EFFECTS OF INCUBATION TEMPERATURE AND TRANSPORTATION STRESS ON YOLK UTILIZATION, SMALL INTESTINE DEVELOPMENT, AND POST-HATCH PERFORMANCE OF HIGH-YIELD BROILER CHICKS

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(ABSTRACT)

Growth and performance parameters of broiler chicks depend on adequate development of the small intestine. Stressors such as elevated or decreased temperatures during incubation and post-hatch transportation may have an effect on the gastrointestinal development of the broiler chick. The objective of the first study was to investigate the effects of elevated embryonic incubation temperature (IT) on post-hatch relative nutrient transporter gene expression, integrity of the intestinal epithelium, organ development, and performance in Ross 308 broiler chickens. Nine hundred fertile eggs were incubated at different egg-shell temperatures during development. Body weights and performance parameters were measured at day of hatch (DOH), d 7, 14, 21, 30, and 42. Small intestine and residual yolk were collected at DOH, d 2, 4, 6, and 10 and weighed individually. The small intestine was evaluated for mucosal morphology measurements and relative nutrient transporter (SGLT1, GLUT2, GLUT5, EAAT3, and PepT1) gene expression. The objective of the second study was to evaluate the effects of embryonic incubation, simulating a multi-stage incubation system, and post-hatch transportation temperatures on post-hatch performance, yolk free body weights, relative nutrient transporter gene expression, yolk utilization, intestinal morphology, and organ development of broiler chickens. Cobb 500 eggs (n=5200) were incubated with egg-shell temperatures, which were combined depending on the early and late development incubation periods as found in multi-stage incubators: Low (L): 36.7°C, Standard (S): 37.5°C, and High (H): 39°C. After hatch, chicks were further separated into 2 transportation groups: control (C; 34°C), and heat-stressed (D; 40°C). The eight resulting experimental groups were: LS-C, SS-C, LH-C, SH-C, LS-D, SS-D, LH-D, and SH-D. Three and two way interactions ($P<0.05$) were observed and discussed in both studies for all the parameters analyzed. These studies present for the first time the effects of altered embryonic IT and stress during transportation of newly hatched chicks, on small intestine morphology, digestive organ development, and expression of nutrient transporters mRNA in high-yield broiler chicks. These results contribute to the understanding of mechanisms by which

either low or high temperatures, as compared to standard recommendations, during incubation and transportation can affect embryonic development and subsequent performance of broiler chicks.

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LIST OF ACRONYMS

BBM- Brush border membrane

BW- Body weight

BWG- Body weight gain

C- Control

cCdx- chicken cad-family

Cdx- cad-family

d- Day

D- Stressed

DOH- Day of hatch

EAAT3- Excitatory amino acid transporter 3 glutamate and aspartate transporter

ED- Embryonic day

EST- Eggshell temperature

FCR- Feed conversion ratio

GALT- Gastrointestinal associated lymphoid tissue

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

GI- Gastrointestinal tract

GLUT2- Solute carrier family 2 member 2

GLUT5- Solute carrier family 2 member 5

H- High

HMB- β-hydroxy-β-methylbutyrate

IEL- Intraepithelial lymphocyte cells

Ig- Immunoglobulins

IO- *In ovo*

IT- Incubation temperature

L- Low

MD- Meckel's diverticulum

MHC-II- Major histocompatibility complex II

mRNA- messenger ribonucleic acid

NRC- National research council

PepT1- Peptide transporter

S- Standard

SGLT1- Solute carrier family 5 member 1

SI- Small intestine

T_3- Triiodothyronine

T_4- Thyroxine

T_a- Ambient temperature

T_b- Body temperature

TCR- T cell receptors

YS- Yolk sac

YSM- Yolk sac membrane

FI- Feed intake

YFB- Yolk free body weights

CHAPTER I

INTRODUCTION

Genetic selection in the poultry industry has resulted in incredibly fast growing chicken lines, whose immediate growth rate is seen as early as during embryonic development. The more rapidly the chick embryo grows, the greater the increase of metabolic heat output by the egg and the faster the oxygen depletion during development. These conditions create problems in current commercial incubation environments of elevated temperature and decreased oxygen levels during later stages of embryonic development. In order to achieve the most productive and efficient production in the poultry industry, it is important to completely understand the needs of the developing embryo during incubation and of the newly hatched chick during the peri-hatch period. As the embryonic incubation and peri-hatch periods of commercial high-yield broiler strains continue to encompass relatively larger portions of the life of the market bird, the incubator, hatcher, and post-hatch environments become increasingly important. Incubation systems have become larger with increased egg capacity without sufficient cooling and ventilation systems to accommodate for the increased metabolic heat production. The elevated temperatures may cause stress to the developing embryo, alter development, and result in poor chick quality and subsequent post-hatch performance. Such stressors may be an important factor for inadequate establishment and maturation of the gastrointestinal tract, which will result in an impaired utilization of nutrients. This will contribute to a decrease in performance parameters causing a significant rise in poultry production costs.

Overall, the goal of this dissertation is to provide information for a better understanding of the mechanisms by which temperature stress during incubation and transportation impacts nutrient utilization, intestinal morphology, and performance of the chicks. This dissertation will focus on the gastrointestinal tract, more specifically the small intestine, as this is together with the yolk sac, the main source by which the chick obtains the necessary nutrients for development and growth during the post-hatch period. To address these objectives, the dissertation is divided into different chapters. The literature review will focus on the incubation systems, with a short inspection back in history and the problems observed currently in the industry. It will also include the evaluation parameters for chick quality and their correlation to good performance,

and particular attention will be given to embryogenesis, morphology, and physiology of the small intestine to attempt to encompass all the pertinent subjects in order for the reader to grasp a better understanding of what the issues are currently in the poultry industry. It will end with a small description of stress and behavioral responses, as well as a brief description of the allocation of resources theory. This will establish the foundation for the rest of the dissertation. The following chapter of the dissertation will focus on the effect of elevated temperature during the last wk of incubation as seen in a single-stage incubator setting. This study included the evaluation of different performance parameters and development of the small intestine through the measurement of mucosal architecture, organ development, and relative nutrient transporter expression in Ross x Ross 308 broiler line, which has been selected for growth rate and traits such as leg strength and cardiovascular health. The remainder of the dissertation will address the effects of multi-stage incubation temperature profiles and early post-hatch transportation stress on performance, relative nutrient transporter gene expression, organ development, and mucosal integrity in a broiler line, Cobb 500, which has been selected specifically for growth and feed efficiency.

There has been a recent increase in papers characterizing the peri-hatch period, yolk nutrient utilization, and external nutrient sources as they are of critical importance for the development of the small intestine and further growth and performance of the precocial bird. Some studies have addressed the importance of incubation in the modern broiler chickens; however, most of these studies have been done in turkeys or have evaluated the incubator temperature rather than the embryonic temperature. Very few studies exist that encompass embryonic temperature during incubation of high-yield type broiler chickens and the further effect of transportation stress on the already stressed chicken. It is the main objective of our laboratory to identify the effects of incubation and transportation stress on the gastrointestinal tract as it represents the most important organ for the assimilation and distribution of nutrients for an optimal lifetime performance of the chicken.

CHAPTER II

LITERATURE REVIEW

Historical Aspects of Incubation

Egyptians. Egg incubation started with ancient Egyptians around 3,000 B. C. (Martin, 2002). The Egyptian hatchery methods were passed down from generation to generation among certain families. The local farmers would bring their fertile eggs to the hatchery, and the hatchery owner was required by law to return two chicks for every three eggs received. Therefore they were required to have at least 75% hatchability of fertile eggs, in order to make a profitable business out of it (Brown, 1979). The Egyptian incubators consisted of thousands of eggs which were placed in piles on the floor of each incubator room (Brown, 1979; Martin, 2002). The temperature control was achieved through the upper chamber of the room, where a continuous fire of camel feces, straw, or charcoal burned in order to provide heat to the eggs below. There was a middle passageway which would give access to each incubator room. The entrance to each incubator room from the middle passageway was through a small manhole. The air was drawn through an opening at the floor, passed through a central hole in the ring of fire, and out through the roof vents which allowed smoke and fumes to escape and at the same time provided some light (Brown, 1979; Martin, 2002). Humidity was controlled by spreading damp jute over the eggs as necessary. The piles of eggs were rearranged and the eggs turned twice a day (Martin, 2002). The middle passageway also served as a warm brooding area for the chicks when they hatched. The hatchery workers lived at the hatchery for monitoring incubation conditions (Martin, 2002). Temperature, humidity, and ventilation were checked and controlled with neither special measuring devices nor thermometers (Brown, 1979; Martin, 2002). The eggs' temperature was measured by the use of the worker's eye-lids, which was considered the most sensitive part of the body for evaluation of temperature (Brown, 1979; Martin, 2002). Humidity and air cell size were measured depending on the sound made by rolling two eggs together in one hand (Brown, 1979). Many millions of chicks were produced by these hatcheries every year in Egypt, and by the mid 1600's the Europeans wanted the same technology. Due to differences of weather between Egypt and Europe, the Egyptian type hatchery was not successful in Europe (Martin, 2002).

Chinese. Artificial incubation of eggs was also practiced in China as early as 246 BC with two successful methods that eventually spread through South East Asia (Brown, 1979). The first method was a very simple one in which they used rotting manure to produce heat. The eggs were mixed with straw and rice hulls and placed on top of the manure (Brown, 1979). Their second method was more widely used and is still functional in the present day. As in Egypt, the basic structure of the hatchery was a cylindrical building, but in these hatcheries, the fire was on the floor. The eggs were placed inside of muslin bags, which were loaded into egg baskets made of woven straw. These baskets were placed in a container that had the shape of an inverted cone which was half filled with ashes. A lid was placed on the top to complete the insulation process and to protect the whole incubator from the rain. Each day, the bags would be rearranged in position, thereby turning the eggs. Temperature was tested through the eyelid of the operator (Brown, 1979; Martin, 2002). The Chinese also utilized a heat transfer method using the heat of older eggs to warm younger eggs. Every seven days a new bag of eggs was added to each basket, and after the first 3 wk, they would turn off the fire and allow the heat of the eggs to continue the incubation process (Brown, 1979). At d 16 of incubation, the eggs were removed to another area in the building where they were covered with a blanket and allowed to hatch from incubation d 19 to 21 (Brown, 1979).

Western. For the following 100 years, there were more promising incubators that used different heating methods (charcoal, steam, oil, or kerosene lamps). The main reason of failure for all of these incubators was that they were incapable of regulating the temperature within the narrow range that was necessary (Brown, 1979; Martin, 2002). It was the invention of thermostats that allowed for temperature regulation in a more accurate way and that allowed the development of modern-style incubators (Martin, 2002). The very first American incubator patent, given in 1843, was for a system with a steam boiler used as a heat source (Arrington, 2008). In the United States, by 1885, there were seven different types of small 'still air' cabinet incubators, which were small incubators without a fan for air circulation, for sale. The company "Cypher Incubator" put a 20,000 duck egg hot air incubator model on the market. By 1900, there were 24 different brands available, and by 1915 about 50 incubators were offered for sale. The incubators were usually small machines designed with a capacity of less than 200 eggs for small-scale poultry producers (Martin, 2002). With these incubators, hatching approximately 50% of all eggs set was considered satisfactory. In the early 1930's the production of a larger

forced-draft incubator, which are incubators with fans for internal circulation and usually very large egg capacity, transformed the industry by improving chick quality and increasing the hatchability to 90% of all eggs set (Martin, 2002). During this decade, the development of one day old chicks sexing by the Japanese was introduced as well. These two events had a great influence on the future of the poultry industry by enabling an increase in hatch of fertile eggs and chick separation by sex as soon as they were hatched. This lead to expanding the industry and significantly reducing its costs. By the 1980's the poultry industry was one of the most efficient industries in the world (Brown, 1979; Martin, 2002).

Modern Incubation Systems. Since the development of the first incubator, the commercial incubator of the early 20th century underwent a variety of changes that improved efficiency and reliability. However, commercial incubators have not changed over the most recent 39 years. The only changes that have been seen are with regard to an increase in computerized control of the environment within the incubator (Deeming, 2002). America and Europe have two different types of incubation systems: single-stage and multi-stage. Single-stage incubators are those in which all the eggs within the incubator are set at the same developmental phase (same embryonic age). In this type of incubation system, there are reduced labor costs, they can more precisely meet the requirements of the embryo, and the equipment can be cleaned and disinfected at the end of each hatching cycle to minimize risks of microbial contamination (Lourens, 2001; Boerjan, 2005; Hulet, 2007). In contrast, the multi-stage system consists of machines that incubate embryos at different developmental stages. They are operated continuously with few eggs being placed and older embryos transferred out of the machine several times a wk. Multi-stage incubation uses the heat generated by developmentally advanced embryos, which produce more metabolic heat, to warm the developmentally immature embryos from the beginning of incubation (Hamidu *et al*., 2007; Hulet, 2007). While in Europe single-stage systems have been more prevalent, in North America, multi-stage incubation systems have been more widely used. However, in recent years hatchery managers in the commercial poultry industry have noticed that the environmental temperature near the last few egg racks in multi-stage incubators is much higher than what it was years ago (Hamidu *et al*., 2007). The assumption is that while the incubators have not changed in the past 30 years or more, the modern broiler strains, selected over the past 5 decades for growth and feed conversion, have drastically changed. There is a greater embryonic heat production in the embryos of these

modern broiler strains (Havenstein *et al*., 2003; Tona *et al*., 2004; Hamidu *et al*., 2007; Hulet, 2007). Since the incubators are not designed to handle this level of embryonic heat production, ventilation is insufficient, thus causing an overheating of specific sections in the incubator. This affects not only the hatchability of the eggs, but also chick quality. In general, modern hatcheries have two main goals: 1) maximize hatchability and 2) synchronize the time of hatch (Hulet, 2007). Due to the inclination towards larger capacity incubators and the selection of high-yield, faster growing chicken lines, the control of temperature and airspeed in a uniform manner for all embryos during the entire incubation process has been very difficult (Van Brecht *et al*., 2003). The identification of the precise requirements of the embryo is essential for the incubators to achieve their full potential in producing good quality broiler chicks.

High-Yield Broiler Chicken Strains

It was during the 1940's and 1950's that different breeding companies started to apply their knowledge in quantitative genetics towards the selection of meat type chickens (Havenstein *et al*., 2003). This selection focused on the increment of growth rate, increasing muscle yield, improvement in feed conversion, and decreased age to slaughter of commercial broiler chickens. The result was "high-yield" chicken strains that require approximately 1/3 of the time and over a threefold decrease in the amount of feed consumed to reach desired slaughter weights as compared to what existed five decades ago (Havenstein *et al*., 2003). High-yield strains exhibit a high rate of growth that is completely different from multipurpose breeds of years ago. Janke *et al*. (2004) demonstrated that select broiler lines (White Plymouth Rock), Ross 208 and 508, have a considerably higher gas exchange and heat production during incubation than a layer line (White Leghorn) from Lohmann. This report was supported by a similar study in which the heat production was compared between a Cobb line broiler chicken and a Julia line (Murata hatchery, Fukuoka, Japan) layer chicken (Sato *et al*., 2006). Another study evaluated embryos from a modern fast growing broiler line, Ross 308, and a slow growing line, Labresse, to compare gas exchange, heat production, and fat oxidation levels. They concluded that the embryos from the modern fast growing line showed a higher metabolic rate and oxidized more fat than the slow growing line of broilers. In 2004, Tona and colleagues compared incubation parameters, one day old chick weight, chick quality, and broiler growth to 7 and 41 d of age, as well as heat production, corticosterone and T_3/T_4 levels in plasma in 3 different commercial lines of broiler

breeders. The differences in heat production before d 18 of incubation and the different levels of corticosterone in plasma after hatch suggested different metabolic rates between the three lines. With these research results and industry observations it has become accepted that the embryos from high-yield broilers have different physiological characteristics and therefore different incubation requirements than classic or genetic lines of previous generations.

Chicken Embryo Development and Nutrition

The chicken egg hatches after 21 d of incubation. The optimal incubation conditions are normally defined as those that will result in the highest hatchability. However, established guidelines indicate that the "appropriate" incubation temperature and conditions, range between 37°C to 38°C with around 55% relative humidity (Smit *et al*., 2008).

Extra-Embryonic Membranes. As early as 72 h of incubation, the embryo is lying on its left side with a fully developed heart, brain, and eyes. It is at the third d of incubation when the gastrointestinal (**GI**) tract and respiratory systems begin differentiation and development. Development of these systems continues until embryonic d 10 to 12 which is the time when the chicken embryo is fully formed (Noble and Cocchi, 1990; Deeming, 2002). For the embryo to successfully complete each developmental stage, it requires the formation of extra-embryonic membranes and fluid compartments that will allow it to survive within the egg. These extra-embryonic membranes consist of the amnion, chorion, allantois, albumen sac, and yolk sac membrane (**YSM;** Romanoff, 1960; Noble and Cocchi, 1990).

The amnion is a fluid-filled environment in which the embryo develops and is formed mainly to protect the embryo. With embryonic growth, the amniotic sac increases in size reaching a maximum volume of 3 to 4 ml at d 15 of incubation (Deeming, 2002). The amniotic fluid level, after reaching its maximum, usually declines to non-existence during the last few days of incubation. The fluctuations in volume of amniotic fluid may be affected when incubation temperature is too high, which will cause an early reduction in the fluid volume followed by a sudden rise to a volume of about 6 ml at d 13 and d 16 of incubation (Romanoff, 1960). The amnion has no blood vessels, but it is a very muscular membrane which aids in the retraction of the YSM into the body cavity just prior to hatching (Deeming, 2002). The muscular tissue of the

amnion responds to changes in environmental or embryonic temperature by changing its contraction capacity (Romanoff, 1960).

The chorion is continuous to the amnion and together with the allantoic membrane forms the chorio-allantoic-membrane, which lines the inner surface of the inner shell membrane and has a very important role in the absorption of calcium ions from the eggshell into the embryo. The albumen sac is formed by d 9 of incubation. It is basically a fold of the chorio-allantois composed of flat, cubical, and cylindrical cells with villus-like projections that extend to the albumen. It has neither blood vessels nor connective tissue, but it plays an active role in the absorption of the albumen (Romanoff, 1960). The albumen, contained by the albumen sac, serves to protect the embryo from microbial infection and thermal changes. It also provides water, mineral ions, and proteins to the developing embryo (Romanoff, 1960; Deeming, 2002). The movement of water from the albumen to the yolk can be altered by incubation temperatures (Deeming, 2002). The albumen proteins move into the amniotic fluid and are swallowed and taken into the alimentary tract by the embryo. The proteins are digested in the intestine or transported into the yolk sac so that they can be used during the peri-hatch period or after hatching. As a result, protein concentration of the yolk is actually higher at the end of development than at the start. By d 16 of incubation, the albumen sac disappears.

From the beginning of incubation, the membranes that will form the YSM split into two layers: the dorsal somatic mesoderm associated with the ectoderm, which consists of flattened cells that will create a supportive membrane, and a ventral splanchnic mesoderm associated with the endoderm, which consists of simple columnar cells where the yolk absorption will take place (Noble and Cocchi, 1990). The function of the YSM is to synthesize, digest, store, and absorb nutrients (Speake *et al*., 1998; Moran, 2007). It is an extension of the GI tract and later in development is attached to the small intestine at the jejunum-ileum junction through a narrow tube like tissue, named the yolk stalk. The YSM acts as the major respiratory membrane until the chorio-allantoic-membrane develops. It surrounds the yolk, replacing the vitelline membrane, and after the fourth d of incubation, the YSM maintains the integrity of the embryonic yolk. It is a highly vascular membrane with an extensive network of blood vessels (Romanoff, 1960; Deeming, 2002; Moran, 2007). These blood vessels are not innervated, thus they do not react to any thermal change in the incubation environment. However, experiments

with embryos at d 4 of incubation showed that the blood vessels in the yolk sac respond to carbon dioxide by contracting (Romanoff, 1960). This suggests that with elevated temperatures, these vessels will respond in an indirect manner by responding to the CO_2 accumulation levels and not to the temperature directly. It was suggested that this sensitivity to CO_2 is a mechanism with which blood flow can be regulated at different temperatures (Romanoff, 1960). As development continues, the YSM epithelium becomes morphologically more complex and extensive folding of the membrane causes it to protrude into the yolk. The YSM is also the main site for ion transport between the albumen and the yolk and is critical in the formation of the embryonic fluid. The major role of the YSM is the transfer of nutrients from the yolk into the blood stream, and it has a key role in lipid metabolism (Speake *et al*., 1998; Speake and Powell, 2003).

The allantois appears at around 65 to 69 h of incubation. It expands and becomes a sac surrounding the amniotic sac, the yolk, and the albumen, resulting in a storage compartment for the waste products from the kidneys (Deeming, 2002). The allantoic fluid decreases in volume from d 14 to 19 of incubation due to water utilization by the embryo. When the incubator temperature is higher than 37°C, the fluid volume will decrease sooner (Romanoff, 1960). As the embryo develops, the body becomes detached from the extra-embryonic tissues, with the exception of a very small area where the body wall and the intestine remain open. This opening is surrounded by a double walled tube, which comprises the yolk stalk (Deeming, 2002).

Yolk Sac. The YSM consists of a lining of simple columnar epithelium. The epithelium is formed by endodermal cells, and it is deeply folded into the lumen. Calcium spherules adhere to the endodermal cells and increase in number as incubation progresses (Bernhard *et al*., 1973; Noy *et al*., 1996). The yolk contains 2 different types of components: the yellow yolk, which consists of globules of 1/3 proteins and 2/3 lipids, and the white yolk, which is formed by smaller globules that consist of 2/3 proteins and 1/3 lipids (Freeman, 1974; Dzoma and Dorrestein, 2001). The stroma is rich in blood vessels; however, it has no lymphatic system (Freeman, 1974). The functions of the membrane include erythropoiesis (Holdsworth and Wilson, 1967; Freeman, 1974; Richards, 1991), synthesizing amino acids and proteins (Cheville and Coignoul, 1984), storing glycogen (Freeman, 1974; Noble and Cocchi, 1990; Speake *et al*., 1998; Speake and Powell, 2003), lipoproteins, minerals, lipophilic vitamins, and

immunoglobulin (**Ig**) G (Holdsworth and Wilson, 1967; Freeman, 1974; Richards, 1991; Kaspers *et al*., 1996) and absorbing and transferring them through the endodermal cells to the blood vessels to supply the embryo (Bernhard *et al*., 1973; Kusuhara and Ishida, 1974; Deeming, 2002). The YSM can modify the nutrients on their way into the circulation (Noble and Cocchi, 1990; Richards, 1991) through digestion of the yolk granules with digestive enzymes that are present in the endodermal cells (Dzoma and Dorrestein, 2001). These functions occur throughout the incubation period and continue until after hatching (Kusuhara and Ishida, 1974; Noble and Cocchi, 1990; Richards, 1991). During the early stages of development, the embryo relies mostly on protein and carbohydrate metabolism from the albumen to supply its needs (Richards, 1991). It is during the last few days in the egg when the embryo is ready to hatch (peri-hatch period), that the yolk is internalized into the body cavity to be absorbed by the chick during hatching and post-hatch periods (Noble and Cocchi, 1990).

Yolk Sac- Nutrient Transport. It is well understood that prior to hatching, nutrient transfer occurs mainly through phagocytosis and pinocytosis within the endodermal cells of the YSM. However, the mechanism by which the transport of nutrients occurs from the residual yolk (yolk inside the abdominal cavity once the chick has hatched) into the chick is still not well understood. It is suggested that the yolk sac in the abdominal cavity of the chick begins to diminish in size by d of hatch (**DOH**) as the yolk is released into the small intestine through the yolk stalk (Romanoff, 1960). In a study it was shown that 5 to 7 d after hatching the yolk sac had almost disappeared (Romanoff, 1960). Conversely, Fritz (1961) reported, after a series of experiments, that all yolk contents are absorbed via the YSM blood vessels even after hatch. Supporting studies demonstrated through deutectomy (surgical removal of the yolk sac) that part of the yolk is transported directly into circulation through the YSM blood vessels as it happens during incubation prior to hatching (Bellairs, 1964; Murakami *et al*., 1992). Yolk nutrients start to be released through the yolk stalk into the chick's gut, after most of the yolk has been absorbed through the endodermal cells (Jordonov and Anastossova, 1963). Esteban *et al*. (1991) made the observation that when the yolk stalk in newly hatched chicks was tied, the majority of yolk contents remained in the yolk sac, in contrast to control chicks whose yolk sac was not tied. The lumen of the yolk sac and possibly the yolk stalk can serve as a passageway for the yolk contents to be transferred into the lumen of the small intestine in newly hatched to 2 d old chicks. Taken together, these reports suggest that the newly hatched chick can obtain residual yolk

contents in two different ways: 1) via blood vessels in the YSM epithelium both by the mechanisms of transport across biological membranes (Fritz, 1961; Bellairs, 1964) and by the phagosome-lysosomal system (Romanoff, 1960), and 2) through the GI tract via the lumen of the yolk stalk, followed by absorption through the intestinal, and possibly the yolk stalk's epithelium (Esteban *et al*. 1991). Approximately 5 to 8 d after the yolk has been drawn into the body cavity of the chicken, the YSM undergoes involution through autolysis and phagocytosis by macrophages from the inner layer of the connective tissue (Muhammad *et al*., 2005). Nonetheless, the rate at which the yolk sac involutes is variable. It has been reported to range from 2 to 4 wk to as much as 34 wk (Deeming, 2002). Retention of the unabsorbed yolk sac has been seen in broilers at the time of processing (6 to 8 wk of age); these yolks were still attached to the small intestine (Buhr *et al*., 2006). Nevertheless, in most cases, after the first wk of age, the remnant yolk sac will detach from the yolk stalk and can be found as a small spherical body in the abdominal cavity (Romanoff, 1960; Buhr *et al*., 2006).

Yolk Sac- Nutrient Utilization. During the peri-hatch period, the embryo begins absorbing the yolk and using lipids as its main energy source, and proteins and amino acids as a source for growth and tissue development (Freeman, 1974; Noble and Cocchi, 1990; Puvadolpirod *et al*., 1997; Speake *et al*., 1998). Approximately 90% of the total energy requirements of the embryo are derived from lipid metabolism, and over 80% of the total yolk lipids are absorbed during the hatching process (Noble and Ogunyemi, 1989; Noble and Cocchi, 1990; Puvadolpirod *et al*., 1997; Vieira and Moran, 1999). During the entire incubation period the yolk mass is maintained constant. Yet, the large mobilization of nutrients into the embryo during the peri-hatch period is evident (Richards, 1991; Vieira and Moran, 1999). The remaining unabsorbed yolk from the hatching process becomes absorbed and utilized post-hatching. Deutectomy in newly hatched chicks had no negative effects on further development despite the lack of absorption of residual yolk nutrients (Richards, 1991). Some studies suggest that the yolk sac is fundamentally important only prior to the last 3 to 5 d before hatching but that it is not essential for the post-hatched chick (Puvadolpirod *et al*., 1997). However, opposite thoughts exist with respect to the importance of the residual yolk sac. Many researchers (Sell, 1996; Uni *et al*., 1998; Chotinsky *et al*., 2001; Geyra *et al*., 2001) agreed that most of the energy and nutrient sources from the yolk are utilized predominantly during the hatching process. Nonetheless, after more research, they realized that the metabolism and nutrition of the newly

hatched chick (usually up to d 4 post-hatch) depends largely on the residual yolk to complement dietary nutrients and maintain efficient utilization of food energy and proteins. Absorption of the yolk is thought to assure GI development and maturation resulting in more successful growth post-hatch. Considering that at the time of hatch the physiological functions show different degrees of maturation, it makes sense that nutrients from the residual yolk will facilitate a progressive adaptation of the bird to oral nutrition, which is influenced by many physiological and environmental factors (Esteban *et al*., 1991). Anthony *et al*. (1989) described the function of the residual yolk as "a temporary energy source which guarantees survival of the newly hatched chick". Murakami *et al*. (1992) reported that the absorption of residual yolk is independent of feed intake in the post-hatch chick. On the contrary, multiple studies have demonstrated that the presence of feed in the intestinal lumen enhances the absorption rate of the residual yolk by stimulating and accelerating development and maturation of the intestinal epithelium (Uni *et al*., 1998; Sklan and Noy, 2000; Noy and Sklan, 2001). Dibner (1999) stated that delayed intake of feed and water by the chicken can impair the development of the GI and immune system, and it has a negative impact on growth performance, susceptibility to stress, and mortality. Thus, the residual yolk can have a beneficial impact on the chick if it is completely absorbed and utilized.

Nutrition of the Embryo- Proteins and Amino Acid Metabolism. The yolk consists of 2% carbohydrates, 17% protein, 33% lipid, and 48% water (Johnson, 2000). These nutrients are located within yolk granules, which are mostly protein complexes (lipoproteins or glycoproteins). The yolk granules are comprised by 2 fractions: the water-non soluble fraction, which is composed of lipovitelline, phosphovitin, and low density lipoprotein, and the water-soluble fraction, which is composed of serum albumen, α2-glycoprotein, and serum globulin. The chicken embryo has 2 different pathways of protein absorption during incubation: 1) through transfer of intact proteins through the YSM (pinocytosis and phagocytosis) and 2) through extracellular digestion within the yolk sac lumen and the absorption of their resulting amino acids through YSM. The amino acids absorbed from albumen and yolk are used for protein synthesis in tissue development, nitrogen transport between tissues, ammonia metabolism, nucleotide synthesis, support of the immune system, and as a source of energy through gluconeogenesis (Pons *et al*., 1986). The embryo metabolism may use the albumen proteins to synthesize glycogen. In some cases, at the end of incubation when oxygen levels decrease, the

embryo or newly hatched chick will deplete the amino acids from muscle protein (Uni *et al*., 2005). This may have a negative effect on growth and performance.

Certain amino acids like glycine and proline were shown to be needed at a higher concentration, then other amino acids in order to support embryonic development (Ohta *et al*., 1999). Glycine is a strictly glucogenic amino acid that is necessary for collagen production, metabolic pathways such as the Krebs cycle, and synthesis of purines and pyrimidines necessary for nucleic acid formation. Proline accounts for 50% of collagen (Murray *et al*., 2006). Since collagen is essential for tissue development, proline and glycine need to be synthesized from other precursor amino acids that are present in higher concentrations in the egg. Proline can be synthesized through many different amino acids. However glycine can only be synthesized from serine or threonine.

Glutamate and glutamine constitute approximately 14% of the egg protein and can donate their carbons towards gluconeogenesis, synthesis of non-essential amino acids, participation in the Krebs cycle, synthesis of nucleic acids, amino-sugars, as well as participation in other metabolic cycles. They both are precursors for proline (Ohta *et al*., 1999).

Ovalbumin or ovomucoid protein is the most abundant protein in the albumen. Ovalbumin gets transported into the yolk, and from there it is transferred into the embryo via the YSM or can be ingested directly from the albumen (Sugimoto *et al*., 1999, Moran, 2007). This protein contains large amounts of carbohydrates and is easily coagulated at elevated incubation temperatures (Sugimoto *et al*., 1999). During migration from the albumen to the embryonic organs, this protein becomes heat stable by modifying its structure (Sugimoto *et al*., 1999). Thus, spontaneous modification of proteins during development can also take place in the YSM in order to protect and maintain embryonic development.

Lipids and Lipid Metabolism. The lipid composition of the yolk consists of very low density lipoprotein fraction which provides 22% of the proteins and 93% of the lipids of the yolk. The very low density lipoprotein fraction components are: 70% triacylglycerides, 25% phospholipids, and 5% free cholesterol and cholesterol esters (Johnson, 2000). The most common fatty acids in the yolk are palmitic, oleic, and linolenic acids. The most common phospholipids are phosphatidylcholine and phosphatidylethanolamine (Speake *et al*., 1998). The

total lipid content of the endodermal cells increases gradually throughout the incubation period, but shows a period of greatest increase between d 15 and 17 of incubation (Noble and Cocchi, 1990). During the last wk of incubation, lipids are being absorbed at very high rates, resulting in decreased levels of lipids in the yolk from 65% on d 13 of incubation to 44% by d 21 (Deeming, 2002). The absorption of acidic lipids is greater by the endodermal cells situated adjacent to the hematopoietic tissues (Speake *et al*., 1998). Yolk lipoprotein particles of a diameter of 30 nm are taken up intact into the endodermal cells of the YSM by receptor-mediated phagocytosis (Kusuhara *et al*., 1974; Speake *et al*., 1998; Moran, 2007). Electron microscopy studies show evidence that at the basal face of the cell, there is active secretion of lipoproteins into the blood capillaries. These lipoproteins enter the embryo's general circulation to be used by the developing tissues. However, the lipoproteins that leave the basal surface of the endodermal cells are not the same as those that entered at the apical surface. The secreted lipoproteins are larger and contain a different range of proteins than the lipoproteins of the yolk (Speake *et al*, 1998; Powell *et al*., 2004). The lipids in the yolk are modified during transport across the YSM (Powell *et al*., 2004). During the transition from pre-hatch to post-hatch periods, metabolic heat is rapidly produced due to the fast rate of oxidation of fatty acids (Moran, 2007). Yalcin *et al*. (2008) reported changes in the composition of fatty acids and their concentrations in high-yield broiler strains, when incubated at elevated temperatures (38.5°C). This probably happens as a mechanism of cell protection given that many of these lipids are involved in the permeability and structure of the developing cells.

Carbohydrates and Carbohydrate Metabolism. Carbohydrates are very important during the development of the embryo, especially during the differentiation phase. They also function through anaerobic catabolism as an energy substrate during the peri-hatch period (Moran, 2007). Approximately 70% of the carbohydrates are in the form of glucose in the albumen and in the form of glycoproteins within the yolk. During early development, there are very low quantities of glucose in the albumen and yolk, but they increase in concentration in the embryonic circulation due to gluconeogenesis, which is active during most of the incubation period. Sugar transport is an active transport against a concentration gradient, and it is available by d 17 of incubation. This transport is inhibited by low sodium concentrations (Holdsworth and Wilson, 1967). During development, the YSM has an increased number of sites for glucose transport, and as the yolk sac involutes, these sites disappear by d 5 to d 6 post-hatch (Holdsworth and

Wilson, 1967). Glycogen provides glucose for energy through anaerobic glycolysis since the oxygen levels are very low until the chick initiates lung respiration (Moran, 2007). Kusuhara and Ishida (1974) observed through electron microscopy that the endodermal cells of chicken YSM are rich in glycogen phosphorylase and glycogen synthase, suggesting the presence of active glycogen metabolism in the yolk. Light microscopy studies have shown the presence of glycogen within the basal endoderm, and electron microscopy reveals glycogen granules to be scattered through the mesodermal layer (Bernhard *et al*., 1973). Gluconeogenic enzymes such as pyruvate carboxylase, phophopyruvate carboxylase, hexose-diphosphatase, and glucose-6-phosphatase reach their maximum concentration levels by d 16 to 17 of incubation and then decrease during the peri-hatch period (Bernhard *et al*., 1973; Kusuhara and Ishida, 1974; Noble and Cocchi, 1990). The enzyme glucose-6-phosphatase allows endodermal cells to produce glucose from glycogen, and these cells supply glucose to the embryo. After d 13 of incubation very little concentration of sugars is left. Consequently, glucose is synthesized from amino acids by gluconeogenesis or by glycolysis from glycogen reserves (Christensen *et al*., 2001; Uni *et al*., 2005). The glycogen shows a peak in synthesis between d 7 and d 9 of incubation. Given that lipid metabolism requires oxygen, at the times of pipping and hatching glycogen becomes critical as a source of carbohydrates until external sources of sugars become available. In the chicken embryo, the major site for glucose synthesis is the liver; however, the kidneys can contribute to gluconeogenesis from 5 to 50% of glycogen; the major reserves for glycogen are liver and muscles (Corssmit *et al*., 2001). Hepatic glycogen starts accumulating at d 6 of incubation. The increase in activity of thyroid hormones and epinephrine, an important hormone in the stress response causes glycogen to decline at d 13 of incubation. By d 18, the accumulation of glycogen in liver increases again up to 400% from what it had earlier in incubation (Hazelwood, 1971). At the time of hatch, the hatching muscles are mostly relying on glycolysis. At DOH, chicks drop their glycogen storage concentrations in liver from 400% to 16% once they have full access to external feed and oxygen (Moran, 2007).

Residual Yolk Utilization. In order for the small intestine to become a fully mature organ, it has to undergo many morphological and physiological changes in the mucosal epithelium. These adaptations, such as dramatic changes in villus size and expression of enzymes and nutrient transporters, will allow the intestine to secrete, digest, and absorb nutrients in a very efficient manner. This will allow the tissue to obtain the energy necessary for

maintenance, development, and growth since energy is required for almost any metabolic and developmental processes (Geyra *et al*, 2001). The chicken's growth is dependent upon efficient transition from yolk to oral nutrition. If successful, it will result in a chick that can reach its maximum growth potential in a shorter period of time, and whose immuno-competence will be completely acquired (Uni *et al*., 1998; Bar-Shira and Friedman, 2006). During the first d of life in the chicken, there is a fast disappearance of protein from the yolk sac due to high intestinal and pancreatic enzyme (trypsin and proteases) activities (Sklan *et al*, 2003). The chicks that do not absorb the residual yolk or have no immediate access to feed, or both, take longer to make an adequate transition from the *In Ovo* environment to the post-hatch life (Sklan, 2003). However, 20% of the residual yolk proteins are Ig, and the lipids are basically triglycerides, phospholipids, and cholesterol (Dibner *et al*., 1998). The use of Ig for nutritional purposes to provide energy for growth to the chick might deprive the chicks from acquiring passive immunity protection, and phospholipids and cholesterol are also more important as major molecules for cell membranes and general cell structure. Consequently, scientists are interested in creating and improving strategies that make the transition for GI development and increase growth rates as efficient and fast as possible. There are 2 strategies that have proved to be successful in facilitating this transition in a shorter period of time 1) *In ovo* feeding (**IO**) and 2) immediate access to feed right after hatch.

In Ovo Feeding. Nutrients available to the chicken embryo are determined by the breeders even before the egg is laid. Thus, the egg has a limited nutrient availability (Foye *et al*., 2006). Given that immediate access to feed post-hatch is essential for the rapid development of the small intestine, and that the glycogen reserves become limited during the peri-hatch period, the access to external nutrients by the embryo will help meet its requirements for energy, block the depletion of amino acids from muscle, and enable the intestinal epithelium to have early utilization of nutrients, thereby accelerating development and allowing the chick to obtain a good key performance indicator. *In ovo* feeding overcomes the delay in access to feed that commonly happens in the industry. The IO consists of injecting a nutritive solution, usually made of maltose, sucrose, dextrin, L-arginine, and β-hydroxy-β-methylbutyrate (HMB), into the amniotic fluid at d 17.5 of incubation. The HMB is a leucine metabolite that allows for maximal growth and cell function. It has been suggested that it can also act indirectly as a stimulator for insulin secretion, which induces glucose uptake into muscles for glycogen synthesis, and has an

important role in stimulation of protein synthesis. Studies have shown that IO in eggs incubated at adequate temperatures (37°C) accelerates the developmental processes (proliferation, differentiation, and maturation) of the intestinal epithelium and increases the chick's body weight at DOH. This occurs by increasing the rates of absorption due to an enlargement in the villi size and the capacity to digest disaccharides (Tako *et al*., 2004)*In ovo* feeding has also been used to improve muscle growth by enhancing the proliferation of satellite cells. This has resulted in an increase of 7 to 8% in pectoral muscle mass (Uni *et al*., 2005). The administration of amino acids *In Ovo* has improved protein accumulation by about 400 mg in the embryo (Ohta *et al*., 1999). The energy status of the late term broiler seems to be improved by IO by aiding the chick in achieving its potential for late embryonic and early post-hatch growth (Uni *et al*., 2005). Two studies have evaluated the performance, up to d 35, of chicks fed *In Ovo* during the last wk of incubation. The research confirmed the idea that early access to feed accelerates gut development by having chicks at DOH with an intestinal morphology of a 2 d old chick. They also demonstrate that these chicks maintain these positive results from *In Ovo* access of feed all the way to 35 d of age as indicated by a 3 to 7% increase in body weight as compared to chicks that did not receive any IO (Uni and Ferket, 2004). Interestingly, Moreno *et al*. (1996) characterized glucose uptake in the chick from d 2 before hatch, until d 7 post-hatch, in the three segments of the small intestine. The rate of glucose transport (V_{max}) significantly increased at DOH in the duodenum, and at d 2 in the jejunum. These results correlate with the morphological development of the small intestine and the presence of luminal nutrients from albumen, residual yolk and external feed sources. Interestingly, a study observed that the ability of the newly hatched chick's small intestine to absorb external carbohydrates is very low due to a limited availability of the main glucose transporter, sodium dependent glucose transporter 1 (**SGLT1**). In this study the expression of SGLT1 starts until d 19 of incubation, increasing abruptly at d 20 and decreasing fast by DOH (Sklan and Noy, 2000). With this the utilization of the residual yolk is important from hatch until d 2 post-hatch, and the external sources of nutrients given in IO have a short window of opportunity to be ingested. In any case, if anything hampers the development of the small intestine histologically and histochemically and impairs its function, the chick will still not get the nutrients necessary. Thus, the IO techniques and its mechanisms of action should be further studied.

Problems with Yolk Sac Absorption. During the peri-hatch period and right after hatching, significant amounts of yolk may pass through the yolk stalk into the intestine. During passage, part of the yolk may be absorbed by the epithelial lining of the yolk stalk or pass directly into the lumen of the small intestine (Esteban *et al*., 1991). Sometimes malformations occur. For example, the mesentery forms loops around the intestine causing strangulation of the yolk stalk, resulting in yolk retention (Barnes *et al*., 2003). At the hatchery chicks that have been incubated at temperatures above or under 37°C to 38°C present a large residual yolk, while those chicks incubated at more optimal conditions (37°C) have a smaller residual yolk. The uptake and secretion of lipids and proteins by the YSM appear to be impaired in situations such as elevated temperatures or decreased oxygen levels during incubation, that lead to high embryonic mortality (Speake and Powell, 2003; Powell *et al*., 2004). Given that there is transfer of passive immunity through the yolk contents into the newly hatched chick during the peri-hatch period, elevated temperatures may hamper the nutritional status as well as the immunocompetence of these chicks.

The umbilicus and the yolk stalk are two structures that are anatomically, histologically, and physiologically intimately related. The umbilicus is part of the ileum, and during its ontogeny it ends up being posterior to the yolk stalk. The retraction of the umbilical loop into the body cavity starts between d 16 to 17 of incubation and ends on d 19, the same time at which the yolk stalk is drawn inside the abdominal cavity. Elevated temperatures during incubation can be correlated with unhealed navels (unhealed umbilicus). This occurrence can lead to omphalitis (inflammation of the umbilicus), which can be infected or not (Kenny and Cambre, 1992). Chicks that present unhealed navels grow much less efficiently than chicks whose navels healed properly. There is a direct correlation between unhealed navels and yolk retention (Kenny and Cambre, 1992; Speers, 1996; Crespo and Shivaprasad, 2003). In severe cases, an unhealed navel may lead to systemic disease and may result in death (Crespo and Shivaprasad, 2003). The physiological explanation for why this happens has not yet been explained. Nonetheless, correlations between yolk sac infection and omphalitis could be explained through the effects of elevated temperatures (higher than 38°C) on the yolk stalk. Given that the umbilicus and the yolk stalk are 2 structures intimately related, studies involving histopathology and functional activity of the yolk stalk should be evaluated.

Meckel's Diverticulum. The yolk stalk is the tissue that connects the yolk sac to the small intestine to allow the vitelline blood vessels to enter the embryo (Romanoff, 1960). It is located in the mid section of the small intestine and is used as a reference point to separate the jejunum from the ileum. The muscle layer of the intestine forms a sphincter-like thickening at the opening of the yolk stalk (Olah and Glick, 1984), which could be thought as the access route of yolk nutrients from the yolk sac to the lumen of the intestine. However, even though the yolk sac is continuous with the endodermal cells of the small intestine, passage of any yolk material into the gut during incubation is prevented by a loop of intestine which protrudes into the yolk and blocks this passageway until just prior to hatching (Noble and Cocchi, 1990). The yolk stalk consists of two walls. The outer wall or somatic stalk connects the embryo and amnion, and the inner wall, or yolk stalk connects the embryo and the yolk sac (Romanoff, 1960). The yolk stalk is totally formed by the end of d 6 of incubation. It is during the last wk of incubation that the endodermal cells form brush bordered simple columnar epithelium containing goblet cells. By d 19 of incubation, a muscle layer composed of longitudinal and circular smooth muscle layers is present. On the luminal side of the muscle, the connective tissue layer produces villus-like folds. The yolk stalk presents a similar basic organization as the small intestine with four histologically different layers. Once the YSM is drawn into the abdominal cavity of the chick, the yolk stalk elongates and remains permanently as Meckel's Diverticulum (**MD**; Olah and Glick, 1984). Between 2 and 5 wk of age, the longitudinal folds of MD are infiltrated with myeloid and lymphopoietic tissues. These are located in different layers of the MD and are separated from each other by the muscle layer. The monocytic cells are found in the lumen were they fuse and form giant cells. Lymphoblasts together with plasma cells form germinal centers (Olah *et al*., 1984). After 7 wk of age, the plasma cells are scattered among a large population of mast cells. MD growth is thought to be related to the absorptive function of its epithelial layer. However, the absorption of yolk is restricted to the first 2 wk of life while significant growth of MD takes place much later (Olah *et al*., 1984). In the germinal centers, secretory cells may serve as follicular dendritic cells. Due to the presence of the lympho-myeloid complex tissues, the MD can be considered, together with the cecal tonsils, Peyer's patches, and aggregated lymphoid tissue in the coprodeum, as part of the GI associated lymphoid tissue (**GALT**; Olah *et al*., 1984). Thus, it can be concluded that the functions of the yolk stalk and yolk sac do not stop after hatching and are not limited to nutritional but immunological functions as well.

Yolk Sac as an Immune Organ. Chicks depend on maternal antibodies to be protected from several diseases at the time of placement on commercial poultry farms. These maternal antibodies are passively transmitted from the hen to the offspring through the yolk. The Ig are transferred from the hen's circulation by the follicular epithelium of the ovary to the developing ova (Patterson *et al*., 1962; Li *et al*., 1998; Wang *et al*., 2004). The maternal antibodies can circulate in the chick for up to 3 wk, hopefully giving the newly hatched chick time to allow for further maturation and development of the immune system (Bermudez and Stewart-Brown, 2003). The immune system of the chicken embryo is not mature enough to produce antibodies prior to hatching, thus there is no de novo synthesis of antibodies by the chicken embryo (Rose and Orlans, 1981; Li *et al*., 1998). The main source for IgA and IgM is the albumen (Kaspers *et al*., 1996). During development, IgA and IgM are transported into the small intestine of the chicken at the time when the embryo swallows the albumen, around d 14 to d 20 of incubation (Kaspers *et al*., 1996). There is no IgA or IgM transferred into the embryo's circulation, but they can be transferred from albumen and found as part of the contents of the yolk sac during the peri-hatch period (Jordonov and Anastossova, 1963). IgG is the only maternal Ig present in the blood of the chick, and it is the main Ig present in the yolk from embryonic d 1 to hatch. It can be found in the albumen at d 4 of incubation and in the amniotic and allantoic fluids from d 12 to d 14 of incubation (Wang *et al*., 2004). The endodermal cells express specific IgG receptors that will allow the IgG to be transferred from the yolk into the blood vessels of the embryo without being degraded or digested (Linden and Roth, 1978; Li *et al*., 1998). IgG can be found in the chicken's serum by d 12 of incubation and in the intestine by d 19 of incubation. Problems during incubation that may affect blood vessels, the absorption of the yolk sac, or the conformation of the MD will not only affect the nutritional condition, but could also negatively impact the immune status of the baby chick.

Incubation and Embryonic Temperature

Incubator Temperature vs. Eggshell Temperature. The temperature surrounding the embryo will determine its metabolic rate and therefore the amount of heat produced (Van Brecht *et al*., 2005). It is therefore very important to take into consideration that the temperature inside the egg is the temperature experienced by the embryo. While the incubator environmental temperature is important, the internal egg temperature should be considered as the direct

determinant for the development of the embryo and the resulting hatchability. However, it is very difficult to measure the internal temperature of the egg without invasive measures that risk damaging the embryo. The measurement of eggshell temperature (**EST**) has been the best non-invasive method to measure the embryo temperature (French, 1997). It has been found that even the EST may have deviations of over 4°C, depending on the egg's location within the incubator and the developmental age (Lourens, 2001; Joseph *et al*., 2006). In measuring variances in EST, Lourens (2001) observed that locations where the air flow was lower in the incubator, the EST were higher. Results showed that hatchability was decreased significantly in those trays with higher EST due to a higher incidence of dead embryos late in the incubation period and an increase in the percentage of chicks culled due to malformations or poor chick quality. In commercial incubators, temperatures higher than 40°C have been observed during the last wk of incubation (Lourens, 2001). The eggs with these high temperatures were those from locations with low airflow between trays. Data from chicken and turkey incubators show that the temperature indicated on the incubator control can be significantly different from the temperature of the air surrounding the egg, meaning that the temperature readings from the incubator environment are not the same for the EST readings (French, 1997; Leksrisompong *et al*., 2007). Leksrisompong *et al* (2007) demonstrated that when the incubator environment temperature was 37.3°C, on d 14 of incubation, the EST at the same time point reached 38.2°C. In order to maintain the EST at 37.9°C on d 19 of incubation, the incubator air temperature had to be decreased to 36.3°C (1.6°C difference), confirming that a difference existed between incubator and embryo temperature. During the first part of incubation, lower EST in multi-stage incubators is common, and during the latter part of incubation EST is commonly higher in both single and multi-stage systems (Joseph *et al*., 2006).

Incubation, Temperature, and Development. Embryonic temperature is considered to be the most important factor during incubation, not only for hatching percentage of fertile eggs but also for post-hatch growth of chicks (Wilson, 1991; Lourens *et al*., 2005; Lourens *et al*., 2007). Heat transfer between the environment and the egg is determined by the airflow direction and its speed around the eggs (Van Brecht *et al*., 2003; 2005), but it is not significantly affected by the humidity level (Deeming, 2002). Since the embryo is not capable of regulating its own temperature until hatching, the embryo body temperature is regulated by the incubator air temperature (Leksrisompong *et al*., 2007). Consequently, the temperature that the embryo

encounters will depend on the incubator temperature, the ability of heat to transfer between the incubator and the embryo, and by its own metabolic heat production (French, 1997). Van Brecht (2005) reported that the thermal conductance of the egg to the environment depends not only on the surrounding air temperature, but on the breed, flock age, porosity and egg characteristics (surface/volume ratio), air humidity, air flow, and ventilation capacity of the incubator. There are two parts involved in converting the yolk and albumen in a fertile egg into an embryo: 1) differentiation and 2) growth (Deeming, 2002). During the first half of incubation (d 1 to d 10.5), chicken embryos go through a differentiation phase involving organ formation. They are capable of absorbing heat from the surrounding air, since they have a lower temperature than the incubator. The second half of incubation (d 10.5 to d 21) is characterized by growth, and embryos must lose heat as their metabolic rate and heat production increases, thereby increasing oxygen consumption and CO_2 levels (Smit *et al*., 2008). In multi-stage incubators it is very common to see the lowest EST (< 37°C) at the beginning of incubation, from embryonic d 1 to d 7, while high EST (> 38°C) readings are common in the late incubation period, from embryonic d 14 to d 21, in both multi-stage and single-stage incubators (Lourens, 2001; Lourens *et al*., 2005; Joseph *et al*., 2006; Lourens *et al*., 2007). Significant metabolic heat production starts around embryonic d 4, resulting by d 9 in a significantly higher embryonic temperature than that of the incubator (French, 1997; Hulet *et al*., 2007). The incubator should efficiently remove the heat accumulated, rather than just distributing the air, so that the EST can be maintained within normal (37°C to 38°C) limits for development (Deeming, 2002; Hulet *et al*., 2007; Smit *et al*., 2008). The increase in metabolic heat within the egg will transfer to the incubator environment rising the incubator temperature. Poor air flow and, therefore, poor removal of the heat within the incubator, will lead to an increase and accumulation of heat, causing the egg temperature to increase further (French, 1997; Lourens *et al*., 2005; Deeming, 2002). In commercial incubators, it is common to find records of egg temperatures 2°C above the set point (temperature at which the equipment is set to maintain incubation; Hulet *et al*., 2007). These conditions make it very difficult to maintain proper egg temperature if the ventilation system within the incubator is not designed to handle such temperature increases (Hulet *et al*., 2007). The temperature conditions during embryo incubation may have a great impact on development of physiological systems, maybe inducing epigenetic adaptations by having an effect on the chicks' development (Decuypere and Bruggeman, 2005). Thus, improving incubation conditions may have positive

long-term effects in the chicken. However, if these conditions are not optimal for embryonic development then negative effects may continue to be observed after hatch. The optimum incubation temperature is defined as the one that will enable us to achieve maximum hatchability (Wilson, 1991). However, the industry has to consider not only maximization of hatchability and synchronization of hatch time, but factors that will determine optimal incubation environment required for high-yield modern broiler strains taking post-hatch characteristics and performance into consideration.

Elevated Temperatures During the Differentiation Phase of Embryogenesis. During the differentiation phase (embryonic d 1 to 14), the tissues are formed, and by d 12 of incubation 90% of the organs are already present. Extra-embryonic tissues are essential to the normal development of the embryo (Deeming, 2002; Deeming, 2005). Hyperthermia in developing tissues or cells has detrimental consequences for embryonic organogenesis. In a study reported by Nilsen (1984), chick embryos that were incubated at 41°C or 42°C, as compared to the standard incubation temperature of 37°C to 38°C, during the first 3 d of development presented both intra- and extra-embryonic vascular abnormalities. Therefore, vascular development is affected by elevated temperatures causing negative effects on further embryonic growth and development. This is confirmed by a report of constant incubation of chicken embryos at 40°C that resulted in malformations of the heart, liver, gizzard, and the small intestine in 63% of the incubated chicks (Delphia and Elliot, 1965). At a cellular level, hyperthermia may cause loss of protein function, which can be attributed to the normal misfolding of proteins with elevated temperatures. As a consequence, protein denaturation may result in formation of insoluble aggregates that result from incorrect folding of the peptide chains (Bensaude *et al*., 1990). A decrease in protein function, together with abnormal modifications that include changes in the phosphorylation state of eukaryote initiation factors and ribosomal proteins, will contribute to disruption of protein synthesis (Bensaude *et al*., 1990). Thus, hyperthermia is a stress factor that, if present during embryonic development, will disrupt the plasma membranes and membrane protein functions, altering cell structure and negatively impacting DNA, RNA, and protein synthesis. The resulting outcome may be seen as decreased hatchability, increased malformations, and possibly poor post-hatch performance due to retarded or defective development of the extra-embryonical membranes and organs. An increased mortality of incubated chicken embryos has been reported with as little as 24 h exposure to temperatures in

the range of 41°C to 42°C (Peterka *et al*., 1996). Elevated temperatures during the avian embryo's early stage of development may have serious detrimental effects on its development and viability. It is important, nevertheless, to consider that there is also an influence of lower temperatures at this critical stage.

Low Temperatures During the Differentiation Phase of Embryogenesis. Temperatures below physiological zero (27°C) cease embryonic cellular differentiation, until warmer temperatures are restored (Feast *et al*., 1998). In the industry, incubation temperatures do not go as low as physiological zero, unless the eggs need to be stored. In multi-stage incubation systems, during the early incubation period (d 1 to d 7), the temperature of the embryo is expected to be really close to the one of the incubator environment due to the differences between heat production and heat transfer from the fertile eggs to the incubator (Lourens *et al*., 2005; Joseph *et al*., 2006). Lourens *et al* (2005) showed that if during the first wk of incubation, the EST is 36.7°C, the embryos will require a higher temperature (38.9°C) during the second and third wk of development to compensate for the alteration in heat production during the early phase of incubation. This is because EST profiles during the first wk of incubation have a great influence on development of thermoregulation abilities of the chick, especially during the first wk post-hatch (Yahav *et al*., 2004a; Yahav *et al*., 2004b). Ross x Ross 308 broiler chicks were incubated at 36.7°C during the first wk of incubation, and results indicated reduced embryonic weight (embryonic d 10), low hatchability, poor chick quality, a decrease in carcass weight, increase in abdominal fat pad yield, reduced fillet weight-yield, and decreased tender weights when compared to embryos incubated at 37.8°C EST (Joseph *et al*., 2006). Earlier studies have also shown that temperatures below 37°C during the first 10 d of incubation result in a lower feed efficiency, increased heat production directly correlated to compensatory growth that starts during incubation and continues at 1, 2, and 3 wk of age post-hatch (Geers *et al*., 1983). This is supported by a study involving chicks incubated during the first 10 d of incubation at 36.8°C that had a lower body mass at d 3 post-hatch. However by d 35, the body mass of the two groups were similar (Decuypere, 1984). Given that it is common to see low temperatures at the beginning of the incubation period in multi-stage incubator systems, possible negative impacts on embryo development and post-hatch performance warrant more detailed monitoring of EST.

Effects of Elevated Temperatures During Growth Phase of Incubation. The growth phase involves the maturation and growth of tissues from embryonic d 15 until the embryo is ready to hatch (Deeming, 2002). Overheated embryos during this phase, will have a low survival rate, reduced chick quality, increased incidences of malpositions and decreased hatchability (French, 1997). The effects of elevated temperatures after the second wk of incubation of turkeys and chickens have been evaluated. Research reports confirm that elevated temperatures during incubation have a negative impact on different physiological systems, as well as a resulting decrease in chick quality and performance. Christensen *et al* (2004a) observed a decrease in intestinal weight and reduction in intestinal maltase and alkaline phosphatase activities suggesting an inhibited function of these in turkey embryos incubated at 39°C during the last three days of incubation (d 25 to d 28;). Other studies reported decreased heart weight and cardiac tissue energy metabolism (Christensen *et al*., 2004b) and reduction in T_3 triiodothyronine (T_3) and thyroxine ($T_{4)}$ concentrations in turkey embryos incubated also at 39°C from d 25 to d 28 (Christensen *et al*., 2005). Interestingly, in a study by Maltby *et al*. (2004) turkey embryos that were incubated at an incubator temperature of 38.5°C from embryonic d 9 to 12 had higher muscle fiber numbers at d 16 post-hatch; and poults incubated at 38.5°C during d 5 to 8 of incubation exhibited higher but delayed myogenin expression, due to an increase in myoblast proliferation but a delayed differentiation. By d 16 post-hatch poults incubated at 38.5°C from embryonic d 5 to 8 had significantly higher muscle nuclei numbers than the control poults that were incubated at 37.5°C during the same period. A greater number of muscle nuclei leads to hyperplasia, which means improved meat quality, however, higher number of muscle fibers leads to hypertrophy, which results in decreased meat quality (Maltby *et al*., 2004). To date, almost all of the published data on incubation temperature and its effects on chick quality, performance and other physiological systems have reported only incubator air temperatures, or have not been done with high-yield broiler strains. Leksrisompong *et al* (2007) showed that Ross X Ross 308 broiler chicks incubated at 40°C EST from embryonic d 19 to 20, had increased weights of yolk sac and liver and decreased gizzard, proventriculus, and small intestine weights, as compared to control chicks incubated at 38.2°C. In another study, 2 high-yield broiler lines (Ross 308 and 508) and a layer line (Lohmann White Leghorn) were evaluated for oxygen consumption to calculate heat production and internal egg temperature used as a measure for body temperature during the second half of incubation (Janke *et al*., 2004). Heat production

was calculated on the basis of egg mass, oxygen consumption, and a caloric heat equivalent (19.7 J/ml^{-1}) obtained from previous research done by Decuypere in 1984. The broiler chicks hatched one d before the layer chicks, which could be interpreted as an indication of faster metabolic rates and thus earlier development. Other studies have shown that during short-term (3 h) increases of incubation temperature, the stimulation of thermoregulatory system development in chick embryos can be induced, whereas a long-term (1 wk) increase of incubation temperature affected the embryo by presenting brain lesions, unhealed navels, and overall reduced chick quality (Yahav *et al*., 2004a). Further research investigating the effects of elevated embryonic temperature of high-yield broilers, on different aspects of development and post-hatch performance, need to be conducted. It is clear that incubation temperature during the first and last wk of incubation has a retarding or accelerating effect on development; however, the effect in lifetime performance is not yet proved or understood. It is necessary to determine optimal EST and monitor it closely, taking into account the long-term effects of incubation parameters during embryonic differentiation and growth.

Chick Quality

There are many factors that may influence chick quality. They are divided into pre-incubation and incubation factors. The pre-incubation factors may determine the characteristics of the egg to be incubated. These are line, strain, or flock age of the breeders, as well as storage conditions of the egg (Decuypere and Bruggeman, 2007). Incubation factors have an impact on the physiology of the developing embryos (Maltby *et al*., 2004). These factors may cause adaptations that alter gene expression or transcription factor levels in a temporal or permanent way affecting them as gene modulators and modifying growth characteristics of specific broiler strains (Decuypere, 1984; Decuypere and Bruggeman, 2005). Incubation conditions increase in importance as selection of different traits in broilers continue, given that the embryo's environment can improve or affect its different stages of development and have an impact on chick quality and growth performance of the post-hatch broiler chick.

The term "chick quality" is still not well defined. However, it could be defined as the grading that a chick obtains at DOH, depending on different characteristics that can categorize them as poor, average, or good quality chicks. The quality of DOH chicks is directly related to the ability of a flock to result in good feed efficiency, high growth performance, and low mortality (Tona *et*

al., 2005). Chick quality evaluation is based and created from data obtained from hatcheries and grower farms. Hatchability cannot be used as a parameter for chick quality, since it does not correlate to the quality of the chicks. Therefore chick quality scoring parameters are related to the DOH chick survival and severity of abnormalities present, such as unhealed navels, malformation of legs, exposed brains, white color, etc (Tona *et al*., 2005). In most hatcheries, a good quality chick will have the following characteristics: yellow color, clean, dry, no malformations, completely sealed and clean navel, no yolk sac or dried membrane sticking out from the navel area, firm body, and alert and responsive to environment sounds (Tona *et al*., 2003; Boerjan, 2005; Deeming, 2005). At the hatchery, chick quality is an "all or none, marketable or not marketable selection" (Decuypere and Bruggeman, 2007). Due to the subjectivity of the evaluation of these physical characteristics, and based on these qualitative parameters, the Pasgar score (Boerjan, 2005) and the Tona or Leuven (Boerjan, 2004; Tona *et al*., 2005) scoring systems were developed. The Leuven and Pasgar scores measure characteristics on different scales. In both, highest numbers qualify as best chick quality. An example of the Leuven score is illustrated in Table 2.1. Both convert visual evaluations into measurable and repeatable parameters. These scores allow for trained workers to be able to score the quality of the DOH chick in a more accurate and repeatable method. These evaluations enabled the conversion of the above mentioned qualitative parameters into quantitative characteristics that allow the chicks to be grouped into poor, average, or good quality categories (Tona *et al*., 2005). Most parameters considered in the Pasgar and Leuven scoring systems have been highly correlated to the conditions of the navel area related to the amount of retracted yolk and to the chick's activity (Fasenko and O'Dea, 2008). Improper navel development and resolution of yolk sac internalization have the highest incidence within the hatcheries, and thus could be parameters of importance in the chicks' growth (Tona *et al*., 2005). However, it has not yet been demonstrated that the Pasgar and Leuven score systems have a strong positive correlation with broiler performance (Vieira and Moran, 1999). Other quantitative measurements that are used to define good quality DOH chicks and that could be correlated to performance are 1) chick's hatching yolk free body mass weight (Boerjan, 2005; Deeming, 2005) and 2) chick length (Hill, 2001; Wolanski *et al*., 2003). The yolk free body mass weight is the body weight without the residual yolk of the post-hatch chick. Chick length measures the spinal length, the shank length, or the entire body length of the out-stretched chick, measured

from tip of the beak to the middle toe (Decuypere and Bruggeman, 2007). A study presented results from eight different Hybro pure lines, showing that chick length correlated positively with yolk-free body mass at hatch (Wolanski *et al*., 2003). They also showed significant positive correlation between chick length at hatch and body weight at six wk of age (Decuypere and Bruggeman, 2007). Body weight at DOH is mostly correlated to the egg's size and weight, but it is not a good indication of chick quality and development because much of the weight includes the unabsorbed yolk sac (Decuypere and Bruggeman, 2007). If a large amount of yolk sac has not been absorbed, it could indicate that the chick is less developed. Considering that the DOH chick's body weight does not represent correctly the chick's development, the yolk free body mass is considered a better indicator of chick quality and proper incubation processes (Decuypere and Bruggeman, 2007). There is no relationship between the weight of a DOH chick and performance at slaughter age (Decuypere, 1984; Tona *et al*., 2004). However, it has been reported that the weights of birds at d 7 to 10 are correlated to the performance at slaughter age. The use of body weight of 7 d old chicks is now considered, in the hatchery industry, as a "Key Performance Indicator" or KPI (Smith, 2005). Thus, part of the hatchery manager's challenge is to increase the possibility of obtaining good quality chicks to allow for the best chance of efficient performance during the first 7 d of life (Smith, 2005). Chick length has been used as a tool for evaluating chick quality. A research group established chick length uniformity profiles. This group measured chick length in chicks hatched from trays localized in different areas of the incubator (poor or good airflow and cool or hot areas) and from different breeder flock ages (prime, young, and old). Results showed that there is a direct correlation between DOH chick lengths with incubation conditions. For example, chicks with short length come from hatcheries that present the highest mortality during the first wk after hatch. Chick length also varies depending on the tray location within the incubator. Chick length in single-stage incubation systems is larger than that obtained in multi-stage incubators, and in multi-stage incubators the chick length is larger during summer than in spring (Hill, 2001).

The Small Intestine

Anatomy. Briefly, as described by Hodges (1974), the small intestine of the chicken presents essentially the same structure throughout its entire length. However, based on size and function it is divided to different sections. The first section called the duodenum, leaves the

gizzard and is doubled upon itself in an elongated loop. The pancreas lies within this loop. From the posterior end of the duodenum the next and longest division of the intestine continues. It begins at the point where the pancreatic and bile ducts enter the intestine, and it has received different names (Meckel's tract, jejunum, ileum, and jejuno-ileum; Romanoff, 1960). For purposes of consistency with most of the scientific community that does research in domestic fowl, this dissertation will refer to the anterior region of this section as the jejunum and the more posterior section as the ileum. Since there is no absolute line of demarcation between these two regions, the MD is commonly used as a point of reference to identify or separate them. The small intestine is supported by the mesentery and ends at the ileo-cecal junction. The ileum leads into the short straight large intestine which comprises the colon and the rectum. The point at which the ileum merges with the large intestine and the two ceca is called the ileo-cecal junction. The ceca are two branches of the intestine which adhere with mesentery anteriorly on either side of the ileum (Romanoff, 1960, Hodges, 1974). The large intestine ends joining together with the cloaca.

Histology. The basic structure of the intestine consists of 5 layers (Hodges, 1974; Karcher and Applegate, 2008):

1) Simple squamous epithelium, underneath which is a thin layer of loose connective tissue containing collagen fibers, blood vessels, and nerves, that thickens to form the mesenteries.

2) Muscularis externa, which also has internal to it a thicker circular muscular layer. Between these two, there is connective tissue formed by elastic fibers.

3) Sub-mucosa is a very thin layer of connective tissue and elastic fibers separating the muscularis externa from the muscularis mucosa.

4) Muscularis mucosa consisting of longitudinal muscle fibers throughout the length of the intestine except in the ceca, where there are two layers.

5) Intestinal mucosa or epithelium formed by villi arranged in a zigzag pattern, which vary considerably in number, shape and size, depending on the region in which they are found. It is formed by the epithelial cells in the villi, the crypts of Lieberkuhn, and the lamina propria (Figure 2.1).

The intestinal mucosa consists of the cells throughout the villi which are: the chief or main epithelial cells (enterocytes), mucus producing goblet cells, enterochromaffin cells, Paneth cells, intraepithelial lymphocyte cells (**IEL**), and the cells from the crypts of Lieberkuhn (Figure 2.2). All these cells are produced at the base of the crypts by mitosis and then gradually migrate up the walls of the crypts and villi and are eventually sloughed off into the lumen from the tips of the villi (Hodges, 1974; Penttila, 1968; Karcher and Applegate, 2008).

The chief cells or enterocytes are arranged in a single layer of tall narrow columnar cells. Filamentous, granular, and rod-like mitochondria can be observed in the apical end of these cells. Different mitochondria present different metabolic rates that may enhance efficiency and functionality of the enterocytes (Hodges, 1974; Karcher and Applegate, 2008). The enterocytes at the base of the villi are shorter than the ones from the mid region or tip of the villi (Hodges, 1974; Fawcett and Jensh, 2002). The function of these cells is to secrete enzymes for hydrolysis, digestion, and absorption of nutrients.

The goblet cells were given this name due to their goblet shape (Rogers, 1994). These are scattered among the main epithelial cells but are much more frequent in the crypts than on the sides of the villi. They can be found either with a swollen appearance or right after they secreted the mucus with a thin dark staining aspect. These cells produce mucin, which is a matrix of glycoproteins or mucus, designed to protect the lining of the intestinal mucosa from insults and pathogens as well as to serve as a buffer from the acidic chime (Karcher and Applegate, 2008).

The Paneth cells are columnar cells found normally at the base of the crypts, and they contain lysosomes, that contain lysozymes, phospholipase A_2 (both digest bacterial cell walls), and defensins (kill bacteria through membrane disruption). Paneth cells do not migrate towards the tip of the villi but stay at the bottom of the crypts, probably strategically in order to protect the stem cells from infection (Fawcett and Jensh, 2002).

The enterochromaffin cells are cells with a pyramidal shape found in the base of the crypts. These cells synthesize and secrete serotonin and histamine under the stimulus of the vagal nerve (Hodges, 1974; Rawdon, 1994).

Intraepithelial cells are T and B-lymphocytes scattered between villi and crypts' cells. These critical effector cells are part of the GALT that also serve to maintain cell integrity and the protective barrier in the mucosal epithelium (Bar-Shira *et al*., 2003).

Crypts of Lieberkuhn open between the villi and occupy most of the lamina propria between the bases of the villi and the muscularis mucosa. The crypts are short, simple slightly sinuous tubular glands that produce secretions which serve as a vehicle for cytokines and a diluting solution for the end products of digestion (Fawcett and Jensh, 2002). There are also stem cells at the bottom of the crypts that undergo frequent divisions and migrate towards the tip of the villi. As they migrate, they mature and differentiate into the different kinds of cells present in the mucosa. Intestinal epithelium cells are short-lived and are constantly sloughed off and replaced. The balance between cell proliferation, migration, and desquamation determines the intestinal epithelial turnover for the maintenance of the villus height. If there is an imbalance in these processes, the villus height will change. The cells' turnover rate will depend on many different factors such as diet, age, stress, pathogens, energy status, etc. Some cells from the crypts also move downwards, and replace the cells of the crypts that undergo apoptosis.

The epithelium of the duodenum consists of plate-like villi with numerous goblet cells, and the remainder of the lamina propria consists of loosely aggregated connective tissue containing blood and lymph vessels, nerves, muscle fibers and both diffuse and nodular accumulations of lymphoid cells (Karcher and Applegate, 2008). Lamina propria passes up within each villus to form the core of the villus with infiltrating B and T lymphocytes, plasma cells, and eosinophils (Hodges, 1974; Fawcett and Jensh, 2002).

The epithelium of the jejunum and ileum is very similar to that of the duodenum. However, there is a decrease in the depth of the mucus membrane, the villi are shorter and broader (leaf-like) and the depth of the crypts decreases considerably. There are fewer goblet cells, which will reduce the mucus barrier thus increasing digestion and absorption. Fewer enterochromaffin cells are found as well (Karcher, and Applegate, 2008).

The ileo-cecal junction consists of a sphincter formed by a thickening and folding of the muscularis mucosa, causing the mucosa to project into the lumen (Hodges, 1974). The ceca are divided in three portions due to their histological differences. The proximal regions of the ceca

have villi well developed in length and width, but the crypts are very shallow. Within the lamina propria many lymphoid cells and some lymphoid nodules are present at the base and among the villi. There are large numbers of goblet cells as well. The middle region of the ceca presents plicae which are well developed folds of the mucus membrane and muscularis mucosa. Villi are very short and blunt, and the lamina propria is infiltrated with lymphoid tissue containing only a few lymphoid aggregates. The distal region of the ceca has the largest diameter from the three sections. The plicae are present and the villi are shorter and blunter. Small lymphoid nodules are still present in the lamina propria or in the sub-mucosa.

The colo-rectum is the shortest part of the intestine and belongs to the large intestine. The mucosa varies depending on the distension or contraction levels of the intestine. When the mucosa is fully distended the villi become flattened, short, thick, and with blunt ends, and the crypts are more shallow and open around the short and flattened villi as well. When the mucosa is fully contracted, the villi look numerous, with a long flat leaf-shape that fill the lumen. The lamina propria is infiltrated with lymphoid cells, and there are more goblet cells than main epithelial cells.

Nutrient Transporters. The nutrient assimilation capacity in an animal depends on the mucosal surface area of the small intestine, digestive enzyme activity, and on functional properties of specific nutrient transporters that are present in the basolateral and brushborder membrane (**BBM;** Amat *et al*., 1996). Transporters are proteins expressed in enterocytes. Transporter systems can be active or passive and are specific for major dietary molecules such as monosaccharides (glucose, fructose, and galactose), peptides or free amino acids, minerals, and vitamins. Passive transport involves simple diffusion, which moves solutes down a concentration gradient without the need of energy (ATP). Active transport mediates the uptake of solutes against an electrochemical gradient and is dependent on ATP. The Na^+/K^+ ATPase pump is the primary exchange mechanism for Na^+ and K^+ in the basolateral membrane.

Carbohydrates can be transported using passive or active mechanisms (Figure 2.3). The hexose-aldoses, such as glucose and galactose are actively transported through a secondary active transporter, the sodium-coupled glucose transporter 1, SGLT1, expressed on the BBM of the enterocytes. This transporter accounts for at least 80% of the glucose absorption and involves sodium-dependent transport. The SGLT1 has binding sites for 2 Na^+ ions/one sugar

molecule, and is expressed in the mature cells located in the upper villus regions (Ferraris, 2001). The hexose-ketose, fructose, is transported through a Na^+-independent facilitated diffusion carrier, the facilitative fructose transporter 5 (**GLUT5**), which is located in the BBM and transports the molecule of fructose into the cell cytoplasm. The GLUT5 transporter is highly stereospecific for fructose (Ferraris, 2001). Once inside the enterocytes, the sugar molecules (glucose, galactose, or fructose) transfer towards the blood stream through the basolateral membrane by simple diffusion using the facilitated transporter Na^+ independent glucose, galactose, and fructose transporter 2 (**GLUT2**; Gilbert *et al*., 2008). Carbohydrate uptake varies depending on the physiological conditions of the animal, starvation, feed intake, diet composition, hypothermia, hyperthermia, or the location or maturation level of the enterocyte along the villus (Ferraris, 2001). The increase in sugar active transport has also been correlated to the time at which histological (morphology) and histochemical (enzyme activity) maturation of the small intestine is reached (Holdsworth and Wilson, 1967). Glucocorticoids induce the expression of glucocorticoid-regulated kinase 1 which enhances glucose transport by increasing SGLT1 abundance in the cell membrane (Carriga *et al*., 2006). The effect of heat stress causes metabolic and physiological changes in the intestinal transport of glucose through the increase of SGLT1, while the basolateral transporter GLUT2 is unaffected (Carriga *et al*., 2006).

The absorption of amino acids also occurs through the action of transport systems that vary in mechanisms for translocation of amino acids (Figure 2.4). Some transporters act as obligatory exchangers ($b^{o,+}AT$ and rBAT) and do not utilize ATP. Others are Na^+-dependent (B^oAT) and require Na^+/ATPase to re-establish the electrochemical gradient, resulting in the use of ATP (secondary active transport). Others use a more complex array of ion dependencies to facilitate uptake of amino acids against a steep gradient (excitatory amino acid transporter 3; **EAAT3**). The oligopeptide transporter 1 (**PepT1**), couples uptake of protons to peptides and is considered a tertiary active transporter (Gilbert *et al*., 2007; Gilbert *et al*., 2008).

Amino acid transporters are classified by their substrate specificity, according to the class of amino acids (basic amino acids, Na^+ independent cationic amino acids, neutral amino acids, and anionic-amino acids; Gilbert *et al*., 2007). Many of these transporters have overlapping and broader substrate specificities than their name suggests. There are several free amino acid transporters characterized in the chicken, however, this review will focus only on the free amino

acid and peptide transporter evaluated in each of the experiments discussed within this dissertation, EAAT3, and PepT1.

The EAAT3 transporter, also known as EAAC1, is a member of the SLC1 family and is specific for anionic amino acids such as aspartate and glutamate (Kanai and Hediger, 2004). Glutamate is vital for the mucosal cellular system which has rapidly proliferating cells, provides energy for T lymphocyte metabolism, and is an obligate requirement for glutamine, which is the provider for one half of the N requirement for both purines and pyrimidines synthesis (Lobley *et al*., 2001; Rudrappa and Humphrey, 2007). Glutamate can replace many of the metabolic functions of glutamine, including energy generation and amino acid synthesis (Reeds and Burrin, 2001). Given the importance of such amino acids, the EAAT3 transporter is crucial for supporting enterocyte metabolism. Its expression is strategically maximized at the villus base and in the crypts, close to the stem cells. It is localized in the BBM of the enterocytes and is highly specific and stereospecific for L-glutamate and D- or L- aspartate (Castagna *et al*., 1997). Transport of glutamate or aspartate, negatively charged amino acids, is "against a steep electrochemical potential gradient" (Kanai and Hediger, 2004). Three Na^+ ions and one H^+ are co-transported with the amino acid in exchange for a K^+/glutamate molecule (Kanai and Hediger, 2004).

Besides free amino acids, di- and tri-peptides are absorbed by the membrane as well. Peptide transport, as compared with free amino acid uptake, is more rapid and energetically efficient. The PepT1 transporter is located in the apical membrane and transports di- and tripeptides into the cell (Daniel, 2004). It is a tertiary transport system, given that it requires the electrochemical potential difference of the H^+ across the membrane, created by the Na^+/H^+ exchangers in the BBM. The H^+ ion enters the cell together with the di- and tri-peptides through the PepT1 transporter. Inside the cell, peptides may be transported out of the cell intact by a basolateral peptide transporter, or be hydrolyzed into free amino acids through the action of peptidases within the cytoplasm. Basolateral transport of free amino acids is mediated by a variety of transporters.

The PepT1 transporter is expressed throughout the different sections of the small intestine and throughout the villi-crypt axis (Chen *et al*., 2005; Li *et al*., 2008). It is a transporter with nutritional relevance in agricultural animals (Gilbert *et al*., 2008). Expression of PepT1 depends

on the developmental stage of the chick, physiological conditions, dietary protein level as well as the substrates present in the intestinal lumen (Chen *et al*., 2005). Insulin as well as leptin, can induce an increase in expression of PepT1 in the membrane by increasing transport from the cytosol to the membrane (Thamotharan *et al*, 1999; Buyse *et al*, 2001). Thyroid hormone on the other hand, decreases the expression of this carrier in the membrane (Ashida *et al*., 2002, 2004).

Ontogenesis of the Small Intestine

Ontogenesis- Gene Expression Regulation. The formation of the chicken embryo requires multiple genes to be involved in pattern formation. The families of regulatory genes involved in the axial determination during gastrulation in embryos include the caudal (***cad***)-family of vertebrate homeobox genes (Frumkin *et al*., 1991; Frumkin *et al*., 1994). Homeobox genes encode nuclear transcription factors involved in patterning and cell differentiation (Sklan *et al*., 2003). The Homeobox genes of the *cad*-family (**Cdx**) characterized in chicken embryos are cCdx-A and cCdx-B, which encode the cdx-A and cdx-B proteins, respectively (Ehrman and Yutzey, 2001). They are part of the regulatory gene complex active during intestinal morphogenesis (Frumkin *et al*., 1991; Ehrman and Yutzey, 2001). The first gene of the *cad*-family reported in chicken was cCdx-A (previously *CHox-cad*), and it was confirmed to be activated at early stages in incubation, during gastrulation, gut closure, initial stages of intestinal epithelium morphogenesis, and even during enterocyte development, maturation, and post-hatch maintenance (Frumkin *et al*., 1991; Frumkin *et al*., 1994; Geyra *et al*., 2002). The second member of the chicken *cad*-family is cCdx-B (Ehrman and Yutzey, 2001). The expression of cCdx-B is higher during neurulation, but it is involved in later stages of incubation as well (Morales *et al*., 1996; Geyra *et al*., 2001). Later during incubation, cdx proteins regulate expression of specific genes during gut development. They bind DNA regulatory sequences through the helix-turn-helix motif rich in basic amino acids, which are the conserved homeodomains. After the differentiation phase of incubation, the cdx-A protein is exclusively expressed in the small intestine of the chick (Geyra *et al*., 2002). It can be detected by d 18 of incubation along the whole length of the villi with higher concentration at the tip. The cCdx-A gene and cdx-A protein are important for the morphogenetic events that lead to the closure of the yolk stalk and to maintain the differentiation of the epithelium and specific epithelium related actions (Frumkin *et al*., 1994). In a study done by Geyra *et al*. (2002), the expression of cCdx-A

and cCdx-B was down-regulated when chicks were starved but immediately up-regulated once the chicks had free access to feed and water. Uni *et al*. (2003) observed that cCdx-A expression increased in the jejunum through developmental stages even after hatching, when it reached a plateau. Geyra *et al*. (2002) observed that cCdx-A mRNA relative to β-actin was low in the chicken embryo at d 15 of incubation, but that it increased its expression from d 17 until hatch and even a few d after. The cCdx genes and cdx proteins therefore have an important role in the differentiation and growth phases of incubation, and even during the early post-hatch period in which the intestine is still undergoing development.

Morphogenesis. According to Grey (1972) and Hodges (1974), the development of folds starts at d 8 of incubation in the duodenum. The folds grow more narrow and straight as they increase in number. The intestinal epithelium becomes triangular in shape with 2 ridges opposite of each other pushing into the lumen and creating the initial pre-villus ridges. The ridge folds increase up to 50% during d 9 and 10 of incubation. At d 13 they change and acquire an undulated form with a zigzag conformation. The zigzag folds begin to break up into villi. Villi formation starts on d 13 and they are elongated with a leaf-like shape. At d 16, the vascular tissue invades the villi, the epithelial cells are simple cuboidal and have very few mitochondria that remain at their basal ends. During the last d of incubation, the villi elongate, and increase in number by dividing from the tip downwards. At this time, the epithelial cells change shape again, into the columnar form, which will remain permanent for the rest of the productive life of the chick. Active mitosis within the epithelium between the villi forms the invagination of the first crypts of Lieberkuhn on d 17. These glands, like the villi, develop first in the duodenum. The enterochromaffin cells begin to differentiate directly from the intestinal epithelial cells at the end of d 11. Goblet cells are not seen in the epithelium until immediately prior to hatch and at the same time, few lymphocytes appear in the intestinal epithelium. When the duodenum is in a functional state, around d 19, the striated epithelial border starts to secrete alkaline phosphatase, at the top of the duodenal loop. Studies show that hydrocortisone causes alkaline phosphatase to appear precociously in the border of the duodenal epithelium and by doing so, it speeds the differentiation of the cells (Hodges, 1974). The muscularis mucosa is present on the 14^{th} d of incubation. Interestingly, research has demonstrated that by incubating the small intestine at 40°C, the muscle in the gut can become active and start contracting as a manner of peristalsis (Romanoff, 1960). For preparation for the peri-hatch period, the villi develop through 5 different

ranks during the last 4 d of incubation, which cannot be identified within the small intestine after d 4 post-hatch (Grey, 1972). In 2003, Uni *et al*. reported corresponding levels or ranks of villi with 3 types of villi being identified in the last 4 d of incubation. According to Karcher and Applegate (2008) these are the same as those described by Grey in 1972.

The differentiation of the mucosal epithelium in the embryo depends on both thyroid and glucocorticoid hormones (Hazelwood, 1971; Uni *et al*., 2003). Studies showed that hydrocortisone regulates the early phase of embryo differentiation (Latour *et al*., 1995; Uni *et al*., 2003). During this period the embryo presents low metabolic rates and has a considerable amount of energy storage. Thyroxin (T_4) alone impacts the embryonic maturation phase of the epithelium by accelerating morphological differentiation of the absorptive cells. During this growth phase, there is utilization of all the energy reserves, an increase in embryonic metabolic rate, as well as an increase in maltase and alkaline phosphatase enzymes (Romanoff, 1960, Black, 1988).

Before the chick starts pipping (the process of breaking through the shell for hatch), the action of swallowing the albumen-amniotic fluid, which contains albumen proteins and a very few carbohydrates, is initiated. The villi enterocytes are ready for absorption of fluid and macromolecular substances. As soon as internal pipping starts, the ingestion and absorption of the albumen-amniotic fluid stops. At the time of hatch, the small intestine is composed of two different types of enterocytes: the embryonic enterocytes, which facilitate the uptake of macromolecular substances, and the normal enterocytes that replace these embryonic cells and allow for efficient digestion and absorption as the chicks mature (Uni *et al*., 1998; Uni *et al*., 2003; Moran, 2007). At hatch, these enterocytes are still immature for such a task and it takes several d for them to become fully efficient in feed utilization. The epithelial differentiation accelerates during d 19 and 21 of incubation. The chicken embryo's intestinal epithelium secretes digestive enzymes and expresses transporters specific for sugar and free amino acids or peptides prior to hatching; however their ontogeny is mediated through different mechanisms (Uni *et al*., 2003). The expression of the enzymes and transporters vary with developmental age and very possibly with the environmental conditions of the embryo. Prior to hatching the chick needs to prepare for the change from YSM digestion and absorption to intestinal utilization of commercial diets. Thus, when chicken embryos are close to hatching, they initiate expression of

enzymes and transporters throughout all intestinal segments. In some studies, a 50 fold increase in mRNA expression levels of PepT1 was observed from d 16 of incubation to DOH (Chen *et al*., 2005). ATPase, maltase, and SGLT1 expression in jejunum increased 9 to 25 fold, from d 15 to 19 of incubation and then decreased by hatch (Uni *et al*., 2003). The most changes in nutrient transporter expression was observed from d 18 of incubation to DOH and there was a 3 to 5 fold increase in EAAT3 and PepT1 and a 7 to 9 fold increase in GLUT2 and SGLT1 respectively (Gilbert *et al*., 2007). The glucose transporter, SGLT1, mRNA expression is detected for the first time in low expression levels by embryonic d 15 and 17, and increases in expression by d 19 of incubation decreasing again by DOH (Uni *et al*., 2003). The embryonic pancreas does have the capacity to secrete enzymes; however, the ovalbumin presents inhibition properties towards several of them. Thus, the capacities of digestion in the intestinal lumen are not completely functional nor mature at hatch and the first few d post-hatch (Moran, 2007).

Transition: from In Ovo to Post-Hatch

It takes approximately 2 wk to make the transition from the *In Ovo* environment to the post-hatch life and embryonic cellular turnover. This transition period is critical for normal development of the digestive, immune, and thermo-regulatory systems of the chicken. These systems, in order to become fully functional and efficient need to differentiate, mature, and go through intense morphological as well as physiological changes after hatch (Karcher and Applegate, 2008).

Digestive System. Once the chick hatches, it requires an adaptation period in order to prepare for the environmental and dietary challenges it will be exposed to. The digestive system of the chick at hatch is anatomically complete; however its functional capacity is immature. The ability to grow efficiently in order to fully reach its genetical potential is reached until d 10 and feed conversion efficiency (gain: feed) reaches its best point between DOH and d 5. During this phase, the mucosal development is based on the increase in size of villi and microvilli, its density (number of enterocytes per villus area), and the amount of functional enterocytes along the villi, all of which will improve the absorptive capacity as the chick ages (Uni *et al*., 1998). Rapid turnover and migration of the intestinal epithelium from crypts to villus tips has been reported in young chicks. Takeuchi *et al* (1998) reported a minimum cell turnover time of 72 h in the duodenum of 7 and 21 d old chicks. Another study observed that enterocyte life-time increases

with age, and migration rates decrease with age. In this study, migration was observed to be completed from cell birth to the tip of the villus in 96 h. in DOH and 7 d old chicks, and reaching only 3 quarters of the villus height in birds older than 14 d of age (Iji *et al*., 2001). Thus there is a reduction in migration rate with age. All the morphological and physiological changes in the intestine that take place during the early post-hatch period are directed to enlargement of the absorptive surface and the differentiation and maturation of the epithelial cells, enzymes, and nutrient carriers (Geyra *et al*., 2001; Karcher and Applegate, 2008). Studies by Uni *et al*. (1998) suggest that the enterocytes in the duodenum are more mature than those from the jejunum and ileum immediately after hatch. This is contradicted, however, by Geyra *et al*. (2001) who found that morphologically the enterocytes in the duodenum and jejunum take longer to mature than those in the ileum, which were by DOH, already mature (Geyra *et al*., 2001). The adaptations, throughout any of the intestinal sections, are extremely sensitive to the nutrient supply but occur regardless of feed presence (Noy and Sklan, 1999). When the nutrients are not supplied by external sources such as feed, the energy required for the intestinal development is supplied from the yolk. However, if nutrients are present in the intestinal lumen, utilization of the residual yolk whose contents supply the substrates for the mucosal epithelium development is accelerated (Uni *et al*., 1998). The mechanism might be through an increase in peristalsis of the intestine (Noy and Sklan, 1998).

On the other hand, physiological changes are also related to an increase in production and activity of basolateral and BBM nutrient transporters. According to Moran (2007), the nutrient transporters are regulated depending on the substrate present in the diets, thus providing possibilities to nutritionists to feed adequate nutrients to the chick at this early post-hatch period. Gilbert *et al*. (2007) observed and confirmed differences not only in enzymes, but also in nutrient transporter gene expression in chicks that were selected based on different diet compositions (corn or wheat based diets). Overall, the results indicated that SGLT1 mRNA was increased 9 fold from d 18 of incubation to DOH followed by a 2 fold increase until d 14 post-hatch. SGLT1, GLUT5, and GLUT2 mRNA increased linearly from d 20 of incubation to d 14 after hatch, and EAAT3 increased more than 3 fold from d 20 of incubation to d 14 post-hatch. Amino acid transport is also influenced by dietary protein level, composition, and quality (Gilbert *et al*., 2008).

Other digestive tract organs such as the gizzard and pancreas do not show the same growth rate as the small intestine (Noy and Sklan, 1998). Starvation (as seen with deutectomy) or feed withholding for 24 h has a negative effect on crypt depth especially in the ileum. These conditions also cause a decrease in villus height in the duodenum and jejunum and depressed mitosis along the villi (Noy and Sklan, 1998). This alters the balance in enterocyte turnover, which further affects assimilation of nutrients and energy utilization required for the development of other systems and organs.

Thermoregulatory System. In a typical biological system, a 10°C increase in temperature results in a two to threefold increase in the rate of many physiological functions (Hiebert and Noveral, 2007). Chicks are homeotherms. In homeotherms, the metabolic rate remains relatively constant within the thermal neutral zone in order to optimize performance (Nichelmann and Tzschentke, 2002). Homeotherms regulate their metabolic rate, biochemically, physiologically, and behaviorally, in order to keep their body at a constant temperature. Thus, as ambient temperature ($\mathbf{T_{a)}}$ varies either above or below the thermal neutral zone, the metabolic rate increases or decreases to maintain a constant tissue or organ temperature (Tzschentke, 2007). The pre-hatch development of thermoregulatory mechanisms positively stimulates the fast maturation of temperature regulation in the early post-hatch period, which is very important for the performance of the chick (Tzschentke, 2007). Newly hatched chicks cannot effectively regulate body temperature. This ability is developed during the first few d after hatching (Dunnington and Siegel, 1984; Tzschentke, 2007). However, thermoregulatory processes start prior to hatching. Respiration movements and panting reactions occur between internal and external pipping. At the same time there are changes in blood flow in the chorio-allantoic membrane with different T_a (Nichelmann and Tzschentke, 2002). The thermoregulatory reactions are a result of epigenetic adaptations to environmental conditions during incubation (Decuypere, 1984). After d 5 post-hatch, increase in body weight, decrease in surface area to body mass ratio, and growth of down and feathers become important in thermoregulation (Dunnington and Siegel, 1984). Consequently, a series of physiological conditions and metabolic changes are responsible for the gradual change from heterothermy to homeothermy in the chick. The ability of the chicken to effectively regulate its body temperature has a direct effect on its ability to grow efficiently in the future.

Broiler chicks need to thermoregulate in all environments to maintain the thermal gradient between the body and their extremities. There are two methods for thermoregulation: heat production and heat dissipation. Heat loss can be divided into evaporative and non evaporative means. The non-evaporative forms of heat loss involve radiation, convection, and conduction. The evaporative form is the only effective form of heat loss when T_a reaches body temperature (T_b). There are two forms of evaporative cooling: cutaneous, which is insignificant in chicks given that they do not have sweat glands, and respiratory, which involves the process of panting (Dawson and Whittow, 2000). Respiratory evaporative cooling is known as tachypnea or thermal polypnea. It is extremely important since it is the only form of evaporative cooling present and efficient in chicks. During panting the chick increases its respiratory frequency (60 to 400 inspirations/expirations per min) and decreases tidal volume. This minimizes gas exchange. Thermal polypnea begins at some temperature above the thermal neutral zone and may increase in intensity as T_a continues to rise in chickens. As T_a approaches body temperature, panting represents essentially the only effective method to cool down. It increases pH in blood if too much CO_2 is blown off by the lungs. Under extremely high environmental temperatures, birds have a specialized form of panting, gullar fluttering, which involves rapid panting to remove heat through evaporation from moist surfaces (Dawson and Whittow, 2000). It takes place within the gullar area (mouth, buccal cavities, and upper alimentary canal/esophagus), and there is no gas exchanged since the trachea is blocked. This involves evaporative heat loss through tissues that do not require gas exchange, and respiratory frequencies range from about 176 to 1000 per min.

Thermoregulation is controlled by the thyroid and adrenal hormones thyroxine (T_4) and corticosterone (Moraes *et al*., 2003). Heat stress, therefore, can induce changes in glucose and uric acid metabolism (Moraes *et al*., 2003). Thermal conditioning during embryonic life of broilers can confer some level of thermo-tolerance post-hatch during the grow-out period. The exposure to high (> 37°C) or low (< 38°C) temperatures during incubation improves the embryo's capacity to adapt to either hot or cold environments (Janke *et al*., 2004; Yahav *et al*., 2004a; Yahav *et al*., 2004b). Uni *et al*. (2001) reported that thermal conditioning at an early age influences triiodothyronine (T_3) concentrations, which in turn alter the intestinal capacity to proliferate, grow, and digest nutrients. However, these changes were not shown to be due to feed intake responses among the treated groups. Yahav *et al*. (2004a) did studies with embryos

incubated at 38.5°C for 3 h each d during d 16, 17, and 18 of incubation. No differences were observed in body weights at hatching, but the thermo-tolerance of birds from the elevated incubation temperature increased when compared to the control group during the grow-out period. For this to happen, the thermoregulatory system needs to be linked to the development of the hypothalamus-hypophysis-thyroid axis to change the heat production threshold response and to the development of the hypothalamus-hypophysis-adrenal axis. It is important to note that thermal manipulation during incubation with the intention of inducing thermo-tolerance post-hatch is accomplished by the exposure of the embryos during the last stage of incubation to elevated temperatures (> 37°C) for very short periods of time, of about 3 or less h/incubation day.

Observations in commercial hatcheries related to problems with elevated incubation temperatures are a different concern. The intestinal epithelium of an 11 d old embryo is very susceptible to injury by heat as compared to other embryonic tissues. Epithelial cells can go through necrotic processes after being incubated at 40°C, although due to the rapid turnover within this tissue, they can easily regenerate. Any necessary regeneration from stressors, such as temperature, will delay development of the GI tract. In summary, as previously stated, incubation at elevated temperatures for short periods of time can induce positive epigenetic changes that can manifest as environmental adaptations in the post-hatch life of the chicken. However, elevated temperatures throughout embryogenesis can be seriously detrimental to the development and possibly post-hatch performance of the chick. It is important to allow the chick to adapt to the post-hatch life to favor the development of those systems that require further differentiation and maturation.

Immune System. At hatch the chick's immune system is not completely mature, it takes at least 10 d for it to completely differentiate and develop into a fully competent system. Thus it is very important to have passive immunity (maternal antibodies) transfer from the yolk into the mucosal epithelium of the chick to protect the chick for the first 2 wk of age (Bar-Shira *et al*., 2003). The GI tract is the central organ for uptake of fluids and nutrients and at the same time it forms the main protective barrier between the sterile environment of the chick's body and the outside world. It is the first line of defense against invasive microorganisms. The majority of pathogens do not enter directly into the bloodstream. Thus the small intestine is an important

organ of the innate immune system. It comprises mechanical, chemical, and cellular mechanisms of defense. Pathogens have to cross the glycocalix mucin matrix, secreted by the goblet cells in the intestinal epithelium, and the mucosal epithelial barrier, before they can reach the blood vessels (Bockman *et al*., 1983). Peristalsis will aid in the elimination of pathogens attached to the mucin. The mucosal tissue is protected by a simple epithelium that separates the localized cells of the immune system from the external antigenic environment. It contains specialized lymphoid structures including the Peyer's patches, lymphoid follicles, dendritic cells, IEL, goblet cells, and microfold or M cells. All of these cells and aggregates make up the GALT (Bockman *et al*., 1983). The M cells are present beyond the entrance to the crypts of Lieberkuhn, but do not reside inside of them. The M cells in the Peyer's patches differentiate from epithelial stem cells in the adjacent crypt which also give rise to enterocytes, goblet cells, and enterochromaffin cells (Owen, 1994). The M cells are seen after d 15 of incubation in the bursa of Fabricius (Bockman and Cooper, 1973). The GALT is a major site for generation and induction of immune responses in the chick, since chickens do not possess lymph nodes. The chicken GALT is organized through immune cells that are spread throughout the epithelium of the GI tract and underlying the lamina propria (Bar-Shira *et al*., 2003). The Peyer's patches are scattered distally in the intestine, including near the ileo-cecal junction, close to MD and within MD. In the ceca there are collections of lymphoid follicles known as the cecal-tonsils. The development of lymphoid follicles in the avian gut is associated with colonization of the intestinal microflora (Honjo *et al*., 1993). The mucosa and sub-mucosa are infiltrated with lymphoid follicles. The IEL, lymphoid cells, and leukocytes of the lamina propria are abundant throughout the gut. Among the enterocytes along the villi, there are IEL, which are a diverse population of lymphocytes including Natural Killer cells, T and B lymphocytes, and heterophils (Bar-Shira *et al*., 2003). Analysis of T cell receptors (TCR) of intraepithelial lymphocytes in chickens suggest that 40% of these cells express $\alpha\beta$ TCR ($CD4^+$) and 45% express α TCR ($CD8^+$) (Kasahara *et al*., 1993). The infiltration of these cells and lymphoid tissue in the GI tract to form the GALT takes place over a period of 14 d at 2 distinct time points or "waves". These waves occur at the first wk post-hatch and the second after the second wk post-hatch, which are demonstrated through the expression of interleukin-2 and Interferon-γ (Bar-Shira *et al*., 2003). The local immune responses are detected as early as d 5 post-hatch in the oral and rectal areas of the chicken. Thus, the adaptive immunity in the chick matures towards the second wk of age,

while the innate immunity reaches maturity around d 5. During the first wk of age, pro-inflammatory cytokines (interleukin-1β) and chemokine (interleukin-8) genes and a group of innate antimicrobial enzymes like β-defensins are expressed in the chick (Bar-Shira and Friedman, 2006). By d 14 post-hatch there is considerable activity of the immune system in MD, cecal tonsils, and the Peyer's patches along the small and large intestines. Additionally, the intestinal mucosa has increased in size and density noticeably by d 28. At the mucosal surface, IgA and secreted dimeric IgA are present. IgA is the primary antibody secreted in the bile and by intestinal tissue. The β-defensins, Gallinacin-1 and Gallinacin-2, were reported to be expressed by chicken heterophils and epithelial cells and increased in expression from hatch throughout the intestine and decreased after the first wk of life.

There are 2 steps in the maturation of the intestine of the chick (Bar-Shira and Friedman, 2006). The first is independent of intestinal exposure to feed and microflora. It is represented by extramedullary granulopoietic process that occurs close to hatch in the small intestine. The second step depends on the exposure of the intestinal environment to feed and microflora; it involves innate pro-inflammatory cytokines and chemokines gene expression (Bar-Shira and Friedman, 2006). Bar-Shira *et al* (2005) have demonstrated that starvation or delayed feed access negatively affects the development and functionality of the immune status of the newly hatch chick up to 2 wk of age. In one study, newly hatched Ross broiler chicks were feed withheld for 72 h after hatch. At d 6 post-hatch, the chicks were immunized with hemocyanin, a respiratory protein commonly used as antigen, via the cloaca. After rectal immunization, there was a decreased systemic and intestinal antibody response, and the colonization of the cecum and colon by T-lymphocytes was delayed (Bar-Shira *et al*., 2005).

Given that the GI tract of the chick is an immune organ as well as a digestive organ, the functional maturation of the GALT will depend on the differentiation and maturation of the small digestive and absorptive capacities of the small intestine. The capacity of a chick to defend itself against intestinal pathogens during the first wk of age relies on maternal antibodies released from the yolk sac directly into the lumen of the chick and on the innate immune system (Bar-Shira *et al*, 2003; Bar-Shira *et al*., 2005; Bar-Shira and Friedman, 2006). According to Mikec *et al*. (2006) stress is the main cause of yolk resorption disorders and consequently of deterioration in the chick's health status. They evaluated the impact of stress on yolk absorption and maternal

anti-infectious bursal disease virus antibodies effectiveness in chicks that were exposed to either cold, heat, or fasting. Their results showed that the stressed chicks had lower levels of maternal antibodies and an increase in energy requirements. Given that development of the small intestine is hampered under stress, the Ig might be used as building blocks for protein synthesis to overcome the delayed development of the intestine instead of as protection against the viral pathogen. Thus, maternal antibodies decreased as a consequence of the increased body energy requirements and their alternative utilization due to nutritional or environmental stressors (Mikec *et al*., 2006).

Stress

Stress is defined as the state of disruption of homeostasis in an organism. The disturbing factors are called "stressors", and the counteracting re-establishment forces are called "adaptive responses" (Chrousos *et al*., 1988). The general adaptation syndrome describes the "flight-fight" responses, which are nonspecific responses to stress (Dohms and Metz, 1991). The flight-fight reaction is a result of physiological changes due to production of catecholamines by the adrenal glands (Carsia and Harvey, 2000). If the stressor continues to be present, then the resistance phase or "conservation withdrawal reaction" is initiated, which is a physiological coping reaction to increasing demands for maintenance or restoration of homeostasis. Chronic stress leads to the exhaustion phase and may lead to disease or even death (Dohms and Metz, 1991). The mechanism of action for this response is through the hypothalamic-hypophysis-adrenal axis, which after the perception of an impulse or stressor releases corticotrophin releasing factor and vasopressin which stimulate the secretion of adrenocorticotropic hormone. The circulating Adrenocorticotropic hormone induces the adrenal cortex to secrete glucocorticoids. Glucocorticoids favor gluconeogenesis converting lipids to glucose for energy supply to the central nervous system and other metabolic pathways or through the catabolism of muscle protein. Glucocorticoids are also known to be immunosuppressive. They can increase susceptibility to certain pathogens, cause lymphoid tissue involution, alter blood profiles and circulation patterns of lympho-myeloid system cells, as well as induce suppression of the MHC-II expression and interleukin-1 and interleukin-2 secretion (Griffin, 1989). Interestingly, glucocorticoids have no effect on body weight. However, low antibody responses, poor feed

conversion, decreased viral resistance, as well as an increased resistance to bacteria, parasites and toxins are all physiological responses to short-term stressors (Gross and Siegel, 1993).

There are different kinds of stressors, which include a large variety of environmental stimuli such as: loud or unusual sounds, lack of food or water, and disease. These induce significant changes in the homeostasis of the animal (Griffin, 1989; Gross and Siegel, 1993). The mechanisms used to maintain or re-establish homeostasis in response to a specific stressor may be specific for that stressor or may be a general response. The participation of the adrenocortical system for secretion of corticosterone is an important characteristic of a nonspecific stress response in chickens (Siegel, 1980). This response results in an increase in cardiovascular system activity and an overall rise in metabolism. The behavioral and adrenocortical responses can occur in conjunction after environmental and metabolic stressors. For example, noises in the environment will induce fear related behavior which will respond, together with adrenocortical activation, to result in immobilization of the animal. Another example would be the increase of aldosterone and corticosterone in response to stressors that affect the Na^+ concentrations in blood (Carsia and Harvey, 2000). Thus, the responses to stressors can be physiological and/or behavioral (Gross and Siegel, 1993; Cheng and Jefferson, 2008). Physiological and behavioral responses are accepted as indicators for disorders in the animal's environment and well being. Vocalizations, for example, are a good way to evaluate the quality in the environment in poultry houses (Barnett and Hemsworth, 1990; Gross and Siegel, 1993). While measurement of lymphocyte: heterophil ratios and levels of corticosterone in blood are the most accepted parameters to evaluate stress in an animal, vocalizations, feed conversion, and physiological responses such as panting or gullar panting can also be reliable indicators of stress in chicks (Gross and Siegel, 1993; Mitchell and Kettlewell, 1998).

The failure to regulate adrenocortical activity has an impact on coping strategies as well as the mobilization of glycogen and triglycerides in response to stressors (Cheng and Jefferson, 2008). According to Cheng and Jefferson (2008), alterations in glucocorticoids and serotonin secretion, concentration levels, or receptor expression are associated with abnormal behaviors in any animal. Stressors will cause different responses in different animals, and the degree of reaction will vary depending on a multitude of factors. These factors include novelty or predictability of the stimulus, if the animals have adapted or are already habituated to such stressors, genetics, and

temperament of the animal (Gross and Siegel, 1993). In a study where broiler chickens were exposed to elevated temperatures, chicks reduced their feed intake and were observed to start panting. However, when these same chickens were exposed for a second time to the same range of elevated temperatures they did not reduce their feed intake. This suggests that there was an acclimation to the stressor (Gross and Siegel, 1993). Overall, an animal has 2 different ways to respond to a stressor: 1) Through behavioral responses such as increased vocalizations, isolation from dangerous stimulus and shivering or huddling in response to cold or panting in response to heat, and 2) If the behavioral response does not allow the chick to adapt or change, significant physiological changes will take place in the autonomic and neuroendocrine systems. However, there is evidence that animals can respond to short term stressors with an increase in glucocorticoids production (Barnett and Hemsworth, 1990). Ethological studies are an important part in veterinary medicine as well. Being able to read the behavior of an animal is very important since it can be used as a diagnostic tool.

Transport Stress. During transportation from the hatchery to grow-out farms, chicks are exposed to different potential stressors, such as thermal stress, acceleration, vibration, motion, impacts, fasting, dehydration, and noise (Mitchell and Kettlewell, 1998). All these and their combinations may result in responses from mild discomfort to death. Several studies on chickens at slaughter age, have acknowledged that 40% (Mitchell and Kettlewell, 1998) of dead on arrivals are due to transport stress, and that the mortality increases as the length of the transport increases (Valros *et al*., 2008). Therefore, transport represents a very important issue from the commercial, public, and even political point of view due to the effect that this has on the welfare of the animals (Mitchell and Kettlewell, 1998). Mitchell *et al* (1992) measured creatine kinase, marker for muscle breakdown, at d 42 in chickens exposed to transport heat stress with "in crate" temperature higher than 45°C, and observed that there was tissue dysfunction and damage due to impairment in muscle membrane integrity. A very important factor involved in stress during transportation is the environmental temperature to which the chicks are exposed. With heat stress if there was enough ventilation or air movement within the vehicle either still or in movement, the stress would decrease. There are no guidelines established for transportation conditions including adequate or acceptable temperature ranges and limits (Cheng and Jefferson, 2008). Most studies realized until today include animals at the end of their productive life as transported for slaughter. Yet, it is acknowledged that no matter

the species, the age, or developmental stage of the animal, transportation is an important factor that potentially induces stress in animals. The effects of transportation stress on the newly hatched chick have not yet been characterized.

Heat stress during pre-slaughter transportation is associated with high mortality and is recognized as the most common problem in the industry (Mitchell and Kettlewell, 1998). In poultry, a lack of airflow through transportation boxes causes an accumulation of heat and moisture that in combination can increase heat stress of the chicks. These environmental conditions stimulate the chicks to respond through panting to achieve heat dissipation. The evaporative respiration increases water loss from the animals. This will add moisture in the environment within the transport truck, and thus increase the heat stress (Mitchell and Kettlewell, 1998). In a study by Valros *et al*. (2008), Hy-line W36 and Hy-line 40 Brown chicks were transported immediately post-hatch for long (14 h) or short time (4 h) periods. The birds transported at both periods of time made distress vocalizations during the times of movement but were completely quiet during the periods of stillness. This indicated that the chicks were hungry, dehydrated, stressed, and tired (Valros *et al*., 2008). There were no differences in growth or feed conversion between both groups. Unfortunately, this study does not report the thermal or ventilation conditions of their transportation treatments. Nonetheless, chicks transported under longer periods of time reduced their ability to compete for feed access, and had a later development of perching behavior. An interesting observation for differences between lines was that the Hy-line 40 Brown chicks were calmer during handling and were easier to catch. This indicates that different animals respond differently to stressors. In several studies, it has been reported that the immune response of chickens is affected by stress (Griffin, 1989; Dohms and Metz, 1991). In a study by Dohms and Metz, (1991) the effect of corticosterone on lymphoid organs (spleen, thymus, and bursa) was evaluated in specific pathogen free leghorn chicks. Injection of corticosterone in chicks had no effects on body weights, however, it lead to apoptosis and involution of the lymphoid tissues (). Therefore, corticosterone causes suppression of humoral and cell-mediated immune responses. Catecholamines can also affect leukocyte activities (Griffin, 1989).

Due to the increase in energy requirements during stress, glycogen is crucial in the adaptation to different stressors (Christensen *et al*., 2004a). During incubation and transportation with

elevated temperatures, heat stress in the embryos and in the newly hatched chicks increases their energy requirements (O'Dea *et al*., 2004). There is activation of the catabolism of muscle protein, where the turnover is induced by activation of gluconeogenesis by glucocorticoids (Puvadolpirod and Thaxton, 2000). Virden *et al*. (2007) conducted 2 experiments to establish the amino acid digestibility coefficients in broilers, Ross X Ross 308, stress induced with corticosterone for stress simulation. Broilers fed a diet containing corticosterone for 10 d had a lower body weight gain and higher liver weight than broilers on the control diet that had no corticosterone. However, there were no differences between treatments for amino acid digestibility. For increased survival, when chicks are exposed to stressors during short or extended durations, they must attempt to adapt to the stressor, utilizing energy available to accomplish such a task. The way in which chicks adjust needs and reallocate that energy is still not well characterized.

Allocation of Resources

The resource allocation theory in livestock production was developed by Beilharz *et al*. in 1993. The theory states that the main limitation to health is the availability of resources in the environment and the efficiency of their use by the organism. Growth, reproduction, and immune status can be considered as investments of protein and energy (Rauw *et al*., 1999). These investments create costs for maintenance and growth. During growth, the different organs, depending on their functions, can be divided into 2 main groups, consuming and supplying. The consuming organs are represented by the breast muscles and feathers in that they basically require investment and maintenance costs. The supplying organs consist of the digestive tract and liver. They are responsible for the production of energy that will be used for growth (Siegel *et al*., 2008). The growth of the animal and the chances of survival will depend upon the function of all organs. The supplying organs are necessary in order to fill the nutritional demands of the growing animal, whereas the consuming organs can be adapted to different functional needs (Siegel *et al*., 2008). Therefore, the animal allocates its resources depending on the requirements or needs for growth, maintenance, reproduction, immune function, and well being in order to establish homeostasis within the organism (Cheema *et al*., 2003). Animals characterized by a rapid early development of supplying organs in relation to consuming organs are also characterized by a high specific growth rate (Rauw *et al*., 1999). Resources during

incubation are fixed by what is available from the egg, while after hatch there is a transition to the utilization of external feed. During the early post-hatch period of birds in general, the supply organs grow faster than the demand organs.

In Ovo. As we have previously seen, growth does not begin after hatch but rather starts at the moment of fecundation of the ovo. The developing embryo requires its energy to be divided between the maintenance of already existing tissues (extra-embryonic membranes) and the synthesis of new tissues (Vleck and Vleck, 1987). Hoyt (1987) proposed that the maintenance energy of a tissue is more costly than the energy required for growth; however Pearson *et al*. (1991) implied that in most cases the energy used for growth in embryonic processes is greater than that used for maintenance. In the egg, because of the developmental mode in which the embryo exists, maintenance costs increase as the incubation period progresses. Approximately 30 to 40% of the embryo's energy is spent in maintaining development, which is an equilibrium between maintenance and growth (Feast *et al*., 1998). There are 2 forms of reallocating the energy available within the embryo: 1) among tissues, where some tissues develop at the expense of others, or 2) within tissues where there is a trade between hyperplasia (increase in cell numbers) and hypertrophia (increase in cell size). When there is a limit in the resources, allocation to hyperplasia is preferred rather than hypertrophia given that cell size is recoverable but cell number is not (Geers *et al*., 1983). The better the allocation of energy towards the digestive system during embryogenesis, the better the baby chick will be prepared to utilize the resources necessary for increased growth and good performance. Once the chick hatches, the nutrient demands and supply change drastically, forcing differentiation and maturation of the intestinal epithelium. The assimilation of nutrients at the intestinal mucosa and allocation of energy and substrates is fundamental for the chicks to obtain energy and building blocks for their different metabolic processes such as locomotion, thermoregulation, GI tract development, and immunocompetence (Obst and Diamond, 1992).

After hatch, increased utilization of external resources becomes an important mechanism for continued development and increased growth. A study compared embryonic development of 2 lines of chickens that exhibited different patterns of post-hatch growth. The lines that had different patterns of post-hatch growth also had different developmental patterns *In Ovo*. The

line with faster post-hatch growth exhibited significantly faster growth earlier during incubation as well (Geers *et al*., 1983).

Post-Hatch. High-yield broiler chicks have an incredibly rapid intestinal growth in the first d after hatch. In the chicks, growth of the intestines is allometric (body mass/metabolic rate). As the chick grows, a progressively smaller fraction of its total body mass is dedicated to intestinal tissue. This development is determined by the distribution of energy between different systems, organs, and tissues (Lilja, 1983). Protein turnover in the GI tract serves as the supplier for drastic changes in the intestinal size and capacity (Cant *et al*., 1996). However, an increase in protein synthesis increases the costs of ATP utilization. According to Lehninger *et al*. (1993), 50 to 90 % of the energy used by all the cells in the intestine is used for protein turnover. There is a positive correlation between protein synthesis and metabolic rate. At least 20% of the total energy of protein synthesis is for metabolic processes involved in the maintenance of the body (Rauw *et al*., 1999). During the peri-hatch period and after hatch, the small intestine increases in size and functionality, in order to assimilate the increase in nutrients present in the intestinal lumen. The energy demands for protein turnover and ion transport are augmented as well. Given that the growth of high-yield broiler chickens has the highest priority over the rest of the physiological functions, the muscular system will continue to grow through the assimilation of nutrients in the GI tract, at the expense of other systems, even to the point that it can result in disease. With a low protein turnover rate there is a decrease in the capability of the animal to adapt or be aware of different stressors. This puts the animal at a higher risk for behavioral, physiological, and immunological pathologies.

Genetic selection for rapid growth and improved feed conversion has led to the disruption of homeostasis in the chicken, thereby creating a need for more available resources for the animal (Beilharz, 1998). In commercial broiler productions, the high-yield broiler strains exhibit higher mortality rates and susceptibility to infectious or metabolic diseases compared to the classic, slower growing broilers (Yunis *et al*., 2000). Havenstein *et al* (2003) reported that while there was an increase in body weight between a high-yield broiler and a slow-growing chicken strain, there was a decrease in the humoral immune response. This observation is supported by another research where a Ross 308 strain was compared to an Athens Canadian Randombred Control strain, a classic strain from 1957 (Cheema *et al*., 2003). The results showed that the Ross 308

strain had lower antibody production against sheep red blood cells than the classic strains. Interestingly, the cell mediated as well as the inflammatory responses, were increased (Cheema *et al*., 2003).

Summary

The studies discussed in this review support the idea that elevated temperatures during incubation and during transportation are 2 major stressors in the pre and post-hatch life of the chicken, respectively. These stressors affect nutrient utilization, and therefore, organ development during incubation and during the peri-hatch period of the chick. These stressors become even more critical in high-yield broiler strains whose growth rate is much faster than that seen in the classic broiler strains. Since the incubation system utilized most commonly in the industry is not the most adequate for the current strains and industry conditions, a consequence of these is that there is an increase in stress. With an increase in stress, comes an increase in energy requirements and the diversion of resources for growth decrease the availability of resources to respond quickly to environmental stimuli. However, the mechanism by which nutrient utilization and organ development is hampered has not yet been described.

The objective of the following experiments is to evaluate the effects of elevated temperatures during incubation and transportation on the development of the gastrointestinal tract, more specifically the small intestine, in high-yield broiler chicken strains. Performance during the grow-out period will also be evaluated. The rationale behind the following experiments is that the stress caused during suboptimal incubation and transportation conditions may lead to an increase in energy requirements in order to overcome the delayed development of the small intestine. The chick will try to obtain that energy from the yolk sac and external sources, however, due to the underdevelopment and probably atrophy of the yolk stalk, there will be yolk retention. Therefore, the few nutrients available will be used for compensatory growth and development thereby decreasing the possibilities of achieving their full genetic potential. In the first trial Ross 308 chicks were incubated under elevated (39.6°C) or control (37.5°C) temperatures. Nutrient transporter gene expression and morphology were surveyed in the small intestine as well as measurement of different organ weights and performance parameters in the grow-out period. In the second trial Cobb 500 chicks were incubated under different temperature profiles imitating a multi-stage incubator and then transported under stress (40°C) or control

(34°C) conditions. Nutrient transporter gene expression and morphology were surveyed in the small intestine as well as measurement of different organ weights and performance parameters during the grow-out period. This dissertation concludes with a discussion of the implications of these experiments and recommendations for future research.

Table 2.1 Leuven scoring system used in chick quality determination[1]

Parameter	Characteristics	Scores
Activity	Good	6
	Weak	0
Down and appearance	Clean and dry	10
	Wet	8
	Dirty and wet	0
Retracted yolk	Normal	12
	Large yolk hard to the touch	0
Eyes	Open and bright	16
	Open, not bright	8
	Closed	0
Legs	Normal legs and toes	16
	One infected leg	8
	Two infected legs	0
Navel	Completely closed and clean	12
	Not closed and not discolored	6
	Not closed and discolored	0
Remaining membranes	No membrane	12
	Small membrane	8
	Large membrane	4
	Very large membrane	0
Remaining yolk	No yolk	16
	Small yolk	12
	Large yolk	8
	Very large yolk	0

[1] Taken from Tona *et al*., 2003 (Poultry Science 82:736-741)

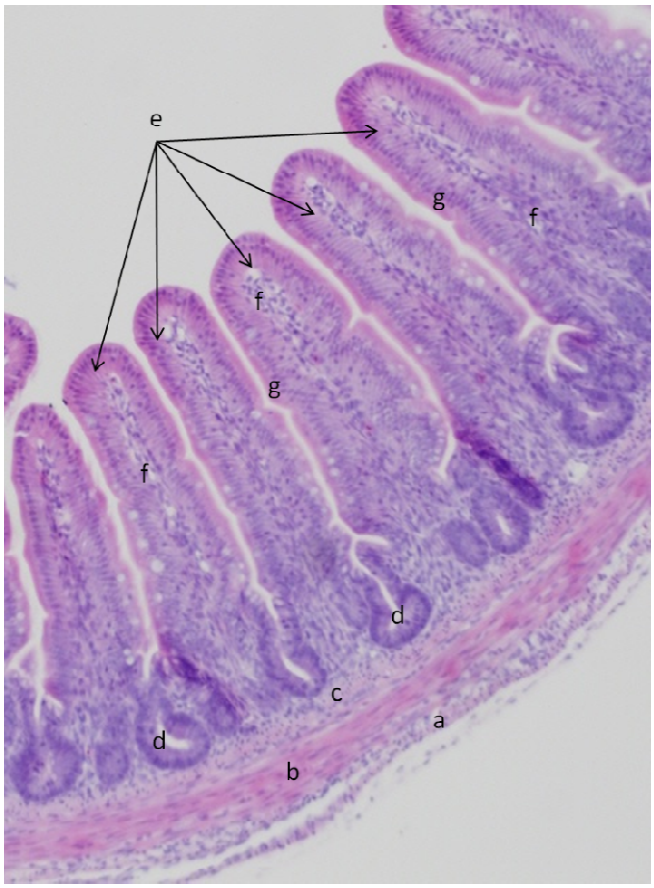

Figure 2.1 Transverse section through the wall of the proximal ileum from a Cobb 500 2 day old chick: a. longitudinal muscle layer, b. circular muscle layer, c. muscularis mucosa, d. crypts e. villi, f. lamina propria, g. epithelium of villi H. and E. 10X (Photo by author)

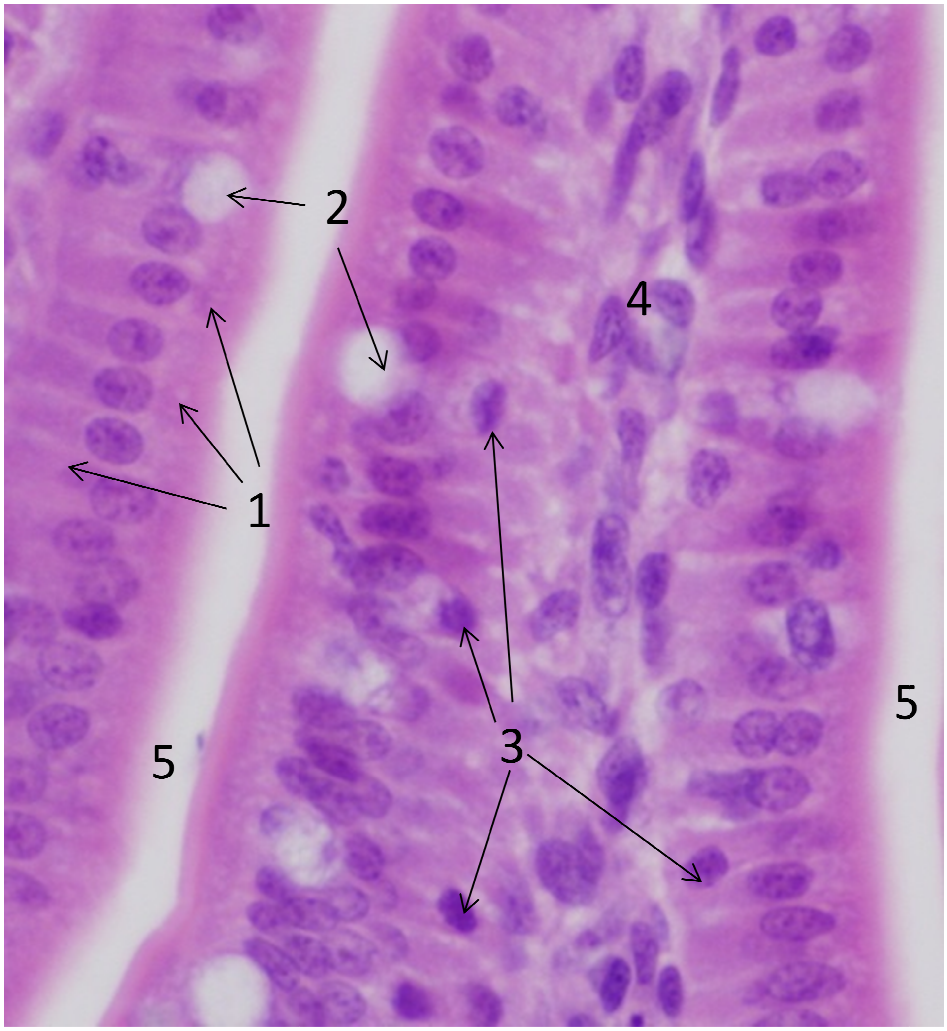

Figure 2.2 Section from part of two ileum villi from a 2 d old Cobb 500 broiler chicken showing the types of cells which make up the mucosal epithelium: 1. Enterocytes, 2. Goblet cells, 3. IEL, 4. Leukocytes within the lamina propria, 5. lumen between villi. H. and E. 60X (Photo by author)

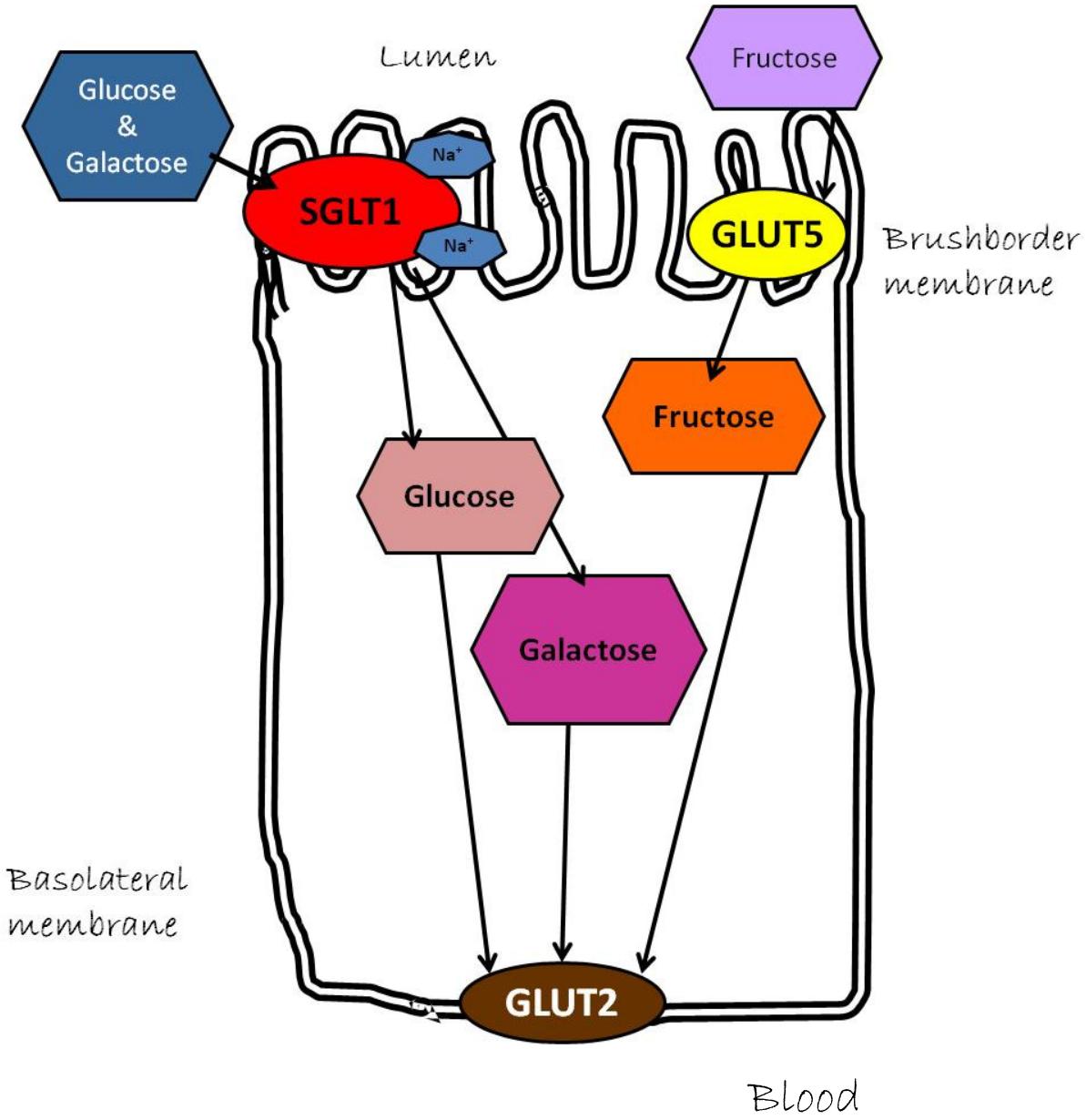

Figure 2.3 Illustration of the mechanisms of glucose, galactose, and fructose uptake in the enterocyte.

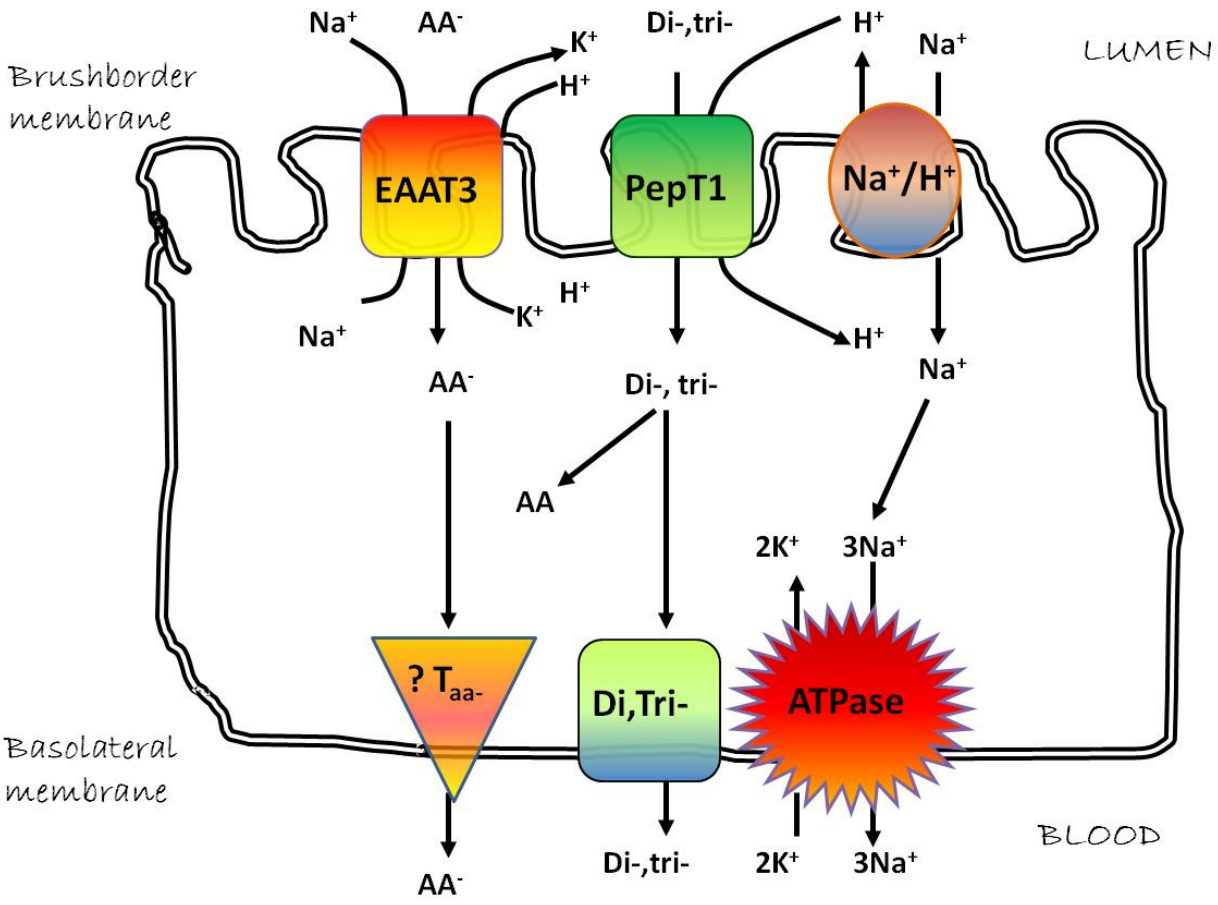

Figure 2.4 Illustration of the nutrient transporters for anionic amino-acids and di- and tri-peptides in the enterocyte.

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CHAPTER III

Effect of Incubation Temperature on Performance, Nutrient Transporters, and Small Intestine Morphology of Broiler Chickens

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ABSTRACT This study evaluated the effects of elevated incubation temperature on post-hatch nutrient transporter gene expression, integrity of the intestinal epithelium, organ development, and performance in Ross 308 broiler chickens. Ross X Ross 308 fertile eggs (n=900) were incubated at different egg-shell temperatures during development. From embryonic day (**ED**) 1 to ED12 all eggs were incubated at 37.1°C, while from ED13 to ED21, the eggs were divided into two groups for incubation at 37.4°C (S) or 39.6 °C (H). Performance parameters were measured at day of hatch (**DOH**), and d 7, 14, 21, 30 and 42. Small intestine and residual yolk sacs were collected at DOH, and d 2, 4, 6, and 10 and weighed individually. Intestinal samples from duodenum, jejunum, and ileum were evaluated for mucosal morphology and relative nutrient transporter gene expression. Nutrient transporters (SGLT1, GLUT2, GLUT5, EAAT3, and PepT1) were evaluated. The intestinal morphology results showed a temperature by age interaction in duodenum villus height (P= 0.02) and crypt depth (P= 0.05) and in ileum villus height: crypt depth ratios (P= 0.02). There was a main effect of temperature resulting in deeper crypts (P= 0.02) in the jejunum of chicks incubated at H compared to S. In the nutrient gene expression evaluation, PepT1 showed a temperature by age interaction where chicks incubated at H had by, d 10, a two-fold lower expression (P= 0.03) than the S group. A temperature by segment interaction resulted in higher expression (P= 0.04) of GLUT2 in the duodenum and jejunum of chicks incubated at the elevated temperature. Conversely, in ileum, GLUT2 expression was lower in chicks incubated at H as compared to chicks from the S temperature group. This study presents for the first time the effects of elevated incubation temperature on small intestine morphology and relative expression of nutrient transporter mRNA in high-yield broiler chicks, which can be critical for the availability of nutrients and distribution of energy in the broiler chicken.

Key words: incubation, broiler, morphology

INTRODUCTION

As the commercial poultry industry changed from slower-growing to high-yield broilers, which are capable of growing muscle much faster, hatchability of fertile eggs began to decline and post-hatch performance decreased (Hulet, 2007). The chicken embryo also has a faster growth rate within the egg with increased heat production during the last stages of development. Currently, chickens spend 30 to 40% of their total life span inside the egg (Hulet *et al*., 2007). Thus, anything that compromises or promotes growth and development during this embryonic period can have a marked effect on overall performance and health post-hatch. The increased growth rate of embryos contributes to an increased metabolic heat production and output from the eggs, which builds up within the incubator. The heat production of embryos of high yield chicken strains is 44% higher than that reported for classic broilers (Hulet *et al*., 2007). Consequently, even though the incubator is set to be at a temperature of 37.4°C, if there is not adequate ventilation, the incubators may reach a temperature of 41.1°C to 41.7°C (Hulet, 2007). As a result, there is decreased hatchability, impaired embryonic development, and poor post-hatch performance. This has been proven through several studies that have evaluated the effect of elevated incubation temperatures on parameters such as hatchability, yolk free body weights, chick length, feed intake, and feed conversion (Lourens *et al*., 2005; Hulet *et al*., 2007; Leksrisompong *et al*., 2007). Other measurements from trials evaluating incubation temperature have included heart and liver weight and carcass and meat yield (Joseph *et al*., 2006; Lourens *et al*., 2007). Interestingly, it has also been observed that chicks incubated at elevated temperatures present white down feathers instead of the normal yellow (Hulet *et al*., 2007; Leksrisompong, *et al*., 2007). This suggests that the elevated temperatures might have a direct impact on gastrointestinal development to negatively influence absorption and digestive physiology of the small intestine. The few physiological parameters evaluated in chicks incubated at elevated temperatures have been focused on the cardiovascular system by measuring heart weight, the endocrine system by measuring the levels of T_3 and T_4 in blood, and the digestive system by measuring maltase and alkaline phosphatase activities in the small intestine (Christensen *et al*., 2004a, 2004b, 2005), however, these studies measured the elevation in incubator temperature as opposed to embryonic temperature. Studies are lacking for evaluating the effect of elevated

temperature during incubation as measured through embryo temperature or eggshell temperature on the development of the gastrointestinal tract, more specifically the small intestine.

Newly hatched broiler chicks have an immature digestive system. The physiological development and maturation (digestive and absorptive function) of the digestive tract occurs through changes in nutrient transporters (Obst and Diamond, 1992) and through remarkable morphological changes such as increases in villus height and crypt depth (Geyra *et al*., 2001). The development and functional maturation of enterocytes is accomplished by the presence of feed in the intestinal lumen and the efficient assimilation of yolk nutrients. During the first 10 d of the broiler chicken's life, the competence of the intestine for nutrient utilization increases to its maximum (Iji *et al*., 2001). During this time, absorption of the yolk sac contents enhances the mucosal growth rate, which will favor further performance parameters given that the growth of the chicken depends on the availability of nutrients. The hypothesis of this research was that elevated incubation temperature (higher than 37.5°C) impairs chick quality and grow-out performance parameters by affecting the absorption of the yolk sac, which in turn would have a detrimental effect on gastrointestinal morphology and physiology. This hypothesis was based on knowledge that the lifetime performance of chickens will depend on competence of gastrointestinal development as reflected in nutrient availability and efficient utilization during the first few d of life, and that this is accomplished or favored by utilization of the yolk nutrients (Geyra *et al*., 2001). A deficiency in nutrient availability during this time would depress mucosal development; impede nutrient transporter expression on the brushborder membrane of the intestinal epithelium, and thus compromise the availability and distribution of energy and resources for growth and optimal performance of the broiler chicken. The goal of this study was to evaluate the effects of elevated embryonic incubation temperature on development of the the small intestine as measured through morphology and expression of nutrient transporters and on post-hatch performance.

MATERIALS AND METHODS

Animal Welfare

This project was approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Design

A total of 900 fertile eggs were obtained from a 31 wk old Ross X Ross 308 broiler breeder flock. Eggs were incubated in single-stage Buckeye Chickmaster incubators (Medina, OH) at Penn State University. Treatments were designated as high temperature (H) of 39.6°C as compared to the conventional standard (S) of 37.4°C. Temperatures were measured on the eggshells. For embryonic day (ED) 1 to ED12, all of the eggs were incubated at 37.1 °C. From ED13 to ED21 half of the eggs were incubated at the S temperature of 37.4°C and the other half at the H temperature of 39.6°C. During incubation, data on shell temperature, incubator temperature, and incubator humidity were logged on a daily basis. For each treatment the percent hatch of fertile eggs and early, mid, and late incubation deads were recorded.

Broilers and Diets

At hatch, 46 chicks per treatment were assigned to floor pens (0.76 ft^2/ chick) with clean pine shavings, nipple drinkers, and hanging feeders in a negative pressure ventilated house. Each treatment was replicated by 7 pens for the 42 d trial. The step lighting program and ventilation were continuously monitored from DOH to d 42. Brooding was accomplished using primary heat from forced air furnaces along with 1 heat lamp per pen as supplemental heat for the first wk of age. All birds were provided with feed and water *ad libitum* throughout the grow-out period. The diets were corn-soybean meal based, and formulated to meet or exceed the NRC (National Research Council, 1994) requirements. The dietary phases consisted of starter (d 0 to d 14), grower (d 14 to d 30), and finisher (d 30 to d 42).

Performance Parameters

Chicks were weighed by pen at DOH, and d 7, 14, 21, 30 and 42. Feed intake (FI) was measured, and feed conversion (FC adjusted for mortality) and body weight gain (BWG) were calculated for each period and cumulatively for the grow-out. Mortality was recorded daily.

Tissue Collection and RNA Extraction

At DOH and d 2, 4, 6, and 10, 4 birds per treatment were randomly selected for evaluation of nutrient transporter gene expression. Each bird for sampling was weighed and euthanized by

cervical dislocation. Tissue samples were immediately collected from duodenum (ascendant loop), jejunum (from the pancreatic duct to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileo-cecal junction). The intestinal segments were rinsed in ice cold PBS, minced with razor blades, and thoroughly homogenized. Duplicate samples of 20mg to 30 mg of minced tissue were collected in 2 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were stored at -80°C until further analysis. Total RNA was extracted from the intestinal tissue samples using the RNeasy Miniprep Kit (Qiagen, Darlington Lab, Valencia, CA) according to the animal tissue extraction protocol provided by the manufacturer. Concentration and purity were determined using the 260/280 and 260/230 ratios obtained from a nanodrop spectrophotometer (Thermo Scientific, NanoDrop™ 1000, Wilmington, DE). The quality of RNA was assessed by visualization of distinct 28S and 18S rRNA bands after gel electrophoresis with ethidium bromide staining.

Primer Design and Validation

Specific oligonucleotide primer pairs were designed by Primer Express software (Applied Biosystems, V. 3, Foster City, CA) and synthesized according to already published chicken sequences (Gilbert *et al*., 2007). Table 3.1 shows the primer sequences used.

cDNA Synthesis and Real-Time PCR

Total RNA was diluted to 0.2 µg/ µL in diethylpyrocarbonate treated water. Reverse transcription into cDNA was performed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The cDNA was diluted 1:30 and stored at -20°C until further analysis.

Relative quantification with real-time PCR was done for 4 samples/treatment with an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). All reactions consisted of 2 uL (10 ng/uL) of reverse transcribed RNA, 12.5 uL SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), 1.5 uL (5uM concentration) forward and reverse primers, and 8.5 uL diethylpyrocarbonate treated water. All samples were run in 96-well plates (Applied Biosystems, Foster City, CA) in duplicate. PCR was performed under the following conditions: 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15s and 60°C for 1 min

(40 cycles). After each run, a dissociation curve was obtained to verify that there was only one product being amplified. The dissociation curve had the following conditions: 95°C for 15 sec, 60°C for 30 min, and 95°C for 15 min. The Ct values for each gene were averaged. The differences between the average Ct values of target and reference gene (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were also calculated (ΔCt). For presentation purposes only, $2^{-\Delta\Delta Ct}$ values were calculated using the ΔΔCt method with the reference gene as the endogenous control and the average Ct value for duodenum from S treatment as the calibrator to express fold changes in gene expression (Livak and Schmittgen, 2001). Since we are interested in the evaluation of the interaction between incubation temperature and segment, we collected samples from the three different segments in the small intestine. In the statistical analysis, the software SAS was used to analyze the segments separately and together to obtain the appropriate two way interactions as well as the main effects. Because the duodenum from S treatment was used as the calibrator, the graphs showing the combined values of the three intestinal segments will not show a value of 1 for S. Significance values for treatment differences were determined from analysis of ΔCt values.

Small Intestine and Yolk Sac Collection

Twenty birds were randomly selected at DOH and d 2, 4, 6, and 10 to determine body weight, yolk sac, and small intestine weights. Each bird was weighed and euthanized by cervical dislocation. The small intestine was collected, digesta was eliminated by gently squeezing the walls of the intestine, and pancreas was excised from the duodenal loop. Once the gut had neither digesta nor pancreas, it was weighed with and without the yolk sac. Measurements of small intestine and yolk sac are reported as percentage of body weight.

Morphological Measurements

Ten birds per treatment were randomly selected at DOH and d 2, 4, 6, and 10 for intestinal morphology evaluation. Each bird was weighed and euthanized by cervical dislocation. Tissue samples (2 cm in length every 4 cm) were collected from duodenum (mid-section of the ascendant loop), jejunum (mid-point from the pancreatic duct to Meckel's diverticulum), and the ileum (mid-point from Meckel's diverticulum to the ileo-cecal junction). The intestinal segments were flushed with cold PBS and fixed in 10% neutral buffered formalin. Each tissue

was cut into 5 sections (10 mm) and placed into a tissue cassette. The tissues were processed by dehydration through a series of graded alcohols, cleared with xylene, and embedded in paraffin. Paraffin sections (5 μm thickness) were mounted onto slides. The slides were stained using routine procedures for Mayer's Hematoxylin and Eosin (H&E) (Luna *et al*., 1960). Measurements of the development of the small intestine were made using SigmaScan Pro 5 software (Olympus America Inc., Melville, NY). Measurement of 3 out of the 5 pieces of each intestinal section for each bird included the villus length (villus tip to crypt opening) and the crypt depth (crypt opening to the base of the crypt right before the lamina propria). Villus length and crypt depth ratios were also calculated. Four villus and 4 crypts were evaluated for each of the 3 intestinal pieces. The average villus length, crypt depth, and villus length to crypt depth ratios per histological slide were analyzed (n=12 measurements/bird, 10 birds/treatment).

Statistical Analysis

Performance data were subjected to ANOVA using the MIXED models for completely randomized design procedure with the SAS program version 9.1 (SAS Institute Inc. Cary, NC). Small intestine morphology, intestine and yolk weights, and relative gene expression data were subjected to ANOVA using the GLM for completely randomized design procedure of the SAS program. All values are expressed as least squares means ± SEM. Intestine and yolk weights expressed as percent of body weight data were arc-sine (square root of percent) transformed prior to analysis. Villus height to crypt depth ratios were log transformed (ln) prior to analysis. The models included main effects of incubation temperature, age at sampling, segment (when appropriate), and all two and three way interactions. Segment by age interactions are not discussed in this study since our interest is mostly on the effect of temperature by age or temperature by segment effects. Differences among treatments were further compared using the Tukey's test. Significance was determined at $P \leq 0.05$. The main effects of age were further tested for linear and quadratic responses using orthogonal contrasts in PROC MIXED. The contrast coefficient matrix for unequal spacing was generated using PROC IML. The model was: $y_{ijk} = \mu + \alpha_i + \tau_j + \varepsilon_{ijk}$ where Y_{jk} = observed dependent variable; μ= overall mean; α_i= the main effect of incubation temperature I; τ_j = the main effect of age J; $(\alpha\tau)_{ij}$ = the interaction between incubation temperature and age; and ε_{ijk} = the random error.

RESULTS

There were no differences in small intestine weights and yolk sac weights or any performance parameters (BW, BWG, FI, or FC) from early post-hatch through the end of the grow-out period. There were two way interactions of incubation temperature and age for both villus height (P= 0.02; Figure 3.1) and crypt depth (P= 0.05; Figure 3.2) in duodenum. At DOH, both groups of chicks had similar villus height. Thereafter, chicks incubated at the elevated temperature had less increase in villus length than the control group until d 2. However, from d 2 to d 4 chicks from the higher incubation temperature had more growth in villus length. The increase in villus length was similar between the two treatment groups from d 4 to d 6. The most marked differential response between the incubation groups occurred from d 6 to d 10. While the villus in chicks incubated at S temperature continued to increase in length, the villus in chicks from eggs incubated at H temperature were decreased in length from d 6 to d 10. At d 10 chicks heat stressed during incubation had shorter villus than the S temperature group. Similar interactions were observed for crypt depth in the duodenum. The crypts in chicks from eggs incubated at H temperature deepened at a faster rate than those of S chicks from DOH to d 4. However, from d 4 to d 10, the crypts in H chicks remained relatively unchanged, while those of the S incubated chicks continued to develop. At d 10, the crypts were deeper in the S group as compared to the H group chicks. A two way interaction of incubation temperature and age was also seen with villus height: crypt depth ratio in the ileum. Ileum villus height: crypt depth ratios (Figure 3.3) in chicks incubated at elevated temperatures decreased significantly from DOH to d 2, maintained a plateau from d 2 to d 4, increased dramatically from d 4 to d 6, and decreased from d 6 to d 10. With S incubation the ratio decreased from DOH to d 4 and then increased steadily from d 4 to d 10 (P= 0.02). In the jejunum (Figure 3.4), only the main effect of temperature was found to affect crypt depths, with deeper (P= 0.02) crypts in chicks incubated at elevated temperatures.

Results of nutrient transporter expression for three sugar transporters (SGLT1, GLUT2 and GLUT5), one amino-acid (EAAT3), and a peptide transporter (PepT1) were variable. No significant differences were found for SGLT1 or GLUT5, and only segment by age interactions were observed for EAAT3 (P= 0.006) and GLUT2 (P= 0.02; data not shown). There was significant temperature by age effects (P= 0.03) on relative PepT1 gene expression (Figure 3.5).

At DOH, PepT1 gene expression was lower in chicks incubated at H temperature than in chicks incubated at the S temperature. While expression decreased in S chicks from DOH to d 2, it increased in H chicks. From d 2 to d 4, relative expression of PepT1 dramatically decreased with a similar slope for both groups, but from d 4 to d 6 the expression in incubation heat stressed chicks increased again as it continued to decrease in S chicks. The S group chicks had dramatically increased PepT1 relative expression from d 6 to d 10, while expression decreased slightly in H chicks. For GLUT2 relative mRNA expression (Figure 3.6), there was a segment by temperature interaction (P= 0.04). In the duodenum and jejunum, GLUT2 expression was significantly higher in chicks incubated at H temperature as compared to S temperature incubated chicks. In contrast, GLUT2 expression in the ileum was higher in S chicks.

DISCUSSION

This study evaluated the effect of incubating high-yield broiler eggs at an elevated temperature (39.6 °C) compared to the standard temperature (37.4°C). No significant differences were observed in post-hatch broiler performance parameters or small intestine and yolk sac weights. However, there were differences in the morphology of the small intestine and in nutrient transporter gene expression. Results of villus height and crypt depth measurements as indices of intestinal post-hatch development suggest that intestinal development was initially increased in chicks from eggs incubated at elevated temperature. However, the accelerated growth reached a plateau as if there was a lack of energy or nutrients to favor the intestinal development and maturation, and by d 10, the duodenal villus height in H chicks decreased and was significantly shorter than villus in the S group. Interestingly, these changes coincide with the timing at which immune cells infiltrate the intestinal mucosa to form the gastrointestinal associated lymphoid tissue (**GALT**). The maturation of the GALT is known to happen in two waves, the first one during the first wk of life and the second from d 7 to d 14 post-hatch (Bar-Shira *et al*., 2003). The energy and nutrients available could be going towards the maturation and formation of the GALT and not into the proliferation, differentiation and maturation of the enterocytes that form the villus. This theory is supported by the duodenal crypt depth data obtained in this experiment, which had a consistent growth deepening until d 6 post-hatch when it reached a plateau in the H chicks. In a study by Uni *et al*. (2000), hyperplasia in the crypts occurs during the first 4 d of life of the chicken and that the growth rate of the intestinal

epithelium decreases after this time to d 14 at which time the intestine is mostly mature and established. Migration of enterocytes takes 2 to 4 d to reach to the tip of the villus, which coincides perfectly with the observations made in this study where there was a drop in villus length by d 10. The effect of elevated temperature on jejunal crypt depth may be explained given that during the first day of age, rapid growth takes place to increase proliferative cells that will later migrate to increase villus length to compensate for stress suffered during incubation.

From d 4 to d 6 post-hatch, there was a steep increase in the villus height to crypt depth ratios in the ileum of those chicks incubated at elevated temperature compared to the standard incubated group, and at this time the residual yolk should have been utilized to its maximum. According to Nitsan *et al*. (1991), the residual yolk contributes 50% and 40% of the total energy and protein supplies, respectively, on the first day post-hatch, and by d 4 there is only negligible amounts of nutrients in the residual yolk with 2% of energy and 6% of protein of the total nutrient supplies. In the past decade the poultry industry has seen huge changes in growth rate and feed conversion in commercial broiler chickens. Differences in yolk absorption were not observed; therefore, it could be assumed that even though the chicks from both groups are obtaining basically similar amounts and quality of nutrients, the utilization and final destination for these are different, or the requirements of the chicks for protein and energy are different. The energy required for mucosal growth is acquired through the nutrients available in the lumen, and for the acquisition of these, efficient absorption must occur.

From the five different nutrient transporters evaluated, only the sugar transporter, GLUT2, and the peptide transporter, PepT1, had differences in relative mRNA expression. GLUT2 is a basolateral Na^+-independent monosaccharide transporter for glucose and fructose with a higher affinity for glucose (Garriga *et al*., 1997). According to Ferraris and Diamond (1989), nutrient transporters are regulated within the small intestine depending on nutrient availability in order to save energy and to direct it where it is needed. The data from this study present relative mRNA expression, which does not necessarily correspond to the activity of functional proteins. The expression of GLUT2 in the duodenum and jejunum of H chicks was significantly higher than S chicks, whereas in ileum it was significantly lower in H chicks when compared to the S group. This could be explained by the fact that the proximal sections of the small intestine, duodenum and jejunum, have a faster mucosal growth rate and structural changes are more profound (Iji *et*

al., 2001). Even though glutamate is the main source of energy for the enterocytes or proliferative cells, the need for glucose as a source of energy to be absorbed and or utilized by the small intestine for the different metabolic pathways is higher in the more rapidly developing duodenum and jejunum of stressed chicks during incubation. PepT1 is a di- and tri-peptide Na^+-dependent transporter expressed in the brushborder membrane of enterocytes. It is more efficient to transport amino-acids through PepT1 than through the free amino-acid transporters (Daniel, 2004). In this study, PepT1 expression in chicks incubated at elevated temperature was lower at DOH, but increased in a steep slope with significantly higher levels of expression by d 2 when compared to standard chicks. From d 2 to d 4 post-hatch, both groups had decreased PepT1 expression, and from d 4 to d 10 post-hatch the incubation temperature groups responded differently. The chicks incubated at elevated temperature had increased expression by d 6 but then expression decreased again by d 10. In contrast, the chicks incubated under standard temperature had increased expression of PepT1 from d 6 to d 10 post-hatch, which resulted in significantly higher PepT1 relative expression levels in S as compared to H temperature chicks at sampling on d 10. This again may correlate to the kinetics of yolk utilization. The chicks incubated at elevated temperatures may need to have increased expression as a means to meet higher requirements for intestinal development, but by d 6 post-hatch there was no more energy available to increase expression and maintain the higher levels of oligopeptide transporters. It has been described previously by Chen *et al*. (2005) that the expression of PepT1 depends on diet changes, feed quality, and bird developmental stage. This could support the observations in our study. The developmental stage of the chick is most likely affected by the elevated temperature during incubation, and the distribution of nutrients from the residual yolk and external diet may be diverted from fulfilling the necessary energy needs for the transport of peptides into other physiological functions. Utilization of residual yolk and external diet is associated with the hypertrophy, hyperplasia, and development of the digestive and absorptive functionality of the small intestine.

The small intestine is not only part of the digestive system, but it is one of the most important organs of the immune system. Infiltration, differentiation, and maturation of the GALT takes place during the first 2 wk of age of the chick. Cytokine and lymphocyte activation require glucose uptake as a source for energy (Fox *et al*., 2005; Rudrappa and Humphrey, 2007). It could also be possible that peptides as a source of amino acids are required as building blocks for

tissue formation and for nucleic acids production for all the cells that are forming part of the small intestine during the early life of the chick. Thus, the results observed with the sugar and amino-acid transporters may indicate different requirements for energy and protein in chicks from eggs incubated at elevated temperature as compared to S temperature.

Most of the studies reported to date have not revealed significant differences in performance parameters of slaughter age broilers as a result of differences in embryonic incubation temperatures. Similarly, we did not observe performance parameter differences. However, the importance of measuring performance in birds has still of economic value to the industry, since it is a way to evaluate whether the chicks are correctly assimilating the diets and utilizing the nutrients provided by the diets for muscle growth. More parameters such as muscle yield and fat deposition in carcass should be observed. However, during the grow-out period, taking measurements such as feed intake and body weights can still be very descriptive of the whole performance of the flock. In this research, although there were no differences in post-hatch performance from alteration of incubation temperature, there were differences in intestinal epithelium development related to incubation temperature. The implications to gut health and susceptibility to disease should be further evaluated. While chicks in this experiment showed no performance differences, they were raised in an entirely clean environment; chicks in the field are normally exposed to different pathogens. The differences in morphology and gene expression might not be seen in performance, but could be translated in chicks with a higher susceptibility to diseases when exposed to dirty environments. It is possible that differences in performance parameters as well as the small intestine and yolk sac weights were not seen as a result of the fact that the Ross X Ross 308 broiler lines' growth rate is different from other broiler lines whose fast growth rate starts earlier post-hatch. Another factor that could affect the results in this study is that the use of a single-stage incubator model with only two temperatures treatments from ED13 to ED21 might not be enough to show the fluctuating changes in temperatures that a normal multi-stage incubator present in the industry may have. Further studies should include an incubation design that mimics multi-stage incubation used at commercial hatcheries and a broiler line selected for faster growth earlier in the grow-out. This study, however, does present the effects of elevated incubation temperature on small intestine development as measured by morphology and nutrient transporter relative gene expression.

Results are indicative of a link between allocation of energy, resources to the immune system, and development of the small intestine.

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Table 3.1 Primers used for relative real-time PCR[1]

Gene	GenBank ID number	Description	Sequence: forward/reverse
GAPDH	NM_204305	Glyceraldehyde-3-phosphate dehydrogenase	GCCGTCCTCTCTGGCAAAG/ TGTAAACCATGTAGTTCA
SGLT1	XM_415247	Solute carrier family 5 (Na^+- glucose co-transporter) member 1	GCCATGGCCAGGGCTTA/ CAATAACCTGATCTGTGCACCAGTA
SLC2A5 (GLUT5)	XM_417596	Solute carrier family 2 (facilitated fructose transporter) member 5	TTGCTGGCTTTGGGTTGTG/ GGAGGTTGAGGGCCAAAGTC
SLC2A2 (GLUT2)	Z22932	Solute carrier family 2 (facilitated glucose and fructose transporter) member 2	CACACTATGGGCGCATGCT/ATTGTC CCTGGAGGTGTTGGTG
PepT1	NM_204365	Peptide transporter	CCCCTGAGGAGGATCACTGTTGGCA GTT/CAAAAGAGCAGCAGCAACGA
SLC1A1 (EAAT3)	XM_424930	Solute carrier family 1 Member 1 (Excitatory amino acid transporter 3 glutamate and aspartate transporter)	TGCTGCTTTGGATTCCAGTGT/AGCA TGACTGTAGTGCAGAAGTAATATAT

[1]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA).

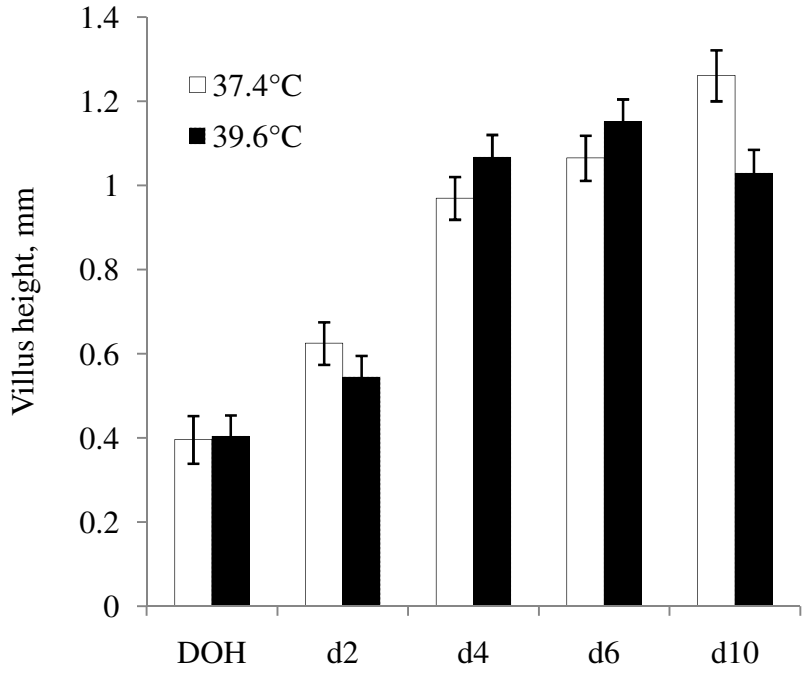

Figure 3.1 Effect of incubation temperature and age on duodenum villus height of Ross 308 broiler chicks incubated at two temperatures, standard (37.4°C) and high (39.6°C). Data are represented as LS Means ± SEM (n=12 measurements/bird, 10 birds/treatment). There was a significant two way interaction (P= 0.02) of incubation temperature and age

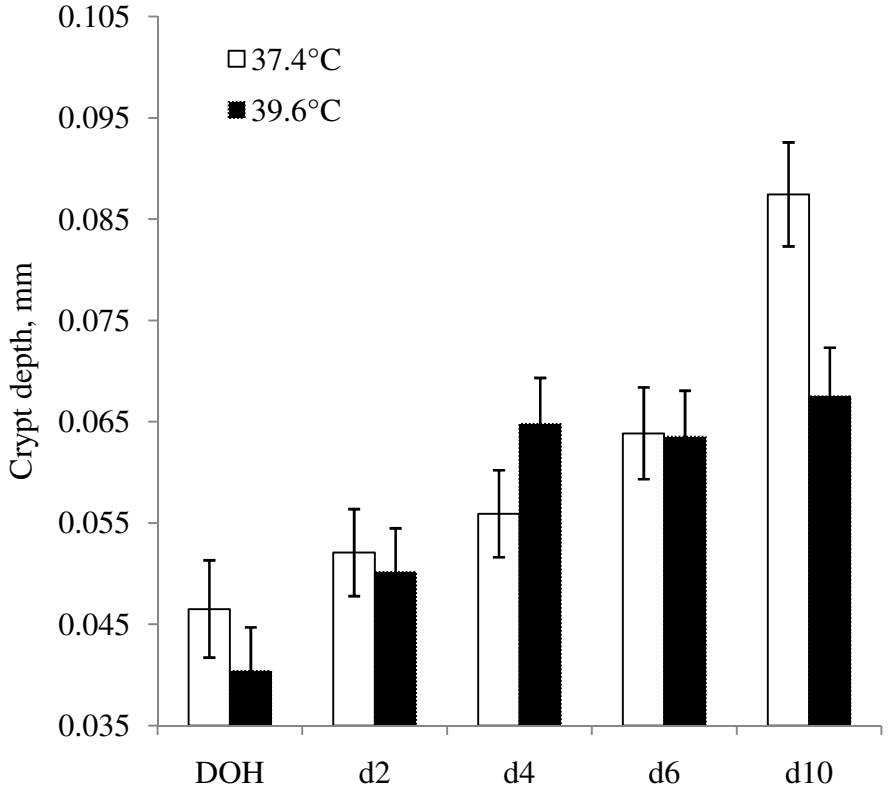

Figure 3.2 Effect of incubation temperature and age on duodenum crypt depth of Ross 308 broiler chicks incubated at two different temperatures, standard (37.4°C) and high (39.6°C). Data are represented as LS Means ± SEM (n=12 measurements/bird, 10 birds/treatment). There was a significant two way interaction (P= 0.05) of incubation temperature and age.

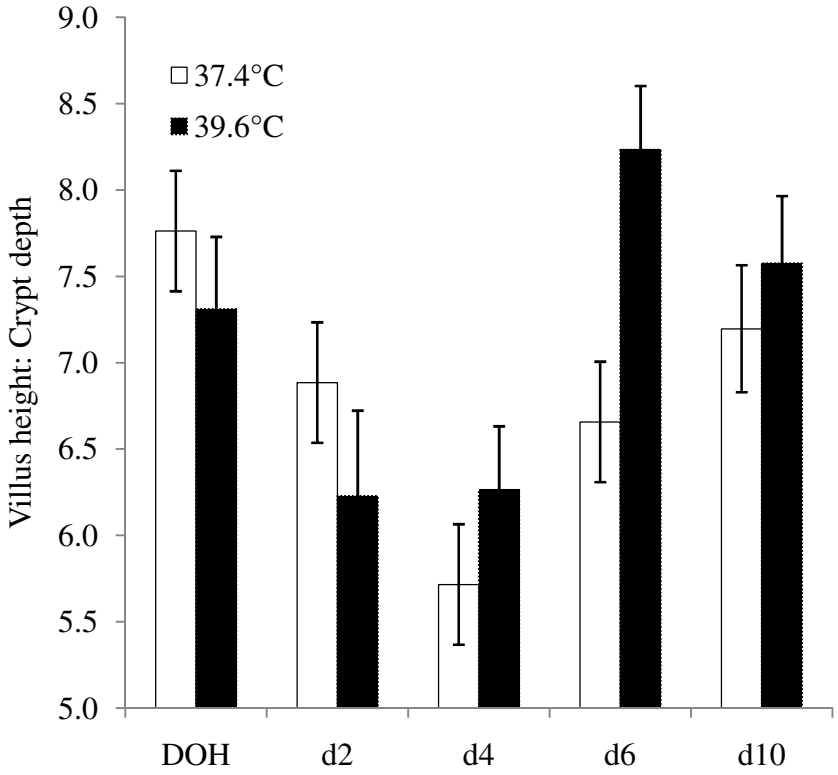

Figure 3.3 Effect of incubation temperature and age on ileum villus height: crypt depth ratios of Ross 308 broiler chicks incubated at two different temperatures, standard (37.4°C) and high (39.6°C). Data are represented as LS Means ± SEM (n=12 measurements/bird, 10 birds/treatment). There was a significant two way interaction (P= 0.02) of incubation temperature and age.

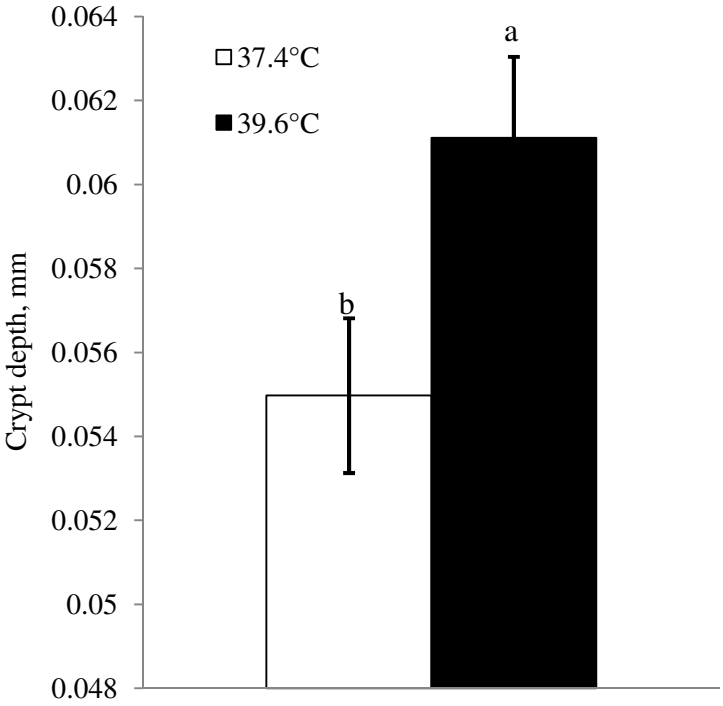

Figure 3.4 Effect of incubation temperature on jejunum crypt depth of Ross 308 broiler chicks incubated at two different temperatures, standard (37.4°C) and high (39.6°C). Data are represented as LS Means ± SEM (n=12 measurements/bird, 10 birds/treatment). There was a significant main effect (P= 0.02) of incubation temperature.

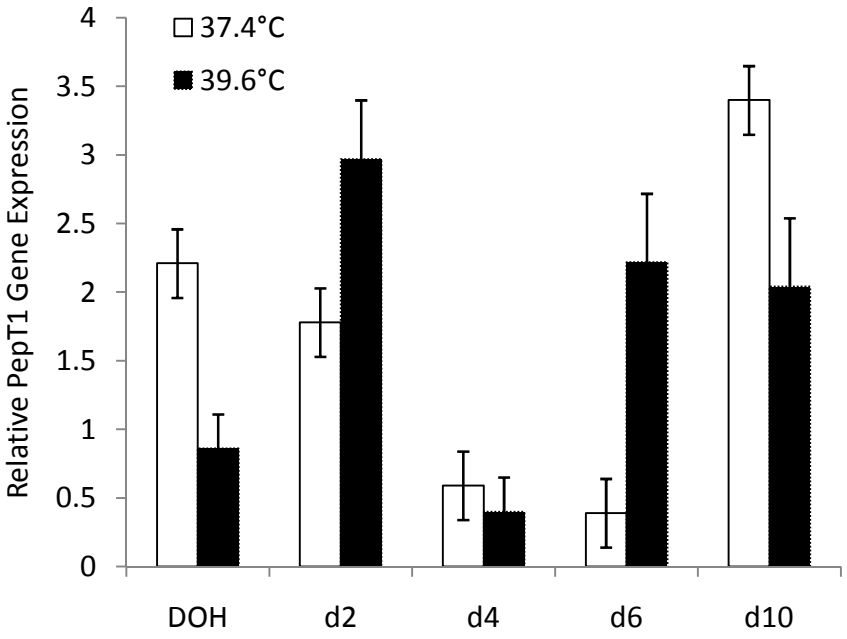

Figure 3.5 Effect of incubation temperature and age on relative PepT1 mRNA expression in the small intestine of Ross 308 broiler chickens incubated at two different temperatures, standard (37.4°C) and high (39.6°C). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from S (37.4°C) treatment as the calibrator. Data are represented as LS Means ± SEM (n=4/treatment). There was a significant two way interaction (P= 0.03) of incubation temperature and age.

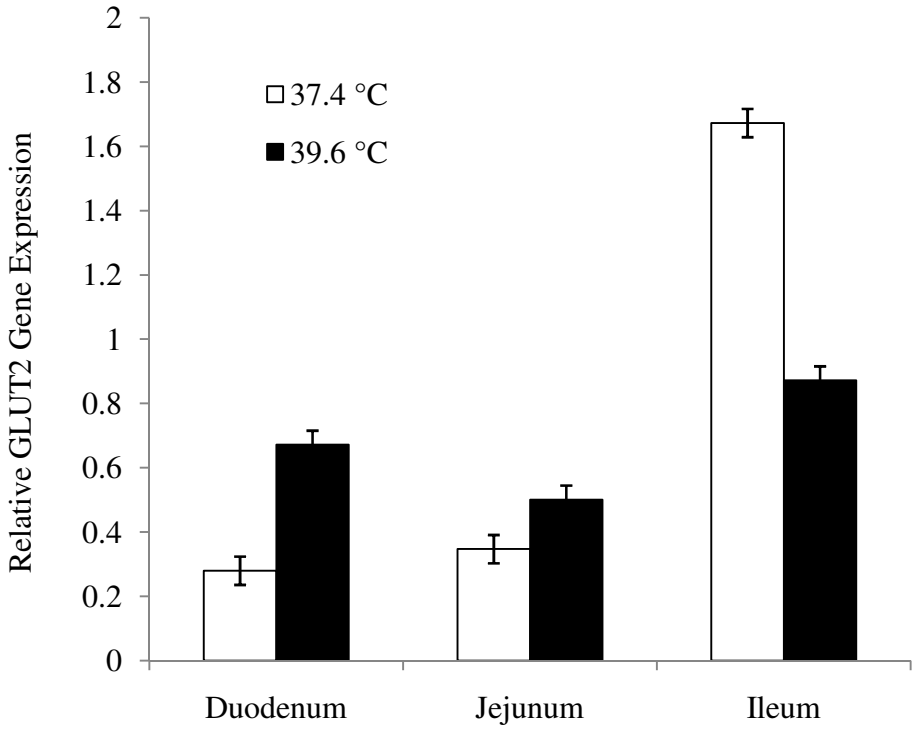

Figure 3.6 Effect of incubation temperature and segment on relative GLUT2 mRNA expression on intestinal segments of Ross 308 broiler chicks incubated at two different temperatures, standard (37.4°C) and high (39.6°C). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from S (37.4°C) treatment as the calibrator. Data are represented as LS Means ± SEM (n=4/treatment). There was a two way interaction (P= 0.04) of incubation temperature and segment.

CHAPTER IV

Effect of Incubation Temperature Profiles and Transportation Stress on Broiler Chicks: I. Performance and Nutrient Transporters

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ABSTRACT High-yield broiler embryos produce more metabolic heat than historic lines of broilers. With insufficient ventilation for heat removal from commercial incubators, excess heat can accumulate and stress embryos in late development stages. Once hatched, the chicks undergo a series of handling procedures, such as sorting, sexing, vaccination, and transportation. Poor incubation environment and post-hatch stressors may be important factors for unfavorable development and maturation of the gastrointestinal tract, which could result in an impaired uptake and utilization of nutrients. This could contribute to a decrease in performance and cause a significant rise in poultry production costs. This study evaluated the effect of embryonic incubation and post-hatch transportation temperatures on post-hatch relative nutrient transporter gene expression and performance of broiler chickens. Cobb 500 eggs (n=5200) were incubated with the following egg-shell temperatures (**EST**), which were combined depending on the early and late temperatures during the incubation period: Low (**L**): 36.7°C, Standard (**S**): 37.5°C, and High (**H**): 39°C. After hatch, chicks were further separated into two transportation groups: control (**C**; 34°C) and heat stressed (**D**; 40°C). The eight resulting experimental groups were: LS-C, SS-C, LH-C, SH-C, LS-D, SS-D, LH-D, and SH-D. Performance data were collected and analyzed at day of hatch (**DOH**) and d 14, 28, and 42. Yolk free body weight (**YFB**) data were collected at DOH (pre- and post-transportation), d 2, 4, and d 6. Intestinal samples were collected from duodenum, jejunum, and ileum at DOH (pre- and post- transportation) and d 4, and 6 for relative gene expression analysis of three sugar transporters (SGLT1, GLUT2, and GLUT5), an amino acid transporter (EAAT3), and the peptide transporter PepT1 through relative quantification real-time PCR analysis. No significant differences were found in feed conversion, mortality, or pre-transportation YFB. There was a three way interaction of incubation temperature (**IT**), transportation, and age for feed intake (**FI**) (P= 0.02). Two way interaction of

IT and age was observed (P= 0.01) for YFB. Body weights (**BW**; P= 0.01) and body weight gain (**BWG**; P= 0.003) presented a two way interaction of transportation and age. There was a main effect of IT on YFB (P= 0.01). No differences were observed in the pre-transportation relative mRNA expression data for any evaluated gene. Differences were seen in relative mRNA expression of SGLT1 with a three way interaction of IT, transportation, and age (P= 0.04) and IT and age (P= 0.0001). For EAAT3 and GLUT5, a two way interaction of IT and age (P= 0.003 and P= 0.008, respectively) was observed. A main effect of IT on the peptide transporter PepT1 (P= 0.02) was also observed. Incubation and transportation temperatures and stress had an effect on the grow-out performance as expressed in feed intake (**FI**), which can directly affect BW of the chicks and subsequently impact development as observed with changes in relative nutrient transporter gene expression in the small intestine. These results contribute to the understanding of mechanisms by which altered temperatures during incubation and transportation can affect embryonic development and subsequently performance of the broiler chick.

Key words: incubation**,** stress, transportation, nutrient transporter, broiler

INTRODUCTION

During the last five decades, meat type chickens have been intensively selected for body weight gain (**BWG**), resulting not only in a higher growth rate but also in an impressive improvement in feed efficiency. With this, today's broilers require approximately 1/3 of the time and over a 3-fold decrease of FI to get to slaughter age (Havenstein *et al*., 2003). The rapid growth rate is not seen only during the grow-out period but as early as during embryonic development. The poultry industry has increased its concern about adequate incubation of these modern commercial broiler embryos so that an optimal environment for embryonic development can be created. The majority of commercial hatcheries use multi-stage incubation systems, where embryos at multiple stages of embryonic development are incubated together. Ventilation plays a major role in maintaining optimal environmental conditions in multi-stage incubators but often the equipment does not possess the capacity to adequately ventilate the different embryonic stages (Smit *et al*., 2008). The industry still encounters many problems in the hatchery with embryonic development and chick quality at hatch, thus it is obvious that incubation parameters are not well optimized. Incubation success relies on multiple factors, such as humidity, CO_2 and

O_2 concentrations, as well as temperature (Decuypere, 1984). Temperature is the most important factor, especially during the late stages of incubation when an increase in temperature may negatively affect growth and increase embryonic mortality (Christensen *et al*., 2004; Hulet, 2007). Since broiler embryos of today produce 44% more heat than embryos of years past, controlling temperature in the incubators requires intensive management (Hulet *et al*., 2007). The incubator temperature (environmental reading) has to be adjusted in order to maintain eggshell temperature (**EST**) and avoid negative effects of high EST during embryonic development (Lourens *et al*., 2005). The embryonic development is directly influenced by the embryo's temperature, so if the incubator conditions are not adjusted to optimize embryonic temperature, maximum hatchability and chick quality will not be attained (Decuypere and Bruggeman, 2005). Overheated embryos can present decreased hatchability, body weight (**BW**), heart size, and yolk absorption, an increase in unhealed navels and late dead embryos, and negative impacts on growth and development of the chick post-hatch (French, 2000; Hulet 2007).

Hatchery treatments such as sorting, sexing, and immunizations, and transportation from the hatchery to the farm can induce further stress for the chick. Heat stress during transportation, in market age birds, is associated with high mortality and is recognized as the most common problem in the industry (Mitchell and Kettlewell, 1998). Transportation of poultry with deficient ventilation causes an accumulation of heat and moisture that in combination can increase the heat stress on the chicks. The heat stress stimulates chicks to pant in order to achieve heat dissipation (Mitchell and Kettlewell, 1998). The evaporative respiration increases water loss from the animals and adds to the moisture in the environment within the transportation truck, thus increasing the heat stress to create a cycle of hyperthermia (Mitchell and Kettlewell, 1998). Therefore, transportation represents a very important issue from the commercial, public, and even political point of view due to the effect that this has on animal welfare (Mitchell and Kettlewell, 1998). To date, there are no guidelines to follow for recommendations on transportation conditions with adequate or acceptable temperature ranges and limits (Cheng and Jefferson, 2008). Several studies have evaluated the effects of stress during transportation on different animals of agricultural value. However, most of them include older animals at the end of their productive life, and only a very few involve newly hatched chicks. Yet, it is known that

no matter the species, age, or developmental stage of the animal, transportation is an important stress factor.

Growth and performance parameters of broiler chicks depend on adequate development of the small intestine. The small intestine is considered to be a supplying organ, which is necessary to cover the nutritional demands of the growing chick (Siegel *et al*., 2008). The animal allocates energy resources depending on the requirements or needs for maintenance, growth, reproduction, and well being in order to establish homeostasis within its body (Cheema *et al*., 2003). High-yield broiler chicks are characterized by rapid early development of the small intestine and a high body growth rate (Rauw *et al*., 1999). When the small intestine matures physiologically, the energy demands for protein turnover and nutrient transportation are augmented as well (Cant *et al*., 1996; Rauw *et al*., 1999). Given that high-yield broiler growth has priority over the rest of the physiological functions, the chicks continue to grow at the expense of the functionality of other systems, even to the point that disease can result (Lilja, 1983; Yunis *et al*., 2000; Havenstein *et al*., 2003). Heat stress can induce changes in glucose and uric acid metabolism (Moraes *et al*., 2003). Glycogen can be crucial in the adaptation to different stressors (Christensen *et al*., 1995). During incubation under elevated temperatures and transportation with poor ventilation, heat stress increases the energy requirements of the chick (O'Dea *et al*., 2004), thus, there is mobilization of glycogen and activation of the catabolism of muscle protein, where the turnover is induced by the activation of gluconeogenesis by glucocorticoids (Puvadolpirod and Thaxton, 2000). The increase in demand for energy causes changes in the intestinal epithelium and nutrient transporter gene expression that may be costly in energy and may happen at the expense of body growth rates and overall performance parameters. A preliminary study was conducted in our laboratory to examine elevated (39.6°C) incubation temperature (**IT**) effects on small intestine development and its relation to performance parameters. Based on preliminary results, the present study was designed to evaluate temperature profiles as seen in a multi-stage incubation system. Multi-stage incubators tend to present low temperatures during the first week of embryonic development and high temperatures during the third week of incubation. A high-yield broiler line selected specifically for rapid growth rate and feed efficiency was used. The objective of this study was to evaluate the relationship between EST, transportation stress, nutrient uptake, and performance parameters. This paper is the first part of an extensive study that consists of incubation temperature profiles

simulating what is seen in commercial multi-stage incubation systems and stress during transportation of newly hatched chicks from a high-yield broiler line. The first part of this study evaluated the effect of these stressors on development of the small intestine as measured through relative gene expression of specific nutrient transporters and their relation to performance parameters.

MATERIALS AND METHODS

Animal Welfare

This project was approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Design

A total of 5200 fertile eggs were obtained from a 31 wk old Cobb 500 flock. Eggs were placed in incubators (Natureform I40, Jacksonville, FL) at North Carolina State University facilities. Based on the profiles of EST observed in commercial hatcheries using multi-stage incubators, there were four treatments designed to simulate the most common range of commercial incubation conditions. Treatments were designated as low temperature (L, 36.7°C) or high temperature (H, 39°C) as compared to the conventional standard temperature (S, 37.5°C). For embryonic day (**ED**) 0 to ED7, half of the eggs were incubated at L and the other half were incubated at S. From ED 7 to ED 14 all eggs from L and S were incubated at S. From ED 15 until ED 21, the eggs were again divided and then incubated at either S or H. This design generated four treatments: eggs incubated at S during both the early and late phases (SS), eggs incubated at S during the early phase and H during the late phase (SH), eggs incubated at the low temperature during the early phase but the standard temperature during the late phase (LS), and eggs incubated at a L during the early phase but the H during the late phase (LH; Figure 4.1). Temperature was regulated by thermistors connected to microprocessors with temperature sensitivity of ± 0.05°C. Humidity was controlled by a similar system using relative humidity sensors. Digital thermometers (Cox, Lexington, NC) were used with each incubator tray to verify set point temperatures. During incubation, data on EST, incubator temperature and

humidity were logged on a daily basis. For each treatment the hatch of fertile eggs, early, mid, and late incubation deads were recorded.

Transportation Design

Once hatched, all chicks were sexed on site, and the incubation groups were further separated into 2 groups for transportation. Two wooden boxes were built in order to control the environment during transportation. Each box had an exhaust fan and two air inlets in order to control the ventilation during transportation (Figures 4.2 A and 4.2 B). The transportation boxes were put in 2 cargo vans, and each box had an equal number of chicks from each incubation treatment (Figure 4.2 C). In the control transportation group (C, 34°C), ventilation was controlled manually to maintain chicks in their thermoneutral zone. For the stressed transportation group (D, 40°C), ventilation was controlled manually in such a way that the chicks showed an increase in their respiratory rate. The panting response in the stressed chicks was an indicator that there was an elevation in temperature. Vocalizations were also used as an indicator of stress during transportation (Figure 4.2 D). The increase in respiratory rate and minimal ventilation caused a decrease in oxygen levels and a possible increase in CO_2. The chicks' behavior was monitored through an observational window throughout the 4 h transportation to Virginia Tech. Actual temperatures in chick boxes were recorded with electronic data collectors during transportation.

Broilers and Diets

After transportation to Virginia Tech, 46 chicks (23 males and 23 females) per treatment were assigned to floor pens (0.76 ft^2/ chick) with clean pine shavings. Each treatment was replicated by 8 pens for the 42 d trial. Birds were reared with a commercial lighting program recommended for Cobb broilers. All birds were provided with feed and water *ad libitum* throughout the grow-out period. Given that feed quality is a factor responsible for assimilation of nutrients in the small intestine and therefore for growth rate differences, chicks received the same quality diet throughout the experiment. The diets were corn-soybean meal based and formulated to meet or exceed the Cobb 500 requirements. The diets were fed in three individual dietary phases. The dietary phases consisted of starter (2,990 Kcal/kg, 21.4% CP), which was fed from d 0 to d 14, grower (3,160 kcal/kg, 19.5% CP), which was fed from d 14 to d 28, and

finisher (3,194 kcal/kg, 17.49% CP), which was fed from d 28 to d 42. Starter and grower diets were medicated with monensin (Coban; Elanco Animal Health, Greenfield, IN), an ionophore commonly used as an antiprotozoan, and with bacitracin methylene disalicylate (Alpharma Animal Health Division, Fort Lee, NJ) an antibiotic commonly known to reduce lactic acid producing bacteria and *clostridium sp.* The finisher diets were medicated with Coban and virginiamicin (Stafac; Phibro Animal Health, Ridgefield, NJ), an antibiotic used to prevent necrotic enteritis.

Performance Parameters

Individual BW were obtained at DOH (post-transportation), d 14, 28, and 42. Feed intake, feed conversion, and BWG were calculated for the three individual feeding phases and cumulatively during the trial. Mortality was recorded daily. Feed conversion was adjusted for daily mortality. At DOH, d 2, 4, and 6, 20 chicks/treatment were randomly selected for yolk free body weight (**YFB**) measurements. Each bird was weighed and euthanized by cervical dislocation. Yolk sacs were collected immediately, and the YFB was obtained.

Tissue Collection and RNA Extraction

At DOH (pre- and post-transportation), d 4, and 6 post-hatch, 3 female birds/treatment were randomly selected for relative nutrient transporter gene expression analysis. Each bird was weighed and euthanized by cervical dislocation. Tissue samples were collected immediately from duodenum (ascendant loop), jejunum (from the pancreatic duct to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileo-cecal junction) without the mesenteric tissue. The pancreas was separated from the duodenal loop and was not used for the analysis. The intestinal segments were rinsed in ice cold PBS, minced with razor blades, and thoroughly homogenized. Twenty to 30 mg of minced tissue were collected in 2 mL micro centrifuge tubes (RNase and DNase free, Ambion, Austin TX.) and were snap frozen in liquid nitrogen. Each sample was collected in duplicate. Samples were stored at -80 °C until further analysis. Total RNA was extracted from the intestinal tissue samples using the RNeasy Miniprep Kit (Qiagen, Darlington Lab. Valencia, CA) according to the animal tissue extraction protocol provided by the manufacturer. Concentration and purity were determined using the 260/280 and 260/230 ratios obtained from a nanodrop spectrophotometer (Thermo Scientific, NanoDrop™ 1000 Wilmington,

DE). The quality of RNA was assessed by visualization of distinct 28S and 18S rRNA bands after gel electrophoresis with ethidium bromide staining.

Primer Design and Validation

Specific oligonucleotide primer pairs were designed by Primer Express software (Applied Biosystems, Version 3, Foster City, CA) and synthesized according to already published chicken sequences (Gilbert *et al*., 2007). Table 4.1 presents the primer sequences used.

cDNA Synthesis and Real-Time PCR

Total RNA was diluted to 0.2 µg/ µL in diethylpyrocarbonate treated water. Reverse transcription into cDNA was done using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The cDNA was diluted 1:30 and stored at -20 °C until further analysis.

Relative quantification real-time PCR was done for 6 samples/treatment with the use of an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). All reactions consisted of 2uL (10 ng/uL) of reverse transcribed RNA, 12.5 uL SYBR Green PCR master mix (Applied Biosystems), 1.5uL (5uM concentration) forward and reverse primers, and 8.5 uL diethylpyrocarbonate treated water. All samples were run in 96-well plates (Applied Biosystems, Foster City, CA) in duplicate. PCR was performed under the following conditions: 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15s and 60°C for 1 min (40 cycles). After each run, a dissociation curve was obtained to verify that there was only one product being amplified. The dissociation curve had the following conditions: 95°C for 15s, 60°C for 30 min, and 95°C for 15 min. The Ct values for each gene were averaged. The differences between the average Ct values of target and reference gene (GAPDH) were also calculated (ΔCt). For presentation purposes only, $2^{-\Delta\Delta Ct}$ values were calculated using the ΔΔCt method with the reference gene as the endogenous control and the average Ct value for duodenum SS-C as the calibrator to express fold changes in gene expression (Livak and Schmittgen, 2001). Since we are interested in the evaluation of the interaction between incubation temperature and segment, we collected samples from the three different segments in the small intestine. In the statistical analysis, the software SAS was used to analyze the segments

separately and together to obtain the appropriate three and two way interactions as well as the main effects. Because the duodenum SS-C was used as the calibrator, the graphs showing the combined values of the three intestinal segments will not show a value of 1 for SS-C. Significance of values for treatment differences was determined from analysis of ΔCt values.

Statistical Analysis

A completely randomized experimental design with a 4 X 2 factorial arrangement of treatments (four levels of incubation temperatures and two transportation conditions) was used. Data were subjected to ANOVA using the MIXED models procedure of the SAS program version 9.1 (SAS Institute Inc. Cary, NC). All values are expressed as least squares (LS) means ± SEM. For BW uniformity (CV), nutrient transporter analysis, and YFB data chicken was the experimental unit. For the other performance parameters the experimental unit was pen. All percentage data were arc-sine (square root of percent) transformed prior to analysis. The model included main effects of incubation temperature, transportation, and age, and all two and three way interactions. Differences among treatments were further compared using the Tukey's test. Significance was determined at $P \leq 0.05$. The contrast coefficient matrix for unequal spacing was generated using PROC IML. The model was: $Y_{ijkl}= \mu+ \alpha_i+\tau_j+\beta_k+(\alpha\tau\beta)_{ijk}+\varepsilon_{ijkl.}$ Where Y_{ijkl} = observed dependent variable; μ= overall mean; α_i= the main effect of incubation temperature I; τ_j = the main effect of transportation J; β_k= the main effect of age k; $(\alpha\tau\beta)_{ijk}$ = the interaction between incubation temperature, transportation, and age; and ε_{ijkl} = the random error. The data from DOH pre-transportation was not included in the statistical model.

RESULTS

Performance Parameters

There were no differences in BW uniformity between incubation temperature or transportation treatments. There was a two way interaction of transportation and age for BW from DOH to d 28 all groups had similar BW (Table 4.2). However, at d 42 the chicks that were transported under controlled conditions were 40 g heavier (P= 0.01) than chicks that were transported under stressed conditions. Yolk free body weights had a two way interaction of IT and age (Table 4.3) and a main effect of IT (Figure A.1). In the two way interaction with YFB,

there were no significant differences at DOH or d 2. However at d 4 and d 6 significant differences (P= 0.01) existed. On d 4 and d 6, chicks from SH incubation had heavier YFB as compared to LS, with SS and LH having intermediate YFB on both days. The only difference in BWG in the trial was seen in the interaction between transportation conditions and age. As observed with BW, birds transported under control or stress conditions had similar BWG from DOH to d 14 and d 14 to d 28. However, from d 28 to d 42, chicks that were transported under stressed conditions presented less weight gain than those transported under control conditions with a 35 g difference (P= 0.003). Three way interactions of IT, transportation, and age with FI (Table 4.4) were observed, with the main changes during d 28 to d 42 (P= 0.02). From DOH to d 14 and d 14 to d 28, there was similar FI for all experimental groups. However, during the period of d 28 to 42, the LS incubation group was the only group that had increased FI when birds were transported under stressed conditions. In contrast, the SH group transported under stressed conditions presented a more dramatic decrease in feed consumption compared to the rest of the experimental groups. In the two way interactions between IT and age and transportation and age for FI, the LS group and transportation stressed group had the lowest FI, respectively (Figures A.2 and A.3). Feed conversion ratios, while not significant (P= 0.07), showed a 2 point improvement in chicks that were transported under control conditions compared to those transported with stress (Figure A.4).

Gene Expression

Numerous main effects and interactions were observed in measurements of the relative expression of nutrient transporters. Interactions of segment by age, as well as main effects of age and segment were observed for all the genes studied. Given the focus of our research, these results are not discussed in this paper.

SGLT1. The three way interaction of IT, transportation, and age, for relative SGLT1 mRNA expression (Figure 4.3) was evident from DOH to d 6 post-hatch (P= 0.04). At DOH groups LS-D, SH-C, SS-C and SS-D had similar initial expression while SH-D had the highest expression of all experimental treatments and LS-C had the lowest expression. From DOH to d 4, most experimental groups had similar response by decreasing gene expression of SGLT1, however, LH-D and LS-C were the only groups that increased expression by 1.7 and 3.9-fold, respectively.

Interestingly, by d 6, most groups had similar SGLT1 gene expression, with the exception of SH-C and SH-D. The group SH-C had the lowest expression when compared to the rest of the experimental groups. The SH-C and SH-D groups were also the only treatments that had continual decreases in SGLT1 expression from DOH to d 6. The two way interaction of IT and age for SGLT1 (Figure 4.4) showed that at DOH, chicks incubated at an early standard temperature (SH and SS) showed around 1.6-fold higher SGLT1 relative mRNA expression than those incubated at an early low temperature (LS and LH). However, at d 4, chicks incubated at low temperatures early in development had increased expression (P= <0.0001), while the chicks incubated at early standard conditions had decreased expression levels. On d 6, all groups had similar expression levels with the exception of the SH group. Groups LH, LS, and SS had around 2.8-fold higher expression than the SH group (P=<0.0001). The main effect of transportation conditions were seen with higher SGLT1 expression in chicks transported with stress (Figure A.5).

EAAT3. The two way interaction between IT and age was evident from DOH to d 6 post-hatch for expression of the EAAT3 transporter (Figure 4.5). The initial expression of EAAT3 in chicks incubated at an early standard temperature was higher than in chicks incubated at early low temperatures. From DOH to d 4 all experimental groups had increased gene expression for the EAAT3 transporter, however the LS group had a much more dramatic increase (2.4-fold higher than other groups at d 4; P= 0.003). The LH group had a 2-fold increase when compared to the 1.2-fold increase of the SS and SH groups. From d 4 to d 6 all experimental groups had decreased mRNA expression for EAAT3, with the LS group showing the most evident change (P= 0.003). All experimental groups had similar expression at d 6 post-hatch.

GLUT5. The initial relative mRNA expression of fructose transporter GLUT5 in chicks incubated at early low temperature (LS and LH) was lower than expression in those incubated at early standard temperatures (SH and SS; Figure 4.6). From DOH to d 4, all the groups had increased expression however the groups incubated at the early low temperatures had a greater increase than the early standard groups (P= 0.008). From d 4 to d 6, all experimental groups had increased gene expression at a similar rate. However, the groups incubated at low temperatures early in development had a final higher GLUT5 expression than those incubated at early standard temperatures.

PepT1. Only a main effect of IT was observed for relative mRNA expression of the PepT1 oligopeptide transporter (Figure 4.7). Chicks incubated at the LH temperature profile had the lowest expression as compared to the rest of the experimental groups. SH had higher (P= 0.02) PepT1 mRNA levels than LH, and significantly lower expression levels than SS group, but were not different from the LS group. LS and SS groups had similar expression levels.

DISCUSSION

Once the chick hatches the nutrient demands change drastically, forcing differentiation and maturation of the intestinal epithelium. The assimilation of nutrients at the embryo's intestinal mucosa and distribution of substrates is fundamental for the chicks to obtain energy for their different physiological processes such as locomotion, thermoregulation, gastrointestinal tract development, and immunocompetence (Obst and Diamond, 1992). After hatching, increased utilization of external resources becomes an important mechanism for continued development and increased growth. Growth, reproduction, performance, and immune status can be considered as an investment of protein and energy (Rauw *et al*., 1999). These investments create costs for maintenance and growth. During growth, the small intestine is responsible for supplying the energy that will be used to fulfill the nutritional demands of the growing animal (Siegel *et al*., 2008). The animal allocates its resources depending on the requirements or needs for maintenance, growth, reproduction, and well being in order to establish and maintain homeostasis (Cheema *et al*., 2003). High-yield broiler chicks are characterized by a rapid early development of small intestine and growth rate. Resources during incubation are fixed by what is available from the egg, but after hatch there is a transition to the utilization of external feed sources. Early low and late high IT and stress during transportation may have detrimental effects on the development of the small intestine, with a resulting negative impact on the utilization of energy and nutrients affecting the chick's lifetime performance.

The objective of this study was to evaluate the effects of several temperature profiles during embryonic incubation and the effects of heat stress during transportation on the development of the small intestine as measured through relative gene expression of specific nutrient transporters and their relation to performance parameters.

In this study, chicks (n=368 chicks/treatment) were weighed on DOH after transportation and prior to placement in pens. No differences in BW from incubation treatment were observed. These data differ from reported results showing chicks (Cobb) incubated at high (38.6°C) temperatures with heavier BW at hatch than those incubated at low (36.5°C) or mid (37.6°C) temperatures. At d 44 the chicks in the high temperature group weighed 100 g less than the mid or low temperature groups (Hulet *et al*., 2007). Previous research done by Decuypere and Bruggeman (2007) showed that BW at DOH was mostly correlated to the egg's size and not to development and performance, because most of the early post-hatch weight includes the unabsorbed yolk sac. Given that the DOH chick's full BW does not directly represent the chick's development, the YFB or BW without the residual yolk is considered a better measurement (Decuypere and Bruggeman, 2007). According to Decuypere (1984) and Tona *et al*. (2004), there is no relationship between the weight of a DOH chick and performance at slaughter age. However, they have reported that the weights at d 7 to d 10 are correlated to the performance at slaughter age (Tona *et al*., 2004). Smith (2005) pointed out that the use of BW at d 7 is now approved in the hatchery industry as a "Key Performance Indicator". In this experiment we collected data from 20 chicks/treatment for YFB measurements at DOH (pre- and post-transportation) and d 2, 4, and 6 post-hatch. No differences were seen at DOH before or after transportation, but differences were observed at d 4 and d 6, with the LS group (always the lightest group) and SH (the heaviest group). Studies have shown that chicks incubated at low temperatures during the first wk of incubation, have no compensatory growth (Geers *et al*., 1982, 1983; Joseph *et al*., 2006), but continue with low BW within the first wk post-hatch. This agrees with reports of Lourens *et al*. (2005) who observed that low EST in the first wk of incubation caused low BW during the first wk post-hatch. Some studies have shown that chicks incubated at low temperatures present a reduced BW and BWG, nonetheless, the chicks recover and reach similar weights during the rearing period (Geers *et al*., 1982; Decuypere, 1984). In our study, the YFB data collected at d 6 showed no transportation effects but only temperature by age interactions or main temperature effects. Conversely, from the performance data, we observed significant BW differences at d 42 with transportation by age interactions. Further research evaluating incubation temperature and transportation stress independently should be performed.

In this study, the interaction of transportation and age was evident in chicks transported under heat stress conditions, which had a decrease in FI as compared to chicks transported with control

conditions. These differences were evident until the last phase of the grow-out period from d 28 to d 42. The LS group had the lowest FI throughout the grow-out period, however it was the only group that showed a higher feed consumption as a response to heat stress during transportation. This is in contrast to the SH group which had drastically decreased FI when transported under stressed conditions. Interestingly, no evident effects on BW, BWG, or FI were seen in chicks incubated with a high temperature during the late phase of incubation. According to Joseph *et al*. (2006) the effects of low temperature during the first part of the incubation period are critical for the chicks' development due to the fact that chicks incubated under such conditions do not have the capacity to present compensatory growth. This may not be the same for embryos incubated at higher than standard temperatures.

There were no significant differences in feed conversion between chicks exposed to different embryonic temperature profiles. However, chicks transported under heat stress conditions, had a 2 point alteration in FCR (1.62 vs. 1.60). Despite the fact that it is not statistically different (P= 0.07), 2 points in FCR will make a big difference in the economy of the poultry industry. The FCR results were an effect of the transportation treatment, but not of IT treatment. Previous studies by Hulet *et al*. (2007) have demonstrated that FCR can be affected by late high IT with differences of 0.05 and 0.07 in cumulative feed conversion between chicks incubated at 37.6°C or 39.7°C, respectively.

In an attempt to understand why differences in BW and other performance parameters may occur with changes in IT profiles, we evaluated indirect indices of nutrient uptake by measuring relative mRNA expression of several nutrient transporter genes. Unfortunately, gene expression in this trial was only measured until d 6 post-hatch. It would be interesting to possibly relate FI and nutrient uptake at each of the feeding phases, in particular from d 28 to d 42, which is where most differences in BW, feed conversion, and FI were found. Given that the nutrient assimilation capacity in an animal depends on the mucosal surface area of the small intestine, digestive enzyme activity, and on functional properties of specific nutrient transporters that are present in the basolateral and brushborder membrane (Amat *et al*., 1996), three sugar transporters (SGLT1, GLUT2, and GLUT5), an amino acid transporter (EAAT3), and the peptide transporter PepT1 were evaluated. Chicks incubated at S temperature early in development had a higher expression of SGLT1 at DOH than chicks incubated at early L temperature incubation profiles.

The chicks from eggs held at S temperature early in incubation also responded differently in expression of SGLT1 from DOH to d 6 when compared to the early L incubation group with decreased SGLT1 gene expression. Overall in this study, SGLT1 relative mRNA expression was increased in chicks transported under heat stressed conditions or incubated at early L temperatures. Research has shown that carbohydrate uptake varies depending on the physiological conditions of the animal, starvation, FI, diet composition, hypothermia, hyperthermia, or the location or maturation level of the enterocyte along the villus (Ferraris, 2001). The increase in active sugar transportation has also been correlated to the age at which maturation of the small intestine is reached (Holdsworth and Wilson, 1967). Thus, suboptimal incubation or stress inducing transportation temperatures may impair differentiation and maturation of the small intestine, which could relate to an increase in SGLT1. Chicks transported with heat stress presented panting and increased vocalizations, indicating that the chicks were undergoing stress. Previous research reported that glucocorticoids, released during stress, induce the expression of glucocorticoid-regulated kinase 1, which enhances glucose transportation by increasing SGLT1 abundance in the cell membrane (Carriga *et al*., 2006). They also found that heat stress causes metabolic and physiological changes in the intestinal transportation of glucose through the increase of SGLT1, while the basolateral transporter GLUT2 is unaffected. We did not find differences in GLUT2, a Na^+-independent transporter for glucose, galactose, and fructose located in the basolateral membrane in the current study. Future studies should consider the measurement of glucocorticoid levels in serum and their correlation to the parameters measured in this research.

Differences in GLUT5 gene expression were found between incubation treatments. GLUT5 is located in the BBM and transports fructose into the cell cytoplasm. Fructose is present as part of sucrose, which is an important disaccharide comprising feedstuffs for poultry (Jozefiak *et al*., 2004). The glycosidic linkage of sucrose can be hydrolyzed by the invertase activity at the BBM of the enterocytes (McNab and Boorman, 2002). Fructose is also present as fructans, which can only be degraded by bacteria in the digestive tract of the chick. Fructans are usually given as prebiotics (Yusrizal and Chen, 2003). In our study, GLUT5 expression between incubation temperature groups was different from DOH to d 6. Chicks incubated at early L temperature, had a lower expression at DOH but had increased expression at a fast rate by d 4 and d 6. The chicks incubated at an early S temperature had a higher expression at DOH, and while still

showing increased expression by d 4 and d 6, they had a significantly lower expression than the early L temperature groups at d 6. The expression of GLUT5 could be used as an indirect measurement of microbial colonization in the small intestine. An immature organ is usually characterized by poor physiological functionality. The small intestine is one the most important immune organs in the chicken, and it functions with both the innate and adaptive branches of the immune system. By d 5 post-hatch the innate immunity in the chick, although immature, is already established, while adaptive immunity takes 2 wk to develop. The innate response of the intestine of the small intestine is comprised of mucin and cells within the intestinal mucosa that contains antimicrobial enzymes. If there is impaired development of the small intestine for competent physiological and immunological properties, the defense systems to pathogens will be compromised. This may allow colonization of a large number of bacteria. The increase in GLUT5 expression could be indicative of more digestion of sucrose and fructans by local microbiota, which could be related to GALT immaturity and bacterial invasion in the small intestine. However, there was no evaluation of the intestinal microflora or mucin presence to support this theory.

Glutamate is vital for the mucosal epithelium since it favors the stimulation of proliferating cells, provides energy for T lymphocyte metabolism, and is the provider for one half of the N requirement for both purine and pyrimidine synthesis (Lobley *et al*., 2001; Rudrappa and Humphrey, 2007). Glutamate can replace many of the metabolic functions of glutamine, including energy generation and amino acid synthesis (Reeds and Burrin, 2001). EAAT3 is localized in the BBM of the enterocytes and is highly specific and stereospecific for L-glutamate (Castagna *et al*., 1997). Given the importance of glutamate for small intestine development, the EAAT3 transporter was evaluated in this study. At DOH, the chicks incubated at the L temperature early in development had a lower expression of EAAT3 than the chicks incubated at early S temperature. It is during this early post-hatch period that chicks are undergoing dramatic changes in the development and maturation of the intestinal epithelium. Most of the cells are proliferating to become migrating enterocytes or protective cells within the crypt area with antimicrobial peptide production. We suggest that the depletion of energy from the small intestine in chicks incubated at sub-optimal conditions decreases the expression of EAAT3 at DOH in the enterocyte membrane. In the case of EAAT3, from d 2 to d 4 the expression levels in LS group increase much faster than in the early S incubation groups, possibly as a manner of

compensatory development. Chicks from LS incubation group seem to require the most expression of EAAT3 from DOH to d 2 to compensate for earlier reduced expression. At d 4, expression reached the maximum levels of expression seen in these results, and by d 6 expression decreased to reach the same expression levels as the rest of the experimental groups.

Besides free amino acids, di- and tri-peptides are absorbed by the brush border membrane as well. Peptide transportation, as compared with free amino acid uptake, is more rapid and energetically efficient. The oligopeptides transporter 1, PepT1, is located in the apical membrane and transports di- and tripeptides into the cell (Daniel, 2004). It is a transporter with nutritional relevance in agricultural animals (Gilbert *et al*., 2008). Expression of PepT1 depends on the developmental stage of the chick, physiological conditions, dietary protein level, as well as the substrates present in the intestinal lumen (Chen *et al*., 2004). Interestingly, the expression of PepT1 was lower in the chicks incubated at late H temperatures. Elevated temperatures are known to increase the incidence of yolk sac retention and decrease FI. Decreases in the amount of nutrients present in the intestinal lumen, may result in lower expression of the peptide transporter. The thyroid hormone decreases the expression of PepT1 in the membrane (Ashida *et al*., 2004). Interestingly, studies by Christensen *et al*. (2005), reported that elevated temperature during incubation can increase T_3 to T_4 ratios in serum.

This study followed previous work done by our laboratory that evaluated the effects of single-stage incubator elevated temperature on small intestine development and post-hatch performance of broilers. In that study, we did not observe any performance parameters to be affected, however, we did observe differences in mucosal epithelium morphology as well as relative gene expression of some nutrient transporters. With that data as a basis, in this research we evaluated multi-stage incubator temperature profiles measuring low EST at the early stage of incubation and high EST at the late stage of incubation, followed by heat stress during transportation of the newly hatched chick. Such stressors had an effect on the grow-out period as expressed in FI, which directly affects BW of the chicks. This can subsequently impact intestinal development as observed with changes in relative nutrient transporter gene expression in the small intestine. Of the transporter genes evaluated, SGLT1 was the most affected by both transportation and incubation heat stress.

This paper is the first part of a study that included incubation temperature profiles similar to that seen in commercial multi-stage incubation systems and stress during post-hatch transportation of a high-yield broiler line to evaluate their effects on performance and nutrient transporter gene expression. In a companion paper we present the second part of this study which evaluates the relationship of the performance data with morphology of the mucosal epithelium, yolk utilization, and digestive organ development. These results contribute to understanding the mechanisms by which altered temperatures during incubation and transportation can impact embryonic development and subsequently post-hatch performance of the broiler chick.

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Table 4.1 Primers[1] used for relative real-time PCR

Gene	GenBank ID number	Description	Sequence: forward/reverse
GAPDH	NM_204305	Glyceraldehyde-3-phosphate dehydrogenase	GCCGTCCTCTCTGGCAAAG/ TGTAAACCATGTAGTTCA
SGLT1	XM_415247	Solute carrier family 5 (Na+- glucose co-transporter) member 1	GCCATGGCCAGGGCTTA/ CAATAACCTGATCTGTGCACCAGTA
SLC2A5 (GLUT5)	XM_417596	Solute carrier family 2 (facilitated fructose transporter) member 5	TTGCTGGCTTTGGGTTGTG/ GGAGGTTGAGGGCCAAAGTC
SLC2A2 (GLUT2)	Z22932	Solute carrier family 2 (facilitated glucose and fructose transporter) member 2	CACACTATGGGCGCATGCT/ATTGTC CCTGGAGGTGTTGGTG
PepT1	NM_204365	Peptide transporter	CCCCTGAGGAGGATCACTGTTGGCA GTT/CAAAAGAGCAGCAGCAACGA
SLC1A1 (EAAT3)	XM_424930	Solute carrier family 1 Member 1 (Excitatory amino acid transporter 3 glutamate and aspartate transporter)	TGCTGCTTTGGATTCCAGTGT/AGCA TGACTGTAGTGCAGAAGTAATATAT

[1]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA).

Table 4.2 Effect of transportation and age on BW[1] ± SEM and coefficient of variation (CV) ± SEM of Cobb 500 broiler chicks transported under control or stressed conditions for 4 h

Transportation	**DOH**		**d 14**		**d 28**		**d 42**	
	BW	CV	BW	CV	BW	CV	BW	CV
Control (34°C)	46.68 ± 6.35	6.88 ±0.34	461.56 ± 6.44	10.61 ±0.35	1568.29 ± 6.56	12.9 ±0.36	2904.70[a] ± 6.79	14.0 ±0.35
Stressed (40°C)	45.79 ± 6.51	7.38 ±0.35	464.38 ± 6.69	10.36 ±0.34	1565.09 ± 6.79	13.1 ±0.35	2864.55[b] ± 6.70	14.83 ±0.35

[1] LS Means ± SEM (n=1376/transportation treatment).

[a-b] Data lacking a common superscript differ significantly (P= 0.01).

Table 4.3 Effect of incubation temperature and age on yolk free BW of Cobb 500 broiler chicks incubated at different temperature profiles[2] during early and late incubation

Temperature Profile	Yolk Free Body Weights (grams)			
	DOH	**d 2**	**d 4**	**d 6**
LH	39.89 ± 1.29	57.75 ± 1.45	92.50[ab]± 1.45	141.33[ab] ± 1.45
LS	40.58 ± 1.33	55.68 ± 1.45	87.28[b]± 1.45	133.21[c] ± 1.45
SH	41.17 ± 1.28	61.19 ± 1.45	94.65[a]± 1.45	143.05[a] ± 1.45
SS	41.80 ± 1.29	59.09 ± 1.45	91.33[ab]± 1.45	135.61[b] ± 1.45

[1] LS Means ± SEM (n=20/treatment)

[2] Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C).

[a-c] Data lacking a common superscript differ significantly (P= 0.01).

Table 4.4 Effect of incubation temperature, transportation, and age on feed intake[1] of Cobb 500 broiler chicks incubated at different temperature profiles[2] similar to multi-stage incubators and transported under stressed or control conditions

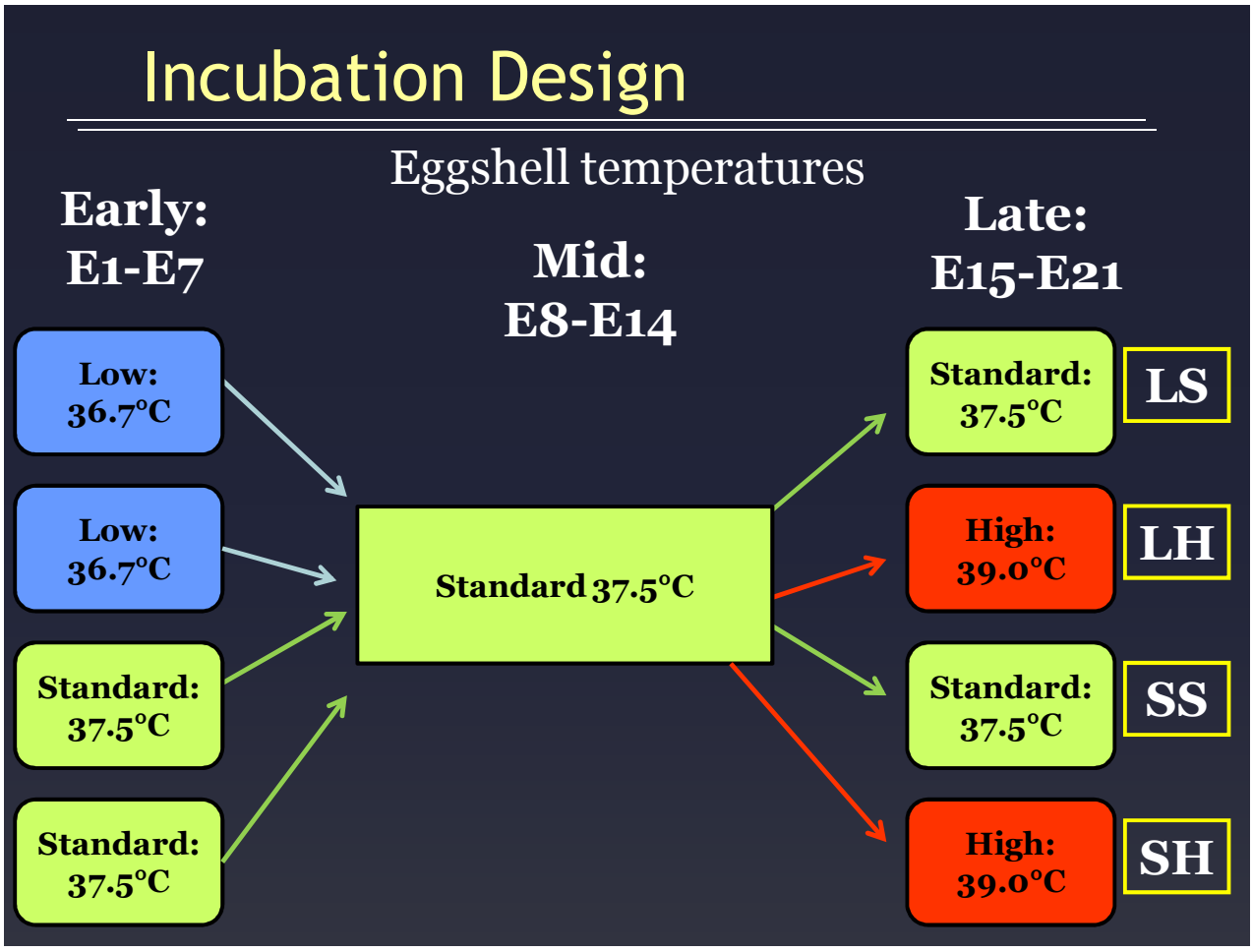
Temperature Profile	Feed Intake (Kg.)		
	d 0 to d 14	**d 14 to d 28**	**d 28 to d 42**
LH-C	0.536 ± 0.022	1.724 ± 0.026	2.556[b] ±0.039
LH-D	0.546 ± 0.022	1.719 ± 0.026	2.549[b] ± 0.239
LS-C	0.525 ± 0.022	1.730 ± 0.026	2.583[b] ± 0.239
LS-D	0.546 ± 0.022	1.728 ± 0.026	2.644[a] ± 0.239
SH-C	0.575 ± 0.022	1.735 ± 0.026	2.630[b] ± 0.239
SH-D	0.570 ± 0.022	1.737 ± 0.026	2.491[c] ± 0.239
SS-C	0.552 ± 0.022	1.727 ± 0.026	2.588[b] ± 0.239
SS-D	0.565 ± 0.022	1.762 ± 0.026	2.536[b] ± 0.239

[1] LS Means ± SEM (n=344/treatment)

[2] Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C) in combination with the transportation treatments control (C, 34°C) or stressed (D, 40°C).

[a-c] Data lacking a common superscript differ significantly (P= 0.02).

Figure 4.1 Incubation design



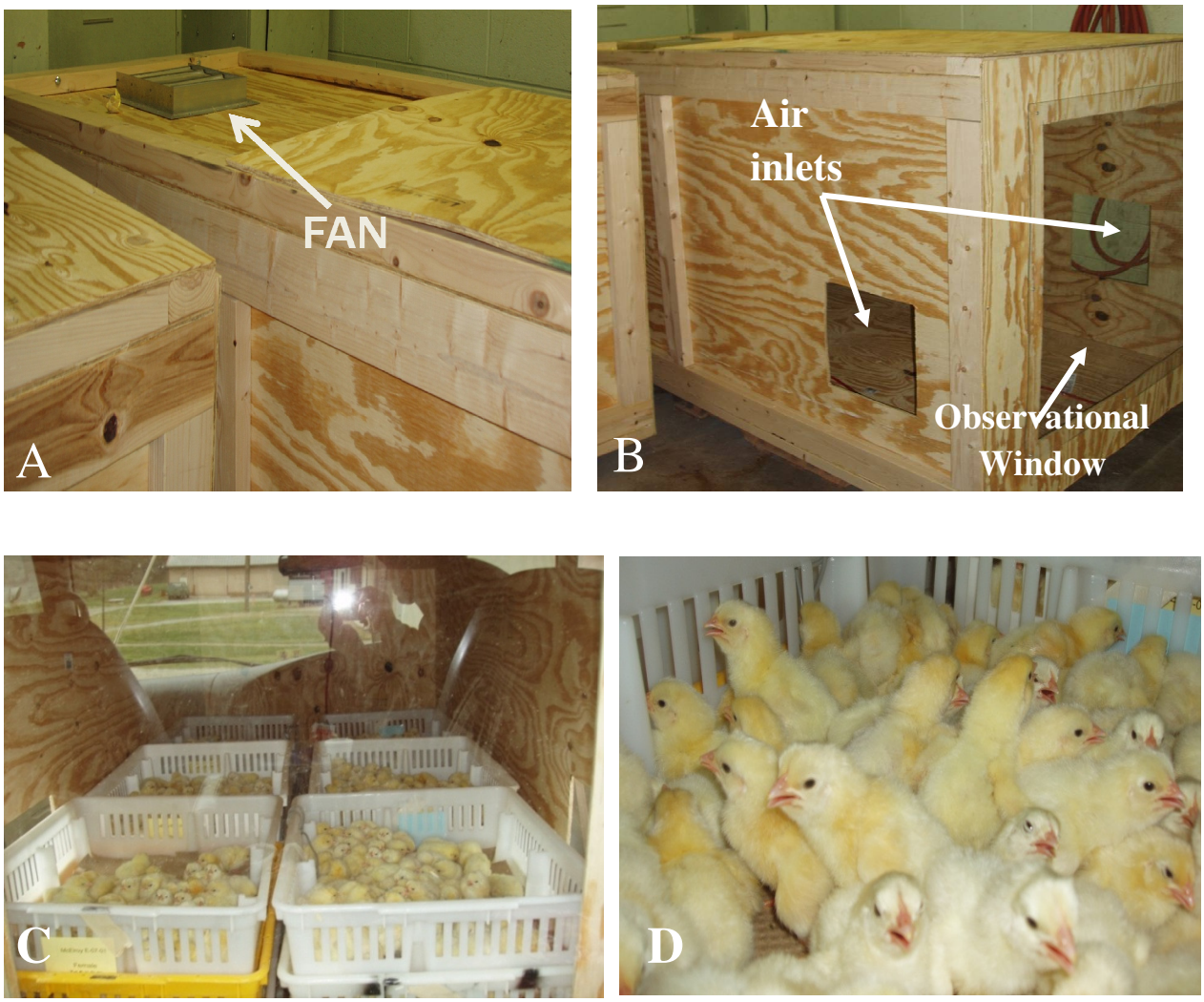


Figure 4.2 Depiction of wooden boxes used for transportation of newly hatched Cobb 500 broiler chicks under control (34°C) or stressed (40°C) conditions for 4 h. **A.** Wood box showing exhaust fan. **B.** Wood box showing air inlets and observational windows. **C**. Chicks within wooden box inside the transportation van. **D**. Stressed chicks panting and vocalizing as an indication of heat stress. (Photos by author)

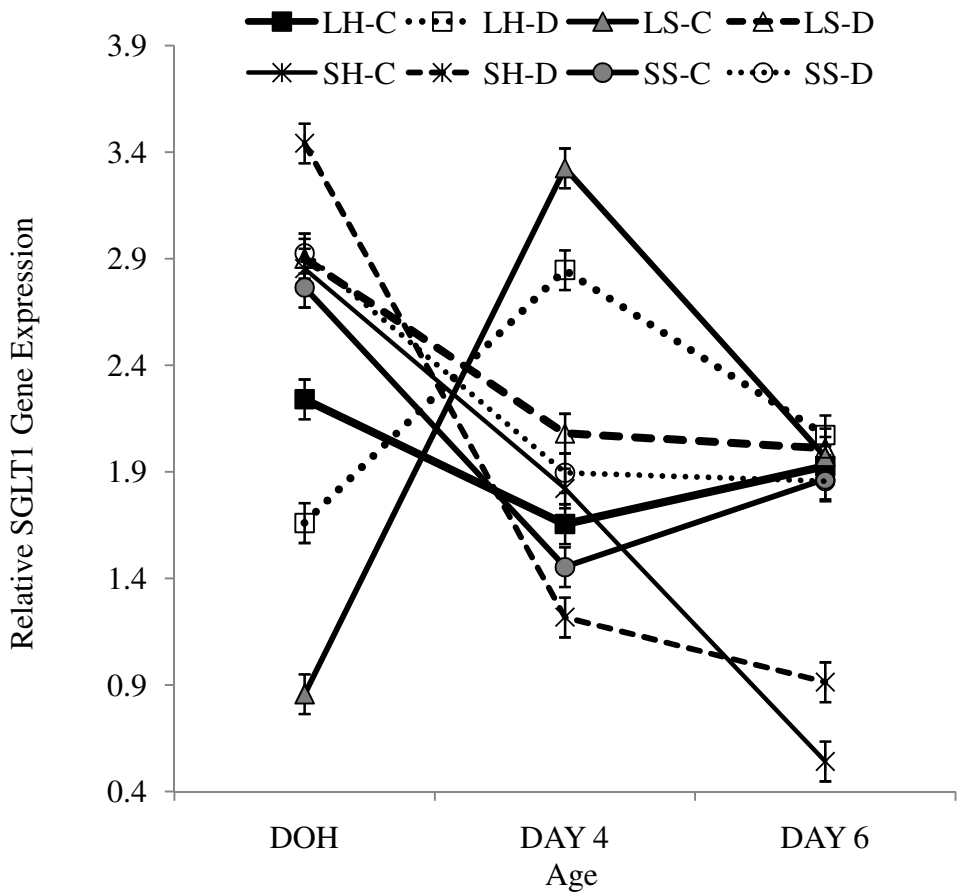


Figure 4.3 Effect of incubation temperature, transportation, and age on relative mRNA expression for SGLT1 in the small intestine of Cobb 500 broiler chicks. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C) in combination with the post-hatch transportation treatments control (C, 34°C) or stressed (D, 40°C).. Relative gene expression for mRNA expression for SGLT1 ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from SS-C group as the calibrator. Data are represented as LS Means ± SEM (n=3/treatment). There was a three way interaction (P= 0.04) of incubation temperature, transportation, and age.

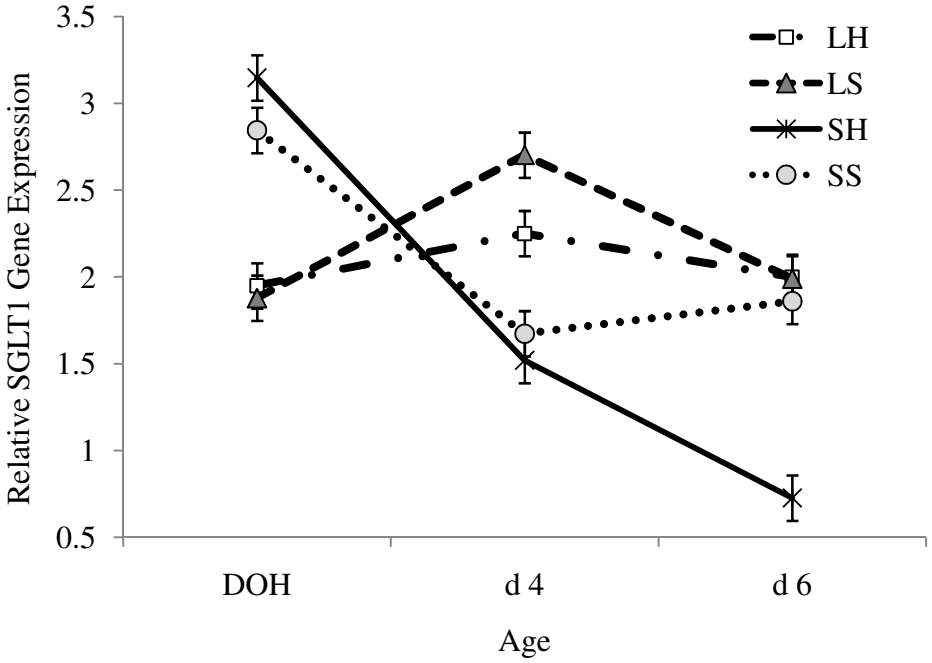


Figure 4.4 Effect of incubation temperature and age on relative mRNA expression for SGLT1 in the small intestine of Cobb 500 broiler chicks incubated at different temperature profiles. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Relative gene expression for mRNA expression for SGLT1 ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from SS-C group as the calibrator. Data are represented as LS Means ± SEM (n=6/treatment). There was a two way interaction (P= 0.0001) of incubation temperature and age.

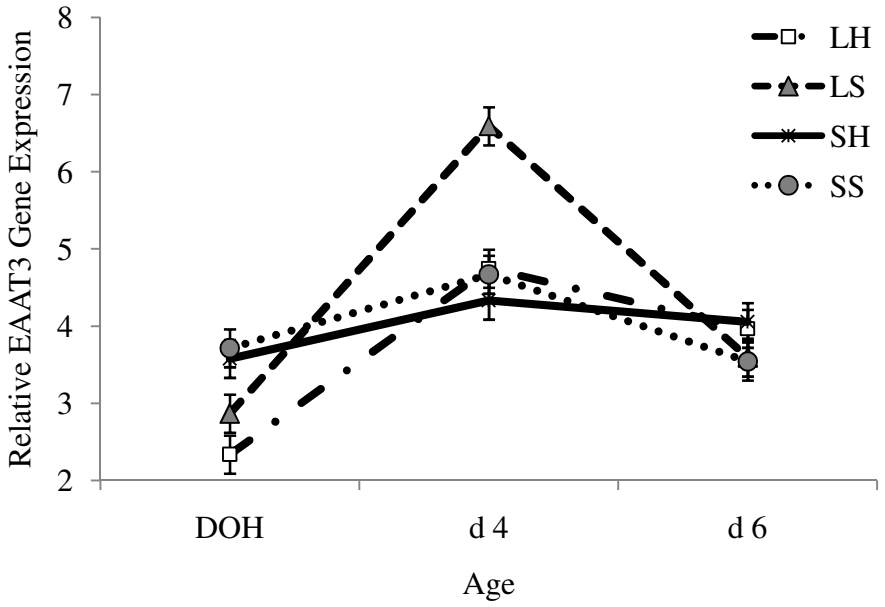


Figure 4.5 Effect of incubation temperature and age on relative mRNA EAAT3 expression in small intestine of Cobb 500 broiler chicks incubated at specific temperature profiles similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Relative gene expression for mRNA expression for EAAT3 ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from SS-C group as the calibrator. Data are represented as LS Means ± SEM (n=6/treatment). There was a two way interaction (P= 0.003) of incubation temperature and age.

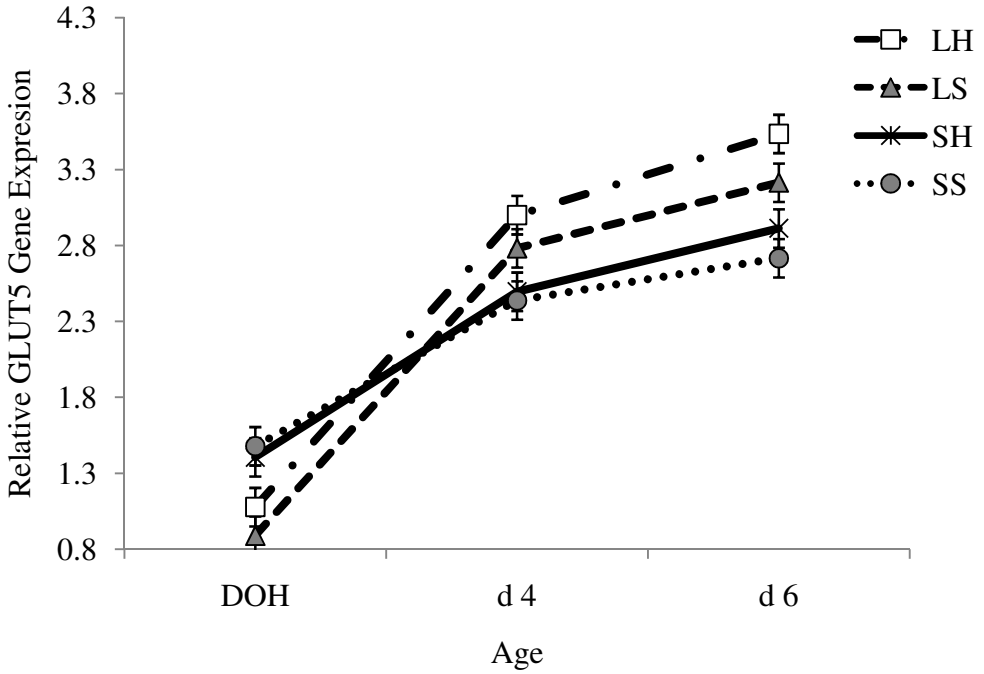


Figure 4.6 Effect of incubation temperature and age on relative mRNA GLUT5 expression in small intestine of Cobb 500 broiler chicks incubated at specific temperatures profiles. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Relative gene expression for mRNA expression for GLUT5 ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum from SS-C group as the calibrator. Data are represented as LS Means ± SEM (n=6/treatment) with a two way interaction (P= 0.008) of incubation temperature and age.

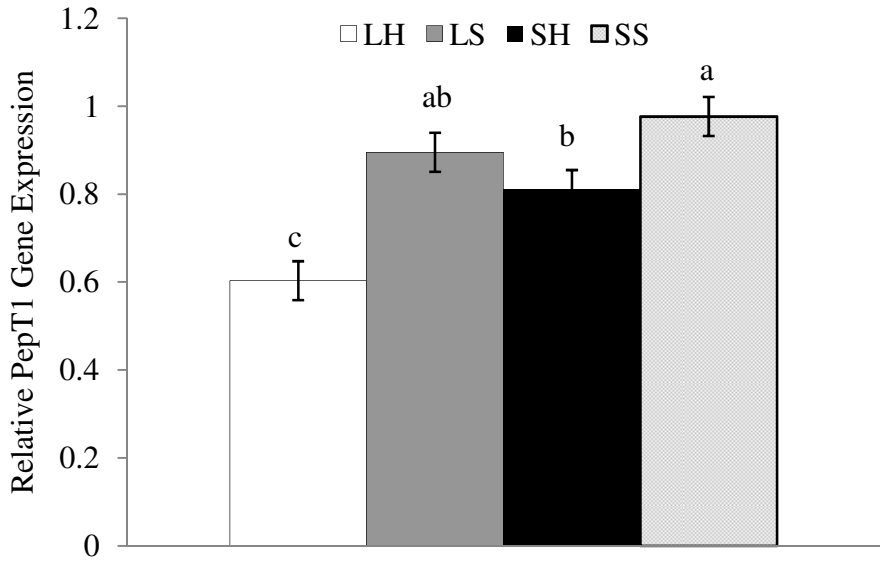


Figure 4.7 Effect of incubation temperature on relative mRNA PepT1 expression in small intestine of Cobb 500 broiler chicks incubated at specific profile temperatures. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Relative gene expression for mRNA expression for PepT1 ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for duodenum SS-C as the calibrator. Data are represented as LS Means ± SEM (n=6/treatment).

[a-c] Data lacking a common superscript differ significantly (P= 0.02).

CHAPTER V

Effect of Incubation Temperature Profiles and Transportation Stress on Broiler Chicks: II. Morphology, Yolk Utilization, and Organ Development

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ABSTRACT Alterations in incubation temperature (**IT**) as well as elevated temperatures during transportation have a direct effect on yolk nutrient utilization and may cause a negative effect on organ and mucosal epithelium development. This study evaluated the effect of embryonic incubation and post-hatch transportation temperatures on broiler chick yolk utilization, organ development, and intestinal mucosal morphology. Cobb-500 eggs (n=5200) were incubated with the following egg-shell temperatures (**EST**), which were combined depending on the incubation period: Low (**L**): 36.7 °C, Standard (**S**): 37.5 °C, and High (**H**): 39 °C. After hatch, chicks were further separated into two transportation groups including control (**C**, 34°C) and heat stressed (**D**, 40°C). The eight resulting experimental groups were: LS-C, SS-C, LH-C, SH-C, LS-D, SS-D, LH-D, and SH-D. Organ weights were collected and analyzed at day of hatch (**DOH**; before and after transportation), d 2, 4, and 6. Organ weights measured included the gastrointestinal tract (**GI**: proventriculus, gizzard, small intestine, without yolk sac), the small intestine (**SI** without yolk sac), and yolk sac (**YS**). Prior to transportation, there were significant differences in GI (P= 0.001), SI (P= 0.007), and YS (P= 0.004), but not intestinal morphology data. For measurements of GI weight, there was a three way interaction (P= 0.02) of IT, transportation, and age as well as two way interactions of IT and age (P= 0.0009), transportation and age (P= 0.0001), and IT and transportation (P= 0.03). As observed with GI weight, measurement of SI weight as a percent of body weights (**BW**) indicated interactions of IT, transportation environment, and age (P= 0.002), and two way effects for IT and age (P= 0.0009) and transportation and age (P= 0.0001). Additionally, three way interactions were observed for YS weight measurements (P= 0.04). For intestinal morphology, there was a three way interaction of IT, transportation, and age on jejunum crypt depth (P= 0.03) and jejunum villus height to crypt depth ratios (P= 0.002). Two way interactions observed included: IT and age on duodenum villus height to crypt depth ratios

(P= 0.01) and ileum crypt depth (P= 0.007), transportation and age on ileum crypt depth (P= 0.006), and IT and transportation on ileal villus height to crypt depth ratios (P= 0.02). The results indicated that the parameters evaluated were differentially influenced by transportation conditions depending on whether the chicks experienced low or standard temperatures early in incubation. Clearly, alone or in combination, the IT and transportation conditions influenced yolk utilization and intestinal morphology.

Key words: small intestine, morphology, broiler, incubation, transportation

INTRODUCTION

The metabolism and nutrition of newly hatched chicks depends largely on absorption of residual yolk to complement dietary nutrients and assure rapid gastrointestinal development and maturation for a successful post-hatch growth (Esteban *et al*., 1991). During the peri-hatch period and right after hatching significant amounts of yolk contents may pass through the yolk stalk into the intestinal lumen, and during passage, part of it may even be absorbed by the epithelial lining of the yolk stalk (Esteban, 1991). The presence of nutrients in the intestinal lumen enhances the absorption rate of the residual yolk by stimulating development and maturation of the intestinal epithelium, and as a consequence it facilitates a progressive adaptation of the bird to oral nutrition (Sklan and Noy, 2000; Noy and Sklan, 2001). The chicks that do not absorb the residual yolk or have no immediate access to feed, or both, take longer to make an adequate transition from the *in ovo* environment to post-hatch life (Sklan, 2003). In order for the small intestine (**SI**) to become a fully developed and mature organ, it has to undergo many morphological and physiological changes of the mucosal epithelium. At hatch the enterocytes are still immature. Between day of hatch (**DOH**) and d 5, mucosal development is based on the increase in size of villus and microvillus, density, and the quantity of functional enterocytes along the villus, all of which will improve the absorptive capacity as the chick ages (Sklan and Noy, 2000). Rapid turnover and migration of the intestinal epithelium from crypts to villus tip have been reported in young chicks; however, as chicks age migration rates decrease (Takeuchi *et al*., 1998; Iji *et al*., 2001). Other digestive tract organs such as the proventriculus, gizzard, and pancreas do not show the same growth rate as the SI (Noy and Sklan, 1998). All the morphological and physiological changes that take place during the early post-hatch period are directed to the enlargement of the absorptive surface and the differentiation and maturation of the

epithelial cells, enzymes, and nutrient carriers (Geyra *et al*., 2001; Karcher and Applegate, 2008). These adaptations will allow the tissue to obtain the energy necessary for growth of the birds (Geyra *et al*., 2001).

At the hatchery, chicks that have been incubated with conditions varying from optimal environments usually present a large residual yolk. It has been shown that elevated temperatures during incubation are usually correlated to unhealed navels, and there is a direct correlation between unhealed navels and yolk retention. Thus, it is believed that chicks with unhealed navels grow much less efficiently than chicks with normal navels. However, the relationship between high temperatures, yolk stalk function impairment and its relation to gut development still needs to be established.

During post-hatch transportation to farms, chicks are exposed to potential stressors such as thermal stress, fasting, dehydration, and noise (Mitchell and Kettlewell, 1998). All these stressors alone and in combination may result in harm to the chick varying from mild discomfort to death. Several studies, in pre-slaughter chickens, have concluded that 40% (Mitchell and Kettlewell, 1998) of dead on arrivals are due to transportation stress, and that the mortality increases as the length of the transportation increases (Valros *et al*., 2008). Some studies measured creatine kinase 1, a marker for muscle breakdown, in pre-slaughter chickens exposed to transportation heat-stress (45°C) and showed that there was tissue dysfunction and damage after transportation due to impairment of muscle membrane integrity (Mitchell *et al*., 1992). The thermal environment to which the chicks are exposed is very important, and if there is not enough ventilation or air movement within the transportation vehicle, the stress increases. Even though it is a problem in the industry, studies to evaluate the effects of heat stress during transportation of newly hatched chicks from different incubation temperature (**IT**) profiles have not existed.

In a previous study we observed that elevated temperature (39.6°C) during later days of incubation, embryonic d (**ED**) 13 to ED21, had a direct effect on intestinal mucosal morphology and relative nutrient transporter gene expression. Given that a lack of access to nutrients, as seen with starvation, deutectomy, or feed withholding for 24 h has a negative effect on crypt depth and causes a decrease in villus height and depressed mitosis along the villus of the SI (Noy and Sklan, 1998), the objective of this study was to evaluate the relation between eggshell

temperatures (**EST**) and transportation stress on SI morphology, yolk utilization, and digestive organs development.

This paper is the second part of an extensive study that was designed to evaluate embryonic incubation temperature profiles, simulating commercial multi-stage incubation systems, and stress during post-hatch transportation of high-yield broilers. The first part of this study evaluated the effect of these conditions on development of the SI as measured through relative gene expression of specific nutrient transporters and grow-out performance parameters. This paper will focus on the SI mucosal architecture and digestive organs development. Together, both papers address factors in incubation and transportation that can have detrimental effects on chick quality and resulting post-hatch performance.

MATERIALS AND METHODS

Animal Welfare

This project was approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation and Transportation Design, Broilers, and Diets

Incubation and transportation treatments were as described in Chapter IV (pages 103 & 104). All experimental design materials and methods for broiler line, chick placement, diets, and rearing conditions were identical to those described in Chapter IV (pages 104 & 105).

Small Intestine and Yolk Sac Collection

Twenty birds from each treatment were randomly selected at DOH (before and after transportation), d 4, and 6 to determine BW, yolk sac (**YS**), gastrointestinal tract (**GI;** proventriculus, gizzard, and SI without YS), and SI (SI without YS) weights. Each bird was weighed and euthanized by cervical dislocation. The YS with contents was carefully separated from the SI and weighed individually. For the GI measurement the intestine was collected, digesta was eliminated by gently squeezing the walls of the intestine, pancreas and mesenteric tissue were excised from the duodenal loop, and the proventriculus and gizzard contents were emptied. Then the proventriculus and gizzard were separated, and the SI was weighed

individually. The small intestine consisted of tissue from the duodenum to the ileo-cecal junction. All weights are reported as percent of BW.

Morphological Measurements

Eight birds per treatment were randomly selected at DOH (before and after transportation), d 2, 4, and 6 for intestinal morphology evaluation. Each bird was weighed and euthanized by cervical dislocation. Tissue samples were collected from duodenum (mid-section of the ascendant loop), jejunum (mid-point from the pancreatic duct to Meckel's diverticulum), and ileum (mid-point from Meckel's diverticulum to the ileo-cecal junction). The intestinal segments (2 cm in length) were flushed with ice cold PBS and fixed in 10% neutral buffered formalin. Each segment was cut into 5 pieces (10 mm), placed into a cassette, and processed through a series of graded alcohols and xylene and embedded in paraffin. Paraffin sections (5 µm thickness) were mounted onto slides. The slides were stained using routine Mayer's Hematoxylin and Eosin (H&E) procedure (Luna *et al*., 1960). Measurements of development of the SI were made using SigmaScan Pro 5 software (Olympus America Inc., Melville, NY) through measurement of the villus height (villus tip to crypt opening) and crypt depth (crypt opening to the base of the crypt, right before the lamina propria). Villus height to crypt depth ratios were calculated. Three out of the 5 sections on each slide were evaluated with 4 villus and 4 crypts measured for each of the intestinal sections. The average per slide was then obtained and used to determine the treatment mean (n=12 measurements/bird, 8 birds/treatment).

Statistical Analysis

A completely randomized experimental design with a 4 X 2 arrangement of treatments (4 levels of incubation temperatures and 2 transportation conditions) was used. Data were subjected to ANOVA procedures for completely randomized designs using the GLM models procedure of the SAS program version 9.1 (SAS Institute Inc., Cary, NC). All values are expressed as LS means ± SEM. Bird was the experimental unit. All ratios data (villus height to crypt depth) were log transformed (ln) prior to analysis. All percent data (intestine weight to BW) were arc-sine (square root of percent) transformed prior to analysis. The model for the morphological analysis included main effects of IT, transportation, segment, and age and all two and three way interactions. The model for intestinal weights analysis included main effects of

IT, transportation, and age and all two and three way interactions. Differences among treatments were further compared using the Tukey's test. Significance was determined at $P \leq 0.05$. The model was: $Y_{ijkl}= \mu+ \alpha_i+\tau_j+\beta_k+(\alpha\tau\beta)_{ijk}+\varepsilon_{ijkl.}$ Where Y_{ijkl} = observed dependent variable; μ= overall mean; α_i= the main effect of IT I; τ_j = the main effect of transportation J; β_k= the main effect of age (or segment) k; $(\alpha\tau\beta)_{ijk}$ = the interaction between IT, transportation, and age (or segment); and ε_{ijkl} = the random error. The data from DOH pre-transportation was not included in the statistical model.

RESULTS

Cobb 500 broiler eggs were incubated at different temperature profiles imitating a multi-stage commercial incubator, and once hatched, the chicks were transported at either control or stressed conditions. The gastrointestinal development through the measurement of mucosal morphology and intestine weights were evaluated.

Intestine Weights

Pre-Transportation. The main effect of temperature resulted in significant differences in YS (P= 0.004), GI (P= 0.001), and SI (P= 0.007) weight as a percent of BW prior to transportation, respectively) between treatment groups (Figure 5.1). The weight of the YS can be related to the amount of yolk nutrients absorbed through either the yolk stalk or the SI. The early L temperature incubation groups (LH and LS) had the heaviest YS when compared to the early standard IT groups (SH and SS); however, the LS group, although heavier, was not significantly different from the early standard incubation groups. The data for GI weights showed that the SS incubation group had heavier GI weight than LS and LH. The SH treatment group had heavier GI weight as percent of body weight than LH groups but was not different from SS or LS. Similarly, incubation at SS conditions resulted in the heaviest SI weight as percent of BW as compared to LH and LS incubation groups, which had similar SI weights. The SH incubation group had similar weights to LH, LS, and SS incubation groups.

Post-Transportation. The weight of YS as a percent of BW (Figure 5.2) was affected by interaction of IT, transportation environment, and age (P= 0.04). All groups had different responses from DOH to d 2 and from d 2 to d 4. At DOH, the early L temperature groups (LH

and LS) had heavier YS weights as percent of BW than the early S groups (SH and SS). From DOH to d 2, the early L temperature groups had a faster decrease in YS weight when transported under stressed conditions, and the early S temperature groups decreased at a similar rate with the exception of the SH group transported under stress, which had a lower yolk absorption rate. From d 2 to d 4 the early S temperature group, regardless of the transportation treatment, had decreased YS weight at the same rate, and the early L group decreased the YS weight at a faster rate when transported under control conditions. From d 4 to d 6 all treatments had the same response and had similar YS weight as percent of BW on d 6. A main effect of temperature was observed as LH incubated chicks had heavier YS weight compared to SH and SS incubated chicks (P= 0.01; Figure A.6).

The three way interaction of IT, transportation, and age for GI weights (P= 0.02) was most evident from d 2 to d 6 post-hatch (Figure 5.3). The initial responses of all groups from DOH to d 2 were similar with a rapid increase in GI weight as a percent of BW. However, from d 2 to d 6, transportation differences appeared to result in differential responses of chicks from the same incubation treatments over time. While chicks incubated at L temperature early in development and transported under control conditions (LH-C and LS-C) had a continued slight increase in GI weight from d 2 to d 6, chicks from those same incubation treatments transported with stress had a decrease or no evident change in GI weights. Chicks from embryos incubated at early S temperatures (SH and SS) and transported in control conditions had little change in GI weight from d 2 to d 6, while chicks from the same incubation treatments transported under stress had decreasing GI weights. Over time, chicks transported with stress responded differently than the respective control transportation from the same IT treatments with all having lower GI weight as a percent of BW by d 6. Two way interactions were also observed with IT and age (P= 0.0009), transportation and age (P= 0.0001), and IT and transportation (P= 0.03). The IT and age interaction for GI weight as a percent of BW (Figure 5.4) was observed in the samplings from d 2 to d 6. On d 2, chicks from early S incubation had heavier GI than that of chicks from early L incubation. At d 4, the chicks from all incubation treatments had similar GI weight. However, at d 6 the opposite results from d 2 were observed with chicks from early S having lighter GI than those from early L. Similar differential results were seen with the interaction of transportation and age from d 2 to d 6 (Figure 5.5). At d 2, chicks transported in stressful conditions had heavier GI than those from control transportation group, however, the opposite results were seen

on d 6. Obvious interactions occurred with IT and transportation (P= 0.03) on GI weights (Figure 5.6). Chicks from early L incubation had lower GI weight when transported with stress as compared to those transported at control conditions, while in contrast, stress transport resulted in higher GI weight, as compared to control, in chicks incubated at early S temperatures.

As observed with GI weight, measurements of SI weight as a percent of BW (Figure 5.7) indicated interactions of IT, transportation environment, and age (P= 0.002). From DOH to d 2, all groups responded similarly with an increase in SI weight. However, numerous differential responses were evident from d 2 to d 4 and d 4 to d 6. From d 2 to d 6, SI weight continued to increase in chicks incubated at early L temperature and transported in control environments (LS and LH). Similarly, chicks from these IT profiles that were transported with stress (LS-D and LH-D) had increased SI with age, but to a lesser extent than those transported in the control environment. Chicks of embryos incubated at early S temperature (SH and SS) had increasing SI weight from d 2 to d 4 regardless of transportation environment, but the responses diverged from d 4 to d 6 with transportation stress resulting in lower SI weights. The SH-C group was the only group with lower SI weights than the other incubation groups transported under the same control conditions, and the SH-D group had the lowest SI weights, when compared to the rest of the experimental groups. As seen with GI weight, regardless of IT, SI weight of birds transported in stress conditions (Figure 5.8) eventually decreased (P= 0.0001) with age in contrast to chicks transported in thermoneutral environmental conditions. The two way interaction between IT and age (P= 0.0009) for SI weights (Figure 5.9) indicated that initial increases in SI weight were similar between incubation treatments, but responses diverged from d 2 to d 6. While chicks from early L incubation (LH and LS) had continued increase in SI, chicks from early S incubation (SH and SS) increased to a lesser extent. Chicks from SH incubation had relatively no change in SI weight from d 4 to d 6, and therefore had the lowest SI weight at d 6.

Intestinal Mucosal Morphology

Samples from duodenum, jejunum, and ileum were collected at DOH (pre- and post-transportation), d 2, 4, and 6 for histology processing and morphological analysis. There were no significant differences observed in the morphology analysis data collected prior to transportation.

In the duodenum, a two way interaction (P= 0.01) of IT and age was observed with villus height to crypt depth ratio (Figure 5.10). Chicks from all incubation groups had increases in villus height to crypt depth ratio from DOH to d 2, however, chicks from SS incubation treatment had larger ratios at DOH and appeared to have the least increase by d 2. All groups had similar villus height to crypt depth ratio on d 2. From d 2 to d 6, the ratios in late H temperature groups (LH and SH) increased linearly resulting in lower ratios at d 4, but larger ratios by d 6 than the late standard IT groups (LS and SS). The ratio in late S temperaute groups increased more from d 2 to d 4 but from d 4 to d 6 decreased in such a way that they ended up with lower villus height to crypt depth ratios.

In the jejunum, a three way interaction of IT, transportation, and age was observed in crypt depth (P= 0.03) and villus height to crypt depth ratios (P= 0.002). The jejunum also was impacted by main effects of IT (Figure A.7; P= 0.04) and transportation (P= 0.02) for villus height, and jejunal villus height to crypt depth ratios responded to main effects (Figure A.8) of IT (P= 0.02) and transportation (P= 0.04). The interaction of IT, transportation, and age in the crypt depth of the jejunum (Figure 5.11) was most evident in the differential responses of the LS-D group. From DOH to d 4, LS-D crypts depth increased linearly. However, from d 4 d to d 6, the crypt depth increased to a larger extent, and by d 6, the LS-D chicks had deeper crypts than all other experimental groups. The SS-D chicks had linearly increased crypt depth from DOH to d 6, while the rest of the treatment groups had similar responses regardless of transportation conditions. For the three way interaction of IT, transportation, and age on villus height to crypt depth ratios in the jejunum (Figure 5.12), all treatments responded differently from DOH to d 6 with the most different responses from DOH to d 4. The SS-C group had slightly increased ratios from DOH to d 2, and from d 2 to d 6 the ratios increased linearly. In contrast, the SS-D group started with one of the highest ratios at DOH and decreased dramatically by d 2, and from d 2 to d 4 the ratio increased again until d 6. From DOH to d 2, LH-C group decreased to have the smallest ratio, and from d 2 to d 6 although ratios increased linearly, this group still had one of the lowest ratio values as compared to the rest of the treatment groups. The LH-D, LS-C, and SH-D ratios had similar responses from DOH to d 2. From d 2 to d 4 these groups differed in response, but from d 4 to d 6 they had similar increases in villus height to crypt depth ratios. The LS-D group had the highest ratios from DOH to d 4, but by d 6 these chicks had one of the lowest ratios.

The ileum measurements indicated two way interactions of IT and age (P= 0.007), transportation and age (P= 0.006), and IT and transportation (P= 0.02). The interaction of IT and age on ileum crypt depth (Figure 5.13) was seen mostly with the LH group whose crypt depth responded differently from the rest of the experimental groups, with a consistent depth from DOH to d 2, increase in depth of the crypt from d 2 to d 4, and no change in depth from d 4 to d 6. The other experimental groups responded similar to one another with linear increases in crypt depth from DOH to d 6. There was also a transportation and age response (Figure 5.14) where the groups transported under stressed conditions had consistently increased ileal crypt depth from DOH to d 6, while the groups transported under control conditions had no change in depth until d 2 and an increase in depth thereafter until d 6. At d 6, chicks transported under stressful conditions had the lowest crypt depths. The interaction between transportation and IT (Figure 5.15) on ileal villus height to crypt depth ratios showed that those chicks incubated at the early L temperature (LS and LH) had larger ratios when transported under stressed conditions, while the early S temperature groups (SH, SS) had greater ratios when transported under control conditions.

DISCUSSION

The metabolism and nutrition of the newly hatched chick (usually up to d 4) depends largely on the residual yolk to complement dietary nutrients and maintain efficient utilization of food energy and proteins to assure rapid gastrointestinal development and maturation. Subsequently, the establishment of a functional intestinal tract will result in more successful growth post-hatch. Nutrients from the residual yolk will facilitate a progressive adaptation of the bird to oral nutrition, which is influenced by many physiological and environmental factors (Esteban *et al*., 1991). Multiple studies have demonstrated that the presence of feed in the intestinal lumen enhances the absorption rate of the residual yolk by stimulating and accelerating development and maturation of the intestinal epithelium (Sklan and Noy, 2000; Noy and Sklan, 2001).

This study represents the second part of an extensive study that evaluated multi-stage incubator temperature profiles by establishing low EST at the early stage of incubation and high EST at the late stage of incubation followed by heat stress during transportation of the newly hatched chick. Such stressors, as presented in the first paper, have an effect on the grow-out

period, as expressed in feed intake, and peri-hatch intestinal development as observed with changes in nutrient transporter gene expression in the SI.

The objective of this study was to evaluate the effects of temperature during embryonic incubation and the effects of heat stress during transportation on the development of the SI as measured through yolk utilization, digestive organ development, and mucosal epithelium morphology.

The YS in the abdominal cavity of the chick begins to diminish in size on DOH as the yolk is released into the SI through the yolk stalk. By d 5 to d 7 after hatching, the YS is almost non-existent (Romanoff, 1960). Data from DOH, prior to transportation, showed a direct relationship between yolk nutrient absorption, as indicated indirectly by YS weight as a percent of BW, and small intestinal weight. The chicks that were incubated at the combination of early L and late H temperature (LH) had the least amount of yolk absorption, and thus a lighter weight of the SI as percent of BW. In previous studies, Ross X Ross 308 broiler chicks incubated at 39°C EST had increased relative weights of YS and liver and decreased gizzard, proventriculus, and SI weights as compared to control chicks incubated at 38.2°C (Leksrisompong *et al*., 2007). Therefore, GI weights can also account for the utilization of the YS since the heaviest GI weights were observed in the SS group, which was the group that had the smallest YS, suggesting more yolk absorption. It could be considered that these data may result from dehydration, water content, or unexplained variability within the yolk, however, by accounting for YS weight as percent of BW these factors were corrected for. In this experiment, YS weight as a percent of BW was most variable between treatments from DOH to d 2, while from d 2 to d 6, treatments had similar YS weight as time progressed. In contrast, the results for SI and GI weight as a percent of BW, were similar from DOH to d 2 and then responses to treatment diverged from d 2 to d 6. Unfortunately, the gizzard and proventriculus were not weighed individually from the intestinal tract; however, by observing the similar responses for both GI and SI weights, we can conclude that the results are due to the absorption of the YS and the direct development of the SI due to the presence of nutrients in the lumen. Yolk absorption will directly impact development of the digestive organs, especially the SI after d 2 post-hatch. For that reason, if a large amount of YS has not been absorbed, it can be assumed that the chick, particularly the intestinal tract, is less developed. Previous studies agree with our observations, and have shown that temperature can

influence the rate of yolk and albumen utilization and therefore chick development (Deeming and Ferguson, 1991). Several studies have concluded that the weight of the residual yolk can be used as a measure of energy used by the chicken embryo during development and during the peri-hatch period (Speer, 1996). Therefore, the high IT may not only reduce the energy available for the embryo to emerge from the eggshell, but may negatively impact the growth and development of the chick (Lourens *et al*., 2006). There is a direct correlation between unhealed navels and yolk retention (Speer, 1996; Crespo and Shivaprasad, 2003). These correlations could be explained through the effects of elevated temperatures on the yolk stalk and delayed yolk utilization. With data from the entire study, we hypothesize that chicks stressed during incubation or during transportation are forced to utilize glycogen from liver and muscle and amino acids from muscle in order to satisfy the energy requirements for maintenance, development, and growth. There is an optimization of luminal nutrient uptake by increasing certain nutrient transporters expression (SGLT1, GLUT5, EAAT3) in the brush border membrane of the SI and intestinal tissue morphological changes, however, by d 6 the results of muscle breakdown and energy deficiency are observed through low yolk free BW, and decreased digestive organ weights.

Transportation had no obvious effect on intestinal weights from DOH to d 4, however, from d 4 to d 6 the responses diverged and chicks transported under stress had lighter intestinal weights than those transported under control conditions. Interestingly, chicks from eggs incubated at the early L incubation profiles transported under stress (LH-D and LS-D) had decreased GI weight, whereas chicks incubated at early S temperature increased their GI weights when transported under the same stressful conditions (SS-D and SH-D). Stress during transportation will induce the release of corticosterone and hydrocortisone in the chicken. Furthermore, the differentiation of the mucosal epithelium in the embryo is affected by both thyroid and glucocorticoid hormones. Thyroxin alone (T_4) impacts the pre-hatching maturation phase of the epithelium by accelerating morphological differentiation of the absorptive cells (Black, 1988). Reports have shown that elevated temperature during incubation can increase T_3 to T_4 ratios in blood (Christensen *et al*., 2005). In this study, chicks transported under stress had an increase in villus height and in villus height to crypt depth ratios until d 4. These results are supported in that if the stress increased the thyroid hormone or glucocorticoids, maturation, and migration of the enterocytes could have been accelerated. These changes would lead to an elongation of the

villus and higher villus height to crypt depth ratios indicative of intestinal tissue development. However, it is important to consider that in order to be considered a differentiated mature organ, the SI has to undergo proliferation, migration, maturation, and apoptosis. It is of common thought that a longer villus will have a larger absorptive surface; however, the enterocytes along the villus might not be mature enough in order to establish such absorption if they are undergoing very rapid turnover. It is understood that in order to arrive at any conclusion, all the intestinal parameters need to be taken into consideration, such as proliferation rate, migration, and turnover rate (apoptosis, and necrosis). The changes in absorption of yolk based on IT and transportation most likely had a direct effect on the morphology data in this experiment.

The LH group presented the shortest villus height and lowest villus height to crypt depth ratios in jejunum, and smallest crypt depth in ileum. During stress, energy requirements increase, so it could also be that during incubation, the stress was such that the chick had to utilize all available energy in an attempt to maintain homeostasis rather than allocating energy to intestinal development. It was also observed that the LS group was among the groups that presented the highest villus height and villus height to crypt depth ratios, so the less stressful late incubation environment for these birds appeared to result in less detriment to intestinal development.

Taken together, we suggest that the single stressor of low temperature early in incubation may not be as detrimental to intestinal development as the combined stress of early low incubation temperature and high temperature late in embryonic development. Furthermore, the jejunal crypt depth measurements from the LS-D group were different from the rest of the experimental groups, which suggests that the additional stress of transportation conditions can compound developmental differences established during incubation. This was also evident with the villus height to crypt depth ratios that were indicative of different responses between control and transportation stressed birds regardless of IT. It is obvious that IT and transportation environment have a direct impact on the development of the mucosal epithelium.

The data strongly suggest that IT and transportation environment had direct and combined effects on YS absorption, which subsequently had a direct impact on intestinal development. In general, the chicks from standard IT (SS and SH) had the most yolk uptake and a corresponding better intestinal development, as measured by intestinal weights and morphological changes.

The SS and SH chicks transported in control conditions had more consistent intestinal morphological changes with age with less erratic differences from one sampling age to the next. The morphological data from these chicks was also indicative of less enterocyte turnover and therefore more mature and established enterocytes. In contrast, the chicks from L temperature incubation conditions early in development (LS and LH) had less yolk absorption in the first few days post-hatch, less intestinal development as indicated by intestinal tissue weight, and morphological measurements suggesting more erratic intestinal changes. The results also indicated that the parameters measured were differentially influenced by transportation conditions depending on whether the chicks experienced L or S temperatures early in incubation. Clearly, alone or in combination, the IT and transportation conditions influenced intestinal development. For all morphological changes, energy requirements increase. This therefore may divert the energy for growth or maybe for immune system development or response in order to maintain integrity of the SI. With the changes observed in intestinal development, yolk utilization, and nutrient transporter expression, it is likely that other physiological development and function are directly or indirectly impacted by these variations from optimal incubation and transportation conditions. Further studies involving evaluation of glycogen utilization and energy metabolism in the embryo and post-hatch chick incubated and transported at sub-optimal conditions will contribute to the understanding of energy availability and utilization of nutrients for intestinal development and growth performance. Additional studies may also evaluate the fact that it is possible that even if the chicks have reached adequate BW, they may be immuno-compromised and susceptible to disease.

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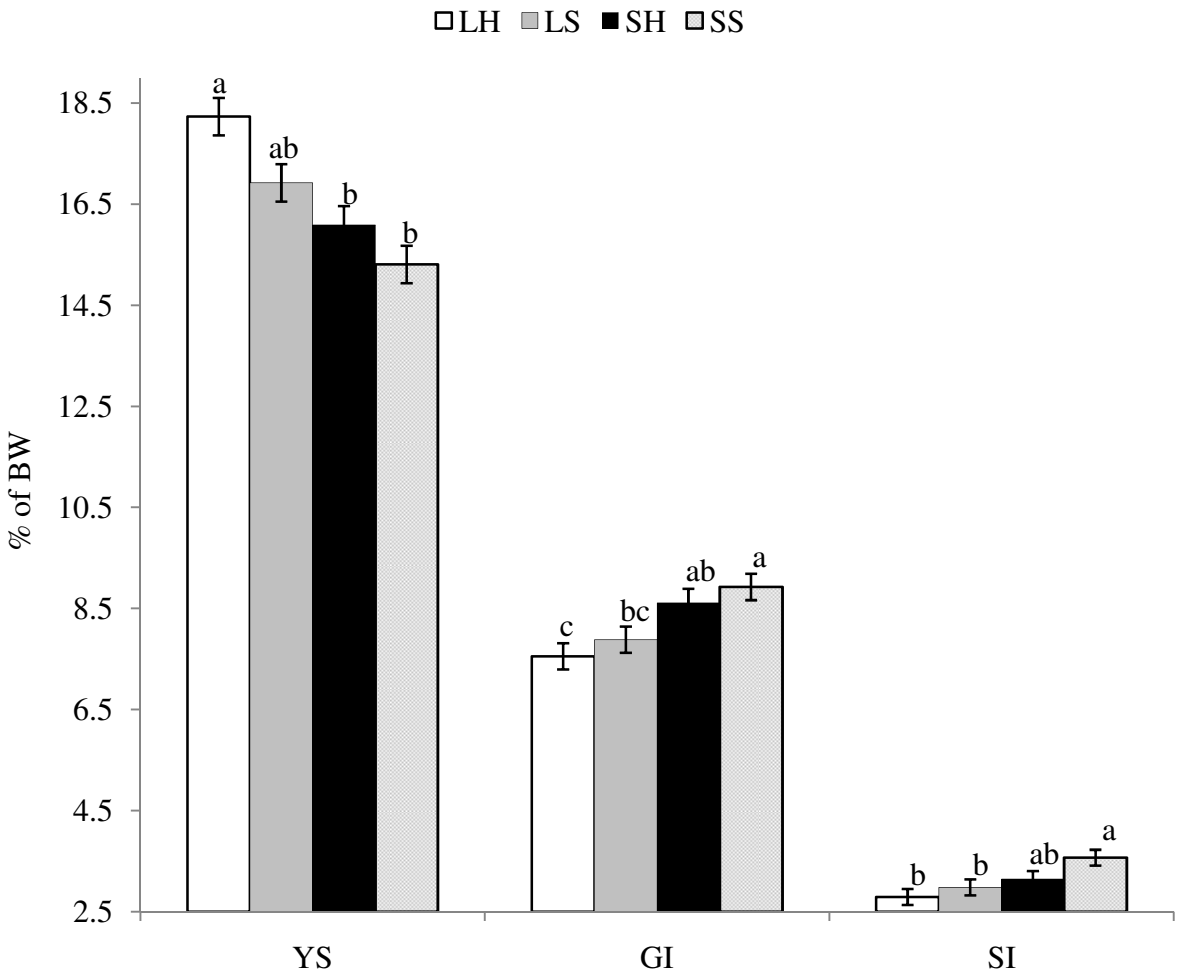

Figure 5.1 Effect of incubation temperature profiles on YS, GI, and SI weights (% of body weight) of Cobb 500 broiler chicks on DOH prior to transportation. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation days. Data are represented as LS means ± SEM (n=20/treatment).

[a-c] Data lacking a common superscript differ significantly YS= Yolk sac, P= 0.004; GI= small intestine with proventriculus and gizzard, P= 0.001; and SI= small intestine alone P= 0.007.

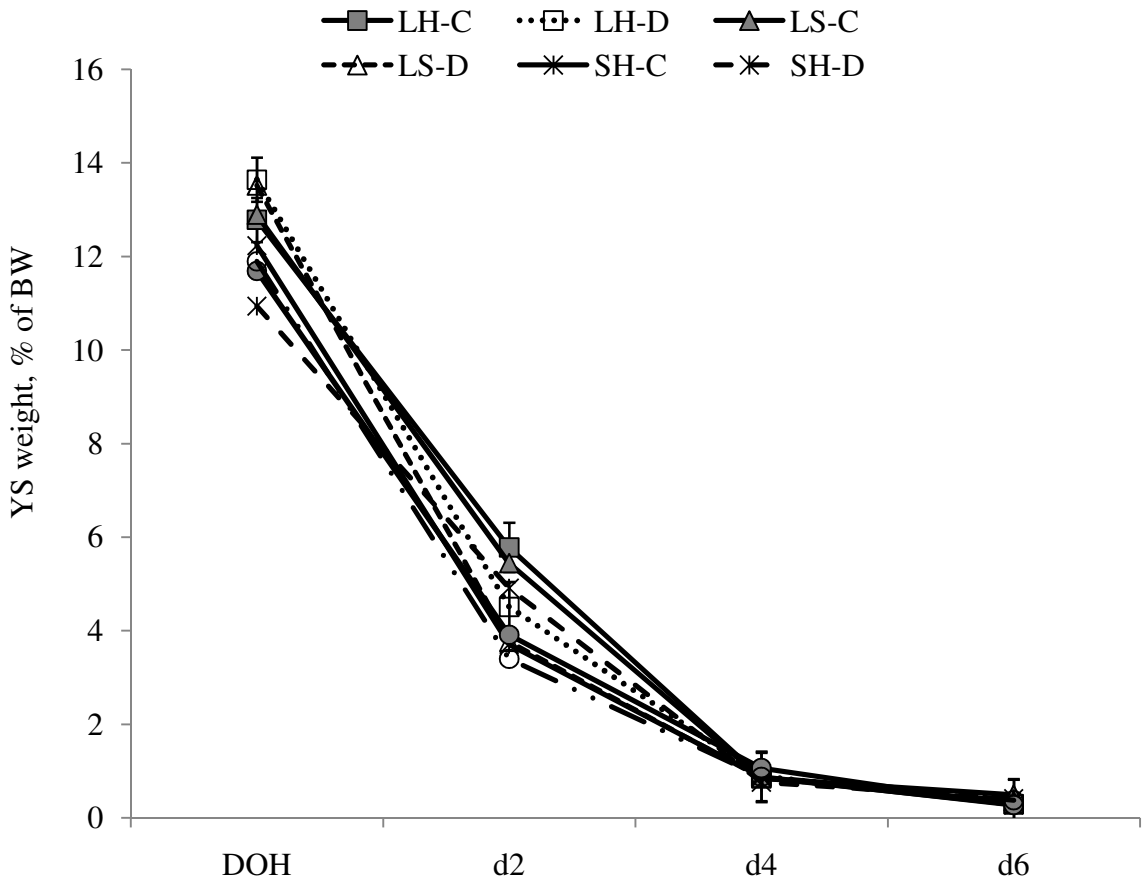

Figure 5.2 Effect of incubation temperature, transportation, and age on yolk sac weight (% of BW) of Cobb 500 broiler chicks. Treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods, in combination with the transportation treatments control (C, 34°C) or stressed (D, 40°C). Results are reported as LS Means ± SEM (n=20/treatment) and showed a three way interaction (P= 0.04) of incubation temperature, transportation, and age.

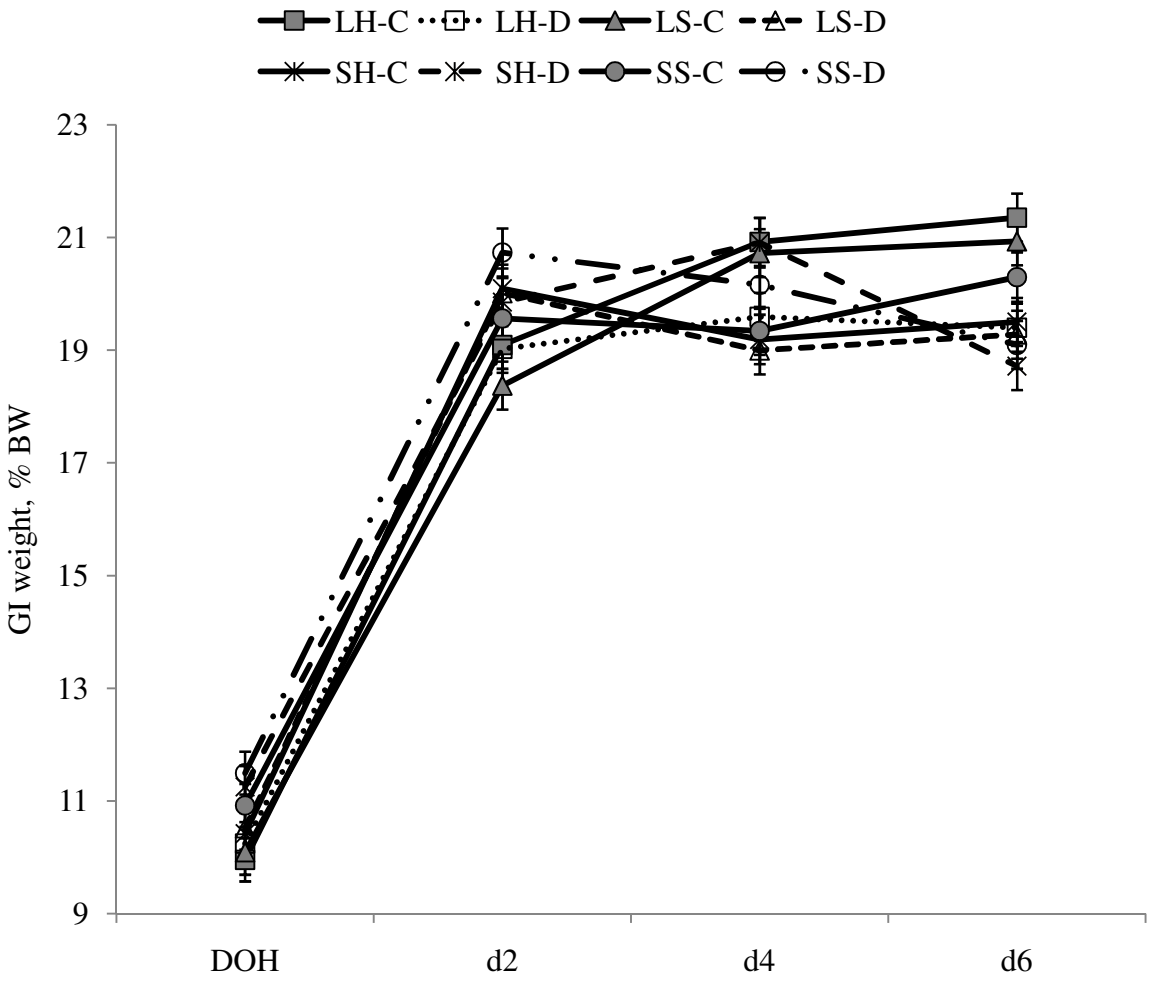

Figure 5.3 Effect of incubation temperature, transportation, and age on gastrointestinal tract (proventriculus, gizzard and small intestine without yolk sac) weights (% of BW) of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods, in combination with the transportation treatments control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=20/treatment). There was a three way interaction (P= 0.02) of incubation temperature, transportation, and age.

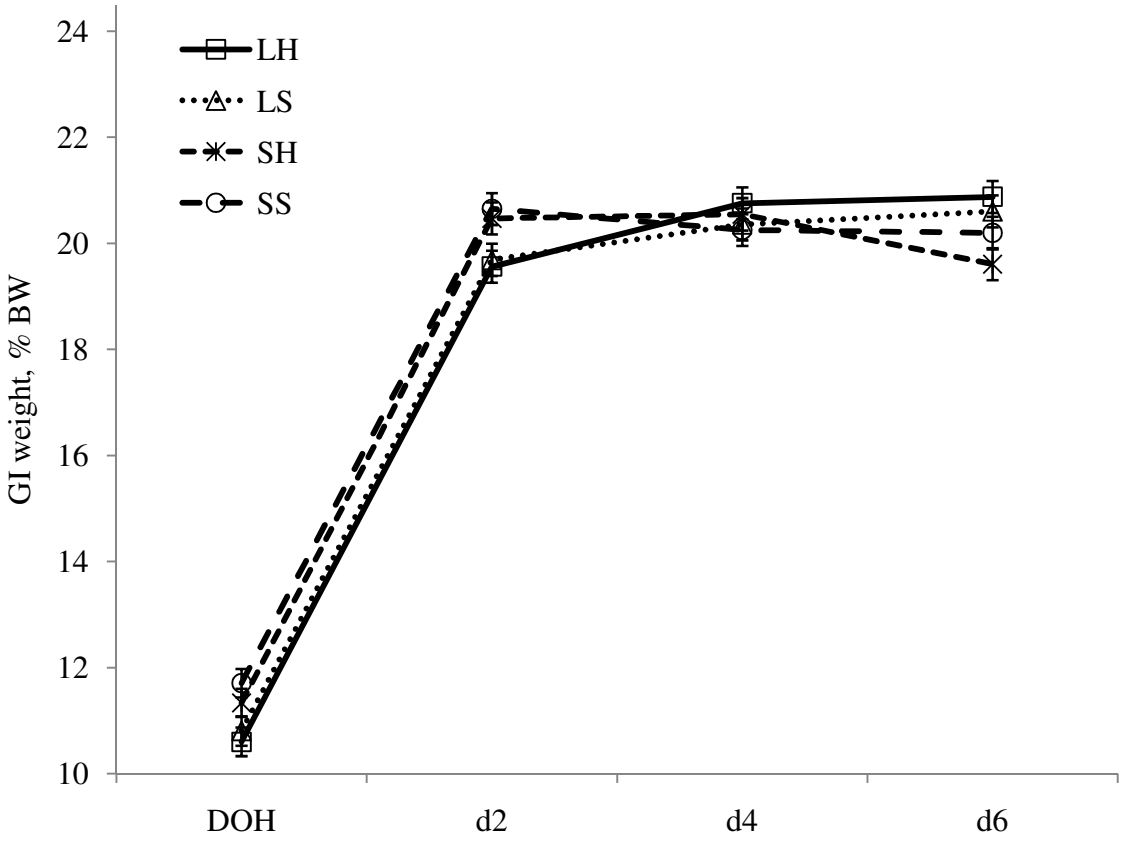

Figure 5.4 Effect of incubation temperature and age on gastrointestinal tract (proventriculus, gizzard and small intestine without yolk sac) weights (% of BW) of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods. Data are represented as LS means ± SEM (n=40/treatment). There was a two way interaction (P= 0.0009) of incubation temperature and age.

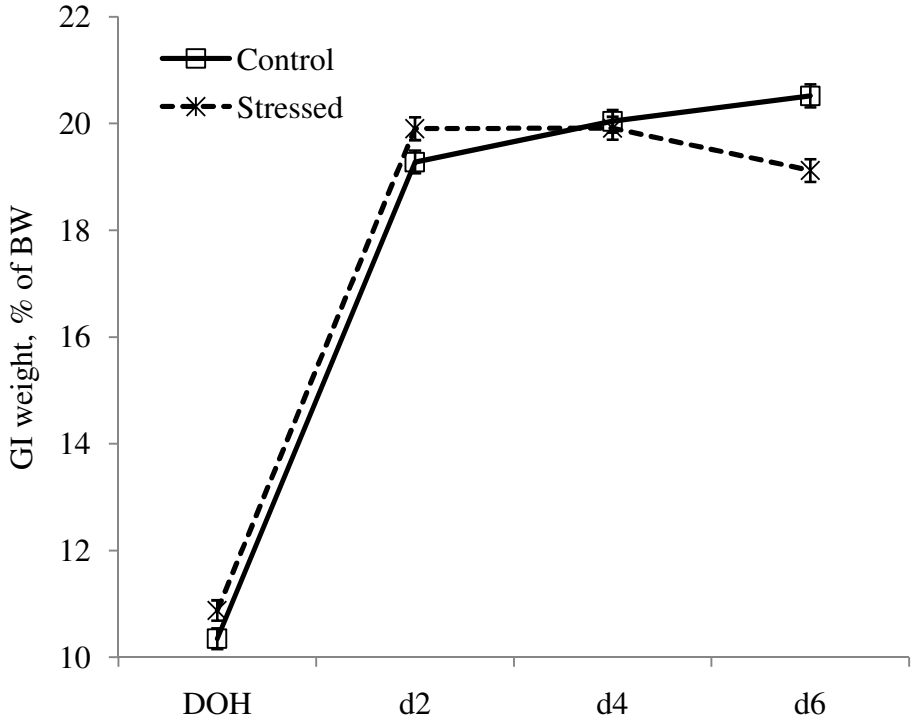

Figure 5.5 Effect of transportation conditions and age on gastrointestinal tract (proventriculus, gizzard and small intestine without yolk sac) weights (% of BW) of Cobb 500 broiler chicks. Transportation treatments were control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=40/treatment). There was a two way interaction (P= 0.0001) of transportation and age.

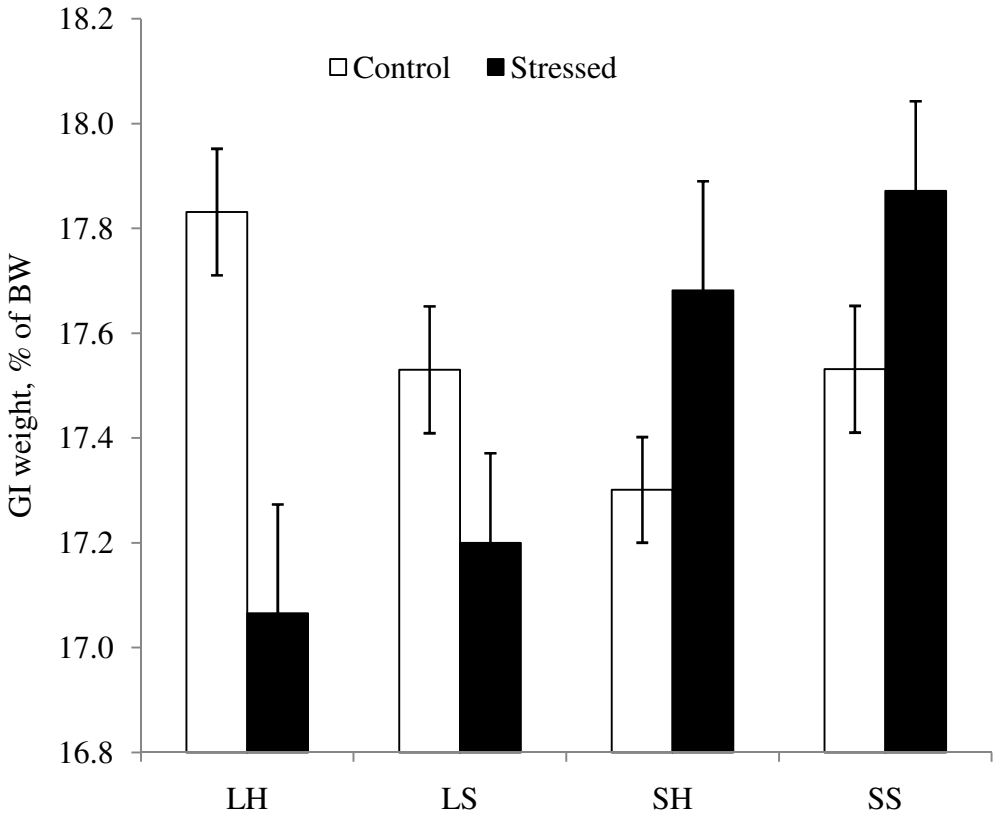

Figure 5.6 Effect of transportation conditions and incubation temperature on gastrointestinal tract (proventriculus, gizzard and small intestine without yolk sac) weights (% of BW) of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) and the transportation treatments control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=40/treatment). There was a two way interaction (P= 0.03) of incubation temperature and transportation.

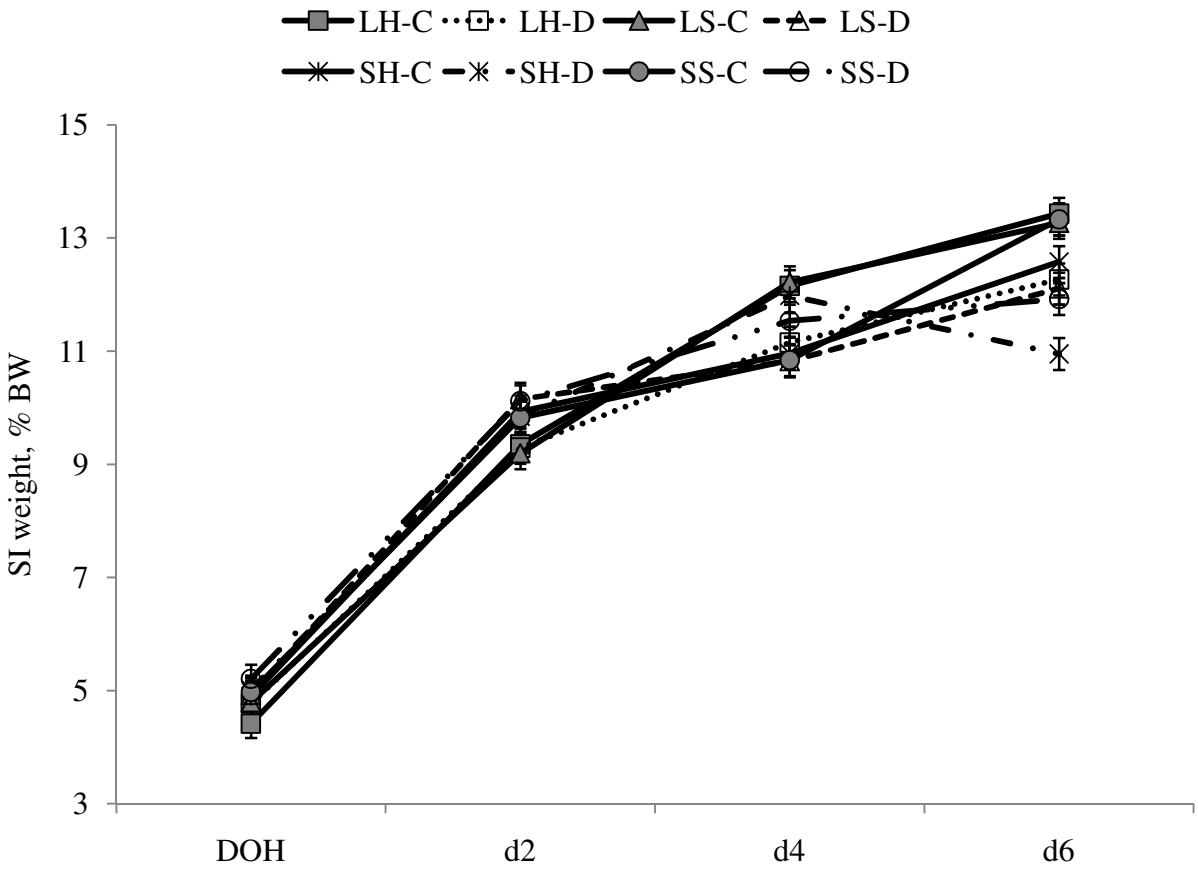

Figure 5.7 Effect of incubation temperature, transportation, and age on small intestine weights (% of BW) of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods, in combination with the transportation treatments control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=20/treatment). There was a three way interaction (P= 0.002) of incubation temperature, transportation and age.

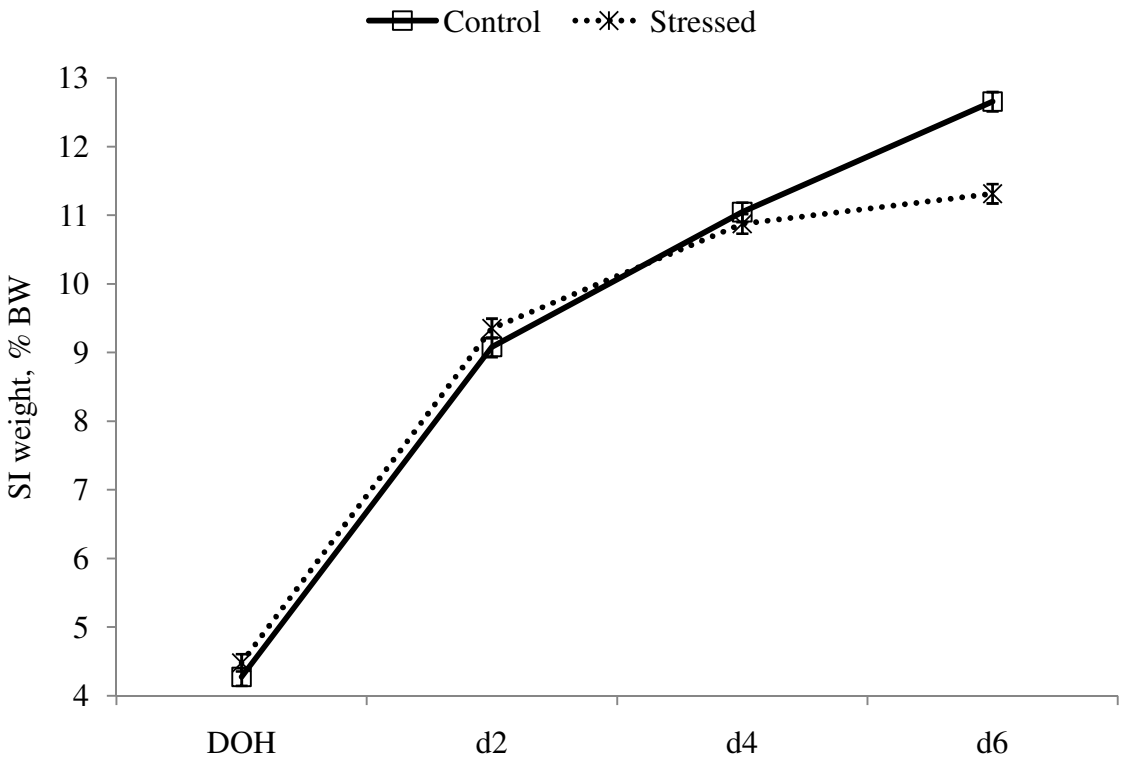

Figure 5.8 Effect of transportation and age on small intestine weight (% of BW) of Cobb 500 broiler chicks transported under control (34°C) or stressed (40°C) conditions. Data are represented as LS Means ± SEM (n=40/ treatment). There was a two way interaction (P= 0.0001) of transportation and age.

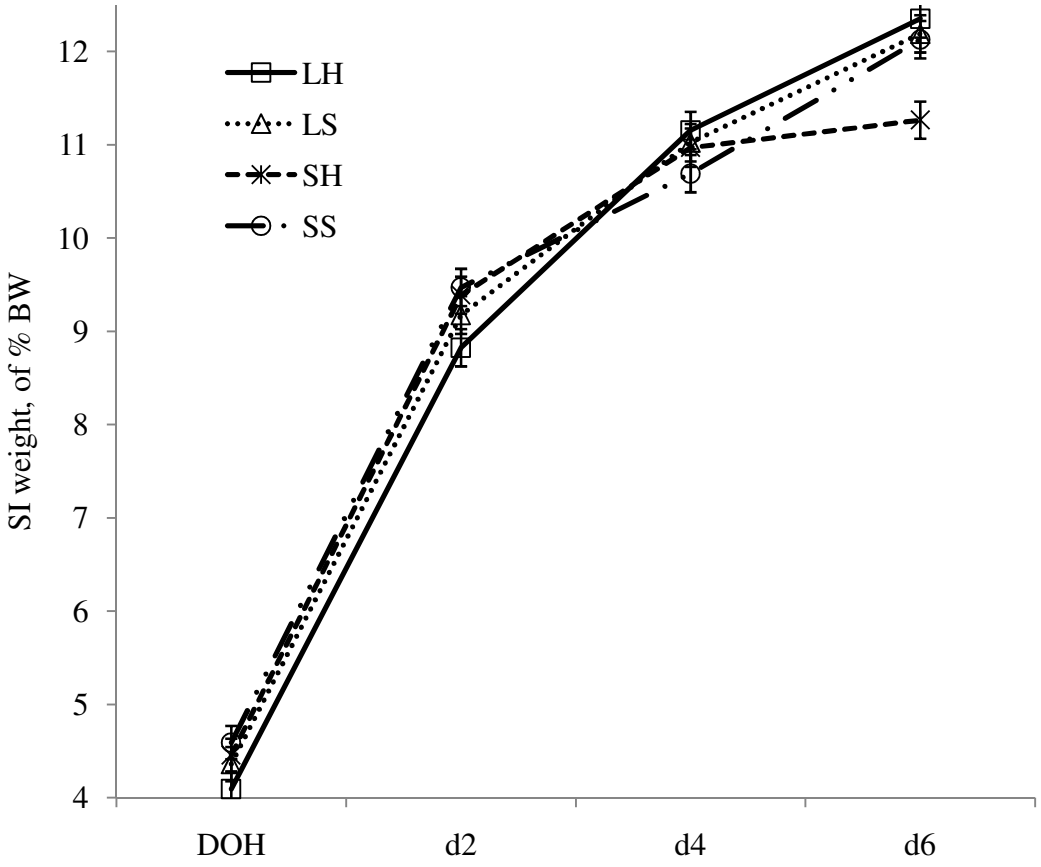

Figure 5.9 Effect of incubation temperature and age on small intestine weight (% of BW) of Cobb 500 broiler chicks. Treatments were designated as low (L, 36.7°C), standard (S, 37.5°C) or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods. Results are reported as LS Means ± SEM (n=40/treatment) and had a two way interaction (P= 0.0009) of incubation temperature and age.

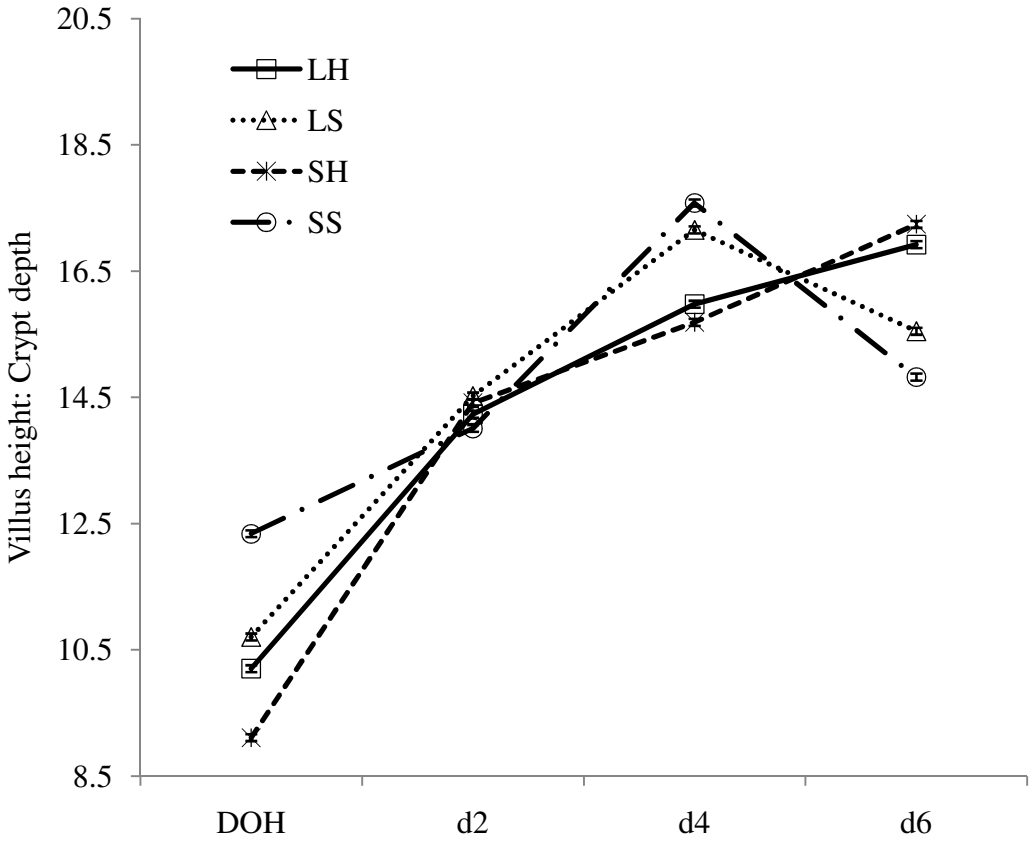

Figure 5.10 Effect of incubation temperature and age on duodenum villus height to crypt depth ratios in Cobb 500 broiler chicks. Treatments were designated as low (L, 36.7°C), standard (S, 37.5°C) or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods. Results are reported as LS Means ± SEM (n=16/treatment) and had a two way interaction (P= 0.01) of incubation temperature and age.

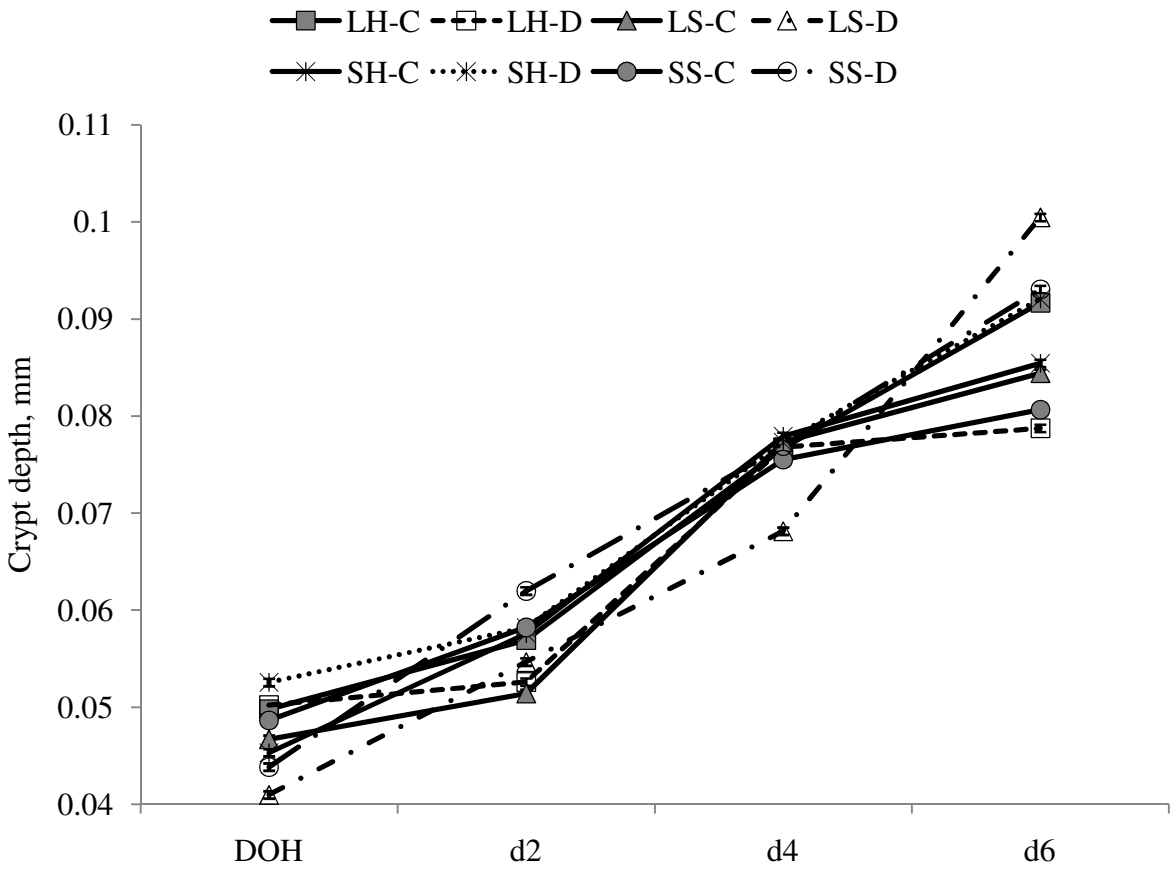

Figure 5.11 Effect of incubation temperature, transportation, and age on jejunum crypt depth of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods, in combination with the transportation treatments control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=8/treatment). There was a three way interaction (P= 0.03) of incubation temperature, transportation, and age.

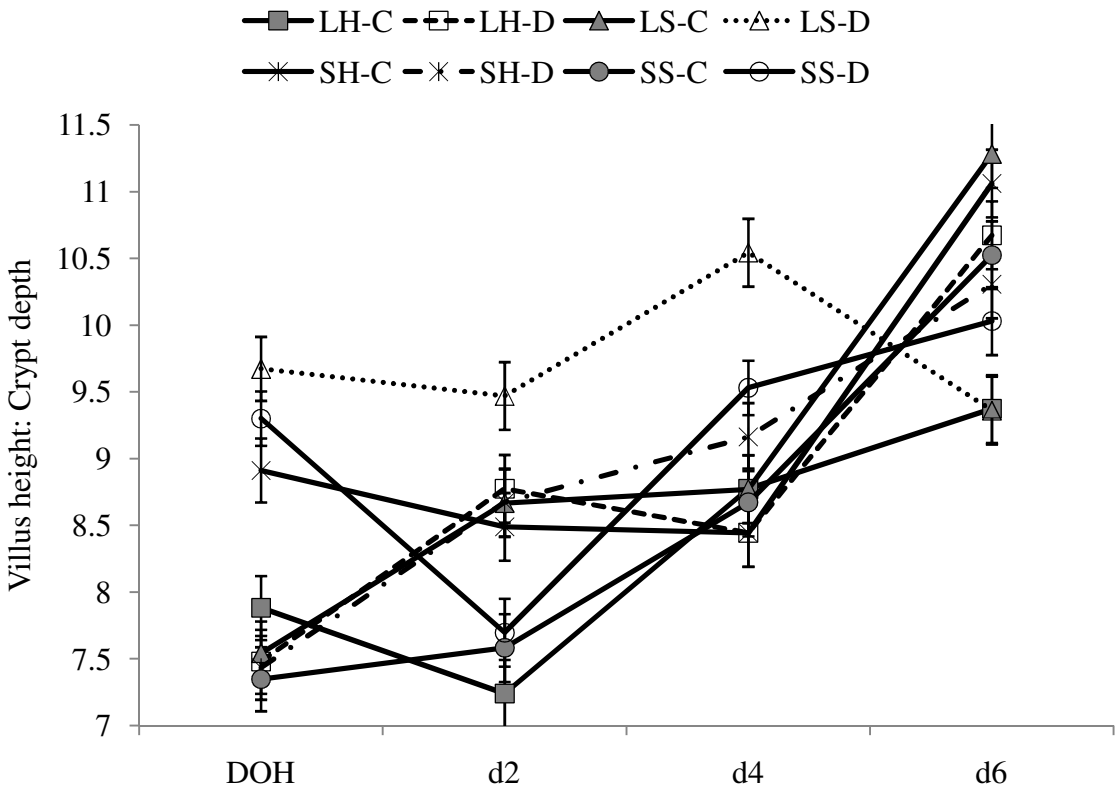

Figure 5.12 Effect of incubation temperature, transportation, and age on jejunum villus height to crypt depth ratios of Cobb 500 broiler chicks. The incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during the early (ED0 to ED7) and late (ED15 to ED21) incubation periods, in combination with the transportation treatments control (C, 34°C) and stressed (D, 40°C). Data are represented as LS means ± SEM (n=8/treatment). There was a three way interaction (P= 0.002) of incubation temperature, transportation, and age.

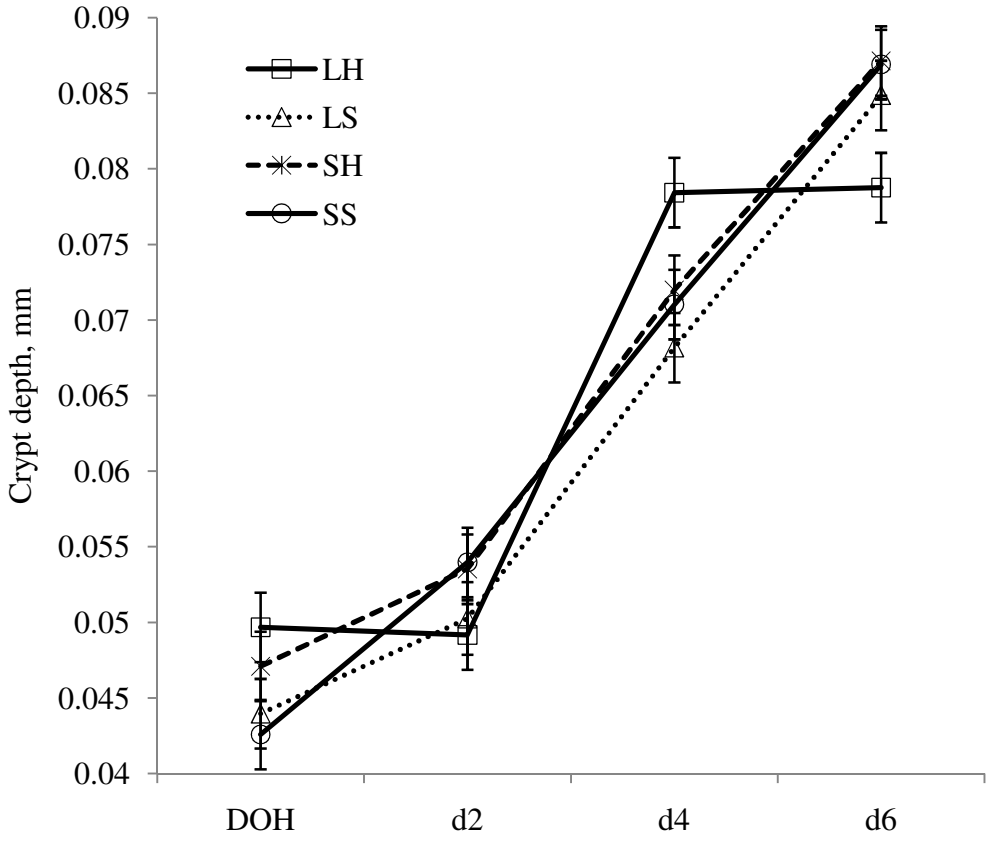

Figure 5.13 Effect of incubation temperature and age on ileum crypt depth in Cobb 500 broiler chicks. Treatments were designated as low (L, 36.7°C), standard (S, 37.5°C) or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods. Results are reported as LS Means ± SEM (n=16/treatment) and had a two way interaction (P= 0.007) of incubation temperature and age.

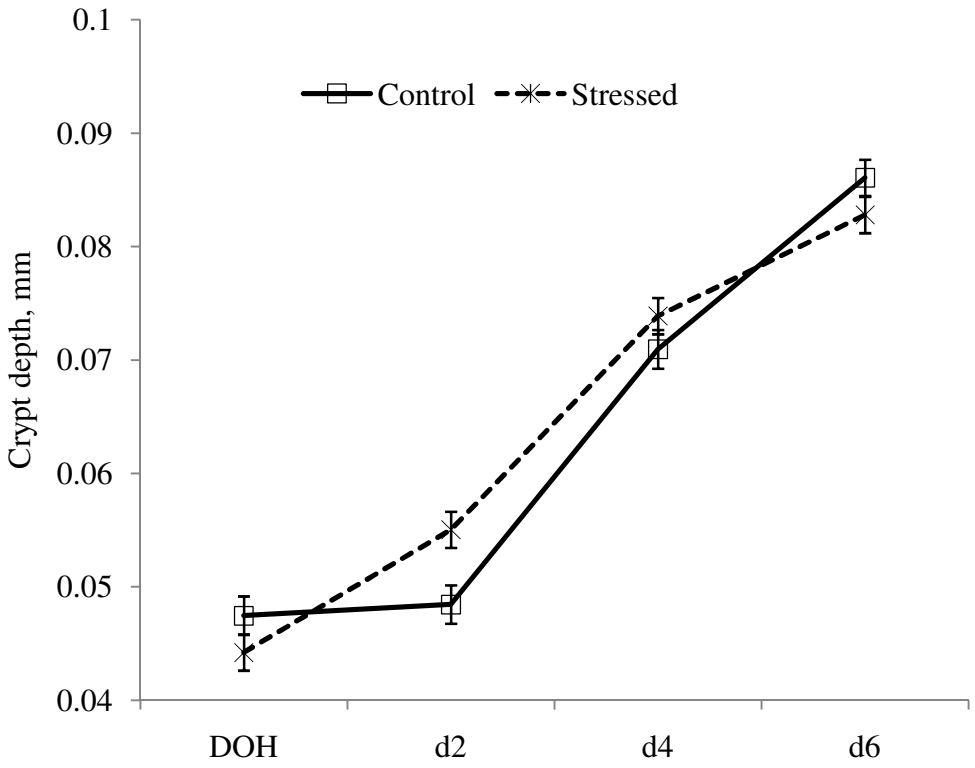

Figure 5.14 Effect of transportation and age on ileum crypt depth of Cobb 500 broiler chicks transported under control (34°C) or stressed (40°C) conditions. Data are represented as LS Means ± SEM (n=16/ treatment). There was a two way interaction (P= 0.006) of transportation and age.

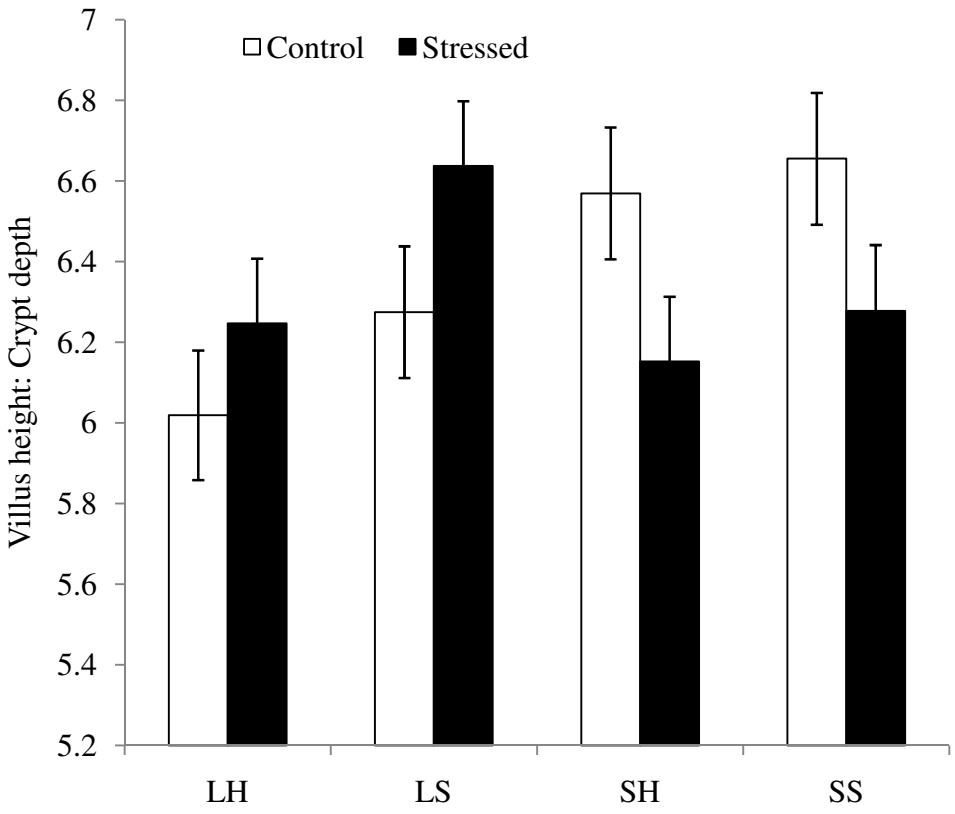

Figure 5.15 Effect of transportation conditions and incubation temperature on villus height to crypt depth ratios in ileum of Cobb 500 broiler chicks. Incubation treatments were designated as low (L, 36.7°C), standard (S, 37.5°C), or high (H, 39°C) during early (ED0 to ED7) and late (ED15 to ED21) incubation periods. Transportation groups were stressed (40°C) or control (34°C). Data are represented as LS means ± SEM (n=16/treatment). There was a two way interaction (P= 0.02) of incubation temperature and transportation.

CHAPTER VI

EPILOGUE

A major challenge in today's poultry industry is the identification of different factors that will determine the optimal incubation temperatures and other parameters required for high-yield modern broiler strains. Ideal incubation parameters should in turn facilitate improved post-hatch characteristics and performance, given that embryos from modern fast growing lines present higher metabolic rates than the slow growing lines of broilers from years past. Embryonic temperature is considered to be the most important factor during incubation, not only for an ideal hatching percentage but also for good chick quality and post-hatch growth. However, there are no guidelines established specifically for the high-yield broiler chick, and incubation equipment follows designs established for classic broiler lineages (Wilson, 1991; Lourens *et al*., 2005; Lourens *et al*., 2007). Previous studies have evaluated the effect of elevated incubation temperatures on thermoregulatory ability, hormone levels, cardiac hypertrophy, and even phenotypic proportion of chick embryos and post-hatched chicks. Additionally, post-hatch transport represents an important issue contributing to chick quality and performance in the poultry industry as well. The optimal thermal environment to which chicks should be exposed during transportation from hatcheries to farms is not well established. It has been observed that lack of ventilation or air movement within the vehicle will increase stress in the birds, possibly resulting in detrimental effects on development and lifetime performance of the chicks. While most of the studies reported about transportation involve different livestock species, the existent data showing the evaluation of transportation in poultry are very few and have included slaughter age chickens. The transportation of newly hatched chicks from the hatchery to the farms has

been for the most part ignored. No previous study has evaluated the effect of multi-stage incubation temperature profiles and transportation stress on the gastrointestinal development and post-hatch performance of high-yield broiler chicks.

The studies presented in this dissertation evaluated incubation temperatures in two independent projects. One study involved evaluation of the Ross X Ross 308 commercial broiler strain and their responses to elevated temperature in a single-stage incubator model. The second study was performed using Cobb 500 commercial broiler chicks and evaluated their response to temperature profiles typical of a commercial multi-stage incubator system and to environmental conditions during transportation directly after hatch. This study was the first to evaluate embryonic temperature during incubation combined with early post-hatch transportation effects on small intestine development by measuring not only tissue weight but also gene expression of specific intestinal nutrient transporters, yolk utilization, and intestinal morphology. In chapter II of this dissertation, performance parameters (BW, BW gain, feed intake, feed conversion, and mortality) were not affected, however we did observe differences in the mucosal epithelial morphology as well as gene expression of some nutrient transporters. The lack of performance differences may be a result of broiler strain. Ross 308 broilers have a rapid growth rate that starts at a later stage of life, and therefore, early post-hatch changes did not impact later performance. Results also indicated that there was a direct effect of incubation temperature stress on the small intestine. These effects were most evident in the early post-hatch period, and the chicks may have recovered from initial deficits and were able to maintain performance by slaughter age. Even lacking performance differences, the data indicated that elevated incubation temperature was likely altering intestinal development and possibly affecting gastrointestinal function.

The hypothesis of our experiments was that elevated incubation temperatures and stress during transportation would decrease or impair the morphological development of the mucosal epithelium as well as the gene expression of nutrient transporters in the small intestine. Interestingly, we observed that the chicks incubated at elevated temperatures and transported under stressful conditions had an increase in certain nutrient transporters, changes in crypt depth, villi length, and villi height to crypt depth ratios, as well as differences in yolk utilization and intestinal weight. These changes suggest modifications and or acceleration of intestinal development in an attempt to compensate for reduced energy availability as a result of the stress impact on metabolism and yolk nutrient availability. These data are not surprising given that the chicks selected for growth will direct much of the nutrients and energy consumed into growth and less towards other important metabolic pathways and or the development of other systems e.g., the immune system. This may have serious implications in a field setting where exposure to different stressors or pathogens exists. In such scenarios, the energy necessary for the development of the immune system, the establishment of the humoral and adaptive responses towards vaccination and further pathogenic challenges, may be compromised. Most of the energy will continue to be diverted toward growth e.g. protein deposition in skeletal muscle and if birds are challenged it may result in increased susceptibility to diseases.

In the second trial we observed differences in feed intake and BW gain from d 28 to 42. All diets from the three different dietary phases were medicated. It would be interesting to evaluate the same parameters that we evaluated early post-hatch, including intestinal morphology and nutrient transporter expression, in the small intestine during all three feeding phases (starter from d 0 to 14, grower from d 14 to 28, and finisher from d 28 to 42) in order to understand if

morphological changes continue throughout grow-out and further our understanding of stress effects on the gastrointestinal tract of the bird and its nutrient utilization.

Compensatory growth is usually described as a rapid growth rate that follows a period of reduced feed intake of an animal (Summers, 2000). It can also be used as an explanation of rapid growth rate that follows a lack of or inappropriate nutrient or energy assimilation in any tissue or organ due to problems during development or disease (Fisher *et al*., 2006). Compensatory growth, while it helps the chick to "catch up" and reach the desired body weight, may result in energy deficits for other systems in the body. Studies on understanding how compensatory growth occurs through changes in the small intestine would be of interest. It would be fascinating to calculate the trade off or expense that compensatory growth has on the small intestine and immune system. I would encourage research focused on the evaluation of energy utilization and its distribution in order to determine when and how compensation takes place and if it is costly to the industry or not. If changes in energy allocation were occurring, it would be interesting to evaluate *in ovo* feeding trials together with incubation temperatures or transportation stress treatments to determine if the same results would occur. It would also be beneficial to evaluate the possibility of feeding chickens with increased levels of specific amino acids necessary for growth and for other systems in order to aid in the compensatory growth after incubation and transportation treatments combined with disease challenges, such as infectious bronchitis, or Newcastle disease virus.

In both trials, alterations in intestinal mucosal morphology and expression of specific nutrient transporters were observed. These measurements do not indicate the presence of a functional protein. However, they are accepted as an indirect indicator of the presence of such proteins. The presence of the actual proteins could be evaluated in order to correlate these with mRNA

expression levels. Western blots or immunohistochemistry analysis could be used to perform such analyses. The production of antibodies against chicken proteins would have to be done due to the general lack of their availability for poultry species. Localization of the nutrient transporters within the enterocytes and along the villi, and quantification of nutrient transporters per area and per total tissue mass should be considered. The nutrient transporters evaluated in this dissertation are by no means the only transporters that exist for transfer of nutrients across the enterocyte. Therefore, physiological studies evaluating the actual nutrient uptake by the tissue should be included in future studies. The use of Ussing chambers to measure ion transport across intestinal epithelial cells would be a start for such studies. Stress during incubation and/or transportation might also affect the protein turnover within cells and tissues. Changes in the nutrient transporters while being transported into the membrane of the enterocyte are also possibilities. Isotope studies tracking the fate of diet nutrients once taken up by the enterocyte would give us an idea of how much and what exactly we need to add to the diet in order to feed for muscle growth and GALT establishment. Following this, the administration of feed, orally and parenterally, would be of interest to identify the final destination of specific nutrients in the experimental chicks. The administration of oral nutrients would identify how much of the nutrients are being absorbed and used locally by the enterocytes. The administration of nutrients parenterally would identify the distribution of nutrients towards different organs other than the small intestine. This technique would allow us to decrease wastage of nutrients in the digesta and/or optimize the local utilization by the small intestine.

Low antibody responses, poor feed conversion, decreased viral resistance, as well as increased resistance to bacteria, parasites and toxins are all physiological responses to short-term stressors that can indirectly affect the lifetime performance of the chick (Gross and Siegel, 1993).

Therefore, determination of the effects of embryonic incubation and transportation stress on lymphocyte populations would be of interest. Chickens do not have lymph nodes but do have other lymphoid structures such as Peyer's patches along the small intestine, germinal centers in the ceca, and cecal tonsils. Studies to evaluate lymphocyte populations should be done through flow cytometry and immunohistochemistry with tissue from cecal tonsils, Peyer's patches, mucosal epithelium (intraepithelial lymphocytes), and spleen. Chicks should also be vaccinated with commercial mucosal vaccines, which are capable of inducing antigen-specific immune responses in both systemic and mucosal compartments, , to evaluate effects of incubation or transportation stress on immune system competency. Mucosal vaccination is considered ideal for the global control of infectious diseases (Nochi *et al*., 2007). Vaccination should be given at DOH with a booster administration on d 14, and specific antibodies should be measured with the use of an ELISA for specific antibody detection. In addition to measuring antibody titer, it would be ideal to challenge the chicks with the same pathogens vaccinated against, since it will be the only way to completely corroborate and compare the immunocompetence of the chicks. Possible small intestine pathogens, such as those involved in runting-stunting syndrome (Astrovirus, Rotavirus) and their relationship to the effects of incubation and transportation stress could be further evaluated. New trials are already under way in our laboratory to examine chicks incubated under the multi-stage system temperature profiles that are vaccinated *in ovo* (ED18) and at DOH. I am confident that the chicks stressed during incubation, either at an early-low or a late-high temperature, or during transportation will have problems establishing a fully functional and competent immune system.

The morphological parameters evaluated in these trials were villus height, crypt depth, and the ratios of the two. Cellular turnover in the small intestine along the villi should also be

considered by measuring migration, cell proliferation, apoptosis, and necrosis to have a more accurate picture of what is happening in the small intestine. I acknowledge the difficulty in measuring cell proliferation, migration, and turnover rates in chicks during the early post-hatch period given that the entire small intestine is going through extensive changes at this time. The techniques utilized for these measurements are usually immunohistochemistry, and the reading of slides is very time consuming, especially when most cells along each villi are positive for the antigen-antibody reaction. The presence or absence of functionally mature enterocytes available throughout the villi that are capable of digestion and absorption should also be evaluated. Again, I acknowledge the difficulty in evaluating cell proliferation at such a young age in the chick when so many morphological changes are occurring, and most cells are under proliferation and migrating at very fast rates. This evaluation should probably be made in chicks after d 10 post-hatch when the small intestine is developed, and fully mature. Overall, I saw that stress increased the expression of certain nutrient transporters, especially glucose transporters. Since stress increases the energy requirements of the animal, the stressed animals will use the energy available for maintenance and growth since that is for what they have been selected. Due to the responses observed in the crypts of the small intestine it could be hypothesized that hyperplasia was occurring. An increase in the number of enterocytes should increase the digestion and absorptive capacity of the villi if they have the time to become functionally mature before turnover. This brings me to different questions: How is the establishment of the gastrointestinal lymphoid tissue (GALT) affected? Does the increase in enterocyte numbers along the villi translate into an increased villi length but a decreased GALT establishment? Is an increase in size of the enterocytes causing the increase in length of the villi but still a decrease in GALT establishment? Is an increased villi length correlated to increased digestion and absorption? The

enterocytes along the villi will not be completely mature and will only be able to digest and absorb until certain time point. Chicken villi, unlike mammals, have cell proliferation along the villi. It is important to consider this factor, since it may affect the interpretation of the results observed in future and past studies. How are all these factors affected during stress or elevated temperatures during development? It was clear from morphological measurements in these studies that incubation temperature and transportation stress impacted development and maintenance of small intestine tissue, which could change energy usage and requirements of the chick. Most likely, incubation under sub-optimal conditions would cause an unnecessary energy expense to the chick and result in a deficit to energy that might have been used to reach the genetic potential for growth and immunocompetence of the animal.

Elevated temperatures during incubation and probably stress during transportation may cause epigenetic changes in gastrointestinal tissue. I suggest the use of microarrays in order to evaluate the genes that are induced or suppressed in expression after being exposed to elevated temperatures. The microarray results would help in the investigation of changes in gene expression, in intestinal morphology (villi length, crypt depth, lamina propria thickness), changes in enterocyte kinetics, as well as the distribution of nutrient transporters throughout the villi. As a result of incubation or transportation stress, post-translational changes might occur in the nutrient transporters or in the protein turnover affecting the transportation of the protein carrier to the cell membrane. Since DNA is the same across cells and tissues, and mRNA may change depending on the tissue type, age of the cell, developmental state, physiological condition, etc. evaluation of the DNA should be taken into consideration. Microarray analyses might provide clues regarding specific gene function. However, microarrays will need validation of the results

through the use of real-time PCR. Microarrays, in situ hybridizations, real-time PCR, and immunohistochemistry should be correlated.

The organ weight differences observed, more specifically the small intestine and yolk sac weights, clearly show the absorption of the yolk related to the small intestine weight. Elevated temperatures may cause a blockage in the passage of the yolk into the intestinal lumen during incubation. The correlations between yolk sac retention, omphalitis, and elevated temperatures are strikingly high, however, the exact cause for this is unknown. Microscopic (histological) and macroscopic studies of the yolk, the yolk stalk, and the small intestine should be conducted to evaluate changes in color, shape, size, content analysis, and histological structure. Yolk stalk blockage, if true, supports our data, as we saw that chicks incubated at LH and LS had the least absorption. Maybe the blockage is not due to temperature per se but to stress, through the effects of glucocorticoids or epinephrine secreted locally in the affected area, since we primarily observed a lack of absorption in the early low incubation temperature groups. Induction of stress with corticosterone should be evaluated to correlate their possible involvement in this theory. Stress hormones can alter proliferation, differentiation, and overall changes in the mucosal epithelium. In our studies we did not collect blood samples to correlate corticosterone or any other stress hormones with the stress present during incubation or transportation. Further studies should analyze corticosterone and lymphocyte: heterophil ratios and correlate these data with morphological and gene expression changes. If the yolk stalk blockage theory is true, it is no surprise that studies done with *in ovo* feeding nutrients through the yolk were not completely successful, but *in ovo* trials administering different nutrients through the amnion were successful. Until recent years the scientific community has focused on feeding the newly hatched chick in order to facilitate the achievement of its full genetic potential.

With data from the entire study, I believe that chicks stressed during incubation with different temperature profiles, or during transportation are forced to utilize glycogen from liver and muscle and amino acids from muscle in order to satisfy the energy requirements for maintenance, development, and growth. We observed that there is a possible improvement of luminal nutrients uptake by increasing certain nutrient transporters expression (SGLT1, GLUT5, EAAT3) in the brushborder membrane of the small intestine and because of the intestinal tissue morphological changes. At DOH we did not observe differences in body weights, however, by d 4 and 6, yolk free body weights were different with the stressed chicks having the lightest weight. It could be that it takes several days to see the effects of the muscle breakdown during the most critical period, which was the perihatch period. Evaluation of glycogen utilization and muscle break down throughout the first 14 d would allow us to further understand if the lack of energy during the perihatch period is compensated for through these sources.

The resource allocation theory should be the impetus for us to pursue more complete studies evaluating the nutrient profiles needed for chicks that have experienced stress during incubation and transportation. This would allow the industry to know what to feed the animal in order to fulfill the energy required to compensate for stress, to continue growth, and to fulfill the nutrient requirements for the immune and other systems as well. Nutrition can impact the incidence of infections and immune response within the lumen. Some nutrients (vitamin E, conjugated linoleic acid) influence the immune system by affecting intercellular communication or by inducing cytokine release during an inflammatory response. If we understand the specific nutrient needs for growth and immunocompetence when chicks are stressed, we would be able to recommend specific diets to encourage better recovery and performance. It may also be possible to improve or alter the metabolism of the gastrointestinal tract. It is important to consider that by

altering or changing the metabolism of one system, it may be at the expense of disrupting another aspect of metabolism in another system. As I mentioned previously, the increase in energy requirements can also force the chick to utilize muscle glycogen and breakdown muscle protein to fulfill such requirements. Elaboration of diets with more available protein or energy, and the addition of enzymes to increase nutrient availability, to meet the requirements of stressed chicks might aid in decreasing the negative effects that incubation or transportation have in the development of the intestinal tract and subsequent performance of the chick. The addition of vitamin E, or conjugated linoleic acid, which are known transcription factors that favor the immune system, are two examples of manipulations of health through nutrition in mammals. Another example is vitamin C. Chickens are capable of synthesizing ascorbic acid. However, under stressful conditions, the requirements for this vitamin increase and synthesis becomes insufficient. Addition of vitamin C might aid in the reduction of the stress induced response imposed on the chicks during incubation or transportation.

The establishment of a link between intestinal homeostasis and food derived bacterial metabolites should also be taken into consideration. As soon as the chick is placed in the farm environment, microflora begin to become established in the small intestine. The bacteria established in the chick's gut will determine the immune and health status of the animal and microflora balance that can contribute to nutrient utilization, since microflora will compete or aid in the digestion and absorption of nutrients. As mentioned in this dissertation, the expression of GLUT5, a nutrient transporter specific for fructose, could be used as an indirect evaluation of microflora activity in the small intestine of the chick. Consequent passage rates and mucin production should also be evaluated. It is possible that the establishment of microflora can also be affected by elevated temperatures or stress during incubation and transportation since the

intestine appears to be undergoing more turnover or delayed yolk utilization at altered temperature profiles.

I believe that with these studies we observed that the gastrointestinal tract is directly affected by both early and late incubation temperatures and transportation stress, which can further impact the lifetime performance of the chick. In some cases, responses were observed to be caused as much by transportation stress as by the incubation temperature profile effects. We also observed in the incubation temperature profiles that not only the elevated incubation temperature during the last week of incubation has an effect on the parameters evaluated, but that the early low incubation temperature has an important impact on them as well. I think that more studies should be pursued in order to understand the mechanisms of action, and to find and establish new incubation and transportation guidelines for the modern high-yield broiler strains. We would have to take into consideration that there are differences in growth rate between different high-yield broiler lines. The industry will probably have to establish independent guidelines for different lines, as they have done with nutrient requirements. In order to be able to understand these mechanisms I would encourage the continuation of this research by separating the evaluation of incubation temperature and transportation into two isolated projects. While the importance of both is of great importance for the poultry industry, together they result in too many interactions that make the interpretation of results complicated. Nonetheless, I believe that this dissertation contributes to the understanding of the incubation and transportation issues facing the industry. Further research should be pursued in order to find the specific parameters and appropriate incubation temperatures needed for the different high-yield broiler lines. Given that turkeys present similar problems or more in the industry, future work in turkey poults under the same conditions should be considered.

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APPENDIX A MAIN EFFECTS

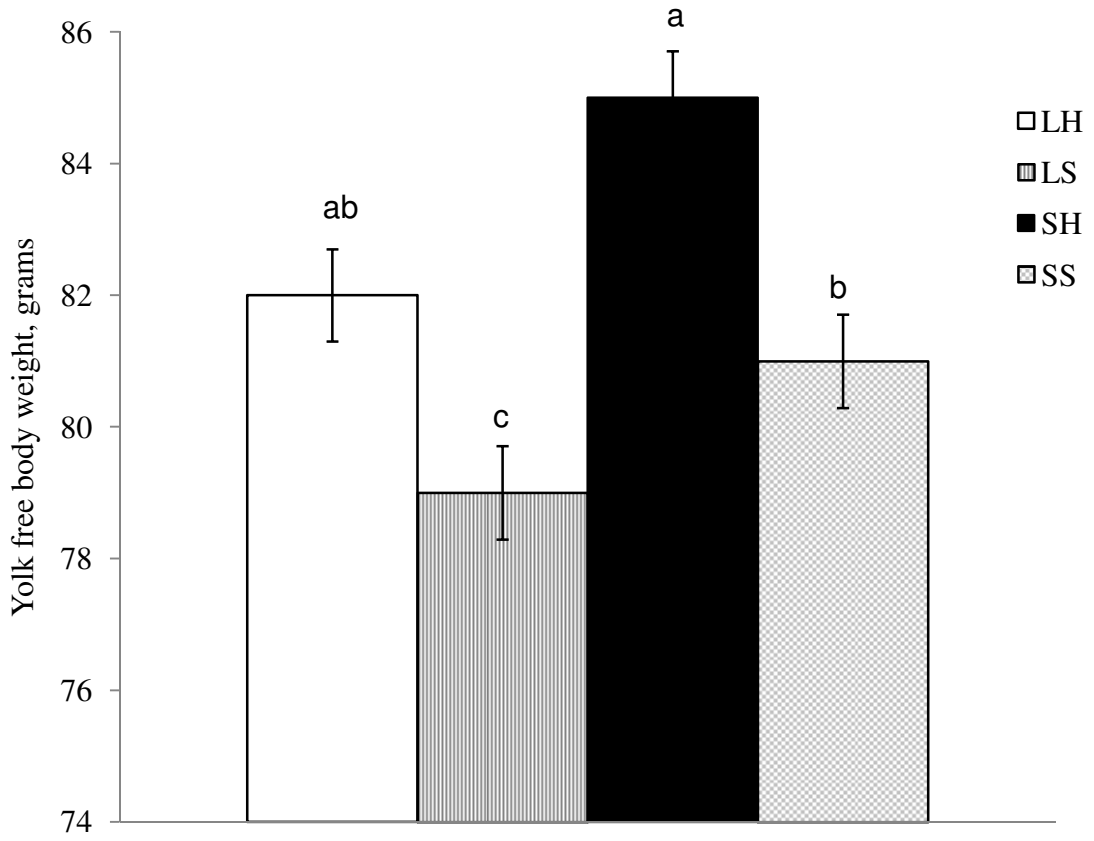

Figure A.1 Effect of incubation temperature on yolk free BW of Cobb 500 broiler chicks incubated at specific profile temperatures similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Data are represented as LS means ± SEM (n=20/treatment).

[a-c] Data lacking a common superscript differ significantly (P= 0.01).

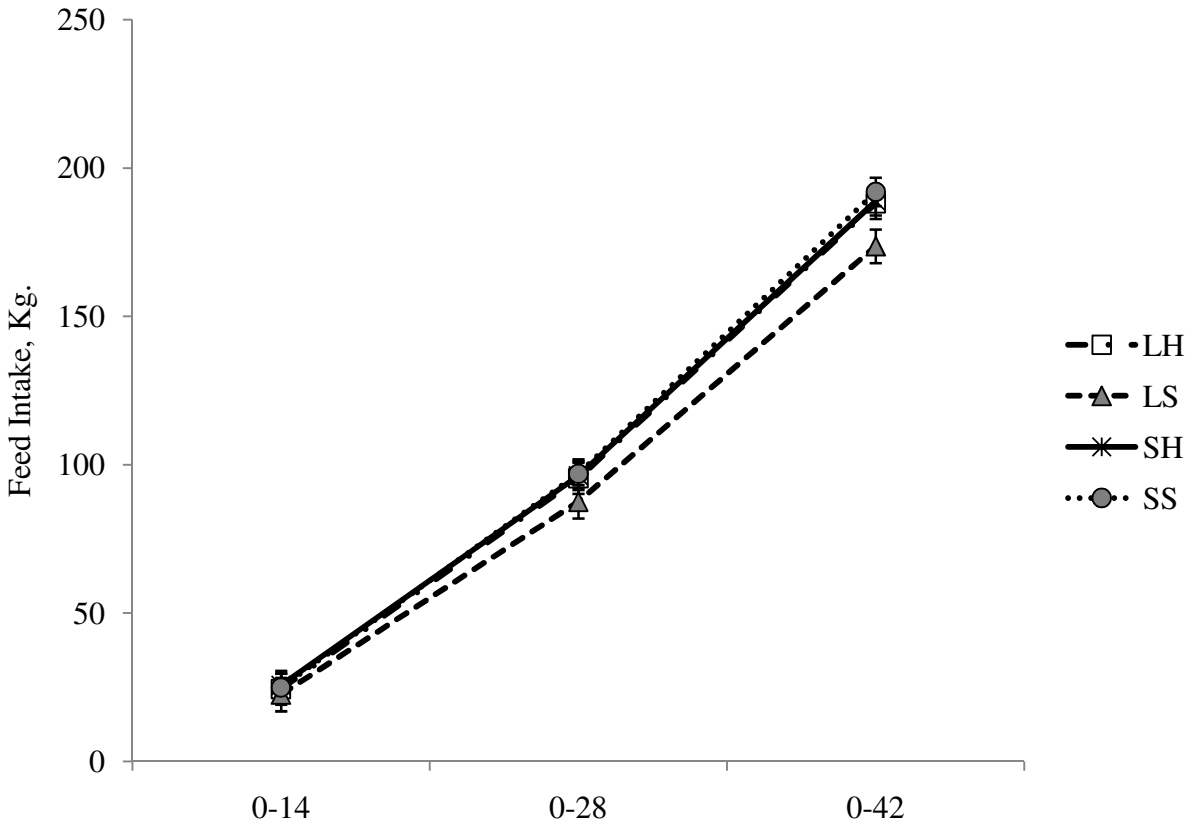

Figure A.2 Effect of incubation temperature and age on feed intake of Cobb 500 broiler chicks incubated at specific profile temperatures similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Data are represented as LS means ± SEM (n=688/treatment) with a two way effect (P= 0.01) of incubation temperature and age.

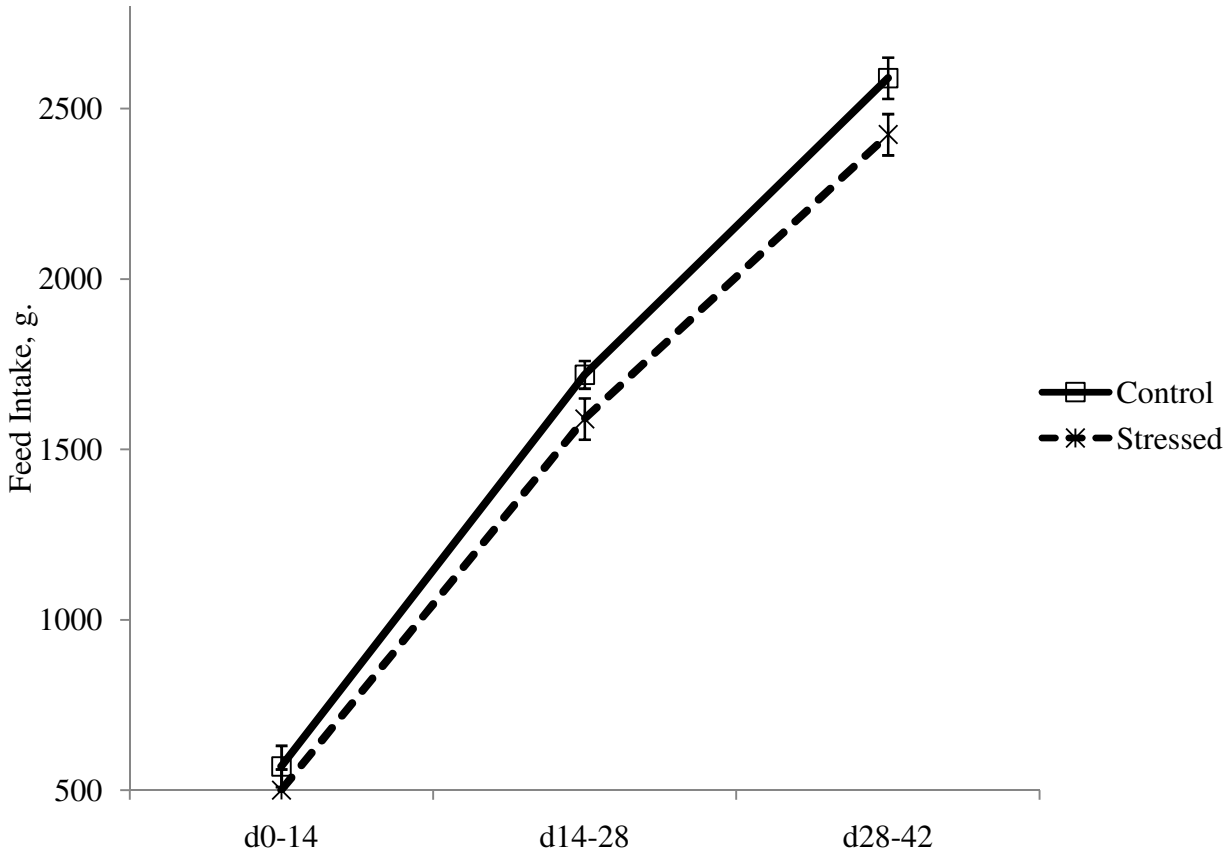

Figure A.3 Effect of transportation and age on feed intake of Cobb 500 broiler chicks transported under control (34°C) or stressed (40°C) conditions. Data are represented as LS means ± SEM (n=1376/treatment) with a two way effect (P= 0.05) of incubation temperature and age.

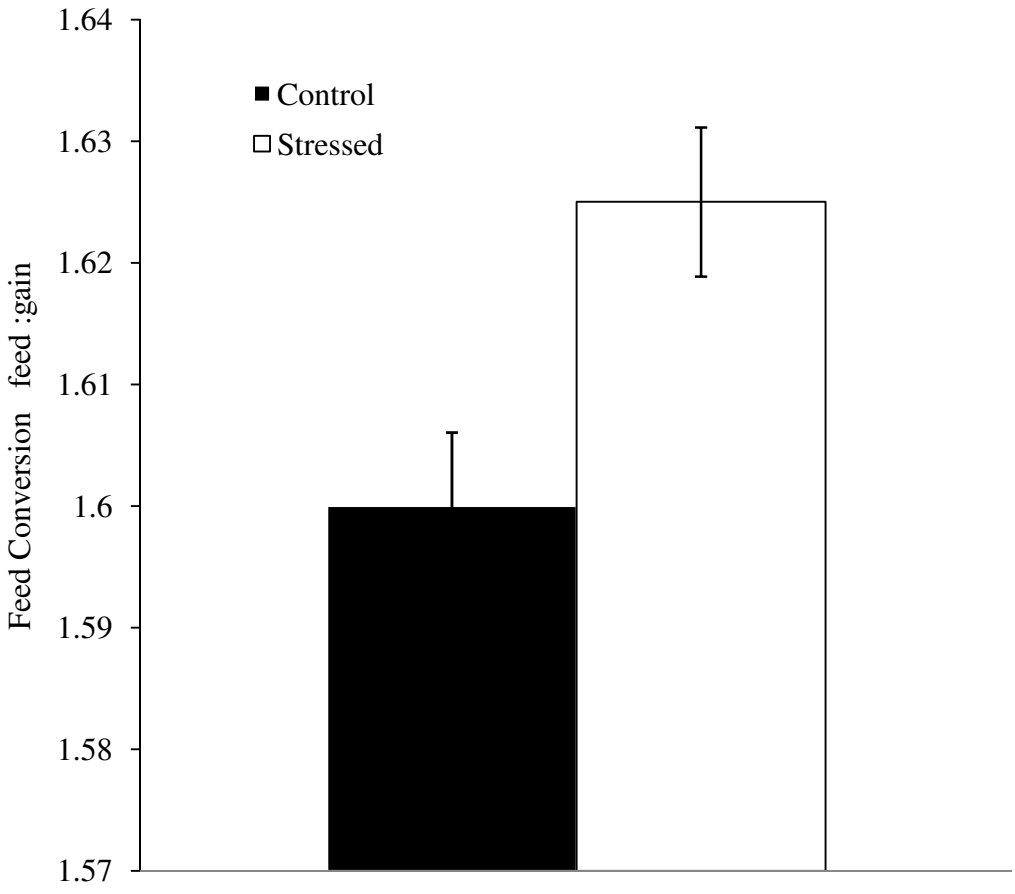

Figure A.4 Effect of transportation on feed conversion of Cobb 500 broiler chicks transported under control (34°C) or stressed (40°C) conditions. Data are represented as LS means ± SEM (n=1376/treatment). Main effect (P= 0.07) of transportation.

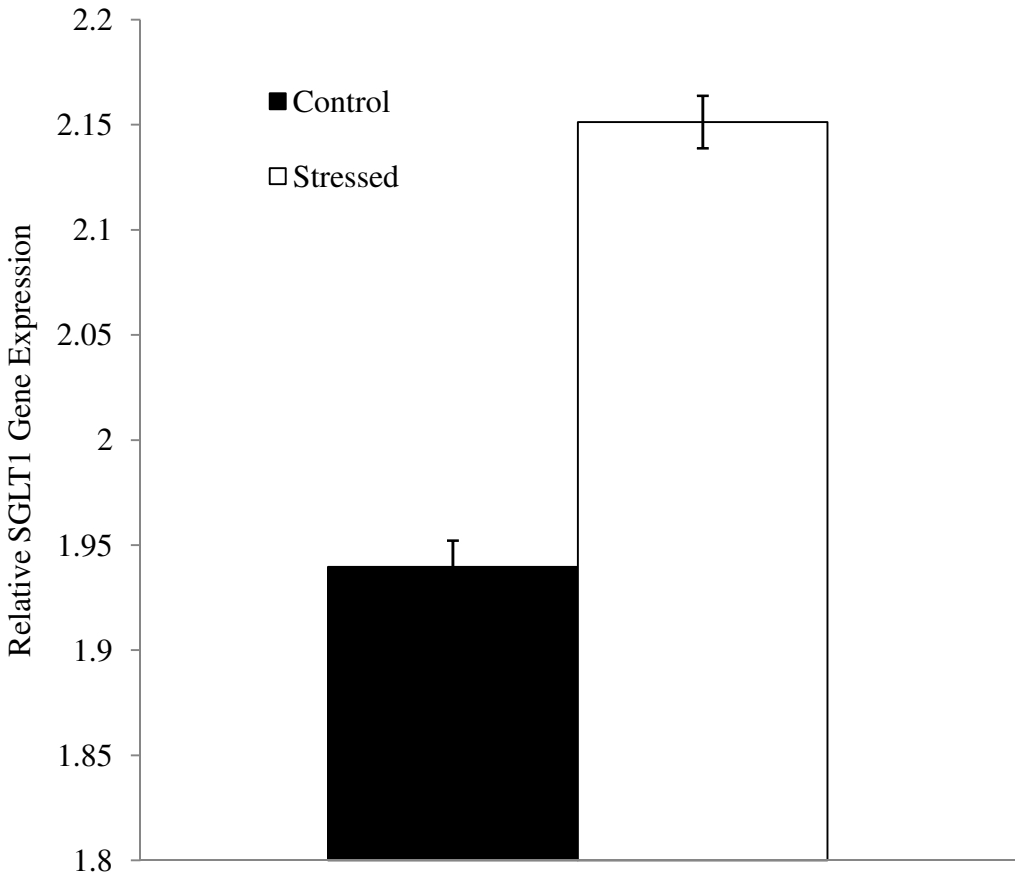

Figure A.5 Effect of transportation on relative SGLT1 mRNA expression in small intestine of Cobb 500 broiler chicks transported under control (34°C) or stressed (40°C) conditions. Data are represented as LS means ± SEM (n=12/treatment). Main effect (P= 0.002) of transportation.

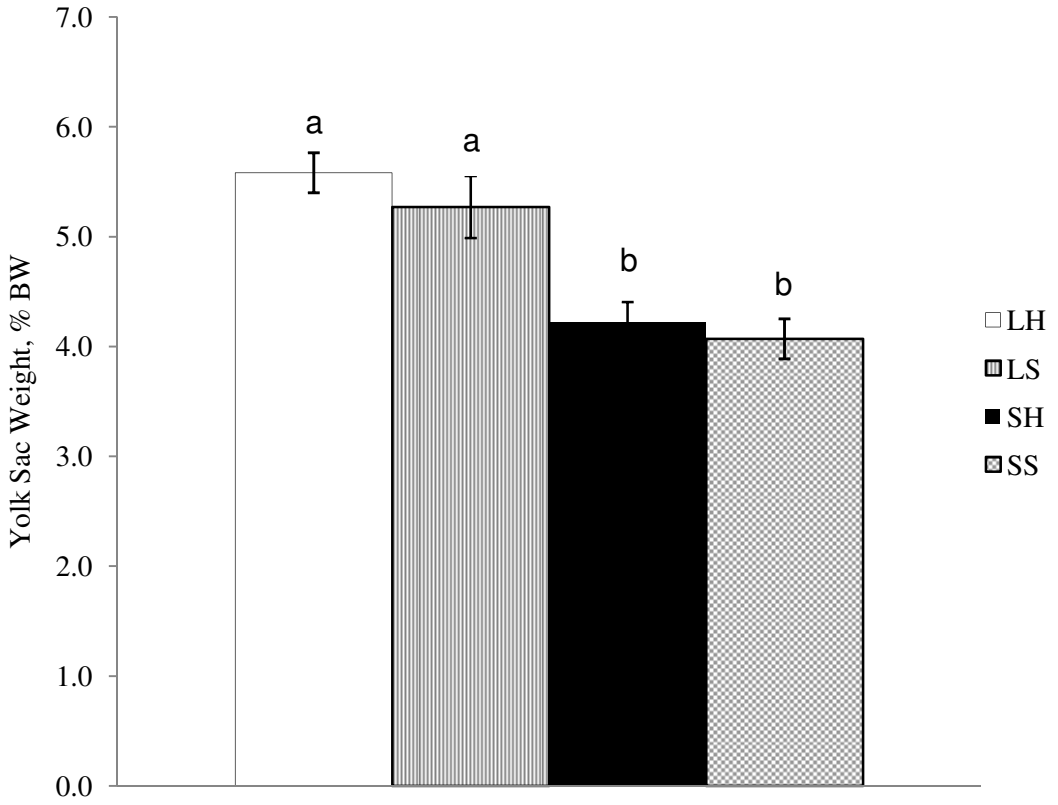

Figure A.6 Effect of incubation temperature on yolk sac (% BW) of Cobb 500 broiler chicks incubated at specific profile temperatures similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Data are represented as LS Means ± SEM (n=20/treatment).

[a-c] Data lacking a common superscript differ significantly (P= 0.01).

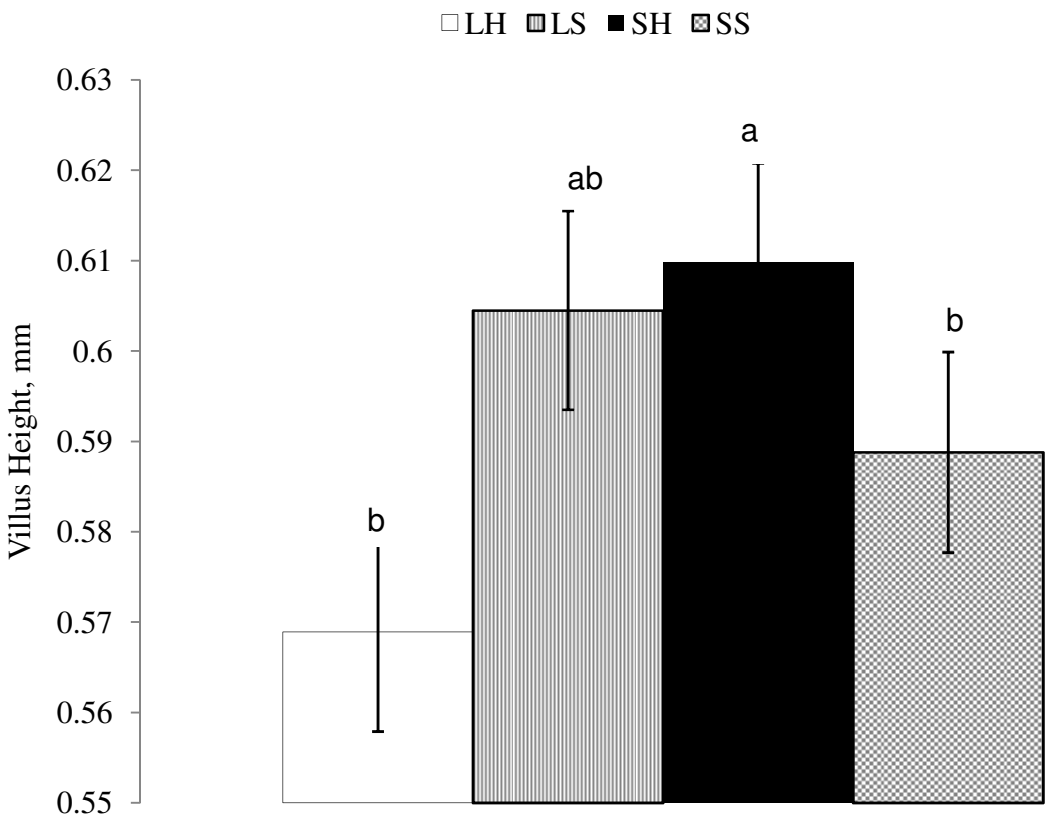

Figure A.7 Effect of incubation temperature on jejunum villus height in Cobb 500 broiler chicks incubated at specific profile temperatures similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Data are represented as LS means ± SEM (n=20/treatment).

[a-c] Data lacking a common superscript differ significantly (P= 0.04).

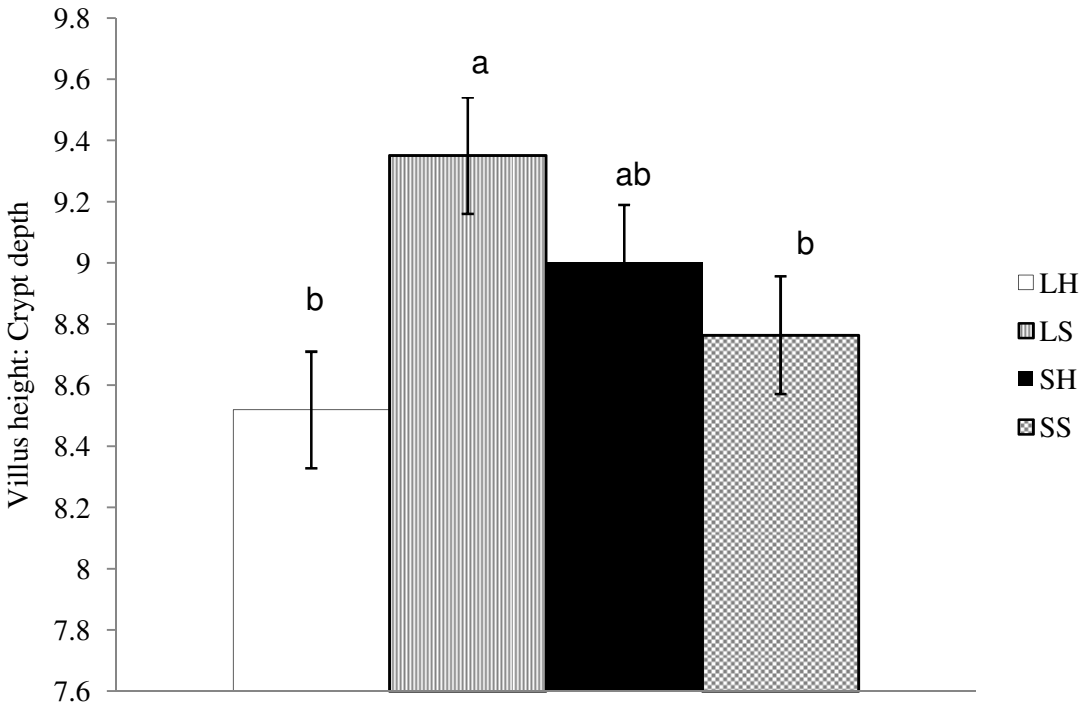

Figure A.8 Effect of incubation temperature on jejunum villus height to crypt depth ratios in Cobb 500 broiler chicks incubated at specific profile temperatures similar to multi-stage incubators. Embryos were incubated with the following eggshell temperatures during early (ED0 to ED7) and late (ED15 to ED21) incubation: low (L, 36.7°C), standard (S, 37.5°C), and high (H, 39°C). Data are represented as LS means ± SEM (n=20/treatment).

[a-c] Data lacking a common superscript differ significantly (P= 0.02).