin

HPGDS- hematopoietic prostaglandin D synthase

TBXAS1- thromboxane A synthase 1 (platelet)

ALDH1A1- aldehyde dehydrogenase 1 family, member A1

C. Integration of energy metabolism

ADCY7- adenylate cyclase 7

PRKAB2- protein kinase, AMP-activated, beta 2 non-catalytic subunit

SNAP25- synaptosomal-associated protein, 25kDa

2. Signal Transduction

A. Signaling by GPCR

TACR1	tachykinin receptor 1
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A
LHCGR	luteinizing hormone/choriogonadotropin receptor
OPRD1	opioid receptor, delta 1
GRM1	glutamate receptor, metabotropic 1
RGS8	regulator of G-protein signaling 8
PLEKHG5	pleckstrin homology domain containing, family G (with RhoGef domain) member 5
CCR8	Chemokine (C-C motif) receptor 8
CCR2	chemokine (C-C motif) receptor 2
CCL5	chemokine (C-C motif) ligand 5
CCL4	chemokine (C-C motif) ligand 4
HBEGF	heparin-binding EGF-like growth factor
POMC	proopiomelanocortin
F2RL1	coagulation factor II (thrombin) receptor-like 1
RLN3	relaxin 3
C3	complement component 3
C5AR1	complement component 5a receptor 1

C3AR1	complement component 3a receptor 1
P2RY10	purinergic receptor P2Y, G-protein coupled, 10
GPR65	G protein-coupled receptor 65
SHH	sonic hedgehog
ADCY7	adenylate cyclase 7
CCL20	chemokine (C-C motif) ligand 20
CCR7	chemokine (C-C motif) receptor 7
CCL19	chemokine (C-C motif) ligand 19
RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)
CX3CR1	chemokine (C-X3-C motif) receptor 1

B. Signaling by NGF

PLEKHG5	pleckstrin homology domain containing, family G (with RhoGef domain) member 5
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
ADCY7	adenylate cyclase 7
LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)

C. Signaling by FGFR

ADCY7	adenylate cyclase 7
LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)

D. Signaling by ERBB2

LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
ADCY7	adenylate cyclase 7

E. Signaling by EGFR

LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
ADCY7	adenylate cyclase 7

F. Signaling by PDGF

LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
ADCY7	adenylate cyclase 7
COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
STAT1	signal transducer and activator of transcription 1, 91kDa

G. Signaling by SCF-KIT

CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
FES	feline sarcoma oncogene
GRAP2	GRB2-related adaptor protein 2
GRAP	GRB2-related adaptor protein
STAT1	signal transducer and activator of transcription 1, 91kDa
SOCS1	suppressor of cytokine signaling 1
LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor

3. Immune system

A. Adaptive Immune system

TRIM36	tripartite motif containing 36
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)
TAPBP	TAP binding protein (tapasin)
CTSS	cathepsin S
CTSA	cathepsin A
CD8A	CD8a molecule
CD8B	CD8b molecule
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
CRTAM	cytotoxic and regulatory T cell molecule
ICOS	inducible T-cell co-stimulator
BTLA	B and T lymphocyte associated
CD40LG	CD40 ligand
CD40	CD40 molecule, TNF receptor superfamily member 5
AMICA1	adhesion molecule, interacts with CXADR antigen 1
C3	complement component 3
CD4	CD4 molecule
CD3E	CD3e molecule, epsilon (CD3-TCR complex)
BLK	B lymphoid tyrosine kinase
CD3D	CD3d molecule, delta (CD3-TCR complex)
CARD11	caspase recruitment domain family, member 11
LCK	lymphocyte-specific protein tyrosine kinase
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
GRAP2	GRB2-related adaptor protein 2
LCP2	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)
ITK	IL2-inducible T-cell kinase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)

DTX3L	Deltex 3-like (Drosophila)
SOCS1	Suppressor of cytokine signaling 1
SOCS3	Suppressor of cytokine signaling 3
ANAPC13	Anaphase promoting complex subunit 13

B. Innate Immune system

NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
LCK	lymphocyte-specific protein tyrosine kinase
HBEGF	heparin-binding EGF-like growth factor
CASP9	caspase 9, apoptosis-related cysteine peptidase
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)
LCP2	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)
GRAP2	GRB2-related adaptor protein 2
ADCY7	adenylate cyclase 7
ITK	IL2-inducible T-cell kinase
TXK	TXK tyrosine kinase
CARD11	caspase recruitment domain family, member 11
TLR3	toll-like receptor 3
LY96	lymphocyte antigen 96
CTSS	cathepsin S
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
IRF7	interferon regulatory factor 7
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
C1QA	complement component 1, q subcomponent, A chain
C1QB	complement component 1, q subcomponent, B chain
C1R	complement component 1, r subcomponent
C1S	complement component 1, s subcomponent
C3	complement component 3
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7
PSTPIP1	proline-serine-threonine phosphatase interacting protein 1
TRIM25	tripartite motif containing 25
IFIH1	interferon induced with helicase C domain 1
NLRC5	NLR family, CARD domain containing 5
IRF1	interferon regulatory factor 1
CD4	CD4 molecule
CCR2	chemokine (C-C motif) receptor 2

C. Cytokine signaling Immune system

IRF4	interferon regulatory factor 4
IL2RG	interleukin 2 receptor, gamma (severe combined immunodeficiency)
SOCS3	suppressor of cytokine signaling 3
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
OASL	2'-5'-oligoadenylate synthetase-like
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
IRF7	interferon regulatory factor 7
IRF1	interferon regulatory factor 1
LCK	lymphocyte-specific protein tyrosine kinase
PML	promyelocytic leukemia
SOCS1	suppressor of cytokine signaling 1
TRIM25	tripartite motif containing 25
USP18	ubiquitin specific peptidase 18
CSF2RA	colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)
STAT1	signal transducer and activator of transcription 1, 91kDa
IL7R	interleukin 7 receptor
INPPL1	inositol polyphosphate phosphatase-like 1
IRF5	interferon regulatory factor 5

4. Neuronal system

A. Transmission across Chemical Synapses

SNAP25	synaptosomal-associated protein, 25kDa
GABRR1	gamma-aminobutyric acid (GABA) receptor, rho 1
SLC32A1	solute carrier family 32 (GABA vesicular transporter), member 1
GRIA3	glutamate receptor, ionotrophic, AMPA 3
ADCY7	adenylate cyclase 7

B. Potassium Channels

KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)
KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1

5. Developmental Biology

A. Axon guidance

CNTN6	contactin 6
CD72	CD72 molecule
RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)
COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
DCX	doublecortin
FES	feline sarcoma oncogene

6. Hemostasis

A. Cell surface interactions at the vascular wall

SLC7A9	solute carrier family 7 (glycoprotein-associated amino acid transporter light chain, bo,+ system), m
CD244	CD244 molecule, natural killer cell receptor 2B4
AMICA1	adhesion molecule, interacts with CXADR antigen 1
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
LCK	lymphocyte-specific protein tyrosine kinase

B. Platelet activation signaling and aggregation

LCK	lymphocyte-specific protein tyrosine kinase
LCP2	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)
SRGN	serglycin
RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)

C. Factors involved in megakaryocyte development and platelet production

GATA5	GATA binding protein 5
HBG1	hemoglobin, gamma A
KIF9	kinesin family member 9
IRF1	interferon regulatory factor 1
IRF7	interferon regulatory factor 7

7. Gene Expression

VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
SLBP	stem-loop binding protein

Appendix B

AACUC APPROVAL FORM

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: (27) 12-22-10R

TITLE OF PROJECT: Scientific Investigation into the response of Broiler Chickens to heat stress by transcriptome analysis

INSTRUCTOR/PRINCIPAL INVESTIGATOR: Carl Schmidt

New or Three Year Review (mark one)

NEW □ **THREE YEAR** x□

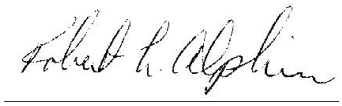
If this is a 3 year renewal, what is the assigned existing protocol number?
_(27) 12-22-10R

(This section for Committee use only)

Application Approved (date): 1-5-2011

Application Rejected (date): _____

Reason for Rejection: _____



Signature, Animal Care and Use Committee **1-5-2011 Date**

APPLICATION INFORMATION:

Title: Scientific Investigation into the response of Broiler Chickens to heat stress by transcriptome analysis

Principal Investigator(Research): Carl J. Schmidt

Address: 107 Allen Lab, 601 Sincock Lane, University of Delaware, Newark, Delaware 19716

Telephone: (302)-831-1334 Email: schmidtc@udel.edu

Proposed start date: February 1 2011 End date: January 31, 2014

Teaching/Outreach □ Research X

If TEACHING box was checked, select from the following:

Demonstration □ Laboratory □ Student Project □

If student project, please define project: _____

Have all participants listed above reviewed the application and is familiar with the proposed work?

YES X□ NO □

If no, identify those needing to review application.

_____ _____ _____

Are all proposed animal care management procedures 1) defined as "pre-approved" by the Animal Care and Use Committee, or 2) part of the Standard Operating Procedures developed by the Animal Care and Use Committee for that particular species?

YES X□ NO □ To be determined by AACUC □

Have all participants been trained? YES X□ NO □

Which participants have not been trained?

_____ _____ _____

Name the person responsible for conducting the training.

If after hours participation is required by students, please describe how this is being handled. (e.g. supervisors, assistants, etc.) Please include the times and days that students may be on site.

ANIMAL INFORMATION:

Common Name of the Animal Requested: Chickens

Amount Being Requested: 1600

Source of Animals: Allen Family Foods and Chet Utterback at the University of Illinois

Where are the animals being held: UD Poultry Farm

Briefly Describe the Goals or Objectives of this Application (use additional space as needed).

The goal of this study is to determine the ability of the modern broiler chicken to handle heat stress compared to the heritage variety. Following treatment, birds will be euthanized by cervical dislocation and organs harvested for transcriptome analysis.

Rationale for scale of study: This is a new area of research, using new genomic approaches to understand how birds respond to heat stress. The large numbers of birds are necessitated in order to achieve statistical significance in our gene mapping studies.

Birds: Heritage birds will be obtained from Chet Utterback at the University of Illinois and the Ross708 birds from a local supplier. Birds will be wing tagged and randomly placed into control and experimental groups as described below (Heat Shock Scheme). In each experiment 100 birds from each line will be included in each experimental group. The size of the facilities at the University of Delaware limit the number of birds per chamber, hence we anticipate multiple replicates over time to a total of 1600 birds per line. Blood will be taken from each bird for DNA extraction prior to heat stress. Also, 12 birds from each group will be removed on post hatch days 2, 7 and 21, euthanized (cervical dislocation) and tissues harvested. Blood biomarker data using the iSTAT will be collected from these birds prior to euthanasia. Chambers will be monitored on a daily basis to insure adequate feed and water and to remove any sick or dead birds.

Heat Shock Scheme: Controls are hatched from eggs incubated at 37^{o}C (99^{o}F) while thermal conditioned embryos will be incubated at 39.6^{o}C (103^{o}F) from embryonic days 10-18, then returned to 37^{o}C. Following hatch through day 21, they will be kept at ambient temperatures. At day 22, the original Control birds will be split into two populations (Control A and B) and the *In Ovo* Heat-conditioned bird also split into two groups (*In Ovo* Heat Conditioned A and B). The A populations will be kept at ambient temperatures while the B populations will be heat stressed at 35^{o}C (95^{o}F) or 7 hours per day for 21 days. There will be 20 birds per chamber. Multiple replicates (hatches) will be conducted. At the end of the trial (6 weeks from hatch), birds will be euthanized and tissues collected.

Attached below is additional protocol information.

Does this procedure involve surgery? YES NO X□

If yes, explain in detail the surgery.

Are drugs, vaccines and/or medications being used? YES □ NO X□

If yes, describe what is being used. Include dosages and routes of administration.

How often are animals monitored and how are sick or injured animals being handled?

The birds will be checked daily and given food and fresh water *ad libidum*. Sick or injured animals will be euthanized by cervical dislocation.

What is the method of euthanasia, if specified in the protocol?

Cervical dislocation as per AVMA Guidelines on Euthanasia 2007

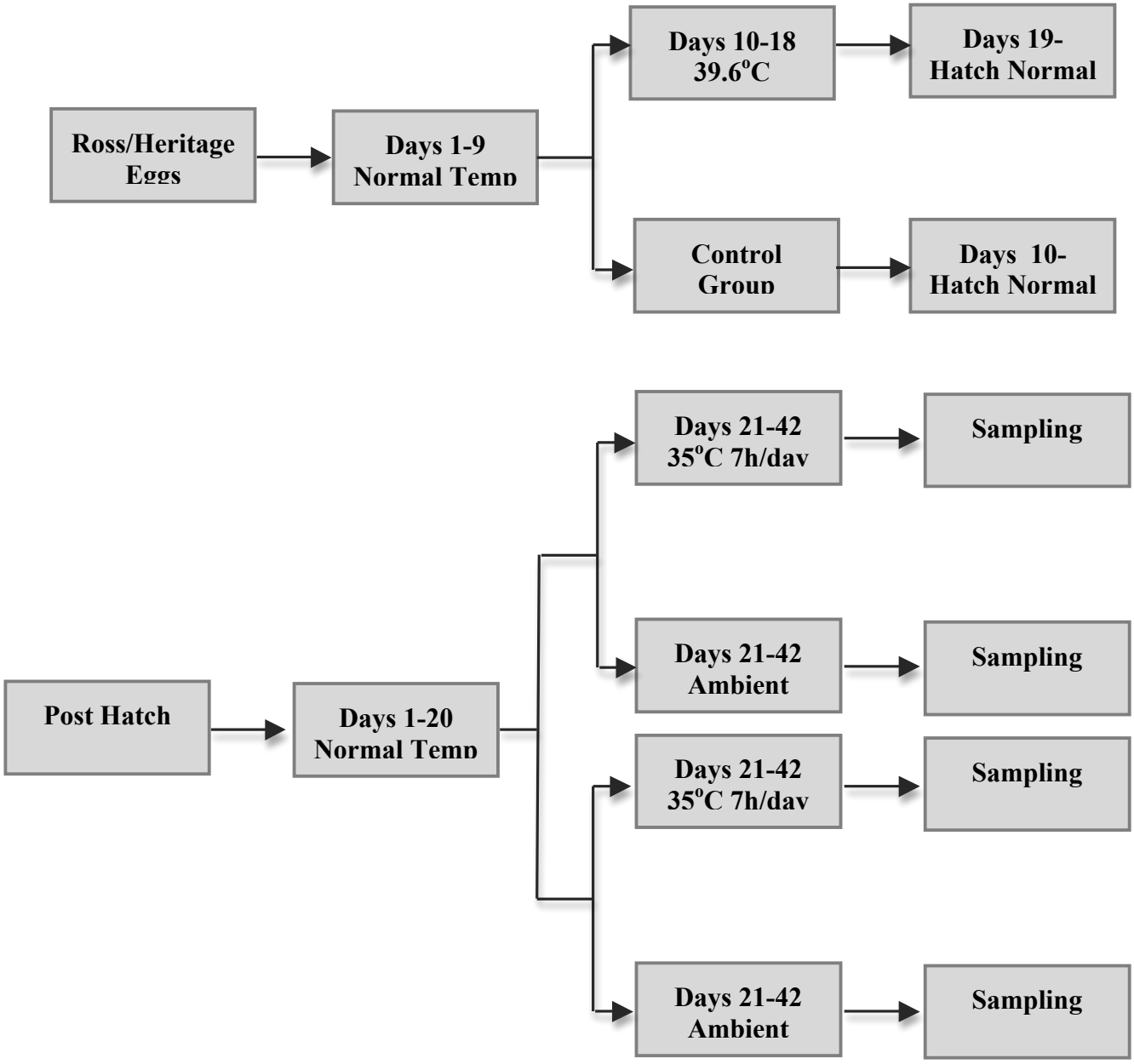
List the veterinarian who is on-call:

Name: Miguel Ruano Telephone: 302-831-1539

Does this application require approval from Occupational Health & Safety (OHS)?
YES □ NO X□

If yes, what form(s) are attached?_____

NOTE: OHS approval is required for experiments involving the use of hazardous substances such as radioactive materials, highly toxic or carcinogenic materials, human reproductive hazards, or zoonotic or human pathogens.



Ross Heritage heat stress experiment: Eggs will be either heat stressed or maintained as controls from embryonic days 10-18, and then returned to normal temperatures. Subsequently, both heat stressed and control birds will be split into two groups each, with one group heat stressed from days 21-42 post-hatch, with the second group kept at ambient temperatures to function as a control. So, there will be a total of 8 groups at the end of each experiment.

Tissue Samples: Genomic DNA & RNA:

- Blood
- Brain
- Heart
- Liver
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Ceca (and contents)
- Fat pad
- Breast muscle
- Spleen

Weekly Measurements:

- iSTAT metabolic measurements
- Weight

Day 21/42

- Shank length
- Shank Width

Morphometric:

- Liver
- Spleen
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Breast muscle
- Heart

Samples are needed for:

- RNA seq
- Micro RNA
- Genomic DNA
    - SNP
    - CVN
    - Epigenetics