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The biochemical and molecular effects of amnionic nutrient administration, “*in ovo* feeding” on intestinal development and function and carbohydrate metabolism in turkey embryos and poults.

(Under the direction of Peter Ferket)

Unlike the mammalian embryo, the avian embryo has a finite amount of energy and nutrients for growth and development. In ovo feeding (IOF), the administration of exogenous nutrients into the amnion, may enhance egg nutriture and early growth performance by improving glycogen status and maturation and function of the gastrointestinal tract (GIT). This hypothesis was challenged by evaluating 5 IOF solutions in 2 experiments. In experiment-1, IOF solutions were protein (EWP), HMB and carbohydrate (CHO). IOF treatments were arranged as a factorial arrangement of 2 levels EWP (0 and 18%), 2 levels HMB (0 and 0.1%). The CHO IOF solution (20% dextrin and 3% maltose) was evaluated for contrast. In experiment 2, IOF solutions were HMB, arginine (ARG), and EWP. Treatments were arranged as a factorial arrangement of 2 levels HMB (0 and 0.1%), 2 levels ARG (0 and 0.7%). EWP (18% EWP + 0.1% HMB + 0.7% ARG) was evaluated for contrast. At 23 d of incubation (23E) 1.5 ml of IOF solution was administered. Upon hatch, poults were provided feed and water *ad libitum*.

In experiment 1, body weights (BW) were determined at hatch, 3 and 7 d, and tissues were sampled for glycogen content of liver and muscle (PC). In experiment 2, BW were determined at hatch and 3, 7, 10, and 14 days and samples were taken to determine glycogen content of liver and PC, hepatic glucose-6-phosphatase (G6P) activity, and jejunal sucrase (S), maltase (M), and leucine aminopeptidase (LAP) activity. Additionally, S, M, LAP, SGLT-1 and Pept-1 expression levels were determined at 25E, hatch, 3, 7, and 14 days.

In experiment 2, poults of EWP, EWP +HMB and HMB alone weighed 6.0%, 2.7% and 3.3% more than the non-injected controls (C) at hatch, respectively ($P < 0.05$), revealing a

significant EWP X HMB interaction. Total hepatic glycogen (HG) was enhanced by the inclusion of EWP in the IOF solution at hatch ($p < 0.05$), whereas total muscle glycogen was enhanced at 7 days by IOF HMB ($p < 0.05$). When contrasting IOF solutions EWP, CHO and C, poult of EWP had the greatest HG reserves at hatch ($p < 0.05$), by 7 days, poult of EWP and CHO had higher HG reserves than C ($p < 0.05$). Additionally, poult of CHO had enhanced total muscle glycogen stores in comparison to C; while poult of EWP had significantly less total muscle glycogen stores than C ($p < 0.05$).

In experiment 2, all in ovo fed poult had 3-4% greater BW than C at hatch and the main effects of ARG and HMB responded independently ($p < 0.05$). Poult of HMB + ARG had BW that were 10-11% greater than C at 10 and 14 days ($p < 0.05$). At hatch, poult of ARG, HMB and ARG + HMB had an 84%, 78.7%, and 75% greater total hepatic glycogen than C ($p < 0.05$), with greater hepatic G6P ($p < 0.05$) activity and a significant ARG X HMB interaction on HG (total (mg), mg/g) and G6P activity ($p < 0.05$).

Jejunal S and M activities were 3-fold greater in poult of ARG + HMB than all other treatments at 14 days ($p < 0.05$). Jejunal LAP activity was improved by IOF HMB + ARG over C at 25E, hatch, 3-d and 14-d with a HMB X ARG effect ($p < 0.05$). There was an HMB X ARG effect on jejunal Pept-1, SGLT-1 and SI expression levels at hatch, 3d, and 7 d ($p < 0.05$). At hatch, Pept-1, SGLT-1 and SI jejunal expression was enhanced by IOF of HMB in comparison to C ($p < 0.05$).

These studies demonstrate that IOF of HMB, ARG and proteins may enhance early growth by improving intestinal function and development, which may provide the nutrients and energy needed for more rapid growth.

“The biochemical and molecular effects of amnionic nutrient administration, “*in ovo* feeding” on intestinal development and function and carbohydrate metabolism in turkey embryos and poults.”

By

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BIOGRAPHY

In 1994, I graduated from North Carolina State University with a Bachelor of Science in Biochemistry and a Bachelor of Arts in Chemistry. While in college, I competed in the Miss America Scholarship Pageants. I was crowned Miss Johnston County 1991, Miss Greater Raleigh 1994, Miss North Carolina top-ten finalist and talent winner. In 2000, I completed a Master of Science Degree in Physiology in the Department of Zoology at North Carolina State University under the direction of Dr. Betty Black. In the spring of 2001, I began a doctoral program in Nutrition under the direction of Dr. Peter Ferket.

My full appreciation for the sciences did not develop until after graduating and entering the workforce. While at Abbott Laboratories, I played a role in the annual training of lab employees. I assisted in the training of hazardous material handling/disposal and laboratory techniques and testing. Education proved to be the key to open the doors of success and promotion. After two years of being employed at Abbott Laboratories, I decided to enter graduate school. I will forever be thankful, for this opportunity to obtain a higher education, in which was denied to my forefathers.

I pray to be a beacon of light for others and share this blessing with others! To whom much is given, much is expected.

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Dissertation:

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I.) Avian Development

A.) Developmental Classifications

Today there is a broad spectrum of developmental patterns observed in contemporary birds. Chicks of various avian species differ markedly in the relative degree of maturation in behavior, anatomy and physiology. The maturity of the postnatal chick is closely related to parental care and environment. Consequently, ornithologists categorize the avian neonate into altricial and precocial developmental types, based largely on the condition of the hatchling. Avian neonates defined as altricial hatch in an almost embryo-like state, eyes closed, lacking feathers and motor activity an inability to leave the nest and find food and feed alone. Precocial avian neonates resemble adult birds, are covered in down feathers and can fly and find food and feed alone from the first day of hatching. There is wide divergence among the avian neonate with hatchlings varying across the altricial-precocial spectrum. Galliforme hatchlings, chicken-like birds, which include grouse, pheasants, turkeys, quail, and domestic chicken, are classified as precocial. Most galliforme hatchling can self-feed, with only some hatchlings relying upon parental guidance for finding food and pointing out food sources during foraging.

As in birds, the mammalian neonate exhibit varying degrees of independence. As compared to birds, the mammalian embryo develops under fundamentally different

physiological and structural conditions. Birds are oviparous; the embryo is surrounded by a calcareous shell and is extruded from the body of the female bird. Thus the egg is laid before the young has developed. Conversely, viviparous animals give birth to a live neonate, after completion of gestational development in utero. Nutrient supply to the mammalian embryo is from the maternal placenta, which is continuous and almost unlimited. The avian embryo has a finite nutrient and energy supply within the closed egg. The intrauterine development of the mammalian embryo enables maternal thermoregulation, whereas the environment must be thermo-regulated for the incubation of the development of the avian embryo. Birds must incubate their eggs throughout the entire embryonic period and are thus they are bound to the nest for extended periods of time. In addition, lactation allows for the nourishment of the mammalian neonate relatively independent of the actual environmental conditions. The nourishment of the avian neonate is dependent upon food abundance and weather conditions.

B.) Extraembryonic Membranes

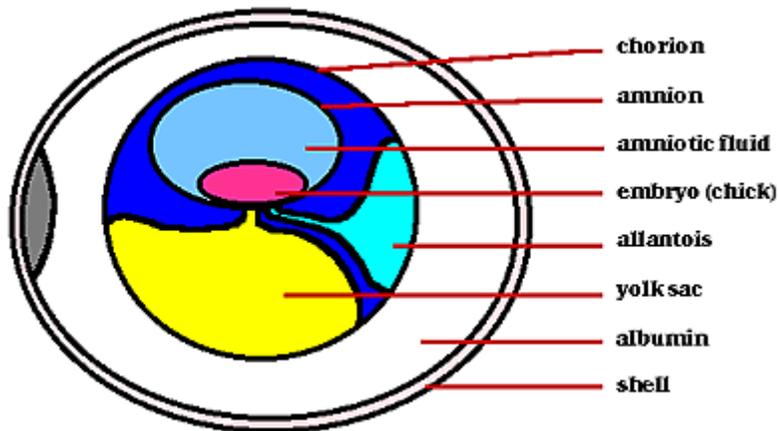


Figure 1.1 Extraembryonic membranes of a viable avian egg (From <http://www.biology.eku.edu/RITCHISO/avianreproduction.html>)

Starck and Ricklets (1998) provided a review of avian embryonic growth. During the course of embryonic development, the embryo increases in size by an exponential function, but the rate of growth decreases as it approaches hatching; which may be due to the reduced rate of nutrient and energy assimilation from the yolk, changes in the gas exchange relative to the oxygen requirements of the embryo and/or changes in the growth potential of the embryonic tissues. The supply of yolk to the developing embryo may also limit embryonic growth, particularly in late in embryonic development. Early in development, the yolk membrane expands and increases in surface area. Acquisition of yolk by the embryo occurs at the surface of the yolk membrane, and it occurs in proportion to the surface area of the yolk. The surface to volume ratio goes down as the

embryo increases in size at any given stage of development. As the embryo, grows the volume and therefore the yolk surface decreases, potentially placing a constraint on the availability of nutrients to the embryo.

All the nutrients and water needed for the developing embryo are stored in the egg. The pores within the egg shell allow for gaseous exchange of oxygen and carbon dioxide. The yolk sac stores nutrients (32% fat, 17% protein and 1% carbohydrates, and 50% water), while the albumin stores water (87%) and protein (11%) (Starck and Ricklets, 1998) . During early embryogenesis, the embryo develops extra embryonic membranes: the yolk sac for nutrient absorption, and the chorioallantois for gas exchange. The functional capacity of these exchange organs is determined by physiological transport mechanisms, the ultra-structural dimensions of transport barriers (gas-blood or yolk-blood barrier) and the overall size of the exchange surface. The yolk and chorioallantois size are limited by egg and thus embryonic growth and development are dependent upon both amount of energy stored within the egg as well as the ratio of the embryo's demand for nutrients and oxygen and the size dependent capacities of the exchange organs. Additionally, embryonic development may be negatively impacted by the accumulation of nitrogenous waste within the egg.

In addition to providing nutritional support, the yolk sac membrane is the primary site for blood vessel growth, blood cell formation, lymphatic cell proliferation and stem cells. After the first week of embryonic development, the absorptive epithelium and dense network of capillaries known as the vitelline vascular system, completely encloses the yolk. The absorptive surface of the yolk membrane does not form villi as in the gut,

absorption occurs across the surface area by diffusion into the vitelline vascular blood supply. Before hatching, the remnants of the yolk sac are internalized into the body cavity of the embryo and may serve as an energy reserve for the first days after hatching.

The extra embryonic membranes, the amnion and chorion are formed by folds of the extra embryonic ectoderm and somatic mesoderm that arise around the embryo. The amnion is the membrane enclosing the embryo. The inner layer of cells of the amnion secretes amniotic fluid, which bathes and protects the embryo. Just prior to hatch, the embryo consumes the amniotic fluid providing water and nutrients. The chorion is surrounds all embryonic structures and serves as a protective membrane. The allantois or allantoic sac serves as a depot for metabolic waste in the form of nontoxic, insoluble uric acid. The allantois increases in size as the embryo grows and eventually fuses with the chorion and becomes the chorio-allantoic membrane. The allantois works in conjunction with the chorion for gas exchange.

C.) Ontogeny of Energy Metabolism

In galliformes (chickens, turkey, quail), the embryonic rate of oxygen consumption increases rapidly during the first 80% of incubation. After this time, there is a plateau phase for several days or even a decline, until the embryo pips the egg when the metabolic rate again increases (Romanoff, 1967; Vleck et al., 1984, Vleck et al., 1979). This plateau in overall growth maybe related to the time required for different levels of tissue maturation prior to hatching. Precocial embryo mass can be obtained at about 80% of the incubation time.

The metabolically active tissues of the egg include the embryo and extra embryonic membranes (yolk sac, chorioallantois, and amnion). In most avian species, the absolute energy consumption of these membranes is relatively small in comparison to the energy cost of the embryo. In the chicken egg, the chorioallantois membrane achieves maximum oxygen consumption on about day 12 of the 21-day incubation period, when the embryo is only 16% of its final size (Ar et al., 1987a). The total oxygen consumption of the extra embryonic membranes during incubation is only about 10% of the total oxygen consumed by the chick embryo (Romanoff, 1967).

Metabolic energy use within an egg can be measured by indirect or direct calorimetry as oxygen consumption or carbon dioxide production throughout the incubation period. In addition, total energy catabolism can be measured as the difference in energy content of the egg at the time of laying and the energy content of the egg at the time of hatching. Both methodologies produce similar results (Vleck et al. 1984), indicating that the yolk supplies the energy needed to fuel embryonic development due to its gradual reduction in mass and energy content throughout embryonic development (Romanoff, 1967; Vleck et al., 1984).

Eggs of precocial species have longer incubation periods and have a 30% higher energy cost of development, and produce hatchlings with greater tissue mass than altricial species (Vleck and Vleck, 1987). Therefore the precocial species, such as the galliformes, have a higher energy cost due to a larger embryo mass during a greater proportion of the incubation than altricial species. Thus, the maintenance cost for a precocial embryo is greater than for an altricial embryo of the same mass (Vleck et al.,

1980; Hoyt, 1987). Therefore the egg of the precocial embryo must provide a higher amount of energy due to high body mass and long incubation period. In the precocial embryo there is an overall 50% transfer of energy from the egg to the embryo during incubation (Vleck et al., 1980; Hoyt, 1987).

There is a marked increase in the mass-specific metabolic rate after pipping of the eggshell, despite decreases in the relative growth rate (Bucher, 1983; Bucher and Bartholomew, 1986; Matsunaga et al., 1989; Bucher et al., 1990; Kuroda et al., 1990; Mathiu et al., 1994). The increases in metabolic rate after pipping and hatching have been attributed both to the removal of physical constraints on oxygen consumption and increases on metabolic demand. Constraints include the shell and membranes as a diffusion barrier (Paganelli and Rahn, 1984; Mathiu et al., 1994) and physical confinement within the shell and its effect on ventilatory capacity (Mathiu et al., 1992). Energy demand increases because of the needed activity during and after hatching and increased metabolic costs of thermoregulation and tissue maturation. The energy cost of hatching increases with the work required to break out of the shell, from the time of pipping to the actual emergence from the shell. In the galliformes, hatching appears to be both fast and energetically inexpensive (Vleck et al., 1984).

D.) Embryonic Metabolism and Energy Use

The mammalian fetus is provided with a constant supply of plasma glucose by maternal blood supply, this monosaccharide can then be used as an energy source for growth and development. After birth, the mammalian neonate is presented with a high fat, low carbohydrate diet (milk) and forms much of the glucose endogenously by

gluconeogenesis. The hepatic enzymes involved in gluconeogenesis have increased activity during this period and decrease in activity after the suckling period (Ballard and Oliver, 1965; Yeung et. al, 1967).

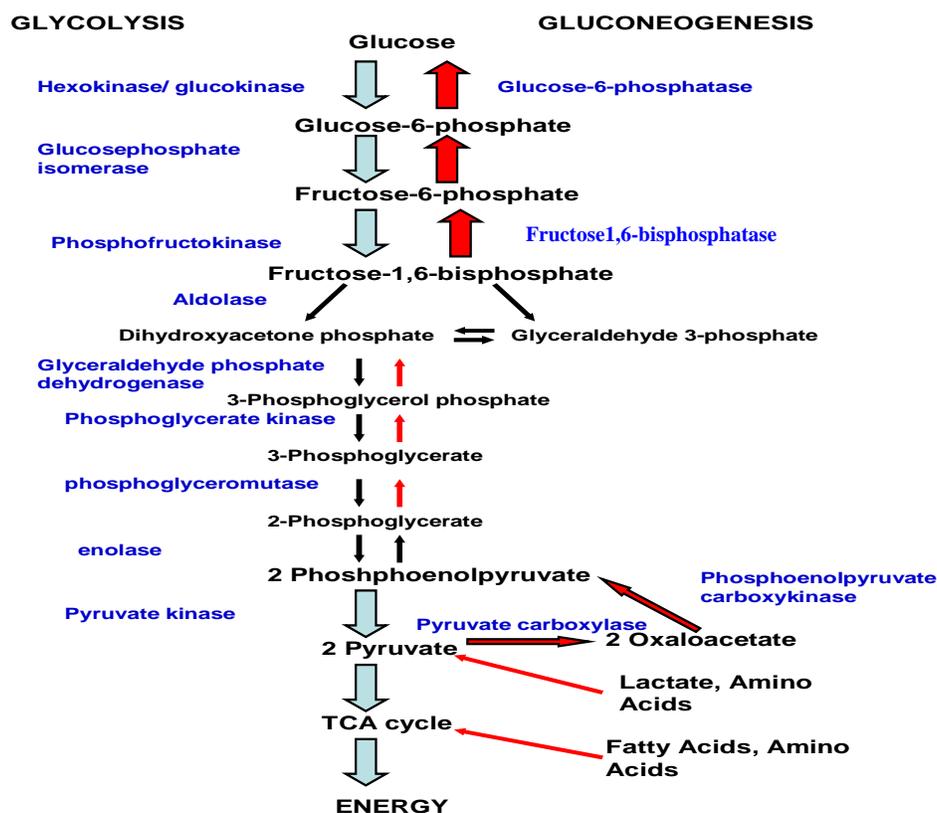
In contrast to mammals, the developing chick embryo must rely upon the nutrients provided by the egg independent of maternal influence. Nutrient transfer from the mother to the embryo is completed before the egg is laid. Thus, the egg contains all of the nutrients needed for the growth and development of the embryo. The only materials exchanged with the environment are water (vapor), oxygen and carbon dioxide. Therefore, the avian embryo is an ideal model for studying nutrient utilization during embryonic development.

In the domestic chicken, nutrients are stored in the egg as yolk (32% fat, 17% protein, 1% carbohydrates, and 50% water) and albumin (87% water and 11% protein) (Mehner, 1983; Starck and Ricklefs, 1998), so the diet of the chick embryo consists of fat and protein, with only traces of carbohydrate. Despite having little carbohydrate, glucose is the primary source of energy needed for embryonic development and growth, in addition to serving as energy these sugars are important components of the cellular membrane, glycoproteins and glycolipids.

Hepatic gluconeogenesis is the primary mechanism for glucose production in the avian embryo and neonate (Romanoff, 1967). Gluconeogenesis in the liver (primary) and kidney (minor) is the endogenous production of new glucose from three-carbon and four carbon precursors, including amino acids, protein and lipid substrates by way of the chemical process gluconeogenesis. The primary gluconeogenic precursors are lactate,

produced from glycolysis in skeletal muscle and erythrocytes; amino acids, and glycerol, derived from the catabolism of lipids. The gluconeogenic pathway is similar to glycolysis in reverse. The three reversible reactions of glycolysis are by-passed by enzymes specific for gluconeogenesis. The remaining seven reactions of gluconeogenesis are catalyzed by glycolytic enzymes, simply running in the reverse direction. There are four key reactions and enzymes of gluconeogenesis that differ from glycolysis. Pyruvate is converted into oxaloacetate by pyruvate carboxylase. The conversion of oxaloacetate is catalyzed by the enzyme phosphoenolpyruvate carboxykinase (PEPCK). The enzyme fructose-1,6-bisphosphatase is responsible for the next key gluconeogenic step, the conversion of fructose-1,6-bisphosphate to fructose-1-phosphate. The final gluconeogenic step is the conversion of glucose-6-phosphate to glucose, which can be stored as glycogen or liberated into the circulation for energy.

Figure 1.2 The sequential biochemical steps of hepatic gluconeogenesis and glycolysis



The gluconeogenic pathways are highly active and decrease after hatching when the chicks are fed a high carbohydrate diet. Earlier studies have demonstrated that the hepatic gluconeogenic enzymes, fructose-1,6-diphosphatase and glucose-6-phosphatase decreased from the 18th day of incubation to about 20 days after hatching and then remained more or less constant up to 6 months of age in chickens (Thind et. al, 1966). Nelson et al. (1966) and Wallace and Newsholme (1967) also found fructose-1,6-

diphosphatase and glucose-6-phosphatase to be less active in adult birds than in embryos. The activity of glucognogenic enzymes increases as the chick embryo developments and it reaches a maximum level of activity at around the time of hatching (Okuno et al., 1964; Felicioli etl., 1967; Sheid and Hirschberg, 1967). In contrast, the activity of gluconeogenic enzymes is very low or absent in rat and sheep fetal liver (Ballard and Oliver, 1963, 1965) and becomes active after birth in many mammalian species (Dawkins, 1963; Ballard and Hanson, 1967; Hahn and Greenberg, 1968).

Endogenously produced glucose is stored in the form of the polymer glycogen in the liver and/or muscles and functions as the body's carbohydrate reserve. Endogenously produced glucose is phosphorylated to glucose-6-phosphate by hexokinase in the muscles, and by glucokinase in the liver. Glucose-6-phosphate is isomerized to glucose-1-phosphate by phosphoglucomutase. Glucose-1-phosphate reacts with uridine triphosphate (UTP) in a reversible reaction catalyzed by uridine diphosphate (UDP)-glucose. UDP-glucose donates a glucosyl residue to the nonreducing end of glycogen branch (n+1). The enzyme glycogen synthase catalyzes each reaction of the addition of a glucosyl residue to a growing glycogen branch within the muscle or liver.

Glycogen is released from the liver into the blood stream when energy is needed by the tissues of the body by the chemical process, glycogenolysis. Liver glycogen can be made available to other tissues by glycogenolysis (the chemical process by which glucose is freed from glycogen) and glucose release. When systemic glucose levels are low, the hormone glucagon is released from the pancreas. In the liver, glucagon exerts an

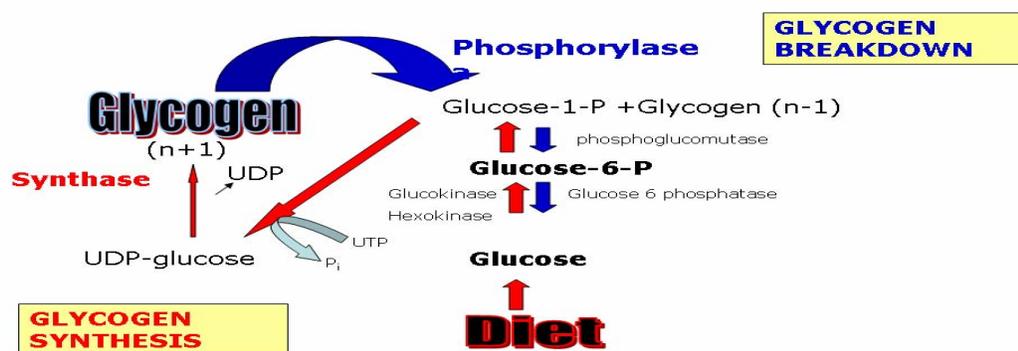
immediate and profound glycogenolytic effect through activation of the enzyme, glycogen phosphorylase, and inactivation of glycogen synthase (Figure 1.1).

Glycogen phosphorylase is present in two interconvertible forms; the active form phosphorylase *a*, and the inactive form phosphorylase *b*. Glycogen phosphorylase consists of two identical polypeptide dimers, which contain a serine residue at position 14 on each dimer. Upon activation these serine residues are phosphorylated, resulting in glycogen phosphorylase *a*. Phosphorylase *a* acts to liberate glucose residues from their storage in glycogen cleavage and phosphorylating a residue from the nonreducing end of glycogen chains in the process of glycogenolysis. Hepatic glucose-1-phosphate is catalyzed by phosphoglucomutase to produce glucose-6-phosphate. Lastly, hepatic glucose-6-phosphate is then converted to glucose by the enzyme glucose-6-phosphatase. Glucose is then released from the liver into the circulation and made available to other tissues and organs. Thus the liver is the supply organ that stores incoming energy as glycogen and provides glucose the primary fuel to the body in times of need.

Muscle tissues lack the enzyme glucose-6-phosphatase, and thus can not manufacture and/or release glucose from glucose-6-phosphate. Muscle glycogen is present in skeletal, smooth and cardiac muscles; but unlike liver glycogen, muscle glycogen can only be utilized by the muscles. In these motor tissues, glycogen serves as fuel that can be accessed more rapidly than other energy stores in active and fast muscles. Carbohydrate (4 kcal/g) in the form of glycogen accounts for a very small amount of the total energy reserves in the body. However this small but readily available energy reserve is critical for support of central nervous system metabolism and for short burst of

energy needed by muscles. Approximately one fourth of the total body glycogen reserves are stored in the liver, with the balance stored in the muscle mass. In addition, skeletal muscles are important for thermogenesis in the avian neonate and developing embryo.

Figure 1.3 The sequential biochemical steps of glycogen synthesis and glycogenolysis



Glycogen first appears in the chick embryo on the 6th day of incubation (Dalton, 1937). Liver glycogen has been observed to increase from 7mg/g of tissue at day 10 of incubation to 26mg/g at day 19 of incubation (Ballard and Oliver, 1963). Ballard and Oliver also demonstrated that the specific activity of glycogenic enzymes increased as incubation progressed. At the end of incubation, the stored glycogen is rapidly mobilized (Muglia and Masseulli, 1934; Gill, 1938; Freeman, 1965, 1969), falling from 19mg/g in the liver of embryos after 18.5 days of incubation to 1.6mg/g in the liver 1 day post-hatch (Freeman, 1965). Glycogen stores are rapidly sequestered to provide energy needed during the hatching process and subsequent growth and development.

Glycogen reserves in the avian embryo provide the critical energy needed for emergence from the egg during the last quarter of incubation. In turkeys, extensive embryonic mortality occurs toward the end of the incubation period when hatching-related events, such as pipping of the egg membrane and shell, beginning of pulmonary respiration, and the actual emergence of the hatching occur (Cherms, 1981; Christensen et al., 1982). Glycogen reserves in the chick embryo is significantly depleted during the perihatch period in order to meet the high energy demand during the process of emergence (Freeman, 1969; Freeman and Manning, 1971).

The pectoral muscle plays a significant role in the hatching process in the chick (George and Iype, 1963; Bakhuis, 1974). In chick embryos, the limbs extended and flexed, then return to the original resting position during every burst of hatching activity (Bakhius, 1974). During the perihatch period, pectoralis muscle glycogen is depleted due to the considerable amount of glycogen utilized by the muscles actively involved in hatching. Additionally, hepatic glycogen reserves are depleted due to carbohydrate utilization for muscular activity during the hatching process. In addition to the pectoralis muscle, the pipping muscle has been found to utilize a large amount of glycogen concurrently (John et al., 1987).

Hatching is the end point for the embryo and the beginning for the chick. During shell emergence, the hatchling's metabolic systems must function at a greatly enhanced rate. Upon hatching, turkey poults have a greater hepatic glycogen phosphorylase/glycogen synthetase ratio up to day-6 post-hatch (Rosebrough et al. 1979). Therefore during the first week post-hatch, poults have a greater propensity for glycogen

breakdown instead of glycogen synthesis. Christensen et al. (2001) demonstrated that glycogen stores decreased to a very low level at hatching and begins to increase in the neonatal chick offered full access to oxygen so it can fully use the lipid stores of the yolk sac and in the glycolytic muscles (Rosebrough et al., 1978a; Rosebrough et al., 1978b). Glycolysis is critical for survival of young hatchlings during the early post-hatch period when poults are adapting to eat an external diet. Poults undergo a metabolic shift in which there is a reduction in the rate of gluconeogenesis and increased glycogen synthesis due to increased specific activity of glycogen synthetase activity (Rosebrough et al. 1979). Excessive depletion of glycogen reserves during hatch adversely affect growth (Donaldson and Christensen, 1992), which may lead to increased early mortality. More recent studies have demonstrated that turkey embryos and hatchlings with elevated blood glucose levels had elevated glycogen concentrations and grew faster than those with depressed blood glucose levels (Christensen et al., 2000).

E.) Regulation of Energy Metabolism

Immediately after hatch, poults must switch from an “in ovo diet” comprising mainly of lipids and protein to an external diet comprising mainly of carbohydrates from corn and other grains. Dietary carbohydrates are broken down into various hexoses within the gastrointestinal tract, the most important of which are glucose and fructose. These hexoses are transported across the epithelium of the intestine into the blood stream. The monosaccharides travel through the blood stream and stored as hepatic and muscle glycogen.

Glycogen metabolism is controlled by the hormones insulin and glucagon. Insulin and glucagons, released from the islets of the pancreas, are powerful regulators of carbohydrate metabolism. Together they coordinate the flow and metabolic fate of endogenous glucose, free fatty acids, amino acids, and other substrates to ensure that energy needs are met in the basal state and during activity. In addition, they coordinate the efficient deposition of the nutrient input from meals. They accomplish these functions primarily by actions on the liver, muscle mass and adipose tissue. Insulin and glucagons are often secreted and act in a reciprocal fashion: when one is needed, the other is not.

Insulin is released in response to elevated blood sugar levels, such as after the consumption of a carbohydrate-rich meal. Within one minute of the release of insulin, glucose transport into muscles and adipose cells is increased up to twenty fold by activation of a glucose carrier system in the plasma membrane (Berne and Levey, 1998). Glucose is the most important stimulant of insulin secretion, but insulin secretion is also stimulated by amino acids that result from digestion of protein in a meal. The basic amino acids, arginine and lysine, are the most potent stimulants; leucine, alanine and others contribute modestly to this effect. Glucose and amino acids are synergistic stimulators of insulin release, such that the plasma insulin rise that follows a meal represents more than the additive effect of its carbohydrate and protein content. Insulin stimulates glucose oxidation and storage as glucose polymer, glycogen, while inhibiting endogenous glucose production via gluconeogenesis (Figure 1.2). Therefore, insulin either lowers the basal circulating glucose levels and/or inhibits the release of stored

glucose. Insulin reduces plasma glucose levels by rapidly recruiting the glucose transporter, GLUT-4, that is specifically expressed in muscle and adipose tissue. In the liver, extra cellular glucose is transported within the hepatocytes by the GLUT-2 transporter. Hepatic glucokinase (Figure 1.2) phosphorylates incoming glucose to glucose-6-phosphate. Insulin then promotes storage of glucose as glycogen by activating the glycogen synthase enzyme complex. In an independent manner, insulin also facilitates the cellular uptake of amino acids that utilizes an amino acid transporter system. After glucose and amino acids are transported into the tissues, insulin then directs the storage or usage of these substances, in which glucose is converted to glycogen or converted by glycolysis to pyruvate and lactate. Insulin stimulates amino acid uptake, and initiates several pathways that lead to protein synthesis. Synthesis of specific proteins, such as albumin, and a number of enzymes from the amino acids, is selectively enhanced, while inhibition of proteolytic and lipolytic enzymes protects protein and lipid stores. Hence, insulin is an anabolic hormone that enhances glycogen reserves, muscle protein deposition and weight gain. Finally, insulin stimulates glucose oxidation and storage, while simultaneously inhibiting hepatic gluconeogenesis. Insulin inhibits gluconeogenesis by decreasing the hepatic uptake of precursor amino acids and by reduction of the activity of the hepatic gluconeogenic enzymes, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-biphosphatase and glucose-6-phosphatase.

F.) Endocrine Control of Development

Glucose and arginine are both potent insulin secretagogues and have been shown to potentiate each others effect both *in vitro* and *in vivo* (Floyd et al, 1970; Efendic et al., 1971; Levin et al., 1971; Pagliara et al., 1974; Gerich et al., 1974). Upon the release of insulin from the islets of Langerhans of the pancreas, glucose and amino acids are transported into the tissues (muscles) where they are either utilized or stored as energy. Thus insulin is known as the “hormone of feasting”, in which it enhances muscle and fat deposition and glycogen reserves.

In addition to its role in carbohydrate metabolism, insulin is involved in the growth and early development of the avian embryo. Studies have demonstrated that exogenous insulin increased the growth (weight, protein, and creatine kinase) between 2 and 5 days of embryonic development (de Pablo et al., 1985; Girbau et al., 1987). Evidence that endogenous insulin exerts this effect comes from the ability of antisera to insulin to depress weight and protein in embryonic growth (de Pablo et al., 1985). Additionally, antisera to the insulin receptor reduces chick embryo growth (bodyweight, DNA, RNA, and protein), suggesting that insulin mediates its effect through the insulin receptors. Based on evidence by immunohistochemistry, insulin is present very early in the embryonic development of the chick embryo (de Pablo et al., 1982), with specific insulin-binding sites in the brain, heart and liver of the chick embryo (Bassas et al., 1987).

Additionally, insulin and growth hormone play a significant role in regulating human fetal development and growth. Growth must be carefully regulated as the human

fetus transforms from a single undifferentiated cell into a large complex organism.

During the last half of gestation, the human fetus grows from about 45g to about 3500g (Berne and Levey, 1998). During this time, fetal growth is dependent on the hormones insulin and thyroxine (T_3).

As in all other vertebrate species, thyroid hormones play important roles in the development of birds. Most of the effects of thyroid hormones are mediated through the action of triiodothyronine (T_3). T_3 effects are mediated through hormone binding to the nuclear thyroid hormone receptors, activation of gene expression, and resultant changes in protein production. Nuclear T_3 receptors are the key to thyroid hormone responsiveness in the target tissues in the developing chickens. In chicken embryos, nuclear T_3 receptors have been detected by labeled hormone-binding studies during the phase of development before the thyroid gland is organized into follicles and is secreting appreciable amounts of hormones. T_3 nuclear receptors are present in embryonic chicken liver by day 9 and in the brain by day 7 of the 21-day incubation period (Bellabarba and Lehoux, 1981; Haidar and Sarkar, 1984). T_3 plasma levels are present at very low concentrations in the embryonic chicken (Thommes and Hylka, 1978). It is unclear whether these circulating hormones are from the small unorganized thyroid gland (Trunnell and Wade, 1955; Daugeras et al., 1976; McNichols and McNabb, 1988) or they are maternal hormones stored in the yolk and absorbed by the embryo. Studies have shown that thyroid hormones are present in the avian egg and yolk and that some hormone enters the embryo (Prati et al., 1992; Wilson and McNabb, 1995).

In general, thyroid hormones, specifically T_3 , act in a permissive or interactive way with other hormones in promoting growth. Thyroid hormones are needed for normal body growth in birds. Thyroid hormones influence neural, skeletal and muscular system development early in embryonic life (Fell and Mellanby, 1955; Lawson 1961; Hall 1973). In addition, thyroid hormones play a critical role in cell differentiation and maturation in a number of tissues, such as lung maturation (Wittmann et al., 1983, 1984), intestinal digestive enzyme production (Black, 1978; Black and Moog, 1978), muscle maturation and contractile protein isoforms (Konigsberg 1958; King and King 1973, 1976, 1978; King et al., 1987). Some of these influences occur during embryonic life (i.e. lung and intestinal development), while others are primarily post-hatch (i.e. muscle development). In addition, thyroid hormones play a critical role in controlling metabolism, thermoregulation, and reproduction throughout the post-hatch life of birds.

The normal growth of birds after hatch requires growth hormone produced from the pituitary gland (King and Scanes 1986). Circulating concentrations of growth hormone are low in embryos and begin to rise just prior to hatch. In chickens, circulating concentrations of growth hormone rise and peak during the early phase of the post-hatch growth and then declines during the mid-growth phase of post-hatch development.

The effects of growth hormone are mediated by growth factors produced locally within the tissues as well as being produced and released into the circulation by the liver. Several lines of evidence indicate that growth hormone exerts much of its effects on growth by increasing insulin-like growth factor-1 (IGF1). Plasma concentrations of both growth hormone and IGF-1 are high during post-hatch growth. In addition, to

stimulating growth, growth hormone exerts both stimulatory and inhibitory effects on lipid metabolism (Campbell and Scanes 1985, 1987), modulates immune system development, and increases plasma T_3 levels during early post-hatch life.

In birds and mammals, there are two forms of IGF's (IGF1 and IGF2) that stimulate the proliferation and differentiation of a number of cells (fibroblasts, myoblasts, enterocytes) in avian embryos. Only one IGF receptor is present in birds and both IGF1 and IGF2 exert their effects through this receptor (Bassas et al., 1988; Kallinocos et al., 1990; Duclos et al., 1991; Armstrong and Hogg, 1992). These IGFs stimulate overall growth of the early chick embryo and growth of specific tissues and organs. IGF's have been shown to be important for muscle cell proliferation and differentiation and growth (Taylor et al., 2001; Langley et al., 2002; Joulia et al., 2003). IGF1 produced locally within the tissues can exert autocrine and/or paracrine effects, thus causing locally mediated growth.

During the early post-hatch period, there are parallel increases in liver growth hormone receptor expression and plasma IGF-I, suggesting that the liver has increased sensitivity to growth hormone during this developmental period (Burnside and Cogburn, 1992). Conversely, hepatic IGF1 receptor mRNA levels decrease between 1-4 weeks post-hatch in chickens (Armstrong and Hogg, 1992), suggesting that the liver becomes less sensitive to IGF1 within this period of high IGF1 production. During embryonic development, IGF1 steadily rises from day 6 to peak at day 15 of incubation, and then declines to low levels at hatch (Robcis et al., 1991). However, IGF1 mRNA is absent in the embryonic liver (Burnside and Cogburn, 1992), thus suggesting that the liver is not

the source of embryonic IGF1. The embryonic development of target tissues show marked tissue specific changes in IGF1 receptor expression: brain peak at day 6; heart peak day 3; limb buds peak day 6 (Bassas et al., 1985, 1988). These differences in timing may play a critical role in the variable timing of differentiation of these tissues during development.

Pro-insulin, the precursor of insulin has high homology in amino acid sequence and three-dimensional structure to insulin and IGF-I (Rinderknecht et al., 1978), thus pro-insulin may act in a similar manner to IGF-I and act as a growth factor. Early studies have shown that pro-insulin stimulates chick fibroblasts growth during embryonic development (Nissley et al., 1976). Administration of pro-insulin and IGF-I *in vitro* stimulated growth in a rat small intestinal crypt-like cell line, IEC-6, three-fold above the controls (Jehle et al., 1999), suggesting that pro-insulin stimulates growth of the small intestinal crypt cells through the IGF family receptors and may play a physiological role in the regulation of intestinal epithelial cell proliferation. Other studies have also demonstrated the importance of IGF's and insulin in gastrointestinal development and especially proliferation and maturation of the enterocytes (Laburthe et al, 1988, Schober et al, 1990, Odle et al, 1996, Menard et al, 1999; Georgiev et al., 2003). In newborn calves, mRNAs for IGF-I and IGF-II, for receptors of growth hormone, IGF-I (IGF-IR), IGF-II (IGF-IIR) and insulin (IR), and for IGF binding proteins 1-3 are found in high density in the ileum of the small intestine (Pfaffl et al., 2002). Therefore IGF's and insulin ingested in colostrum (Odle et al 1996; Blum and Baumrucker 2002), IGF's produced in the gastrointestinal wall, and IGF's and insulin circulating in blood plasma

can affect gastrointestinal morphology and function after binding to their respective mucosal receptors (Lund, 1994; Fholenhag et al, 1997; Jehle et al, 1999; Blum and Baumrucker 2002; Georgiev et al., 2003).

Nutrient supply controls overall body and muscle growth and may do so directly or indirectly through its influence on regulatory factors, such as IGF-I and IGF-II and insulin (Thissen et al., 1994; Guernec et al., 2003). Under fasting conditions, circulating IGF-I and IGF-II levels are reduced as well as hepatic IGF-I mRNA levels (Beccavin et al., 2001; Guernec et al., 2003), which may hinder muscle and gastrointestinal development and early growth.

Mammalian milk and colostrum contain IGF-I and IGF-II which may play a vital role in regulating gastrointestinal development, functioning and glucose uptake during the suckling period (Donovan and Rosenfeld, 1991). Parenteral administration of IGF-I increases jejunal glucose absorption in adult rats (Zhang et al., 1995), while oral IGF-I administration increases jejunal uptake of the non-hydrolysable form of glucose, 3-0-methylglucose in neonatal pigs (Alexander and Carey, 1999). Studies by Lane et al (2002) demonstrated that rat pups given a milk replacer supplemented with IGF-I had increased jejunal uptake of glucose, increased plasma glucose levels and increased expression of the jejunal glucose transporters above the controls. Thus IGF's may play a critical role in regulating tissue maturation, development, function and somatic growth by indirect and direct mechanisms.

G.) Avian Enteric physiology and development

Precocial hatchlings have a relative gut size (percentage of neonate mass) ranging from 6% to 10%, while altricial hatchlings range between 8% to 15% (Portmann and Vischer, 1942; Portmann 1955; Neff, 1972). Neff (1972) describes a 15- to 37-fold increase of intestinal mass as precocial birds' transition from neonate to adult, whereas altricial birds increase intestinal mass 7- to 19-fold. In the young galliformes, the gastrointestinal tract grows at a much greater rate than other organs, especially during the first 7-10 days (Baranylova and Holman, 1976; Lilja et al., 1985). Additionally, the patterns of intestinal growth appear to be correlated with body growth rate (i.e. rapidly growing birds exhibit rapid early development of digestive organs; Lilja et al 1985, Konarzewski et al 1989). Therefore, it appears that selection has created changes in the patterns of organ growth, which result in high rates of total body growth (Lilja et al., 1985). Smith et al. (1990) investigated the relationship between accelerated weight gain and major changes in intestinal function using 4-wk-old female chickens from three lines exposed to different degrees of genetic selection pressure or rapid growth rate and feed conversion efficiency. Chickens intensively selected for growth had a 40% increase in villus surface area, crypt size and enterocyte migration rate as compared to chickens subjected to relaxed selection. Smith et al. (1990) suggested that the increases in villus surface area enable faster growing birds to sustain increased demands for nutrient digestion, absorption and assimilation

The major functions of the gastrointestinal tract (GIT) are hydrolyzing, transporting and absorbing nutrients. The GIT presents the first barrier to metabolism of

dietary nutrients, and the major physiological functions of the gastrointestinal system are to digest foodstuffs and absorb nutrient molecules into the bloodstream. The GIT carries out these functions by modulating enteric motility, secretions, digestion and absorption. Motility refers to the movements of the GIT to as to mix and convey the contents along the length of the GIT. Secretion refers to the processes by which the accessory glands, liver and pancreas, produce and release secretions into the GIT that aid in the digestion of food. Digestion is the process by which macronutrients are chemically degraded into smaller molecules. Absorption refers to the process by which nutrient molecules are absorbed by the cells that line the GIT and enter the blood.

The typical diet of the avian neonate consists of a mixture of carbohydrates and proteins. These large macronutrients contain large insoluble organic molecules that must be hydrolyzed into small absorbable molecules. Typical dietary carbohydrates are composed of long chains of simple sugars called polysaccharides. Starches, as found in grain and potatoes, are polysaccharide chains composed of linked glucose molecules. Upon digestion, polysaccharides are hydrolyzed into disaccharides (two simple sugars joined together) and/or monosaccharides. Sucrose and maltose are typical disaccharides found within the diet. Upon hydrolysis, sucrose generates the monosaccharides fructose and glucose, while the hydrolysis of maltose generates two glucose molecules. Glucose is the most important metabolic “fuel” for the body.

Dietary proteins are composed of long peptide chains, with each peptide chain consisting of amino acid residues linked by peptide bonds. These polypeptide chains have very complex three-dimensional structures. The first step in protein digestion is the

disruption of the three-dimensional structure, so that the proteolytic enzymes can attack the peptide chain. Hydrolysis of the polypeptide chain produces dipeptides, tripeptides and free amino acids, which are absorbed across the intestinal epithelium into the portal blood. Free amino acids and small peptide chains can provide the building blocks needed for rapidly growing and developing tissues.

Hence, protein is a very essential dietary component for the avian neonate. Soybean meal provides the major dietary protein in the diet of young hatchlings. Young growing turkey poults need at least a 26% protein diet that supplies all of the essential amino acids needed for growth (i.e. isoleucine, leucine, phenylalanine, valine, methionine, lysine, arginine, threonine, and tryptophan) (Klasing, 1998). Essential amino acids must be provided by the diet due to the body's inability to synthesize them at adequate levels to support growth and development. Histidine, glycine, and proline can be endogenously produced, but at a rate that is insufficient to meet dietary requirements. Thus, histidine and glycine can also be considered to be essential amino acids in growing birds. Amino acids that can be endogenously produced are defined as nonessential amino acids. The production of nonessential amino acids is dependent upon an adequate supply of dietary nitrogen.

Dietary carbohydrates provide glucose, the primary source of energy needed under all physiological conditions. In addition to serving as energy, these sugars are important components of the cellular membrane, glycoproteins, glycolipids and glycopeptides. Blood sugar below physiological levels is accompanied by exhaustion, fatigue, and impaired neural and physical functioning. This amount if not replaced could

not sustain life. Thus, dietary carbohydrates must be consumed to replenish the body's carbohydrate stores, hepatic and muscle glycogen. Corn provides the main source of carbohydrates in the diet of young hatchlings.

Upon feed consumption, digesta transits from the mouth, esophagus, crop, proventriculus, gizzard, small and large intestine. Once in the mouth, feed is moistened by mucous secretions of the mouth. The bolus of feed enters the crop or it transits directly to the proventriculus and gizzard. The crop acts as a temporary storage organ when the gizzard contains feed. Due to their high metabolic rate, birds require a continuous supply of food material to the digestive tract, which is met by the presence of stored food in the crop. Crop function is less critical when food is always available as in commercial broiler and turkey production facilities.

Upon emptying of the gizzard, ingested feed transits from the crop into the proventriculus. The main function of the proventriculus is the production of gastric juice and the propulsion of juice and food into the gizzard. The proventriculus is similar to the glandular pyloric region of the stomach in mammals. The major secretions of the proventriculus are hydrochloric acid (HCl), pepsins, gastrin and bicarbonate. Upon the entrance of food into the proventriculus, gastrin is released from the G-cells, which signals the release of HCl from the parietal cells. Subsequently, pepsinogen (the proenzyme form of pepsin) from the chief cells is activated by cleavage of acid-labile linkages to produce pepsin. The conversion of pepsinogen to pepsin increases as the pH decreases in the proventriculus-gizzard. Pepsin, an endopeptidase, is a very potent protease that may digest as much of 20% of the protein present in a meal into smaller

peptides that can be further digested by exopeptidases in the small intestine, such as trypsin and chymotrypsin. Therefore, dietary protein digestion begins within the proventriculus.

The proventriculus empties the contents (now called chyme) into the gizzard. In the gizzard, the chyme is mixed and ground by the contractions of the gizzard, assisted by the presence of small stones or grit to ensure that peptide bonds are sufficiently exposed to proteolytic enzyme activity. Repeated oscillations of chyme between the gizzard and proventriculus assist in the digestion process. The retention time of food in the gizzard is variable; finely ground chyme passes to the duodenum within minutes, while hard grains may remain in the gizzard for several hours.

Once in the duodenum, luminal digestion begins as chyme is mixed by the peristaltic action of the duodenum with pancreatic secretions. The pancreatic secretions contains enzymes that breakdown dietary sugars and starches into simple sugars, monosaccharides, fats into fatty acids and glycerol, and peptides into free amino acids and short peptide chains. The proteases of pancreatic juice are secreted in the inactive form as trypsinogen, chymotrypsinogen, and procarboxypeptidase. Trypsinogen is activated to trypsin, by enterokinase which is secreted by the duodenal mucosa. Trypsin then activates chymotrypsinogen, and procarboxypeptidase to chymotrypsin and carboxypeptidase, respectively. Pancreatic amylase cleaves starch molecules into oligosaccharides. In the avian species, carbohydrate digestion begins within the duodenum of the small intestine. In addition, pancreatic secretions contain lipases which

hydrolyze dietary lipids. If pancreatic enzymes are absent, the absorption of lipids, proteins and carbohydrates is abnormal.

Additionally, the liver secretes bile into the small intestine through the bile duct. Bile emulsifies large fat globules into small droplets so as to facilitate lypolysis in the small intestine can act upon. Final digestion and absorption occurs within the jejunum, the second portion of the small intestine. Thus partially hydrolyzed feedstuffs from the duodenum are subjected to the final proteolytic digestion of the brush border enzymes present within epithelium cells, enterocytes of the of the jejunal epithelium. The jejunum contains finger-like projections known as villi, which project into the lumen. Cells covering the villi are known as enterocytes. Enterocytes are specialized for the digestion and transport of nutrients. Each enterocyte is covered with microvilli, which are very small projections into the lumen that greatly increases the absorptive surface area. The absorptive area of the jejuna is also known as the brush border membrane. The oligosaccharidases and disaccharidases of the brush border membrane are important for hydrolyzing oligosaccharides and dissacharides into one six carbon units known as monosaccharides.

The major brush border disaccharidases are sucrase, which splits the disaccharides sucrose into glucose and fructose, and maltase, which splits maltose into two glucose molecules. These disaccharidases appear complexed as sucrase-isomaltase within the brush border membrane. Brush border peptidases cleave peptide bonds at the amino terminus of a peptide, releasing a free amino acid. The peptidase, leucine aminopeptidase (LAP,) cleaves small peptides at leucine residues, yielding free amino

acids and shorter peptide chains. The digestive products, glucose, fructose, amino acids, dipeptides and tri-peptides are then transported across the jejunal epithelium by specific nutrient transporters. Food materials which escape enzymatic breakdown along the tract pass into the ceecal tubes and are subjected to bacterial breakdown.

Disaccharidases are present in the 12-day old chick embryo in low concentrations and show marked increase during the last few days of incubation, at a time when there is elongation and increase in the number of jejunal villi (Bell, 1971; Sell et. al. 1989, Uni et al., 2000, 2003). Siddons (1969) and Matsushita (1985) found that the activities of maltase and sucrase increased rapidly from Days 19 to 21 of incubation and 21 days after chicks hatched, before reaching a plateau. The newly hatched chick therefore possesses marked maltase activity and high sucrase activity due to the sucrase-isomaltase complex (Siddons, 1970). The high maltase activity present in the intestine of the young chick ensures that rapid breakdown of carbohydrate occurs in the young hatchling and develops in a way that allows utilization of dietary carbohydrate with the appearance of grains and starches in the diet.

Apart from digestion, some of the brush border enzymes may be involved in other functions, including nutrient transport from the intestine, reception of signals into cells and regulation of cell growth and differentiation. The activity of the brush border enzymes is dependent upon age and diet. Dietary carbohydrate concentrations elicit a response in avian intestinal disaccharidase activity. Lei and Slinger (1970) observed an increase in sucrase, but not in maltase activity of 2-week old chicks fed a high starch, low fat diet as compared to those fed a low starch, high fat diet. Siddons (1972) found that

the activities of maltase, sucrase and palatinase (a type of maltase in the intestinal mucosa that hydrolyzes palatinose, a structural isomer of sucrose with α 1-6 and α 1-4 linkages) were much greater when 16-day old chicks were fed a high carbohydrate than chicks fed a low carbohydrate diet. Rodriguez and Rodriguez (1982) reported that the specific activities of maltase and sucrase of chick intestines increased in proportion to increases in the dietary concentration (0%, 30%, and 60% of the dry matter) of cane molasses. Similarly, Sell et al. (1989) found significant effects of feeding diets differing in carbohydrate and fat content on disaccharidase activities of turkeys. These effects were seen after 5 days of feeding, especially in the proximal jejunum. When the dietary carbohydrate concentration and intake were high, the specific and total activities of maltase, sucrase, isomaltase, and trehalase were high.

H.) Intestinal Nutrient Transport

After hydrolysis by the brush border enzymes (peptidases, disaccharidases, oligosaccharidases) the small peptide chains, free amino acids and hexoses are free for nutrient uptake within the small intestine. The intestinal lining consists of tight junctions between the cells, making it impermeable to even small solute molecules. Therefore proteins are present within the epithelium, in which function as nutrient carriers of monosaccharides and free amino acids, vitamins and minerals across the intestinal epithelium to the bloodstream. Of the nutrient carriers, the amino acid and peptide transporters are the most diverse. Each carrier protein transports a class of amino acids (i.e. neutral, cationic, aromatic, amino amide) rather than one specific amino acid. Transport of amino acids across the apical and basolateral membrane occurs primarily by

different sets of transporters. Some of the nutrient carriers are found in both membranes and are sodium dependent, while others are sodium independent (Berne and Levy, 1998). The intestinal transporters found within the basolateral membrane are ubiquitous within tissues, while the transporters found within the apical membrane are unique to the intestinal epithelium and nutrient transport. Therefore the nutrient transporters of the apical membrane are of primary research interest within the field of nutrition.

These protein nutrient carriers, known as transporters, convey nutrients across the intestinal epithelium by passive or active means. Passive transport involves simple diffusion, which moves substances down a concentration gradient and is not energy (ATP) dependent. Active transport is capable of transporting a substrate across the membrane against an electrochemical gradient and is directly dependent on metabolic energy. Primary active transport takes place within the intestinal cells in the transport of Na^+ and K^+ by the Na^+/K^+ ATPase pump. The uptake of amino acids and glucose across the apical membrane of the enterocytes requires the energy created due to this electrochemical gradient produced by the Na^+/K^+ ATPase pump. Thus nutrient uptake relies upon secondary active transport and is known as the Na^+ co-transport system. The immediate source of metabolic energy is the hydrolysis of ATP. Sodium is actively pumped out of the enterocytes and potassium is pumped into the cells across the basolateral membrane.

With the Na^+/K^+ ATPase pump, the rate of extrusion of sodium exceeds the rate of potassium uptake (3 sodium ions out for each 2 potassium ions in). Due to this electrogenic pump, the interior of the cell becomes electronegative to the exterior. Thus

the Na^+/K^+ dependent ATPase pump maintains an electrochemical gradient that is conducive to re-entry of the Na^+ ion. This re-entry occurs in the brush border membrane, which also brings about Na^+ -coupled entry of amino acids and other nutrients. Sodium, which has been pumped out of the cell, reaches the luminal side of the epithelium *via* the intracellular space.

Seven brush border amino acid nutrient transporters have been identified of the apical intestinal membrane (Berne and Levy, 1998). Five nutrient carriers are sodium-dependent and mediate secondary active uptake of amino acids; (B transporter carrier of neutral amino acids, B^{0+} transporter carrier of neutral amino acids, basic amino acids and cystine, XAB^- carrier of acidic amino acids, β carrier of β amino acids (taurine). Two transporters are sodium-independent (b^{0+} carrier of neutral amino acids, basic amino acids and cystine; y^+ carrier of basic amino acids) and their uptake of amino acids is mediated by facilitated transport (Berne and Levy, 1998). While these intestinal transporters have specificity, there is an extensive amount of overlap between the structural requirements for active transport of amino acids across the brush border membrane by these systems.

The L-amino acids are termed the natural occurring isomers due to their presence in foodstuffs or in the tissues of higher animals. All amino acids share a common amino and carboxyl termini. Classification of the amino acid side chain (aromatic, neutral, acidic, basic) is the major factor determining the method of intestinal transport. Stereochemical configuration is extremely important in determining transport. D-forms of amino acids are transported very slowly in relation to L-forms of the same amino acid.

Neutral amino acids have very little or no net charge at or near neutral pH, thus electrical potential is relatively unimportant in determining the net movement against an electrochemical gradient. Therefore, the net movement of neutral amino acids is determined by the concentration gradient. Basic amino acids, such as lysine and arginine, have a net positive charge in the physiological pH range, while the interior of the enterocyte is electronegative. Thus, basic amino acids can be concentrated in the interior of the cell due to the difference in the electrical potential. The amino acids, glutamine, glutamate and aspartate are the preferred energy source by enterocytes. Most of the glutamine, glutamate, and aspartate that enter the cells across the brush border are shunted to the pathways of energy metabolism, and thus are not transported into the blood (Berne and Levy, 1998).

Often the intestinal peptidases of the brush border membrane do not hydrolyze dietary proteins fully into free amino acids, producing small peptide chains (dipeptides, tripeptides) within the luminal contents of the jejunum. The intestinal Pept-1 transporter of the apical membrane is responsible for transporting these small peptide chains across the lumen into the blood stream. Recent studies have demonstrated the nutritional importance of the jejunal Pept-1 transporter for whole-animal protein nutrition (Fei et al., 2000; Matthews, 2000; Wenzel et al., 2001). The intestinal Pept-1 transporter had been previously identified for its primary role in absorption and drug therapy (Tsuji and Tamai, 1996).

The rate of transport of these small peptides across the jejunal epithelium usually exceeds the rate of uptake of free amino acids (Berne and Levey, 1998). Experiments

have demonstrated that glycine was absorbed by the jejunum less rapidly as a single amino acid than as a glycylglycine or glycylglycylglycine peptides. The Pept-1 transporter has a high affinity for dipeptides and tripeptides, and a low affinity for peptides of four or more amino acid residues. The transport system is stereospecific and prefers L-amino acid residues with bulky side chains. The transport of small peptides is powered by the electrochemical potential difference of the H^+ across the membrane and thus is a secondary active process. The H^+ gradient is created by the Na^+/H^+ exchangers in the brush border membrane. Consequently, H^+ and small peptides enter the cell *via* the Pept-1 transporter simultaneously. Once within the cytosol of the epithelium, the cytosolic peptidases cleave most of the dipeptides and tripeptides into single amino acids, which leave the cell at the basolateral membrane by facilitated transport. The jejunum is more active in small peptide transport than the ileum.

The nutrient binding site of the nutrient carrier alternates between the exterior and interior surfaces of the membrane, thus providing a gated channel. The protein nutrient carrier spans the membrane. The nutrient-substrate binds to the site on the luminal surface and the gate-opening mechanism linked to the recognition process allows sodium (for amino acid and glucose) and/or H^+ (for small peptides) to enter a hypothetical channel penetrating the transporter, with the flow of cations coupled to the nutrient being transported (Mathews 1991). Then conformational change of the complex results in delivery of the cations and nutrients into the cytoplasm of the cell. The translocation rate is thought to be in the order of nanoseconds. Once within the cell the nutrients are

transported into the blood stream by the fore mentioned ubiquitous amino acid nutrient transporters or by the GLUT2 transporter for hexoses across the basolateral membrane.

The diet of the domestic turkey and/or chicken is primarily corn, which when subjected to luminal digestion, produces primarily glucose and fructose available for nutrient transport. The glucose transporter of the apical membrane of the intestine, SGLT-1, is a specialized high affinity Na⁺ co-transport protein, which is expressed in high abundance in the brush border membrane. The SGLT-1 transporter has binding sites for 2 Na⁺ ions and 1 glucose molecule. Fructose has its own transport mechanism that is a Na⁺-independent facilitated diffusion by the protein carrier GLUT5 of the apical membrane. Therefore fructose accumulation into the cell occurs down a concentration gradient. Once within the cell, both fructose and glucose exit the cell by facilitated diffusion by the GLUT2 transporter of the basolateral membrane.

Diamond and Karasov (1987) demonstrated that intestinal nutrient transporters show adaptive regulation to diet. To study regulation of sugar transport, mice were fed isocaloric diets containing carbohydrate levels varied from 0% to 68% in a low versus high carbohydrate diet, respectively. Subsequent studies were performed to determine regulation of amino acid transport. A high (72%) versus a low (4%) protein diet was fed to mice using rations of protein and/or amino acids. Mice were fed for 5-14 days and nutrient transport was measured using an isotopic accumulation method *in vitro* at 37°C. Dietary substrate levels reversibly regulated transport in the proximal and mid small intestine for every solute studied. Nutrient uptake in the distal small intestine was the lowest and few solutes elicited regulatory response. Amino acid and sugar transport was

up-regulated in response to moderate to high levels of substrates (Diamond and Karasov, 1987; Diamond and Ferraris, 1993). Increased intestinal absorption was solely due to specific, parallel increases in the number of intestinal brush border apical membrane co-transporters and in number and activity (Ferraris and Diamond 1993). Diamond and Karasov concluded that the amino acid and sugar transport systems are regulated and function independently. Intestinal transporters show no response to dietary changes for up to 12 hours, yet reach a new steady state with 1-3 days. Up-regulation (≈ 1 day) of amino acid and sugar transport systems is faster than down-regulation (≈ 3 days).

Diamond and Karasov theorized that a basal activity level in the intestine function to enable the animal to utilize a brief input of dietary substrate. Due to slowness of the regulatory responses, the dietary components would exit the small intestine prior to the triggering of the regulatory mechanisms. Thus a nutrient present in a single meal could only be absorbed or metabolized if the nutrient carrier is present and maintained at basal levels even in the substrates absence. In conclusion, Diamond and Karasov stated that regulation serves to match intestinal nutrient carriers to a running average of dietary composition of several meals.

The intestinal epithelial tissue carrying out absorption is in a continuous state of renewal. Enterocytes produced in the intestinal crypt migrate to the tips of the villi where they are shed into the intestinal lumen (Cheng and Leblond, 1974). This leads to complete cell renewal every 2-3 days in rodents (Ferraris and Diamond, 1993), 3-4 days in ovine (Attaix and Meslin, 1991), 5-6 days in human (Traber, 1990), and 2-3 days in chicken (Uni et al., 2003). During this migration process the enterocytes acquire

differentiated functions specifically needed for digestion, absorption and mucin secretion (Imondi et al., 1969; Ferraris et al., 1992b; Weiser 1973; Traber et al, 1992; Meddings et al., 1990; Thomson et al., 1994; Uni et al., 2000, 2003; Geyra et al., 2001).

Experiments by Scharrer et al. (1967, 1971) demonstrated that amino acid transport of rat small intestine is enhanced by an increase in dietary protein. These experiments proved that this intestinal change was due to specific amino acid transport. The transport of L-leucine was compared to D-galactose transport. Forty-two adult male Sprague Dawley rats were fed diets of either low (13% casein) or high (88% casein) protein for 2 weeks. The results indicated that rats fed the high protein diet transported significantly larger amounts of L-leucine against a concentration gradient than did rats fed the low protein diet. There were no differences in galactose transport between both feeding groups.

Adaptation of amino acid transporters is more complex than that for sugar transport. Karasov et al. (1987) formulated a diet that lacked all the non-essential amino acids but contained the essential amino acids. Animals consuming this diet maintained in a healthy condition during the duration of the experiment. The activities of the acidic (probed with aspartate) and imino acid transporter (probed with proline) increased linearly as dietary nitrogen increased. This increase in amino acid uptake was approximately 80% greater in animals consuming a high protein diet (Karasov et al. 1987). The pattern was more complex for the other amino acid transporters. Amino acid transport of the essential amino acids was unchanged or enhanced as a consequence of low dietary protein. With a high protein diet, the transport of these essential amino acids

was enhanced, but not to as great an extent as aspartate or leucine. Alanine transport followed an adaptive pattern similar to that of the essential amino acids. The imino acid and acidic amino acid transporters are induced by high dietary protein and repressed by nitrogen deficient diets. The neutral amino acid transporter (substrates including the essential and non-essential amino acids) is only slightly induced by high protein diets and is relatively insensitive to low protein diets. A similar pattern applies to the essential basic amino acid transporter, which can also carry some essential neutral amino acids.

The uptake of the dipeptide, carnosine, is related linearly to dietary protein content (Ferraris et al. 1988a). Thus this transporter is regulated in a similar manner to the acidic and imino acid transporters. This may suggest that the dipeptide carrier is generally used for the absorption of non-essential amino acids or amino acids to be used for energy. Indeed, we might expect that repression of the dipeptide carrier at low dietary protein levels would be compensated for by the activity of the specific amino acid carriers. At low luminal protein content it might be expected that digestion of protein would be more complete, thus affording less use for the dipeptide carrier (Hirst, 1993). Stein et al. (1987) compared the effectiveness of seven different amino acids in inducing the transport of five different solutes, which were substrates for separate transporters. These studies proved that the amino acid transporters are regulated independently of sugar transport and semi-independently of each other. Aspartate was a good inducer of the uptake of aspartate (acidic transporter) and lysine (basic transporter), but not leucine (neutral transporter) or methylaminoisobutyrate (imino acid transporter). Arginine induced aspartate uptake and to a lesser extent leucine uptake, but not uptake of the other

substrates. Valine only stimulated leucine uptake. Thus acidic amino acids appear to be regulated by their substrates. Whereas dietary amino acids that are not specific substrates of glucose, basic amino acid and imino acid transporters appear to be the best regulators of these transport systems. Free amino acids and peptides are potent stimulants for regulation of peptide transporters. Conversely, peptides have a very influential effect on regulating amino acid transporters (Ferraris and Diamond, 1989).

Diamond and Karasov (1987) proposed four factors to be considered in explanation of transporter regulation: 1) energetic cost of transporter synthesis and maintenance; 2) caloric payoff from a metabolizable nutrient; 3) absolute requirement for the compound; and 4) potential toxicity to the animal. Up-regulation of nutrient carriers is an apparent attempt to completely absorb dietary nutrients. Thus Ferraris et al (1990) have suggested that transporter activity is regulated to maintain a slight excess capacity over intake. Ferraris et al. (1990) introduced the theory that the maximal uptake capacity of the small intestine for glucose is only slightly greater than the delivery of glucose at a fixed physiological state. Cant et al. (1996) hypothesized this narrow margin between capacity and delivery is due to the small intestine being an open system. As the total number of glucose transporters increase, glucose uptake increases until uptake equals intake. Beyond this point, the cost of synthesis and maintenance outweigh the caloric benefit.

Regulation serves to match transporter and enzymatic activity to a running average of diet composition over many days; it does not switch transporters on and off with each meal. In the absence of food, the intestinal brush border enzymes and nutrient

transporter activity decrease during starvation (Ferraris and Diamond, 1989). Adaptation to diet in birds and mammals allows the conservation of energy by regulation of nutrient transport proteins and brush border digestive enzymes. Specific adaptive mechanisms include changes in site density of transporters and in the affinity constants (Ferraris and Diamond, 1997). Thus in the absence of a dietary component, a specific nutrient carrier will be down regulated in an effort to conserve vital energy needed for the growth and development in other tissues. Diet is also known to influence intestinal organ length, mass and surface area. Thus non-specific adaptive mechanisms include changes in mucosal surface area and in the ratio of transporting to non-transporting cells (Ferraris and Diamond, 1997).

In the neonatal hatchling, the physiological cost of immune function may compete for nutrients that would otherwise be allocated to growth or other functions. Therefore, antibiotic-treatment of feed or food is a common practice within the poultry industry to increase growth rates. Antibiotics can reduce the microbial activity in the gut, which could make more nutrients available for the host. Antibiotics can also reduce the immunogenic stimulation of the gut immune system, permitting more feed to be utilized for growth. It has been shown that feeding antibiotic-medicated feeds was associated with reduced level of immune activity in the host's gut (Roura et al., 1992).

I.) Intestinal Gene Expression Development and Regulation

Intestinal genes are specifically expressed and produce the proteins of the brush border membrane that digest and absorb the diverse nutrient molecules of the diet. Intestinal gene expression may be dependent upon age (developmental stage) and the

luminal presence of nutrient molecules. Intestinal phenotype is determined by pre-wired developmental patterns, which predict the appearance of specific sets of digestive and absorptive proteins within the gut correlative with age (Traber, 1997). The activity and RNA expression of brush border enzymes, which digest disaccharides and small peptides and the major nutrient transporters (sodium-glucose transporter) are found at 15 days of incubation and begin to rise on the 19th day of incubation and increase further at hatch in broiler embryos (Uni et al., 2003). In murine species, the level of sucrase-isomaltase transcripts is low until the suckling-weaning transition, when pups are transitioning from a milk-based diet to a diet of mainly carbohydrate. SI represents on the most extensively studied genes activated during this developmental transitions (Traber, 1998). The SI protein and mRNA concentrations are first detectable in the mouse embryonic intestine and remains stable through the first two weeks of postnatal life. At the suckling-weaning transition there is an induction of SI mRNA and protein observed, with highest levels found within the jejunum and no detectable levels in the colon (Rings et. al, 1994; Krasinski et al., 1994; Tung et al., 1997, Boudreau et al., 2002).

Conversely, lactase, (the brush border enzyme responsible for digestion of lactose found in milk) intestinal expression remains high until weaning in mammalian species. Studies have shown that these developmental patterns of gene expression are hard-wired and are present independently of dietary stimulus (Motohashi et al. 1997; Traber, 1997; Tung et al., 1997; Boudreau et al., 2002; Oesterreicher et al., 1998). Declining patterns of lactase protein and mRNA concentrations during weaning were not due to termination of milk ingestion (Motohashi et al., 1997; Shaw-Smith and Walters, 1997). In this study,

prolonged nursing rats showed the same developmental patterns of lactase gene expression as weaned rats. Additionally during this transition from suckling-weaning, there is a change in the activation of transcription of distinct genes related to specific functions of the intestinal epithelium (Rings et al., 1994).

The regulation and ontogeny of the brush border enzyme responsible for carbohydrate digestion, sucrase-isomaltase (SI) is not simple. Studies by Yeh and Holt (1986) used intestinal isografts that had no contact with ingested food which showed the timing of sucrase appearance in rodents to be independent of luminal contents. When these isografts were transplanted into animals 5 days younger than the donors, sucrase appeared earlier in the transplanted intestine than in the host. Suggesting that sucrase is controlled by an internal biological clock within the intestine itself. However, luminal contents can have a marked effect on the activity of sucrase, which is separate from this internal mechanism.

Earlier studies (Markowitz et al., 1993, 1995; Tung et al., 1997; Suh et al., 1994; Wu et al., 1994) found a SI promoter upstream of the SI gene. The promoter is the region upstream of the gene and binding of regulatory elements and DNA binding proteins (transcriptional factors) are important for gene regulation. Thus, intestinal-specific gene expression is determined by transcriptional regulation of these DNA elements and binding proteins. Therefore, intestinal-specific genes are up-regulated in response to increasing concentrations of these transcriptional factors. Thus nutrient stimuli are indirectly responsible for intestinal-specific gene expression, while transcriptional

regulation is directly responsible for the appearance of the mRNA transcripts of intestinal specific genes.

The short SI promoter region has three identified regulatory sites: sucrase isomaltase footprint 1 (SIF1), SIF3 and GATA. The SIF1 element interacts with the Cdx transcription factors (Suh et al., 1994; Taylor et al., 1997, Boudreau et al., 2002) that belong to the homeobox gene family. The expression of Cdx1 and Cdx2 are restricted to the intestinal epithelium (Silberg et al., 2000). Cdx1 protein is found mainly in the crypt compartment of the intestinal epithelium, whereas the Cdx2 protein is detected in both the crypt and villus. Cdx2 stimulates differentiation and expression of the SI in the intestinal epithelial cells (Suh and Traber, 1996). Goda et al. (2000) demonstrated that force-feeding a fructose diet gave rise to an increase in the binding of dimeric Cdx2 protein to the SIF1 element, thus leading the carbohydrate-induced transcription of the SI gene. However the specific role of the SIF1 element in the regulation of the SI promoter activity *in vivo* is unclear.

In the chicken, the Cdx genes differ from the mammalian genes, with two of the homeobox genes termed CdxA (Frumkin et al., 1991) and Cdx B (Morales et al., 1996). CdxA has 95% homology to the mouse Cdx1 and Cdx2 in the amino acid sequence of the homeobox domain; while the CdxB has 95% amino acid sequence homology to the mouse Cdx4. Thus it is assumed that the CdxA transcription factor functions in the same capacity that the mammalian Cdx1 does (Frumkin et al., 1994). Geyra et al., (2002) demonstrated that CdxA has a role in enterocyte maturation and SI gene regulation. CdxA plays a major role in gut development during embryogenesis and post-hatch and is

expressed exclusively in the chick small intestine (Frumkin et al., 1994). Expression and protein concentrations of CdxA in chicken increased from 15-d of incubation until hatch, after which further changes were small (Sklan et al., 2003). Additionally, CdxA protein was shown to bind to the promoter region of the SI in the chick indicating that CdxA is similar to the mammalian Cdx2. The mRNA SI was observed at 15-d incubation, and increased from 17-d of incubation to a peak on day 19, decreased at hatch and had a further peak of expression 2-d post-hatch (Sklan et al., 2003). Sklan et al. (2003) also demonstrated a correlation between the expression of the transcription factor CdxA and SI gene expression; thus suggesting that CdxA may be responsible for SI gene expression and developmental patterns. In contrast, mRNA SGLT-1 was not detected until 19-d of incubation when a major peak of expression was observed followed by a decrease to low levels at hatch and small increases post-hatch (Sklan et al., 2003); thus implying that SI and SGLT-1 are regulated by different mechanisms and exhibit developmental patterns prior to hatch.

The GATA element is localized in the proximal region of the SI promoter (Boudreau et al., 2002b; Krasinski et al., 2001). GATA-4, 5, and 6 are expressed in the intestine as well as the mesoderm and endoderm derived tissues, such as heart, liver and lungs, and they play a critical role in the regulation of tissue-specific gene expression (Molkentin et al., 2000). GATA-4 transcripts were detected in the villi of the small intestine of mice (Arceci et al., 1993) and chicken (Gao et al., 1998), whereas GATA-6 mRNA was localized to the crypt-villus region (Gao et al., 1998).

The SIF3 elements interact with the hepatocyte nuclear factors (HNF) transcriptional factors. Despite the name, HNF is not liver specific and is found within the intestine, spleen, thymus and kidney. The HNF transcription factors, HNF1- α and HNF-1 β , bind to the DNA as either heterodimers or homodimers. HNF1- α stimulates the SI promoter activity via the SIF3 element, whereas the HNF-1 β down-regulates this effect (Boudreau et al., 2001). HNF1- α mRNA has been localized to the crypts of the villi, but its exact distribution is not known (Serfas et al., 1993).

Recent studies by Bourdreau et al (2002) have demonstrated the interaction of the HNF1- α , GATA-4 and Cdx2 transcriptional factors *in vitro* and *in vivo*. These factors activated the promoter activity in cotransfected studies where GATA-4 requires the presence of both HNF1- α and Cdx2. These findings imply a combinatorial role of HNF1- α , Cdx2 and GATA-4 for the time and position dependent regulation of SI transcription during development.

Studies by Uni et al. (2003a) have demonstrated the intestinal developmental patterns for other intestinal-specific genes responsible for digestion and absorption in the chick embryo. Maltase, aminopeptidase (AP), SGLT-1 and ATPase activities began to increase at 19-d of incubation and increased at the day of hatch. These intestinal transcripts were first detected at 15-d of incubation. The relative expression (gene expression/ β -actin gene expression) of these genes was low at 15 and 17 days of incubation and increased 9 to 25 fold at day 19 of incubation and declined at the day of hatch. However, gene expression was not correlated to protein activity. While these experiments have identified the developmental patterns and activities of intestinal-

specific genes it has been yet to be determined the exact molecular mechanisms involved in their regulation.

Experiments by Gal-Garber et al. (2003) have demonstrated nutrient induced expression and activity of the brush border proteins of chickens. Expression of the sodium-potassium ATPase pump was enhanced by low dietary sodium and was two-fold higher in chicks receiving the low sodium diet, but was unchanged at high dietary sodium. The V_{max} of the sodium-potassium ATPase pump was unchanged, but the affinity was altered by dietary sodium concentrations. Expression of the sodium-potassium ATPase pump of chicks receiving the high dietary sodium and the controls was similar, with a decreased affinity. Additionally, previous studies have shown that the expression, activity and affinity of the sodium glucose transporter is altered by dietary concentrations of carbohydrate (Gal-Garber et. al, 2000a).

Regulation of the digestive and absorptive systems of the GIT is very important for post-hatch survival. The GIT is an energetically costly tissue due to the amount of energy needed for nutrient transport, rapid enterocyte turnover and maintenance. Therefore it is a common practice within the poultry industry to feed antibiotic treated feed, which results in increased growth rates. Antibiotics can reduce the microbial activity in the gut, which could make more nutrients available for the host. Antibiotics can also reduce the immunogenic stimulation of the gut immune system, permitting more feed to be utilized for growth. It has been shown that feeding antibiotic-medicated feeds was associated with reduced level of immune activity in the host's gut (Roura et al.,

1992). In the neonatal hatchling, the physiological cost of immune function may compete for nutrients that would otherwise be allocated to growth or other functions.

J.) Constraints on Growth

The domestic turkey and broiler chicken has been selected for decades for rapid growth, greater feed conversion efficiency, and enhanced breast yield; the faster the rate of growth, the greater the demand of nutrients relative to energy. Therefore, modern poultry must consume additional nutrients relative to metabolizable energy to meet the demands of rapid growth. Consequently, there must be a compromise for the partitioning of energy between body growth and tissue maturation (growth defined as increase in size; maturation defined as changes in tissues approaching more closely to adult morphology and function). Thus, the rate of growth may be limited physiologically by nutrient and energy availability, physiological capacity and organ maturation and/or function.

When energy reserves are limited and dietary metabolizable energy is restricted as often occurs in the young hatchlings, the energy demands of thermoregulation, maintenance and activity must be met first and body growth suffers. For a short time, energy deficits may be made up from energy reserves, provided that energy storage is a part of growth the strategy. In addition, energy maybe obtained from nutrients that would otherwise accumulate in tissues. Ultimately, chicks may obtain energy by breaking down tissues. In this case, body protein is catabolized as a supplemental energy source or to provide the building blocks for structural growth elsewhere (Dunham et al, 1989, Ricklefs, 1991).

Ricklefs et al. (1969, 1979) categorized the constraints of growth into three levels. Level one constraints on growth are related to the availability of food resources. Level two constraints on growth are related to the chick's capacity to utilize the available resources. Level three constraints on growth are related to the compromise between somatic growth and tissue maturation and function.

Unlike the mammalian embryo *in utero*, the avian embryo is faced with a significant level one constraint because it possesses a finite amount of *in ovo* energy and nutrients to support embryonic growth and hatching. As the embryo grows and develops *in ovo*, the yolk reserves and albumin steadily decline. The lipids from the yolk and protein from albumin provide the gluconeogenic precursors needed for endogenous production of glucose, the primary fuel. Endogenously produced glucose is then stored as glycogen in the liver and muscles. Therefore the glycogen reserves provide the primary fuel as glucose needed for growth and development and are thus critical for the survival of the avian embryo. A great deal of the glycogen reserves are utilized for hatching, which is relatively energetically expensive. By the end of incubation, the yolk stores are nearly depleted and the residual yolk is internalized within the body cavity. Therefore, hatchlings have limited energy reserves due to diminished glycogen and yolk stores, and they cannot gain external energy resources because they do not have immediate access to feed upon hatching. In commercial hatcheries, it is a common practice that young hatchlings may go for 24-48 hours after hatch without feed (Moran and Reinhart, 1980). As a result, hatchlings must mobilize critical body resources (primarily muscle) to provide the energy needed for maintenance, thermoregulation and

activity. Delaying access to feed for 24 hours after hatch increases mortality rates and stunted growth of broilers, which persisted until market age (Vieira and Moran, 1999). Additionally, studies have shown that birds denied access to feed did not have compensatory growth equal to the birds that were fed early (Misra, 1978; Hager and Beane, 1983; Wyatt et al., 1985; Nir and Levanon et. al, 1993; Noy et al., 2001b).

Modziak et al. (1997) suggested that breast muscle size appears to be permanently reduced by the inhibition of satellite cell mitotic activity after poor post-hatch nutritional status. As a consequence, approximately 2% to 5% of hatchlings do not survive this critical post-hatch period because of limited or depleted body reserves. Modziak et al. (2002b) demonstrated that denied access to feed resulted in decreased cross sectional surface area of muscle microfibers, which may be resultant from an increased number of apoptotic cells versus the fed controls; thus suggesting that breast muscle size might be permanently reduced by poor post-hatch nutritional status. Halevy et al. (2000) demonstrated that long-term starvation (i.e. denied access to feed) resulted in nearly complete cell-cycle arrest and a decrease in satellite cells of the muscle, whereas short-term starvation may enhance muscle satellite cell number. Thus, alterations in muscle satellite cell activity may lead to subsequent delayed cell hyperplasia and muscle maturation. Studies by Halevy et al (2000) also demonstrated that starvation caused a significant reduction in bodyweight and relative muscle weight and meat yield, in comparison to the controls which persisted for the duration of the study. As a consequence, approximately 2% to 5% of hatchlings do not survive this critical post-hatch period because of limited or depleted body reserves (Phelps, 1987).

Level two constraints on growth, which are related to the chick's capacity to utilize the available resources, is common among poults and chicks that are unable to self-initiate feed intake immediately after hatch. The time to self-initiate feed intake may vary from immediate to as long as 48 hours in some poults. Such long delays in the time to self-initiate feed intake may result in stunted growth, morbidity and increased mortality. Therefore young hatchlings may experience or continue to experience a negative nutritional status, even when feed is readily available.

Additionally, the avian neonate hatches with an immature GIT. During the first seventy-two hours post-hatch, the GIT undergoes rapid morphological, biochemical and cellular development in order to better digest and absorb incoming nutrients (Uni et. al, 1998, 2000, 2003a; Noy et. al., 2001a). It has been hypothesized that the digestive and absorptive capacity of the gut may limit the energy supply needed for the growing chick (Kirkwood, 1983; Lilja 1983; Kirkwood and Prescott 1984; Konarzewski et al. 1989), due to a low absorptive and digestive capacity. Therefore, hatchlings may experience mal-absorption of dietary nutrients. The GIT is the major supply organ of the body, which provides the nutrients and energy needed for growth, development, thermoregulation, maintenance, and for the replenishment of energy reserves. Avian neonatal growth and development is dependent upon nutrient digestion and absorption, which is directly related to intestinal function and morphological development (Baranylova and Holman, 1976; Sell et al., 1991; Akiba and Murakami, 1995; Yamauchi et al., 1996; Uni, 1999). Therefore, rapid and early maturation of these tissues post-hatch is of paramount importance during the critical post-hatch period.

The delay in the access of feed in the poultry industry may adversely delay the development of the digestive and/or absorptive activity of the GI tract. Early studies by Yamauchi et al (1996) demonstrated that birds denied access to first feed for 24 to 48 hours have decreased villi length. Studies by Geyra et al. (2001) have shown that 0-48 hours of delayed access to feed after hatching adversely affected the GIT due to a reduction in the intestinal surface area, number of cells/ crypt, and the % of proliferating cells in particularly the duodenum and jejunum. Additionally, Geyra et al. (2002) have demonstrated that the intestinal expression of the transcriptional factors CdxA and CdxB was depressed by 0-48 hours of starvation in comparison to fed chicks post-hatch. Delayed access to feed for 48 hours post-hatch, also resulted in an increase in intestinal intracellular mucins, which might have been due to impaired mucin secretion or enhanced mucin production. Changes in mucin dynamics may affect the absorptive, digestive and protective functions of the gut (Uni et.al., 2003b; Smirnov et al., 2004).

Level three constraints on growth are related to the compromise between somatic growth and tissue maturation and function. Thus, the growth rate of the organism would be determined by the proportion of its tissue devoted to growth in contrast to mature function. For example, precocial chicks grow more slowly than altricial chicks because they partition more energy towards tissue maturation than somatic growth. In addition, chicks of all species grow more slowly as they develop because they are becoming more adult-like in morphology and function. Therefore, body growth is constrained and a greater proportion of energy is partitioned for the maturation and development of the organ systems (skeletal, muscular, and gastrointestinal) to meet the metabolic needs of a

larger organism. During the first week post-hatch, hatchlings are under microbial challenge and rapid maturation of the immune system is critical for post-hatch growth. Additionally, the skeletal and muscular systems must mature at a rapid rate to meet the need for locomotion and thermoregulation.

As a consequence, these three constraint levels on growth may plausibly limit the rate of postnatal growth in the hatchlings. To maximize growth potential and tissue/organ maturation, hatchlings must have immediate access to feed and water and must promptly self-initiate feed intake to prevent the depletion of energy reserves (glycogen). With immediate access to feed, hatchlings may replenish their glycogen reserves, which may provide the energy needed for optimal organ growth and development and growth performance post-hatch. Denied immediate access to feed, would compromise somatic growth, due to the allocation of energy to the rapidly growing and developing organs (Konarzewski et al., 1989, 1990). The gastrointestinal tract is an energetically expensive organ, so energy must be allocated between body growth, maintenance, thermoregulation, activity, storage and tissue maturation.

K.) In Ovo Feeding (IOF)-Early Nutrient Administration

Definition of “In Ovo Feeding”

Poultry producers experience early poult mortality (EPM) of 3 to 6%, annually (Phelps, 1987). Mortality of young hatchlings is greatest during the first two weeks post-hatch. The causative agents for EPM are unknown, but it may be related to limited energy reserves, decreased feed and water consumption, and environmental stressors.

New innovations by Uni and Ferket (Enhancement of development of oviparous species

by *in ovo* feeding. Patent # US 6,592,878 B2, Jul 2003; Uni and Ferket, 2004) involve the administration of exogenous critical nutrients by day 23 of incubation into the amnion of developing turkey and chick embryos. The late term embryo orally consumes the amniotic fluid before hatching and therefore the “*in ovo*” administered nutrients are presented to the enteric tissues for digestion and absorption and then can be utilized or stored as energy. Consequently, *in ovo* feeding is fundamentally feeding the embryo an external diet before to hatch (Uni and Ferket, 2003 Patent # US 6,592,878 B2, Uni and Ferket, 2004). Therefore *in ovo* feeding may serve as a tool to overcome the level one, two and three constraints (Ricklefs et al. 1969; 1979) on growth during embryonic and post-hatch development in domestic poultry by: a) enhancing and sparing the body’s energy reserves, b) enhancing the development and functioning of the gut, and c) providing enough energy needed for optimal growth and organ maturation. Thus, the central hypothesis is that “*in ovo* feeding” will enhance enteric development of the embryo and neonate, improve hatchability, and increase post-hatch growth, body weight and muscle gains, and survivability of commercial turkeys.

In Ovo Feeding Background

Previous studies have been conducted experimenting with administration of substances within the egg (in the air cell, yolk) of the developing embryo, but none of these studies have involved the administration of nutrients into the amnion and therefore have not examined the effects of fundamentally “feeding” the avian embryo. In 1978, Al-Murrani attempted to alter the composition of the yolk of broiler breeder eggs. He hypothesized that the egg yolk was mainly composed of lipids and moisture, with a small

ratio of proteins. He thought that an increased protein ratio in the yolk would provide hatchlings with increased energy and provide the building blocks for weight gain. He fed broiler breeders a low energy-high protein diet in hopes of obtaining a higher protein ratio in the yolk of the progeny, proving to be very costly and resulting in reduced hatchability. Subsequently, Al-Murrani (1982) injected an amino acid mixture resembling the amino acid profile of egg protein into the yolk sac of developing embryos of broiler breeder eggs. *In ovo* injection of the amino acid mixture into the yolk sac resulted in increased bodyweights at hatch and at 56 d of age in comparison with the control group.

Studies by Ohta et. al (1999) studied the effects of amino acid injection in broiler breeder eggs on embryonic growth and hatchability. Treatments include a non-injected control and injected control of 0.5mL sterile water or 0.5mL of amino acid suspension matching the amino acid profile of egg protein. Injections were made into the yolk sac or air cell of 15 eggs weighing an average of 68.7 ± 3.8 g on days 0 and 7 of incubation. *In ovo* injection into the air cell at day 0 or 7 of incubation greatly reduced hatchability. While there was no statistical difference in the bodyweights at hatch between the treatments, injection of amino acid into the yolk sac at day 7 resulted in a 4.0% increased bodyweights in comparison to the controls, while hatchability was similar between all groups injected into the yolk sac at day 7. The hatching bodyweight relative to egg weight was improved when amino acid solution was injected into the yolk sac at day 7 of incubation. This experiment was repeated by Ohta et. al. (2001) and the chick bodyweight relative to initial egg weight was also higher ($P < 0.05$) in eggs injected with

amino acids at day 7 of incubation as compared to the negative controls (non-injected eggs) and positive controls (eggs injected with water). This data demonstrates that amino acid injection into eggs affects embryonic growth of broilers with little impact on hatchability. Research regarding *in ovo* nutrient administration is extremely minimal. Therefore, the work of Uni and Ferket (2001) would provide critical data identifying the optimal target site and feeding formulas ideal for increased growth and enteric development of young poults and chicks.

Initially, Uni and Ferket (2003 Patent # US 6,592,878 B2) proposed to optimize the *in ovo* feeding solutions by identifying nutrients, metabolic effectors and enteric modulators that may directly or indirectly enhance growth and/or energy reserves. Dietary carbohydrates and proteins are the essential components of the commercial starter diet. Dietary carbohydrates provide the needed energy for maintenance, growth and development, while proteins supply amino acids, the building blocks needed for hormone synthesis, tissue growth and muscle development. Thus, protein and carbohydrates were identified as critical *in ovo* feeding solution components. Because avian saliva is deficient in amylase, carbohydrate digestion cannot begin until these *in ovo* fed nutrients enter the small intestine and are subjected to hydrolytic cleavage by the pancreatic and mucosal enzymes. Thus, simpler sugars (such as maltose, sucrose, and dextrin) were chosen as a carbohydrate sources for *in ovo* feeding solutions.

For over a decade, β -hydroxy- β -methylbutyrate (HMB) has been used as a dietary supplement for the enhancement of muscle deposition. HMB is a metabolite of the essential amino acid, leucine. Approximately 5% of leucine metabolism follows this

pathway, thus producing small amounts of HMB. Multiple studies have revealed that when dietary HMB is consumed multiple physiological enhancements occur. Early studies by Van Koeveering et. al, (1994) demonstrated that HMB supplementation in steers caused an increase in carcass quality, and upon shipping stressed calves were fed HMB and morbidity was decreased by 40% and mortality by 50%. Nonspecific early mortality in male broilers was reduced when HMB was supplemented between 0.003% and 0.01% of the diet (Nissen et. al, 1994). In addition, increases in bodyweight, breast yield, and hot carcass yield were observed, resulting in higher kilograms of broiler produced per broiler placed (Fuller et.al, 1994).

After identification of the key nutrients and effectors, Uni and Ferket (2003 Patent # US 6,592,878 B2) sought to identify the optimal solution volume and osmolality, and stage of embryonic development suitable, for *in ovo* feeding. One hundred fertile Hybrid turkey eggs were obtained from Prestage Farms (Clinton, NC) at 19 days of incubation. The eggs were then incubated at 99.9 °F-100.0 °F until hatching. The amniotic volume, pH and osmolality were measured at days 21, 22, 23, 24, 25, 26, 27 of incubation with a total of 10 eggs per day. Each egg was candled and the amniotic fluid was extracted by creating a window or small hole within the egg so that the amniotic sac could be visualized. A 23 gauge hypodermic needle was used to extract the amniotic fluid. At day 21 of incubation, the average (n=10) amniotic volume was 3.5mL, pH=6.5, and the osmolality was 322 mOsm. By day 22 of incubation, the average (n=10) amniotic volume was 2.0 mL, pH=6.5, and the osmolality was 322 mOsm. By day 23 of incubation, the average (n=10) amniotic volume was 1.0 mL, pH=7.3, and the osmolality

was 318 mOsm. By day 24 of incubation, 8 of the 10 embryos had orally consumed the amniotic fluid and internally pipped, the amniotic volume present in the remaining two eggs were negligible. By day 25 of incubation, all embryos had internally pipped and orally consumed the amniotic fluid. Evidently, the embryo orally consumes the amniotic fluid beginning at day 21-day 23 and continues slowly through incubation. Therefore, our initial *in ovo* feeding trials targeted these days for *in ovo* nutrient administration. The volume identified as suitable for *in ovo* nutrient injections during this stage of development was 1.0-2.0mL for turkey embryos. During the onset of my doctoral studies several experiments were conducted in which we attempted to inject 2.0mL of *in ovo* nutrient solutions at 21-d of incubation, which resulted in high embryonic mortality (unpublished). Therefore, *in ovo* feeding solution volume was reduced to 1.5mL of solution (or 1.6% of the weight of the egg or 10-20 calories) and the injections were administered on 23-d of incubation for all subsequent *in ovo* feeding experiments.

One of the primary obstacles to overcome with *in ovo* feeding is osmotic challenge. Avian embryos have limited means of osmo-regulation within the closed environment of the egg. Therefore, the osmotic challenge presented with *in ovo* feeding solutions could threaten embryonic survival. Thus, a series of small experiments (Uni and Ferket, 2003 Patent # US 6,592,878 B2) were conducted to determine the effects of *in ovo* feeding solutions of various levels of osmolality on hatchability. Osmolality ranged from 150 milliosmoles (mOsm) to 1500 mOsm. Osmotic pressure was modified by varying the NaCl inclusion level in an isocaloric *in ovo* feeding solutions containing 25% dextrin. Hatchability was 90% or greater with the *in ovo* solutions of less than 700

mOsm, with an optimum hatchability observed at about 400 to 600 mOsm (Uni and Ferket, 2003 Patent # US 6,592,878 B2). Unacceptable hatching rates were observed when the *in ovo* feeding solution exceeded 800 mOsm (Uni and Ferket, 2003 Patent # US 6,592,878 B2). Hatchability was directly affected by increasing or decreasing osmolality of the *in ovo* feeding solutions ($R^2=0.97$) (Uni and Ferket, 2003 Patent # US 6,592,878 B2). Therefore, the osmolality of all *in ovo* feeding solutions were measured prior to the onset of experimentation. The osmolality of all *in ovo* feeding solutions used in subsequent *in ovo* feeding trials ranged from 400 to 500 mOsm.

Optimal levels of nutrients (protein, carbohydrate), HMB (Uni and Ferket, 2003 Patent # US 6,592,878 B2) and arginine (Table 1.1) to be fed *in ovo* were determined by preliminary experimentation. Three hundred fertile commercial turkey eggs were divided into 5 treatment groups of 50 eggs. At 24-d of incubation, each group was then administered *in ovo* either 1mL of increasing levels of HMB 0ug (non-injected control), 0.1ug, 1.0ug, 10ug, 100ug, or 0.9% saline (injected-control). Hatchability was enhanced by *in ovo* feeding HMB at the 0.1ug and 1.0ug levels, over the controls. Additionally, liver glycogen was increased approximately 40% in all HMB levels as compared to the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2). There was a significant quadratic response ($P<0.05$) as the level of HMB injected *in ovo* increased. Hatchability rate was positively correlated with liver glycogen content of turkey and chick embryos before hatch ((Uni and Ferket, 2003 Patent # US 6,592,878 B2).

For both broilers and turkeys, *in ovo* feeding experiments were conducted to evaluate the effects of varying levels of simple and complex carbohydrates and proteins

fed *in ovo* (Uni and Ferket, 2003 Patent # US 6,592,878 B2). *In ovo* feeding solutions were formulated to have a similar calorie: protein ratio as in a conventional chick or poulter starter diet. The average hatchling consumes 14.285 g feed per day (NRC). The hatchling must consume a diet containing about 28% crude protein with 2800 kcal metabolizable energy (ME) (kcal/kg feed (2.8 kcal/g feed) for adequate growth and development. Thus hatchlings must consume approximately 40 kcal ME/day (14.285 g feed/day * 2.8 kcal ME/g feed= 39.998 kcal ME/day), with approximately 11% of this from protein (39.998 ME/day * 28% protein= 11.19944 kcalME/day from protein). As a result, the calorie: protein ratio for the week old hatchling is 4: 1. Because the protein requirement for the developing embryo may be higher than the requirement for growing hatchling, the protein inclusion rate was greater than a 4: 1 (calorie: protein).

In preliminary experiments by Uni and Ferket (2003 Patent # US 6,592,878 B2) 200 broilers (100 eggs/treatment) were either *in ovo* fed 1mL of 17% carbohydrate (2.5% sucrose, 2.5% maltose, 12% dextrin) and 8.25% egg white protein or injected with 1 mL of 0.9% saline at 18-d of incubation. Egg white protein was chosen as the ideal protein source because it is the predominant protein present in the amniotic fluid. The experimental *in ovo* fed group had a 3-8% increase in bodyweights at hatch, day 7 and day 14 post-hatch, the *in ovo* fed birds weigh 5% more than the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2). Similar preliminary experiments by Uni and Ferket were conducted in which there were increases in bodyweights of 3-8% post-hatch when broilers were *in ovo* fed a combination of varying levels of carbohydrates (10% sucrose, 10% maltose, 5% dextrin or 5% sucrose, 5% maltose, 15% dextrin or 2.5% sucrose, 2.5%

maltose, 12% dextrin) and was sustained for up to 35, 25 and 14 days, respectively. Additionally, there was a significant increase in the jejunal villi height by over 45% only 48 hours after *in ovo* feeding of 25% carbohydrate (10% sucrose, 10% maltose, 5% dextrin) in comparison to the controls.

Turkey poults that were *in ovo* fed 2mL of 24% egg white protein in 0.9% saline at 22-d of incubation had a 4-8% increase in bodyweights through 24 days post-hatch over the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2). When turkeys were *in ovo* fed 25% carbohydrate solution hatchability and hatching weight (7.5%) was significantly improved over the controls (78.7% versus 67.0%, $P < 0.05$) (Uni and Ferket, 2003 Patent # US 6,592,878 B2). Additionally, chicks *in ovo* fed a combination of carbohydrate and HMB, and turkey poults *in ovo* fed egg white protein and HMB had significantly increased bodyweights and intestinal villus surface areas in comparison to the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2).

More recent experimentation by Tako et al. (2004) demonstrated that *in ovo* feeding of carbohydrates and/or HMB increased intestinal villus width and surface area in comparison to the controls in broiler chicks at hatch. Chicks *in ovo* fed HMB had an increase of the villus surface area of 45% greater than the controls, while *in ovo* feeding of carbohydrate alone or in combination with HMB resulted in a 33% increase in the villus surface area in comparison to the controls at 3 days post-hatch (Tako et al., 2004). Additionally, jejunal sucrase-isomaltase activity was higher in embryo chicks *in ovo* fed either carbohydrates or HMB alone or in combination over the controls at 19 days of incubation. Also, bodyweights of chicks *in ovo* fed either carbohydrates or HMB alone

were significantly greater than the controls at hatch through 10 days post-hatch (Tako et al., 2004).

Additional studies by Uni and Ferket (2004) revealed that *in ovo* feeding of solutions containing carbohydrates and protein increased broiler hatching weights by 3% to 7%, over the controls, which was sustained up to 35 days of age. Also, *in ovo* fed broiler hatchlings had intestinal development similar to the development of a 2-day old conventional chick offered feed immediately post-hatch. In addition, *in ovo* fed chick embryos and hatchlings receiving carbohydrates (dextrin, maltose, and sucrose) had enhanced hepatic glycogen stores in comparison to the controls.

Based on research reported by Chevalley et al. (1998) and Kim et al (2004), arginine was identified as a good candidate for *in ovo* feeding nutrient administration. Chevalley et al. demonstrated that pharmacological doses of arginine *in vitro* enhanced collagen and bone formation by stimulating IGF-I production in osteoblasts. Arginine supplementation (0.4%) in artificially reared piglets significantly increased plasma concentrations of insulin and growth hormone by 24-27% in piglets in comparison to the non-supplemented controls (Kim et al., 2004). Also, 0.2% and 0.4% arginine supplementation to artificially reared piglets significantly enhanced the average daily weight gain by 28% and 66%, and body weight by 15% and 32%, respectively over the controls (Kim et al., 2004). Studies (Flakoll et. al, 2004) have demonstrated that when 23 elderly women were given a supplementation of 2g HMB, 5g arginine and 1.5g lysine daily they had significantly greater limb circumference, leg strength, and handgrip strength in comparison to the placebo group. In addition, the experimental group

experienced a 20% increase in protein synthesis as opposed to the placebo group. Experiments by Budford and Koch (2004) indicated that supplementation with glycine, arginine and α -ketosiocaproic acid (a leucine metabolite) enhanced anaerobic cycling performance of male cyclists. Thus, HMB and the amino acid arginine were identified as *in ovo* feeding components, which may enhance indirectly energy reserves, muscle deposition and growth.

Thus a growth dose response arginine and HMB *in ovo* feeding trial was conducted to determine the optimal levels of arginine and HMB conducive to increased growth (Foye et al (un-published), Table 1.1)The National Research Council (NRC) recommends that at hatch young poults should be fed a diet containing 28% protein and 1.6% arginine. At one week post-hatch, the average turkey poult will weigh 0.12kg and consume 0.10kg of feed per week or 14.285g/day of feed and 0.2288g/day of arginine (NRC). A young hatchling weighing approximately 60g will consume 7.2g of feed/day and 0.1152g/day of arginine. Therefore the arginine *in ovo* levels was graded based upon these values, by either increasing or decreasing in dosage by two. The final arginine *in ovo* levels were as follows 0%, 0.2%, 0.7%, 7.7%, 15.4%, 23.0%, and 30.7%. The bodyweights were taken on the day of hatch, 3, 5, 8, 10, 14, 21, and 28 days post-hatch. The graded HMB *in ovo* levels were chosen based upon previous feeding trials conducted by Nissen et al 1994 and Uni and Ferket, 2004 (0.01%, 0.03%, 0.1%, 0.3%, 1.0%, 3.0%). Bodyweights for the non-injected and injected controls were very similar at the time points measured (Table 1.1). The arginine levels of 7.7% or greater were lethal to the embryo upon *in ovo* administration. Poults *in ovo* fed 0.2% arginine had bodyweights

that were 3%, 18%, 24% and 15% greater than the controls at days 10, 14, 21 and 28 days post-hatch (Table 1.1). Poults *in ovo* fed 0.7% arginine had bodyweights that were 1%, 2%, 5%, 7%, 17%, 9% and 3% greater than the controls at days 3, 5, 8, 10, 14, 21, and 28 days post-hatch (Table 1.1). Hatchability of 0.2% and 0.7% arginine *in ovo* fed poults was 73% and 91%, respectively, with a hatchability of 82% for the injected controls and 65% for the non-injected controls. Therefore 0.7% arginine was identified as an optimal level for *in ovo* feeding due to improved hatchability and bodyweights in comparison to the 0.2% arginine *in ovo* feeding group.

Based on these preliminary results the procedures and methodologies for each *in ovo* feeding experiment were standardized. For each experiment, 19-d fertile Hybrid turkey eggs were donated by Prestage Farms (Clinton, NC). Approximately five hundred fertile turkey eggs were placed in temperature and humidity regulated incubators. During incubation, the temperature was held between 99.9-100.0°F and the eggs rotated every two hours. At day 21 of incubation, the eggs were weighed and eggs of 65g to 85g were evenly distributed within approximately five treatment groups. At 23-day of incubation, each egg was candled and the location of the amnion was identified and marked on the egg. A pilot *in ovo* injection hole was then made within the area of the amnion. Each egg was disinfected with 70% isopropyl alcohol and injected with 1.5mL of *in ovo* feeding solution with one treatment being a non-injected control. After *in ovo* nutrient administration each egg was disinfected and the hole sealed with a small piece of tape and transferred to hatching baskets. Upon hatching, each poult was marked for identification and the body weight recorded. Poults were given water and feed based

upon the NRC requirements for turkeys *ad libitum*. The bodyweights and tissue samples were taken prior to hatch, at hatch and post-hatch.

Table 1.1 The dose effects of in ovo feeding (IOF) arginine or HMB on growth at hatch through four-weeks post-hatch in turkeys¹.

	Hatch	Dy 3	Dy 5	Dy 8	Dy 10	Dy 14	Day 21	Day 28
IOF ² treatment	Bodyweights (g)							
Inj control	63.76±1.87	66.47±3.10	90.50±3.36	144.52±7.41	187.90±11.71	258.68±21.03	522.22±44.22	735.56±46.06
Non-Inj control	63.31±1.04	79.67±1.82	99.93±2.48	152.12±4.27	193.91±6.02	267.68±7.13	516.21±27.29	742.33±31.97
0.2% ARG	61.42±1.35	75.87±2.75	98.02±3.78	152.27±8.10	200.32±11.47	317.11±20.62	637.10±42.37	858.33±48.87
0.7% ARG	61.74±1.76	80.17±2.86	101.50±3.75	159.40±9.63	206.60±12.96	312.57±27.02	564.57±62.58	763.57±76.62
0.01% HMB	60.01±1.40	66.47±3.87	90.50±4.43	147.03±11.00	196.17±15.98	295.20±38.17	519.60±68.44	799.00±65.96
0.03% HMB	63.36±1.42	77.62±1.81	97.02±2.19	151.37±7.01	193.62±12.79	317.06±14.63	606.88±38.55	814.38±24.32
0.1% HMB	60.61±1.44	75.03±2.47	98.93±3.71	162.15±7.93	215.44±12.24	338.89±25.07	645.60±52.26	836.00±62.66
0.3% HMB	62.34±1.89	76.42±2.54	96.93±2.49	159.42±5.45	207.27±10.54	325.98±19.29	610.78±51.55	768.89±57.09
1.0% HMB	60.89±0.96	74.42±2.86	95.68±4.66	156.42±7.33	206.18±10.32	321.71±19.47	617.00±37.33	796.67±36.40
3.0% HMB	64.44±1.21	78.93±1.94	99.64±3.24	156.44±6.63	199.96±10.53	305.61±20.63	570.71±50.62	819.29±58.17

¹All data represents the mean value ± standard error of 20 sample birds per treatment.

² IOF treatments ARG=0.7% arginine and 0.2% arginine in 0.4% saline; % HMB IOF treatments in 0.4% saline.

Table 1.2 The dose effects of *in ovo* feeding (IOF) arginine or HMB on hatchability in turkeys

IOF ¹ treatment	% Hatchability (# eggs hatched/total # eggs set X100)
Inj control	82%
Non-Inj control	65%
0.2% ARG	73%
0.7% ARG	91%
0.01% HMB	82%
0.03% HMB	82%
0.1% HMB	91%
0.3% HMB	91%
1.0% HMB	82%
3.0% HMB	73%

¹IOF treatments ARG=arginine in 0.4% saline; % HMB IOF treatments in 0.4% saline.

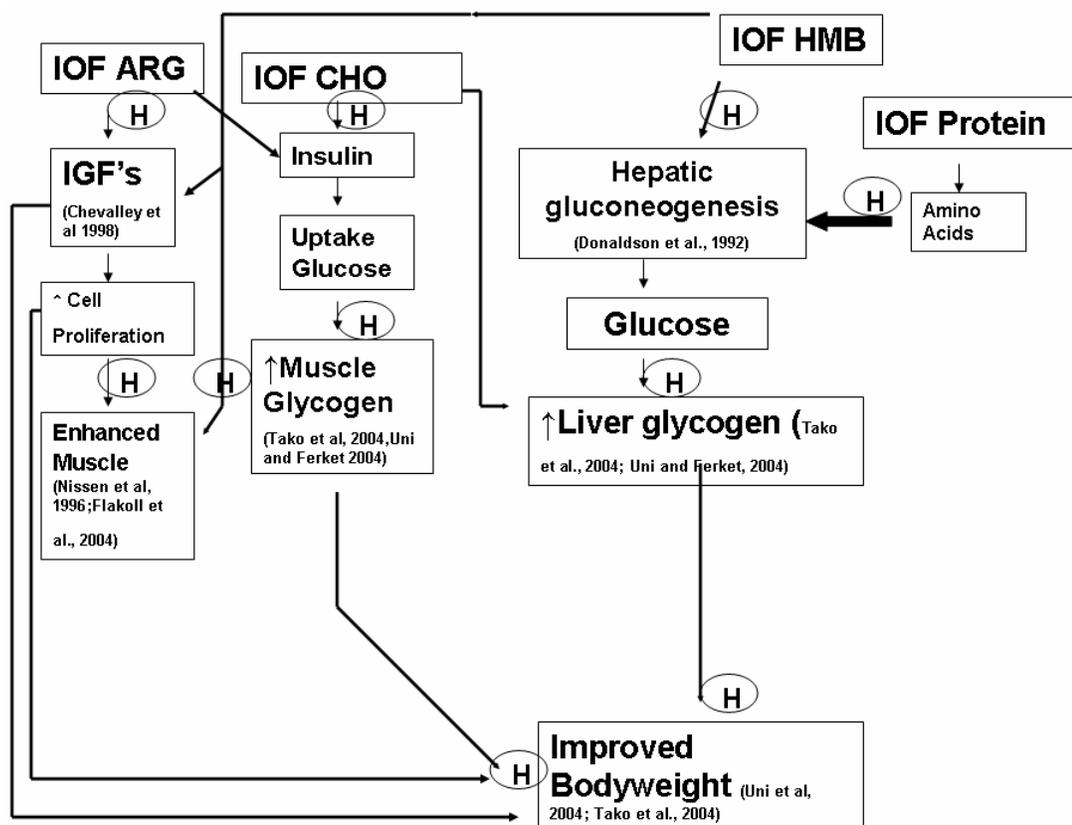
Development of IOF Experimental Hypothesis

Previous studies by Donaldson et al (1992) have shown that administration of the amino acid during the early post-hatch period enhanced hepatic glycogen reserves, as did glucose, whereas glucose inhibited hepatic gluconeogenesis. Hepatic gluconeogenesis is the primary mechanism for glucose production in the avian embryo and neonate and is critical for the supply of glucose, the primary fuel for growth and development (Romanoff, 1967). Based upon these findings, I hypothesize that *in ovo* feeding (IOF) of dietary protein will enhance hepatic gluconeogenesis and thus enhance hepatic glycogen reserves (Figure 1.4). Conversely, I hypothesize that IOF of carbohydrates will depress hepatic gluconeogenesis due to the action of insulin and thus depress hepatic glycogen storage. Previous studies by Uni and Ferket (2004) and Tako et al (2004) have shown

that IOF of carbohydrates and/or HMB significantly enhanced hepatic glycogen reserves and bodyweights in chick embryos and hatchlings. While the mechanisms of action for HMB are not known, I hypothesize that *IOF* of HMB will enhance bodyweights and hepatic glycogen reserves by enhancing hepatic gluconeogenesis in turkey embryos and poults (Figure 1.4). I hypothesize that *IOF* of carbohydrates and arginine will enhance muscle glycogen reserves, indirectly by the action of insulin, which will potentiate the uptake, and storage of glucose in the muscles.

In ovo administered nutrients may enhance the limited energy and nutrient supply of the egg, thus overcoming the level one constraint of limited egg nutriture. Upon oral consumption, digestion and absorption of the *in ovo* administered nutrients; the nutrients can either be utilized for energy, substrates for tissue development, and/or stored as energy (glycogen) (Figure 1.3). The enhanced glycogen stores may provide supplemental energy needed to fuel growth, development, maturation of organ systems, and hatching; or it may spare residual yolk and/or body protein (muscle) during the embryonic and peri-hatch periods of development (Figure 1.4). Thus *IOF* may serve to supplement the amount of energy and nutrients available in the egg of the avian embryo. Therefore, even if poults or chicks do not consume feed immediately after hatch, they have enough available energy and nutrients to overcome the delay in the time to self-initiate feed intake and/or denied access to feed and water, and consequently do not experience a negative nutritional status. Such enhancements may improve early post-hatch growth performance parameters and survivability.

Figure 1.4 The hypothesized effects of *in ovo* feeding (IOF) of protein, HMB, arginine and carbohydrates (CHO) on glycogen status and somatic growth in turkey poults.



(H) =hypothesis

Under typical hatchery practices, the avian neonate maybe denied access to feed and water for up to 72 hours post-hatch. This delayed access to feed may be a hindrance to enteric development. Studies have shown that incoming nutrients aid in stimulating enteric development, function and maturation (Zarling and Mobarhan, 1987; Butzner and

Gall, 1990). Studies have shown that immediate access to feed after hatch results in more rapid development of the intestine during the post-hatch period (Baranylova and Holman, 1976; Geyra et al., 2001a; Uni et al., 1998). Therefore, early post-hatch deprivation, may developmentally delay enteric maturation post-hatch. Additionally, when young hatchlings are given immediate access to feed and water, they often experience mal-absorption of incoming nutrients. Avian neonates hatch with an immature GIT, and therefore have a low capacity to digest and absorb incoming nutrients early in the post-hatch period. Within the first 72 hours post-hatch, the GIT is undergoing rapid morphological changes in which it is increasing in size, function and digestive and absorptive capacity (Uni et al., 1998a, 1998b, 1999a, 1999b, 2000, 2003a). It is within this window of time in which poult experience mal-absorption of incoming nutrients.

In ovo feeding may serve as a tool to overcome level two constraints on growth. Firstly, *in ovo* feeding may expedite GIT maturation before hatch thus chicks and poults hatch with a more mature gut. Secondly, *in ovo* feeding may prevent post-hatch mal-absorption of nutrients by causing the compensatory changes conducive to an external diet to occur prior to hatching. Thus, at hatch the expression and activity of the carbohydrate and protein digestive and absorptive systems are enhanced and mature functioning and poults can adequately digest, absorb and utilize nutrients from feed (Figure 1.5). Studies by Tako et al (2004) demonstrated that *IOF* of carbohydrate and/or HMB significantly enhanced the villus surface area of chick embryos by approximately 33% over the controls, thus upon hatching the *in ovo* fed chick had a greater absorptive and digestive surface area than the conventional chick. Rapid and early enteric

maturation is critical due to the transition from a lipid-based diet to a carbohydrate based external diet post-hatch. Intestinal development maybe a rate determining step in growth, and the brush border enzymes may have different developmental timetables that influence digestion in post-hatch birds. Thus, the brush border enzymes of the intestine may play a rate-limiting role in providing substrates for growth. Activity of the brush border enzyme responsible for carbohydrate digestion, sucrase-isomaltase (SI) was significantly enhanced by *IOF* carbohydrate and/or HMB in broiler chicks and embryos over the controls (Tako et al, 2004, Uni and Ferket, 2004), thus the *IOF* chicks had a greater capacity to digest incoming dietary carbohydrates than the conventional chick. Early maturation and functioning of the GIT is of extreme importance because the GIT is one of the primary supply organs of the body and it must function optimally very early in life to ensure survival. These incoming nutrients are utilized to meet the metabolic requirements needed for more rapid growth and development in young poult.

Numerous studies have shown that dietary sugars, proteins, and amino acids enhance the expression and activity of brush border enzymes and nutrient transporters within the small intestine (Diamond et al., 1984; Diamond and Karasov, 1987; Karasov and Debnam, 1987; Ferraris and Diamond., 1997; Ferraris et al., 1992a; Ferraris et al., 1992b.). Thus, I hypothesize that *IOF* of protein will directly enhance the activity of the brush border enzyme, leucine aminopeptidase (LAP) responsible for peptide hydrolysis, and the activity and expression of the alanine transporter (B^{+0}) responsible for uptake of neutral amino acids, and the peptide transporter (Pept-1) responsible for the uptake of di-peptides and tri-peptides (Figure 1.5). I hypothesize that *IOF* of carbohydrates will

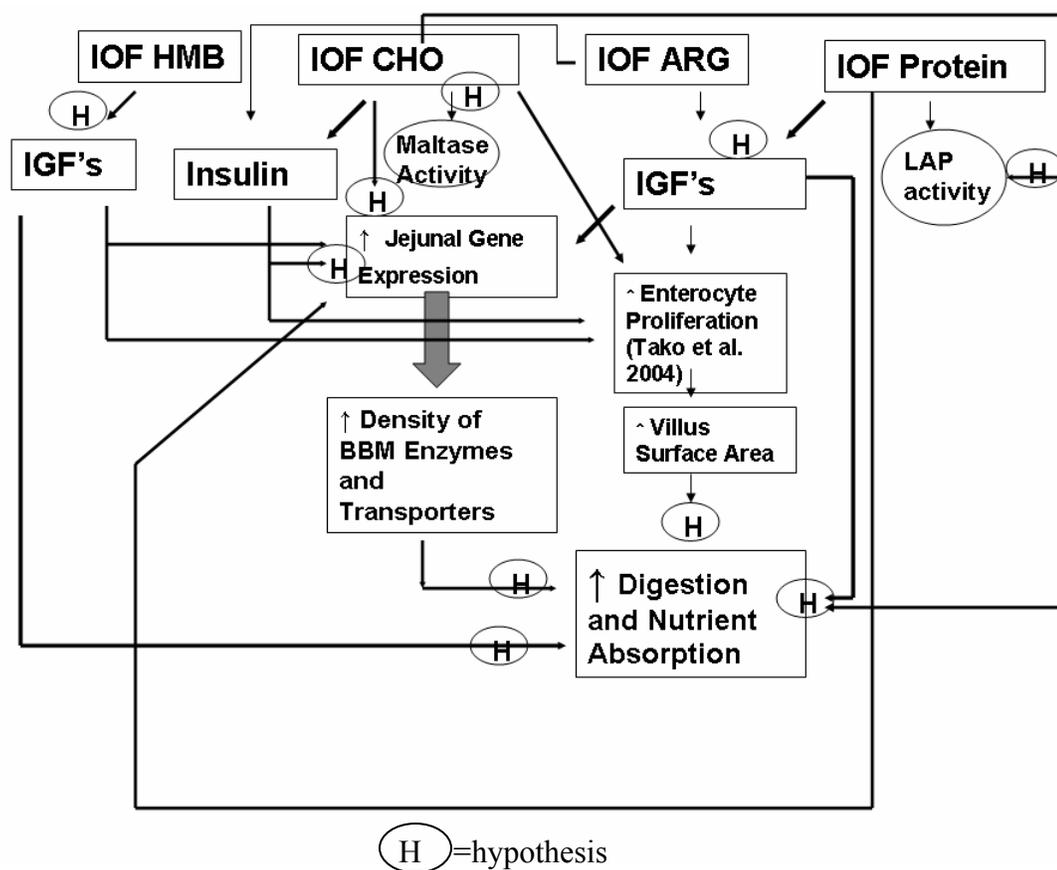
directly enhance the activity and expression of the sodium glucose transporter (SGLT-1), responsible for uptake of glucose in the jejunum and the brush border enzyme, sucrase-isomaltase (SI), responsible for the hydrolytic cleavage of maltose and sucrose in the jejunum (Figure 1.5).

Pharmacological doses of arginine have been shown to stimulate IGF-I production (Chevalley et al, 1998). IGF-I mediates local and systemic growth by enhancing cell proliferation and protein synthesis. Studies have shown that IGF-II and insulin stimulates enterocyte proliferation, while IGF-I stimulates proliferation of the cells of the crypts (Jehle et al., 1999). IGF-I has also been shown to enhance the activity and expression of the SGLT-1 of the small intestine. Additionally, arginine is a potent insulin secretagogue. Insulin and pro-insulin have been shown to enhance intestinal cell proliferation, thereby increasing the villus absorptive area of the jejunum (Laburthe et al, 1988, Schober et al, 1990, Odle et al, 1996, MacDonald 1999, Menard et al, 1999; Jehle et al., 1999). Thus, I hypothesize that *IOF* of arginine will enhance intestinal development and function by increasing the villus absorptive and digestive surface area of the jejunum and by increasing the activity and expression of SGLT-1, Pept-1, B⁺, SI and LAP indirectly by the action of IGF's and insulin (Figure 1.5). Previous studies (Nissen et al, 1994; Fuller et al., 1994; Flakoll et al 2004) have demonstrated that HMB supplementation enhances muscle deposition and increases bodyweights. It is known that muscle deposition and somatic growth are regulated by IGF's. While the mechanism of action for HMB is not known, I hypothesize that *IOF* HMB will enhance muscle

deposition and bodyweight indirectly by altering protein synthesis by the action of IGF's (Figure 1.4)

Lastly, level three constraints on growth can be overcome indirectly by *in ovo* feeding. *In ovo* feeding enhances glycogen reserves and spares residual yolk and body protein (muscle) stores. Additionally, *in ovo* feeding enhances GIT development, capacity and functioning during embryonic and post-hatch development. Therefore poults have an increased capacity for nutrient digestion and uptake and incoming nutrients can be used or stored as energy. Thus, young hatchlings have enough energy to support both growth and tissue and organ maturation, and overall growth is not compromised. Adequate energy can be allocated for the development and maturation of the GIT, immune system, muscular and skeletal systems. For example, poults are not immuno-compromised due to an immature immune system, and they can overcome microbial challenge. Adequate energy must be supplied to the skeletal and muscular systems to ensure that locomotion, support and activity are correlative to a rapidly increasing body size.

Figure 1.5. The hypothesized effects of *in ovo* feeding (IOF) of protein, HMB, arginine and carbohydrates (CHO) on intestinal morphology, function and gene expression in turkey poults.



The purpose of this dissertation was to investigate the effects of *in ovo* feeding of various macronutrients (carbohydrates and proteins) and metabolic and enteric modulators (amino acids or their metabolites) on energy metabolism, growth performance, enteric development and functioning, and plasma IGF's during avian development. The objectives were to determine the direct and indirect effects of *in ovo*

feeding on avian embryonic and neonatal growth and development. The second and third chapters (manuscripts) present the effects of *in ovo* feeding of macronutrients (proteins and/or carbohydrates) and/or HMB and/or arginine on energy metabolism and muscle and body weight gains. The fourth and fifth chapters (manuscripts) presents the effects of *in ovo* feeding of protein and/or HMB and/or arginine on the activity of the brush border enzymes (SI, LAP), the activity of the nutrient transporters (SGLT-1, B⁺), and the expression of the brush border enzymes and nutrient transporters, respectively. The sixth chapter presents the effects of *in ovo* feeding of protein and/or HMB and/or arginine on plasma IGF1 and IGF2 during the early post-hatch period of turkeys. The final chapter examines the effects of *IOF* protein and/or HMB and/or arginine on jejunal gene expression in turkey embryos and poults.

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Chapter 2

Effect of In Ovo Feeding Solutions Containing Egg White Protein, β -hydroxy- β -methylbutyrate (HMB), and Carbohydrates on Glycogen Status and Neonatal Growth of Turkeys.

ABSTRACT *In-ovo* feeding (IOF), injecting dietary components into the amnion prior to internal pipping, may enhance early growth performance by altering glycogen status. This hypothesis was challenged by evaluating five *IOF* solutions containing protein, HMB and carbohydrate. Four *IOF* treatments were arranged as a factorial of two levels of egg white protein (0 and 18%) and two levels of HMB (0 and .1%). An additional *IOF* solution containing carbohydrates (20% dextrin and 3% maltose) was evaluated for contrast purposes. All the *IOF* feed solutions was prepared in .4% saline. At 23 d of incubation 1.5 ml of each *IOF* treatment solution was injected into the amnion and returned to the hatching incubator until they were pulled at 28d of incubation. All poults were given *ad libitum* access to feed within 24 hours after hatch. Body weight (BW) were determined on all poults at hatch and at 3 and 7 d of age, and 10 poults per treatment were sampled at the same times to evaluate weights and glycogen content of liver and pectoralis muscle (PC).

The poults *in ovo* fed treatments A (protein), B (protein +HMB), and D (HMB) weighed 6.0%, 2.7% and 3.3% more than the controls at hatch, respectively ($P < 0.05$), revealing a significant protein X HMB interaction effect. But at 3 and 7 d, only HMB-treated poults had significantly greater BW ($P < 0.005$). At hatch, % PC was greatest among treatments A and D ($P < 0.05$), but HMB increased % PC at 7 d. Total hepatic

glycogen was enhanced by treatments A and B at 7-d post-hatch ($P < 0.05$), over the controls, whereas total muscle glycogen reserves were enhanced at one week post-hatch by *in ovo* feeding of treatment D ($P < 0.05$). Contrasting treatments A (protein) and S (20% dextrin + 3% maltose) with controls, revealed that they had had greater BW than the controls at hatch ($P < 0.05$) but not subsequently. Treatment A resulted in the greatest hepatic glycogen reserves at hatch ($P < 0.05$), but by 7 d post-hatch both treatments A and S had higher hepatic glycogen reserves than the controls ($P < 0.05$). Additionally, poult *in ovo* fed treatment S had enhanced total muscle glycogen stores in comparison to the controls; while *in ovo* fed poult of treatment A had significantly less total muscle glycogen stores than the controls ($P < 0.05$). The results of this experiment demonstrate that *in ovo* feeding of protein or carbohydrate may enhance hatch weight and glycogen status, but this effect can be modulated during the neonatal period by inclusion of HMB.

INTRODUCTION

Embryonic development among mammals and birds differ mainly by their embryonic source of glucose nourishment and energy metabolism. The mammalian fetus is provided with a constant supply of plasma glucose by maternal blood supply, this monosaccharide can then be used as an energy source for growth and development. Mammals then switch from a carbohydrate-based energy nutriture to a more fat-based nutriture after birth, when the neonate is presented with a high fat, low carbohydrate diet (milk) and forms much of the glucose endogenously by gluconeogenesis. The hepatic enzymes involved in gluconeogenesis have increased activity during this period and

decrease in activity after the suckling period (Ballard and Oliver, 1965; Yeung et. al, 1967). In contrast, the developing chick embryo must rely upon the nutrients provided by the egg independent of maternal influence. Nutrient transfer from the mother to the embryo is completed before the egg is laid. Thus the egg contains all of the nutrients needed for the growth and development of the embryo. The only materials exchanged with the environment are water (vapor), oxygen and carbon dioxide. The in ovo nutriture of the chick embryo consists mainly of yolk fat with only traces of carbohydrate (Mehner, 1983; Starck and Ricklefs, 1998). However, glucose is the primary source of carbohydrate energy needed for development, growth and maintenance, and it is an important component of the cellular membranes, glycoproteins and glycolipids. After hatch, birds switch from a fat-based energy nutriture to more carbohydrate-based as the neonatal chick adapts to a high-carbohydrate diet.

Glycogen first appears in the chick embryo on the 6th day of incubation (Dalton, 1937). Ballard and Oliver (1963) observed liver glycogen increased from 7mg/g of tissue at 10-d of incubation to 26mg/g at 19-d of incubation, and the specific activity of glycogenic enzymes increased concurrently. At the end of incubation the stored glycogen is rapidly mobilized, falling from 19mg/g in the liver of embryos after 18.5 days of incubation to 1.6mg/g in the liver 1 day post-hatch (Freeman, 1965, 1969). Glycogen stores are rapidly sequestered to provide energy needed during the hatching process and subsequent growth and development.

Glycogen reserves in the avian embryo provide the critical energy needed for emergence from the egg during the last quarter of incubation. In turkeys, extensive

embryonic mortality occurs toward the end of the incubation period when hatching-related events occur, such as pipping of the egg membrane and shell, beginning of pulmonary respiration, and the actual egg emergence (Christensen et al., 1992, 2000, 2001). During this time glycogen reserves in the chick embryo is significantly depleted during the peri-hatch period in order to meet the high energy demand during the process of emergence (Freeman, 1969; Freeman and Manning, 1971). Hepatic and muscle glycogen reserves are depleted due to carbohydrate utilization for muscular activity during the hatching process (George and Iype, 1963; Bakhuis, 1974; John et al., 1987).

Additionally, upon hatching turkey poults have a greater hepatic glycogen phosphorylase/glycogen synthetase ratio up to day-6 post-hatch (Rosebrough et al. 1979). Therefore during the first week post-hatch, poults have a greater propensity for glycogen hydrolysis and not glycogen synthesis. This diminished or depleted glycogen stores may lead to a negative nutritional status, which may lead to reduced or stunted growth and increased mortality during the early post-hatch period. Mortality rate of young hatchlings is greatest during the first two weeks post-hatch, averaging between 3 and 6% (Phelps, 1987).

A novel method of supplementing the *in ovo* nutriture of oviparous species is described as “*in ovo* feeding” within the US Patent (6,592,878) of Uni and Ferket (2003) involves the administration of exogenous nutrients into the amnion of the developing embryo of chickens and turkeys at about 17 and 23 days of incubation, respectively. Because the late term embryo orally consumes the amniotic fluid (comprised primarily of water and albumen protein) prior to pipping of the air cell, this *in ovo* feeding technology

is a cleaver means of presenting exogenous nutrients to the enteric tissues for absorption and utilization for growth and stored energy as glycogen. Therefore, *in ovo* feeding a supplemental source of protein and carbohydrate may help overcome the constraint of limited egg nutriture. Consequently, the enhanced glycogen stores may provide supplemental energy needed for better neonatal survival, fuel more rapid growth, and spare body protein (muscle) reserves post-hatch. Hatchlings with enhanced glycogen reserves have improved bodyweights, decreased mortality and improved performance (Moran, Jr., 1988).

Uni and Ferket (2003) injected turkey embryos with varying levels of HMB at 24 days of incubation, of HMB 0ug (non-injected control), 0.1ug, 1.0ug, 10ug, 100ug, or 0.9% saline (injected-control). Hatchability was enhanced by *in ovo* feeding HMB at the 0.1ug and 1.0ug levels, over the controls. Additionally, liver glycogen was increased approximately 40% in all HMB levels as compared to the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2), with a quadratic response ($P < 0.05$) as the level of HMB injected *in ovo* increased. Moreover, hatchability rates were positively correlated with liver glycogen content of turkey and chick embryos before hatch ((Uni and Ferket, 2003 Patent # US 6,592,878 B2). Also, *in ovo* feeding of a 24% egg white protein solution enhanced the bodyweights of turkey poults 4% to 8% over the controls at hatch, 3 days post-hatch and 12 days post-hatch over the controls (Uni and Ferket, 2003). Also, poults *in ovo* fed a 24% egg white protein and HMB solution had significantly increased bodyweights of poults by 4% to 6% in comparison to the controls from hatch through 24 days post-hatch (Uni and Ferket, 2003).

Additional studies by Uni and Ferket (2004) revealed that *in ovo* feeding of solutions containing carbohydrates and protein increased broiler hatching weights by 3% to 7%, over the controls, which was sustained up to 35 days of age. Also, *in ovo* fed broiler hatchlings had intestinal developmentally similar to the 2-day old conventional chick offered feed immediately post-hatch. In addition, *in ovo* fed chick embryos and hatchlings receiving carbohydrates (dextrin, maltose, and sucrose) had enhanced hepatic glycogen stores in comparison to the controls day 20 of incubation and hatch (Uni and Ferket, 2004).

β -hydroxy- β -methylbutyrate (HMB), a metabolite of leucine, has been hypothesized to be responsible for leucine's ability to stimulate protein synthesis or prevent proteolysis (Nissen et al, 1996, 1997; Ostaszewski et al, 1996). It has been reported that the addition of HMB to the bathing medium containing muscle strips of rats and chicks increased protein synthesis by approximately 20%, while proteolysis was inhibited by approximately 80% (Ostaszewski et al, 1996). Uni and Ferket (2003) reported that the *in ovo* administration of HMB to turkey embryos prior to pipping increased glycogen status at hatch. HMB may augment the beneficial effects of *in ovo* feeding protein on glycogen status of the turkey hatchling. Additionally, we hypothesize that *in ovo* feeding of HMB will enhance the development of pectoralis muscle in the avian neonate by sparing the use of muscle protein for gluconeogenesis. Schultz and Mistry (1981) demonstrated that non-gluconeogenic substrates, such as beta-hydroxybutyrate or ethanol in the culture medium of isolated chicken hepatocytes increased the formation of glucose by 80% and 200%, respectively. These substrates

may enhance gluconeogenesis due to their beneficial effect of increased reducing equivalents which can significantly affect a change in the oxidation-reduction (pyruvate/lactate) ratio, in hepatocytes. We hypothesize that HMB fed *in ovo* may enhance hepatic and muscle glycogen reserves in the avian embryo and neonate.

To test the hypotheses described above, a study was designed to evaluate the effect of *in ovo* feeding solutions containing egg white protein (predominate protein source within the egg) and/or HMB on growth and energy status of the neonatal turkey, and contrasting it with an *in ovo* feeding solution containing a highly digestible source of carbohydrate (dextrin and maltose) that would not exceed osmolality limits. Our objective was to determine the total hepatic and muscle glycogen, body and organ weights as a result of *in ovo* feeding of the fore mentioned treatments in turkeys. The turkey was chosen as an animal model due to its economic importance within the poultry industry. Additionally, the turkey embryo develops in a closed environment, independent of maternal influences and is thus an ideal animal system in which to study the effects of exogenous nutrients on embryonic growth, development and energy metabolism.

MATERIALS AND METHODS

Incubation and in ovo feeding (IOF)

Viable Hybrid® turkey eggs were obtained at 19 d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). At 21 d of incubation 500 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65 g to 85 g per egg. These eggs were then evenly distributed among five treatment groups of 100 eggs each, such

that the weight distribution profile among all 5 treatment groups was identical. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was then punched using a 23 gauge needle and 1.5 ml of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15 mm. The injection hole area was disinfected with an ethyl alcohol-laden swab, sealed with a cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as ascetically as possible such that the *in ovo* feeding treatment solutions contained the following: A) 18% egg white protein (EWP) in 0.9% saline; B) 18% EWP + 0.1% HMB in 0.9% saline); D) 0.1% HMB in 0.9% saline); and S) 20% dextrin + 3% maltose in 0.9% saline). The controls (treatment C) were not injected but they were subjected to the same handling procedures as the *in ovo* feeding treatment groups. The HMB used in this study was the calcium salt kindly provided by Metabolic Technologies, Inc. (Ames, IA). The egg white protein (egg whites from chicken catalog # E0500), dextrin (Type I from corn catalog # D2006) and maltose monohydrate catalog #M5885 used in this study was purchased from Sigma (St. Louis, MO)

Animal Husbandry

Upon hatching, each poult was identified by neck tag number and body weights recorded at hatch and 7 d post-hatch. Hatchability rate of viable eggs was >95%, and did not differ significantly among treatment groups. About 12 poult were randomly assigned to 7 replicate pens per treatment. All the birds were housed in a total confinement building with supplemental heat from propane-fired heaters to maintain about 27C. Each floor pen bedded with soft pine wood shavings, and equipped with automatic nipple drinkers, a

manual self-feeder, and supplemental incandescent heat lamp to maintain a spot brooding temperature of about 40°C. Each pen of poults were given *ad libitum* access to a typical turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat) that met or exceeded National Research Council (1994) nutrient requirements for turkeys. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Tissue Sampling and Glycogen Analysis

At hatch and 7 d post-hatch, 10 birds per treatment were euthanized by cervical dislocation and within 2 minutes the whole liver and pectoralis muscle was dissected and placed on ice before freezing for subsequent glycogen analysis. Frozen liver or muscle samples were then thawed in groups such that all sample days and treatments were equally represented so as to account for errors associated with glycogen analysis. These pectoralis muscle and liver samples were then homogenized in 8% perchloric acid (1g/4mL) and glycogen content was determined using methods described by Dreiling et al (1987). After homogenization, the samples were centrifuged at 14,000 rpm, 4°C for 30 minutes. One milliliter of the supernatant was transferred to a clean polypropylene tube and 2.0 mLs of petroleum ether was added to each sample and vortexed. The samples were centrifuged at 2000 rpm, 4°C for 15 minutes. Subsequently, 0.4mL of 8% perchloric acid and 2.6mL of iodine color reagent (1.3 mL of solution A in 100 mL of 67.8% saturated calcium chloride (anhydrous) solution; (solution A= 0.26g iodine + 2.6g potassium iodide dissolved in 10 mL of distilled water)) was added to a 10 μ L aliquot of the sample (bottom layer) in a disposable cuvette. All samples were read at a wavelength

of 460nm. The amount of glycogen present in a 10uL sample is determined by preparation of a known glycogen standard curve.

Statistical Analysis

All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1996). Each bird served as an experimental unit for statistical analysis. Because highly significant age effects were observed, the treatment effects were evaluated by neonatal age (i.e. hatch, 3 d and 7 d of age). Data from *in ovo* treatments A, B, C, and D were analyzed as a 2X2 factorial arrangement, with two levels of egg white protein (0% and 18%) and two levels of HMB (0% and 0.1%). Variables having different F-test were compared using the least-squares-means function in SAS (1996) and the treatment effects were considered significant at $P < 0.05$. An additional analysis was conducted to contrast the effects of dietary carbohydrates *versus* dietary protein on hepatic and muscle glycogen content. These data were analyzed in as a one-way ANOVA with treatments A (18% EWP in 0.9% saline), C (non-injected control) and S (20% dextrin and 3% maltose in 0.9% saline). When ANOVA tests were significant ($P < 0.05$), the treatments were separated by least squares means (t-test). All experiments were conducted with an equal frequency of variables within each treatment.

RESULTS

The temporal changes of glycogen status, relative liver and pectoralis muscle weight from the day of hatch to one week post-hatch

At hatch, the hepatic and pectoralis muscle glycogen stores were depleted in the conventional turkey poult (Figure 2.1). Conversely, after one week of consuming a

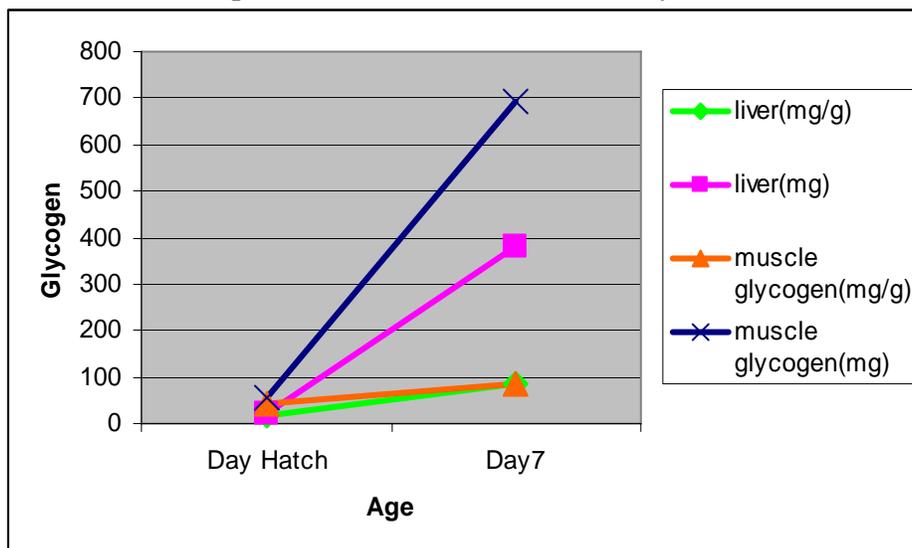
carbohydrate-rich diet, the total hepatic and pectoralis muscle glycogen status was vastly improved in the conventional poult. On the contrary, the glycogen concentration (mg/g) of the liver and pectoralis muscle was roughly unchanged. At 7-d post-hatch, the total pectoralis muscle glycogen (mg) was approximately 1.75 fold greater than the total hepatic glycogen (mg) content in the conventional poult (Figure 2.1), which was primarily due to pectoralis muscle mass (Figure 2.2). There was approximately a two-fold increase in the relative pectoralis (% of the bodyweight g/g) muscle mass between the day of hatch and 7-d post-hatch, while there was very little increase in the relative liver mass (% of the bodyweight g/g) during the first week post-hatching (Figure 2.2) .

The effects of IOF of two levels of egg white protein (0% and 18%) and two levels of HMB (0% and 0.1%)

At hatch, the bodyweights of all *in ovo* fed poult were significantly greater than the controls ($p < 0.05$, Table 2.1), and there was a highly significant ($P < .005$) protein X HMB effect observed. Poult *in ovo* fed 18% EWP, a combination of 18% EWP + HMB, or 0.1% HMB had bodyweights that were 6.0%, 2.7% and 3.3% greater than the controls, respectively ($p < 0.05$, Table 2.1). This effect was lost by 3 d post-hatch as a significant main effect of HMB appeared ($P < .05$) and persisted until 7 d: the EWP + HMB and HMB *in ovo*-fed poult were larger than the controls, their values were statistically similar. At 7 d post-hatch, only the HMB *in ovo* fed poult had significantly greater bodyweights in comparison to the controls ($p < 0.05$ Table 2.1). Moreover, those poult *in*

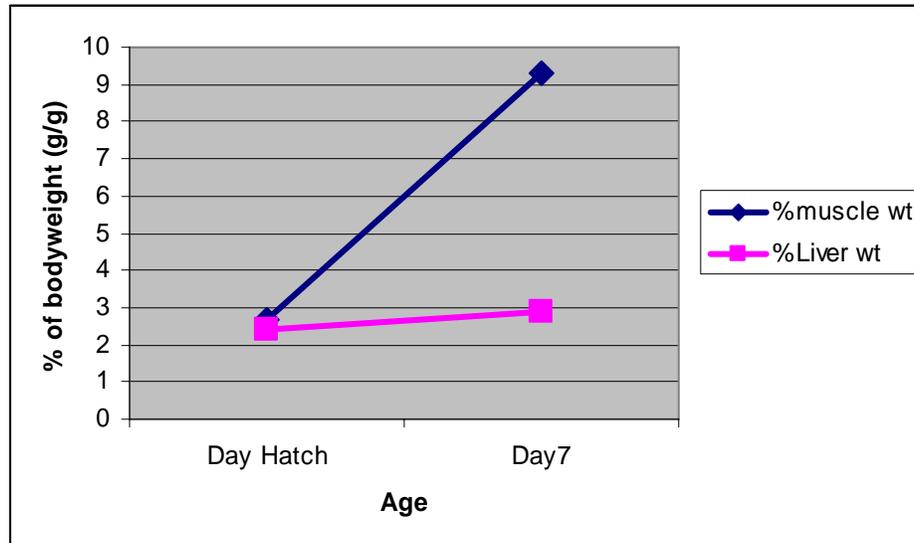
ovo fed the 18% EWP and 0.1% HMB *in ovo* fed poult had significantly greater pectoralis muscle size relative to body weight at hatch in comparison to the controls ($p < 0.05$, Table 2.2). At 3 d post-hatch, there were marginal differences in the relative pectoralis mas between the *in ovo* fed poult and controls, but a significant positive effect of HMB on relative pectoralis mass of poult was observed (Table 2.2). Additionally, there were no significant differences in the relative pectoralis mass between poult that were *in ovo* fed EWP or 20% dextrin + 3% maltose at hatch or 7d post-hatch (data not shown).

Figure 2.1 The temporal changes of glycogen status from the day of hatch to one week post-hatch in conventional turkeys¹



¹Each value represents the mean of the non-injected controls (n=10)

Figure 2.2 *The temporal changes of relative muscle and liver weights from the day of hatch to one week post-hatch in conventional turkeys¹*



¹Each value represents the mean of the non-injected controls (n=10)

Table 2.1 The effects of *in ovo* feeding (IOF) of β -hydrox- β -methylbutyrate (HMB) and egg white protein on the bodyweights of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Bodyweight		
	Hatch	Day 3	Day 7
	------(g) -----		
A(protein)	56.02 ^a	73.23 ^a	131.91 ^c
B(protein + HMB)	54.09 ^b	76.63 ^a	140.93 ^{ab}
control	52.64 ^c	73.64 ^a	135.47 ^{bc}
D(HMB)	54.46 ^b	75.84 ^a	145.75 ^a
Source of Variation	----- P-Value -----		
Protein	0.0008	0.882	0.157
HMB	0.905	0.0324	0.001
Protein X HMB	<0.0001	0.642	0.831
SEM (DF) ³	0.217 (227)	0.630 (186)	1.44(185)

¹All data represents the mean value \pm standard error of 7 replicate pens per treatment containing 12 sample birds per pen.

² Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline.

³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis.

^{a,b}Means within a column with different superscript are significantly different (P<.05).

Poults *in ovo* fed EWP had the heaviest bodyweights and the greatest relative pectoralis muscle mass at hatch (p<0.05, Table 2.1 and Table 2.2, respectively).

Evidently, neither body weight nor pectoralis muscle yield was enhanced further at hatch by the addition of HMB to the IOF solution. Poults *in ovo* fed EWP + HMB and HMB alone had similar body weights and pectoralis muscle yields, revealing a significant protein X HMB interaction effect (p<0.05). But this significant protein X HMB effect was no longer observed 3 and 7 d post-hatch. In contrast, *in ovo* feeding of HMB alone

had the greatest effect on bodyweight and relative pectoralis mass 3 and 7 d post-hatch ($p < 0.05$ Table 2.1 and Table 2.2, respectively).

Table 2.2 The effects of *in ovo* feeding (IOF) of β -hydrox- β -methylbutyrate (HMB) and egg white protein on relative pectoralis muscle and liver mass of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Pectoralis Muscle		Liver	
	Hatch	Day 7	Hatch	Day 7
	-----(% of Body Weight) -----			
A (protein)	3.3 0 ^a	9.20 ^a	2.60 ^a	3.00 ^a
B (protein + HMB)	2.80 ^b	9.80 ^a	2.40 ^a	3.20 ^a
C (control)	2.70 ^b	9.30 ^a	2.40 ^a	2.90 ^a
D (HMB)	3.10 ^{ab}	10.00 ^a	2.40 ^a	3.00 ^a
Source of Variation	----- P-Value -----			
Protein	0.241	0.588	0.255	0.262
HMB	0.713	0.050	0.172	0.090
Protein X HMB	0.002	0.873	0.334	0.550
SEM (DF) ³	0.06(36)	0.167(36)	0.083(36)	0.088(36)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline.

³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis.

^{a,b}Means within a column with different superscript are significantly different ($P < .05$).

Total hepatic glycogen and hepatic glycogen concentrations (mg/g) were enhanced significantly at hatch over the controls by *in ovo* feeding of EWP ($p < 0.05$, Table 2.3) with a significant protein X HMB interaction ($p < 0.05$, Table 2.3). Additionally, *in ovo* feeding of HMB significantly enhanced the concentration of hepatic glycogen over the controls at hatch ($p < 0.05$, Table 2.3). There was a significant protein effect ($P < .005$), such that poult *in ovo* fed EWP alone and EWP + HMB had

significantly enhanced total hepatic glycogen reserves and concentrations at one week post-hatch ($p < 0.05$, Table 2.3), in comparison to the controls. Total pectoral muscle glycogen was significantly enhanced at hatch and 7 d post-hatch by *in ovo* feeding of HMB alone ($p < 0.05$, Table 2.4) over the controls, with HMB and protein independently having significant main effects ($p < 0.05$, Table 2.4); with the inclusion of protein in the *in ovo* nutrient solution total pectoralis muscle glycogen was depressed, while HMB inclusion in the *in ovo* solution enhanced total pectoralis muscle glycogen content relative to the controls (Table 2.4). Conversely, *in ovo* fed poult of treatment EWP alone and EWP + HMB had significantly greater pectoral muscle glycogen concentrations in comparison to the control and HMB treatment groups at 7 d post-hatch ($p < 0.05$, Table 2.4).

Poult *in ovo* fed either EWP alone or HMB alone had enhanced hepatic glycogen reserves in comparison to the controls at hatch ($p < 0.05$, Table 2.3). Nevertheless, as the significant ($P < .05$) protein X HMB interaction revealed, the effects of EWP and HMB were not additive when shown by the depressed hepatic glycogen status observed when these two components were combined in the *IOF* solution. By 7 d post-hatch, protein had the main effect on hepatic glycogen reserves ($p < 0.05$, Table 2.3). At one week post-hatch, there was no interactive effect of protein and HMB on hepatic glycogen status.

Glycogen index, calculated as the sum of the total hepatic and muscle glycogen divided by body mass (mg/g), is a relative indicator of energy status to support metabolism and growth. Poult *in ovo* fed HMB had a significantly higher glycogen

index than the control, EWP, or EWP + HMB *in ovo* treatments ($p < 0.05$, Table 2.5).

This effect was diminished by 7 d post-hatch. Similarly, glycogen index at 7 d post-hatch did not differ significantly among the controls and those poultts *in ovo* fed the EWP and S (20% dextrin + 3% maltose) treatments (Table 2.7).

Table 2.3 The effects of *in ovo* feeding (IOF) of β -hydrox- β -methylbutyrate (HMB) and egg white protein on hepatic glycogen concentration and total hepatic glycogen of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Glycogen Concentration(mg/g)		Total Glycogen (mg)	
	Hatch	Day 7	Hatch	Day 7
A (protein)	36.18 ^a	140.54 ^a	52.50 ^a	590.53 ^a
B (protein + HMB)	22.00 ^{bc}	154.91 ^a	28.30 ^b	682.93 ^a
C (control)	16.44 ^c	84.68 ^b	20.30 ^b	378.64 ^b
D (HMB)	25.69 ^{ab}	66.83 ^c	32.50 ^b	296.90 ^b
Source of Variation	----- P-Value -----			
Protein	0.123	0.004	0.061	0.009
HMB	0.631	0.940	0.414	0.961
Protein X HMB	0.027	0.492	0.017	0.424
SEM (DF) ³	2.54(36)	11.60(36)	3.62(36)	53.83 (36)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline.³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis.

^{a,b}Means within a column with different superscript are significantly different ($P < .05$).

Table 2.4 The effects of *in ovo* feeding (IOF) of β -hydrox- β -methylbutyrate (HMB) and egg white protein on pectoralis muscle glycogen concentration and total pectoralis muscle glycogen of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Glycogen Concentration(mg/g)		Total Glycogen (mg)	
	Hatch	Day 7	Hatch	Day 7
	-----(% of Body Weight) -----			
A (protein)	9.18 ^c	140.54 ^a	16.88 ^d	469.59 ^d
B (protein + HMB)	24.05 ^b	154.91 ^a	36.49 ^c	545.92 ^c
C (control)	40.74 ^a	84.68 ^b	56.12 ^b	693.50 ^b
D (HMB)	42.96 ^a	66.83 ^b	70.32 ^a	790.76 ^a
Source of Variation	----- P-Value -----			
Protein	<0.0001	<0.0001	<0.0001	<0.001
HMB	0.0002	0.072	<0.0001	0.0016
Protein X HMB	0.0046	0.978	0.483	0.691
SEM (DF) ³	1.05(36)	0.937(36)	1.92(36)	12.71(36)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

² Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline. ³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis.

^{a,b}Means within a column with different superscript are significantly different (P<.05).

Table 2.5 The effects of *in ovo* feeding (IOF) of β -hydrox- β -methylbutyrate (HMB) and egg white protein on the glycogen index of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Glycogen Index	
	total liver glycogen + total muscle glycogen(mg)/ bodymass (g)	
	Hatch	Day 7
A (protein)	1.27 ^b	7.82 ^a
B (protein + HMB)	1.23 ^b	9.04 ^a
C (control)	1.50 ^b	7.46 ^a
D (HMB)	1.92 ^a	7.62 ^a
Source of Variation	-----p-value-----	
Protein	0.005	0.277
HMB	0.216	0.397
Protein*HMB	0.144	0.514
SEM (DF) ³	0.150(36)	0.810(36)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline.

³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis. ^{a,b}Means within a column with different superscript are significantly different (P<.05).

Contrast between IOF of 18% egg white protein (EWP) and 23% carbohydrate (S)

Poults *in ovo* fed either the EWP or 20% dextrin + 3% maltose (S) had significantly greater bodyweights than the controls at hatch (p<0.05, Table 2.6). By 7 d post-hatch, there were no significant differences in body weights or relative pectoralis mass among the treatments. At hatch, poults *in ovo* fed the EWP had significantly greater total hepatic glycogen reserves than the control or S treatment poults (p<0.05, Figure 2.7). At 7 d post-hatch, poults of EWP and S treatment groups had similar yet significantly greater hepatic glycogen reserves than the controls (Figure 2.1). Hatchlings *in ovo* fed EWP had significantly less total muscle glycogen reserves than the control and S-treated poults. At 7 d post-hatch, the S *in ovo* fed poults had significantly greater total muscle glycogen

than the control and EWP-treated poult; while the EWP-treated poult had significantly less total muscle glycogen than the controls ($p < 0.05$, Figure 2.1). Poults *in ovo* fed S had a significantly higher glycogen index (mg/g) than the non-injected controls and EWP *in ovo* fed poult ($p < 0.05$, Table 2.7).

Table 2.6 The effects of *in ovo* feeding (IOF) of egg white protein and carbohydrate on the bodyweights of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Bodyweight		
	Hatch	Day 3	Day 7
	------(g)-----		
A(protein)	56.02 ^a	73.23 ^a	131.91 ^a
C(control)	52.64 ^b	73.64 ^a	135.47 ^a
S(sugar)	56.65 ^a	74.98 ^a	139.27 ^a
P-value	<0.001	0.634	0.293
SEM(DF) ³	0.265(176)	0.709(146)	1.74(146)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment C is the non-injected control. Treatment S contained 20% dextrin + 3% maltose in .9% NaCl saline. ³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom is represented in parenthesis. ^{a,b}Means within a column with different superscript are significantly different ($P < 0.05$).

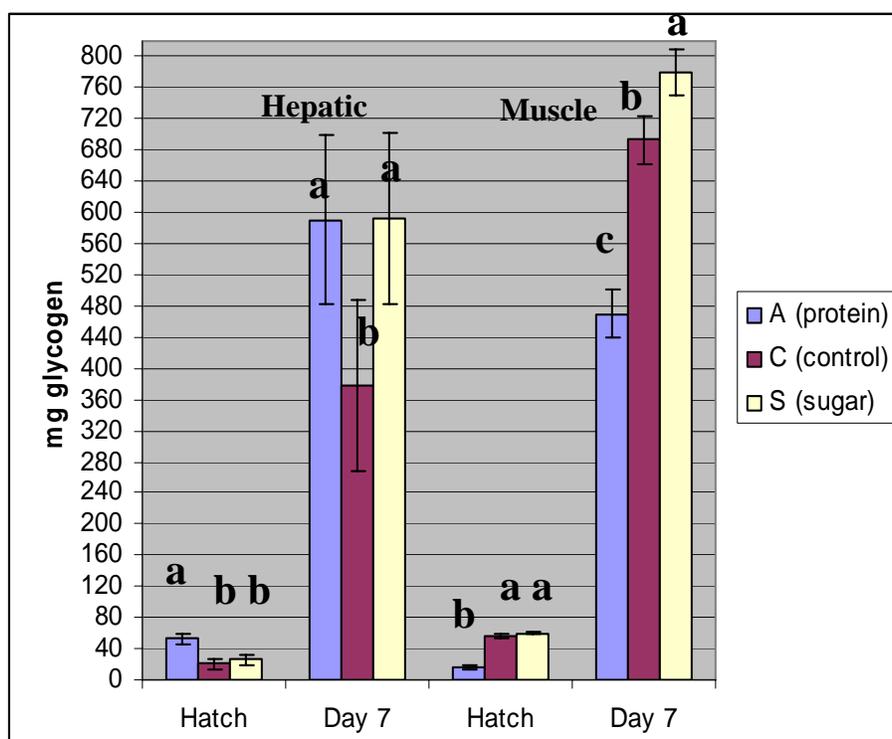
Table 2.7 The effects of *in ovo* feeding (IOF) of egg white protein and carbohydrate on the glycogen index of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	Glycogen Index	
	total liver glycogen + total muscle glycogen(mg)/ bodymass (g)	
	Hatch	Day 7
A(protein)	1.27 ^a	7.46 ^{ab}
C(control)	1.50 ^a	7.82 ^b
S(sugar)	1.59 ^a	9.80 ^a
P-value	0.292	0.081
SEM(DF) ³	0.084(27)	0.439(27)

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment C is the non-injected control. Treatment S contained 20% dextrin + 3% maltose in .9% NaCl saline. ³SEM(DF) represents the pooled standard error of the mean and the degrees of freedom are represented in parenthesis. ^{a,b}Means within a column with different superscript are significantly different (P<.05).

Figure 2.3 The effects of *in ovo* feeding (IOF) of egg white protein and carbohydrate on total hepatic and pectoralis muscle glycogen of turkeys at hatch and 7 days post-hatch.¹



¹All data represents the mean value \pm pooled standard error of 10 sample birds per treatment. ² Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment C is the non-injected control. Treatment S contained 20% dextrin + 3% maltose in .9% NaCl saline. ^{a,b}Means with different superscripts are significantly different ($P < .05$).

DISCUSSION

The effects of IOF of two levels of egg white protein (0% and 18%) and two levels of HMB (0% and 0.1%)

In ovo feeding of EWP had a direct, substrate-mediated affect on hepatic glycogen at hatch and 7 d post-hatch. Digested proteins provide free amino acids, which are the substrates of hepatic gluconeogenesis, whereas dietary HMB does not have a substrate-mediated affect of enhancing hepatic glycogen. The primary substrates needed for gluconeogenesis are lactate, glucogenic amino acids, and glycerol. *In ovo* feeding of HMB enhanced total muscle glycogen reserves at hatch and 7 d post-hatch. The uptake of glucose and amino acids by skeletal muscle is mediated by the action of insulin. While the mechanism of action of HMB have not been identified, these data suggest that *in ovo* feeding of HMB stimulated the release of insulin, which resulted in increased uptake of endogenously produced glucose from the liver and the formation of glycogen within the muscles. Hence, energy (glycogen) is re-partitioned from the liver to muscles stores. Therefore, HMB had an indirect, hormone-mediated affect on total muscle glycogen.

When EWP and HMB were fed *in ovo* together, there were no additive improvements in total hepatic or muscle glycogen. Several experiments have demonstrated that HMB supplementation increased muscle deposition (Fuller et.al, 1994; Flakoll et. al, 2004). Thus HMB, when fed in combination with the egg white protein, may have enhanced the usage of absorbed amino acids for improved muscle deposition

and not for use as gluconeogenic precursors for glucose and ultimately glycogen formation.

The data indicate that *in ovo* feeding of HMB alone may have stimulated the release of insulin or insulin-like growth factors, which are potent inhibitors of hepatic gluconeogenesis. Therefore, HMB in combination with protein would have prevented the incoming amino acids liberated from protein digestion from serving as gluconeogenic precursors in the liver. These amino acids and peptides would have been absorbed by the muscles due to the action of insulin and incorporated into protein and/or utilized for immediate energy needed for tissue growth and development. In addition, insulin would have increased the glycolytic activity of the muscles. Therefore, endogenously produced glucose would be absorbed by the muscles and also utilized for energy. Further studies must be conducted to determine synergistic effects of *in ovo* feeding nutrients on glycogen metabolism.

In summary, *in ovo* feeding of either EWP and/or HMB improved the amount of available energy needed for rapid growth and development, which was evident by improved bodyweights at hatch. *In ovo* feeding may serve as an effective method to improve early post-hatch growth and muscle deposition, and it may prevent post-hatch mortality by enhancing hepatic and muscle glycogen reserves that can be used to endure energy deficits until sufficient energy is consumed upon the initiation of feed intake.

Contrast between IOF of 18% egg white protein (EWP) and 23% carbohydrate (S)

In ovo feeding of dietary protein enhanced total hepatic glycogen reserves at the day of hatch, whereas dietary carbohydrate fed *in ovo* had no effect. Previous studies

(Romanoff, 1967; Donaldson et al, 1992, 1993; Christensen et. al., 1992, 2000, 2001, 2003) have demonstrated the importance of gluconeogenesis in the carbohydrate metabolism of the avian embryo and neonate. The research reported herein demonstrates the importance of protein substrates for hepatic glucose production. At 7 d post-hatch, the glycogen status of poult *in ovo* fed proteins and carbohydrates were similar. These results parallel with earlier experiments (Romanoff, A.L., 1967; Rosebrough et.al, 1979; Donaldson et. al,1992, 1993; Christensen, 1992, 2000, 2001) that demonstrated that by 7 d post-hatch, young poult and chicks undergo a metabolic shift by which gluconeogenesis is not the primary mechanism of glucose production. By 7 d post-hatch, young poult are nearly adapted to consume a carbohydrate-rich diet. The intake of dietary carbohydrates results in insulin surges and insulin is a powerful inhibitor of hepatic gluconeogenesis. Thus after one week of eating a carbohydrate-rich corn diet, the effects of *in ovo* feeding of protein on hepatic glycogen would be lost.

Unlike the liver, skeletal muscle requires the action of insulin for the uptake of glucose from the blood. Insulin release occurs with the consumption of a carbohydrate-rich meal and the resultant rise in blood sugar. Thus, pectoralis muscle glycogen was not enhanced by *in ovo* feeding of protein, but it was enhanced by *in ovo* feeding of carbohydrates. As expected at day 7, *in ovo* feeding of carbohydrate had a profound affect on muscle glycogen. *In ovo* feeding of carbohydrates most likely resulted in the release of insulin and the uptake and storage of glucose in the form of glycogen in the muscles. Unlike the liver, skeletal muscle lacks the gluconeogenic enzymes needed for the conversion of proteins and amino acids into glucose and therefore would have been

unable to use protein *in ovo* fed for enhancement of muscle glycogen stores. *In ovo* feeding of dietary protein targets hepatic glycogen enhancement by taking advantage of high hepatic gluconeogenic rates in the avian neonate; whereas *in ovo* feeding of dietary sugars enhanced muscle glycogen, which ultimately responds as improved bodyweights.

The avian embryo and neonate is similar to an endurance athlete. The endurance athlete maximizes muscle glycogen levels by “glycogen loading” before an exercise event to help prevent fatigue. Exercise of high intensity, long duration causes depletion of glycogen reserves, known as “hitting the wall”, which results in fatigue and exhaustion. Additionally, HMB supplementation in trained athletes has been shown to increase muscle mass and strength (Kreider et. al, 1999; Panton et al., 2000) and endurance (Vukovich et al., 2001). Among birds, the hatching process is similar to an exercise event of high intensity and long duration for the endurance athlete. Glycogen stores become depleted during the hatching process, due to the high amount of energy expenditure needed.

In ovo feeding of proteins and carbohydrates may help alleviate glucose depletion by glycogen loading before hatch. Thus, the injected nutrients are available for use and storage which may provide the fuel needed for hatching and subsequent growth and development.

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Chapter 3

The Effects of In Ovo Feeding of Arginine and/or β -hydroxy- β -methylbutyrate (HMB) with Egg White Protein on Glycogen Metabolism and Growth in Turkey Poults.

ABSTRACT *In-ovo* feeding (IOF), injecting dietary components into the amnion prior to internal pipping, may enhance early growth performance by enhancing hepatic gluconeogenesis and altering glycogen status. The effect of in-ovo feeding arginine, HMB, and with protein (EWP) on bodyweights, organ weights, total liver and pectoralis major muscle glycogen of Hybrid® poults were studied. At 23 d of incubation, 100 eggs were each were subjected to 4 IOF treatments consisting of 1.5 ml of .4% saline solutions containing a factorial arrangement of two levels of arginine (ARG 0% or 0.7%) and two levels of HMB (0% and .1%). As a contrast, an IOF solution containing .1% HMB+.7% ARG+18% egg white protein (EWP) was also tested. All poults were given ad libitum access to feed and water within 24 hours after hatch. Body weights (BW) were determined at hatch and 3, 7, 10, and 14 d post-hatch. Poults were also sampled at 25 days of incubation (25E), hatch, 3, 7, and 14 days post-hatch to determine breast muscle yield, and hepatic glycogen content and glucose-6-phosphatase (G6P) activity.

All *in ovo* fed poults had 3-4% greater BW than the controls at hatch. Three days post-hatch, the HMB and HMB + ARG-treated poults had 4.2% and 5.1% greater BW than the controls, respectively ($P<0.05$). At 10-d and 14-d post-hatch, BW of poults *in ovo* HMB + ARG were 10.2% and 10.7% greater than controls, respectively ($P<0.05$). Breast muscle yield (% of BW) was enhanced at hatch by *in ovo* feeding of HMB ($P<0.05$) over the controls. At hatch, poults *in ovo* fed ARG, HMB and ARG + HMB had a 84%, 78.7%, and 75% greater total hepatic glycogen than controls ($P<0.05$), and

they had significant greater hepatic glucose-6-phosphatase activity ($P < 0.05$). When the non-injected controls, HMB + ARG and HMB + ARG + EWP *in ovo* feeding treatments were contrasted, poults *in ovo* fed HMB + ARG and HMB + ARG + EWP had approximately 6.0%, 3.2%, and 8.9% greater bodyweights at hatch, 3 d and 10 d post-hatch, respectively ($P < 0.05$) over the controls. Weight gains were only sustained in poults *in ovo* fed HMB + ARG for up to two weeks post-hatch ($P < 0.05$). Total hepatic glycogen was significantly greater in poults *in ovo* fed HMB + ARG and HMB + ARG + EWP over the controls ($P < 0.05$). Hepatic glucose-6-phosphatase activity was enhanced by *in ovo* feeding of treatments HMB + ARG and HMB + ARG + EWP at hatch and was diminished by one week post-hatch ($P < 0.05$). This study demonstrates that administration of HMB and arginine *in ovo* enhances hepatic glycogen reserves, which may provide the fuel needed for more rapid subsequent growth during the critical post-hatch period.

INTRODUCTION

Due to a limited supply of carbohydrate within the egg, hepatic gluconeogenesis is the primary mechanism for glucose production in the avian embryo and neonate (Romanoff, 1967). The gluconeogenic pathways are highly active and decrease after hatching when the chicks are fed a high carbohydrate diet. The glucogenic enzymes increase in activity as the chick embryo development progresses and they reach a maximum activity about the time of hatching (Okuno et al., 1964; Felicioli etl., 1967; Sheid and Hirschberg, 1967). Hepatic gluconeogenesis is very active in the avian neonate (Donaldson and Christensen, 1992a) and may be a contributing factor needed in hepatic glycogen accretion.

Raheja et. al (1971) observed an accumulation of hepatic glycogen from the day of hatch until four weeks of age, which maybe due to increased activities of the gluconeogenic enzymes and the appearance of glycogen synthase rather than phosphorylase. The enzymes related to gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), fructose di-phosphatase, and glucose-6-phosphatase, reached their maximum activity at hatch and then decreased over the course of seventeen weeks of age. More specifically, glucose-6-phosphatase activity decreased for the first 6 weeks and then subsequently showed little change. PEPCK activity decreased during the first 3 weeks, changed little for the next 3 weeks, then declined and showed a significant rise again after 14 weeks.

Early studies by Yarnell et.al (1966) have demonstrated that the radiolabeled carbon skeletons of the amino acids, C¹⁴-glutamate and C¹⁴-alanine were incorporated into endogenously produced glucose, thus demonstrating the overall activity of the gluconeogenic pathway in avian embryonic tissues. The key enzymes of gluconeogenesis have been detected in the avian liver by the 6th day of incubation (Kilsheimer et al., 1960), thus demonstrating the embryonic capability to utilize amino acids as gluconeogenic substrates. Schultz and Mistry (1981) demonstrated that non-gluconeogenic substrates, such as beta-hydroxybutyrate or ethanol in the culture medium of isolated chicken hepatocytes increased the formation of glucose by 80% and 200%, respectively; thus suggesting that these substrates may enhance gluconeogenesis due to their beneficial effect of increased reducing equivalents which can significantly affect a change in the oxidation-reduction (pyruvate/lactate) ratio, in hepatocytes.

A novel method of supplementing the *in ovo* nutrition of oviparous species is described as “*in ovo* feeding” within the US Patent (6,592,878) of Uni and Ferket (2003) involves the administration of exogenous nutrients into the amnion of the developing embryo of chickens and turkeys at about 17 and 23 days of incubation, respectively. Because the late term embryo naturally orally consumes the amniotic fluid (comprised primarily of water and albumen protein) prior to pipping of the air cell, this *in ovo* feeding technology is a means of presenting exogenous nutrients to the enteric tissues for absorption and utilization for growth and stored energy as glycogen. Therefore, *in ovo* feeding of supplemental nutrients may help overcome the constraint of limited egg nutrition. Consequently, the enhanced glycogen stores may provide supplemental energy needed for better neonatal survival, fuel more rapid growth, and spare body protein (muscle) reserves post-hatch. Poultry producers experience early poulter mortality (EPM) of 3 to 6%, (Phelps, 1987). Mortality of young hatchlings is greatest during the first two weeks post-hatch. The causative agents for EPM are unknown, but it may be related to limited energy reserves, decreased feed and water consumption, and environmental stressors. Hatchlings with enhanced glycogen reserves have improved body weights, decreased mortality and improved performance (Moran, Jr., 1988). Therefore, *in ovo* feeding may be of economic advantage to poultry producers (broilers and turkeys).

In ovo feeding of amino acids and amino acid metabolites, such as arginine and β -hydroxy- β -methylbutyrate (HMB), may facilitate gluconeogenic precursors to enhance hepatic and muscle glycogen stores. Hatchlings with enhanced glycogen reserves have improved bodyweights, decreased mortality and improved performance (Moran, Jr.,

1988). HMB, a metabolite of leucine, has been hypothesized to be responsible for leucine's ability to stimulate protein synthesis or prevent proteolysis (Nissen et al, 1996, 1997; Ostaszewski et al, 1996). It has been reported that the addition of HMB to the bathing medium containing muscle strips of rats and chicks resulted in an approximately 20% increase in protein synthesis, while proteolysis was inhibited by approximately 80% (Ostaszewski et al, 1996).

Additionally, earlier studies by Uni and Ferket (2003, 2004) and Foye et al. (Dissertation Chapter 2, Table 2.3) demonstrated that HMB fed *in ovo* enhanced hepatic glycogen stores. Experimentation by Foye et al. (Dissertation Chapter 2) revealed that turkey poults *in ovo* fed egg white protein and a combination of HMB and egg white protein had enhanced total hepatic glycogen reserves over the controls at 7-d post-hatch ($P<0.05$), whereas total muscle glycogen reserves were enhanced at one week post-hatch by *in ovo* feeding of HMB ($P<0.05$). Also, studies by Foye et al. (Dissertation Chapter 2 Table 2.7, Figure 2.3) demonstrated that poults *in ovo* fed egg white protein had significantly enhanced total hepatic glycogen reserves at hatch, over the controls and poults *in ovo* fed a 23% sugar solution. Uni and Ferket (2003) injected turkey embryos with varying levels of HMB at 24-d of incubation, of HMB 0ug (non-injected control), 0.1 μ g, 1.0 μ g, 10 μ g, 100 μ g, or 0.9% saline (injected-control). Hatchability was enhanced by *in ovo* feeding HMB at the 0.1 μ g and 1.0 μ g levels, over the controls. Additionally, liver glycogen was increased approximately 40% in all HMB levels as compared to the controls (Uni and Ferket, 2003 Patent # US 6,592,878 B2), with a quadratic response ($P<0.05$) as the level of HMB injected *in ovo* increased. Additionally, hatchability rates

were positively correlated with liver glycogen content of turkey and chick embryos before hatch ((Uni and Ferket, 2003 Patent # US 6,592,878 B2).

Based on research reported by Chevalley et al. (1998) arginine was identified as a potential candidate for *in ovo* feeding nutrient administration. Chevalley et al. (1998) demonstrated that pharmacological doses of arginine *in vitro* enhanced collagen and bone formation by stimulating IGF-I production in osteoblasts. Additional studies (Flakoll et al, 2004) have demonstrated that elderly women supplemented with 2g HMB, 5g arginine and 1.5g lysine daily had significantly greater limb circumference, leg strength, and handgrip strength in comparison to the placebo group. In addition, the experimental group experienced a 20% increase in protein synthesis as opposed to the placebo group. Experiments by Budford and Koch (2004) indicated that supplementation with glycine, arginine, and α -ketosiocaproic acid (a leucine metabolite) enhanced anaerobic cycling performance of male cyclists. Thus, HMB and the amino acid arginine were identified as *in ovo* feeding components, which may enhance glycogen reserves, muscle deposition and growth.

We hypothesize that *in ovo* feeding of the amino acid, arginine and HMB with or without egg white protein will enhance hepatic and muscle glycogen reserves due to an increased hepatic gluconeogenic activity. Thus with enhanced glycogen reserves poult will have increased post-hatch growth performance, hatchability and survivability. Additionally, we hypothesize that *in ovo* feeding of HMB will enhance breast muscle mass relative to body weight in the avian neonate. The primary objective of this study was to determine the effects of *in ovo* feeding of arginine and HMB with and without egg

white protein on growth, breast yield, liver and pectoralis muscle glycogen status at 25-d of incubation and up to 14 days post-hatch. Hepatic glucose-6-phosphatase activity was also evaluated as an indicator of gluconeogenesis during the late-term embryo and neonatal turkey.

The turkey was chosen as an animal model due to its economic importance within the poultry industry. Additionally, the turkey embryo develops in a closed environment, independent of maternal influences and is thus an ideal animal system in which to study the effects of exogenous nutrients on embryonic growth, development and energy metabolism.

MATERIALS AND METHODS

Incubation and In Ovo Feeding (IOF)

Viable Hybrid® turkey eggs were obtained at 19d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). Twenty eggs per treatment were placed within one of five trays, such that each treatment was equally distributed within the incubator. At 21 d of incubation 500 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65 g to 85 g per egg. These eggs were then evenly distributed among five treatment groups of 100 eggs each, such that the weight distribution profile among all 5 treatment groups was identical. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was then punched using a 23 gauge needle and 1.5 ml of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15 mm. The injection hole area was disinfected with an ethyl alcohol-

laden swab, sealed with a cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as aseptically as possible such that the *in ovo* feeding treatment solutions contained the following: A) 0.1% HMB in 0.4% saline; B) 0.7% arginine in 0.4% saline; D) 0.1% HMB + 0.7% arginine in 0.4% saline; and E) 18% egg white protein (EWP) + 0.1% HMB + 0.7% arginine in 0.4% saline. The controls (C) were not injected with a solution, but they were subjected to the same handling procedures as the *in ovo* feeding treatment groups. Preliminary experimentation was conducted in our laboratory indicating that *in ovo* injection of 2.0 mL of 0.9% saline did not affect embryo and poult bodyweights, breast yield or glycogen status. The HMB used in this study was the calcium salt, kindly provided by Metabolic Technologies, Inc. (Ames, IA). Free-base L-arginine and egg white protein (egg whites from chicken catalog # E0500) was purchased from Sigma (St. Louis, MO).

Animal husbandry and tissue sampling

Upon hatching, each poult was marked for identification and the body weight recorded at hatch, 3 d, 7 d, 10 d and 14 d post-hatch. Hatchability for all treatments was equal to or greater than 96%. Poults were randomly assigned to four rooms of approximately two hundred square feet each at Dearstyne Avian Research Facility, North Carolina State University. Twenty-five poults from each treatment were randomly assigned to each of four rooms. Each pen was equipped with manual self-feeders and drinkers. The concrete floor was bedded with wood shavings and supplemental heat was provided. Poults were given a turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat) based upon the National Research Council (1994) requirements for turkeys *ad libitum*. At hatch 3 d, 7 d

and 10 d post-hatch, 10 poults were randomly selected for sampling from each treatment (≈ 2 poults/room/treatment). The 10 poults per treatment were euthanized by cervical dislocation and within 2 minutes the whole liver and pectoralis muscle was dissected and placed on ice before freezing for subsequent glycogen analysis. The entire pectoralis muscle and livers were sampled at 25 d of incubation, hatch, 3 d, 7 d and 14 d post-hatch and stored at -20°C for future analysis. Frozen liver or muscle samples were then thawed in groups such that all sample days and treatments were equally represented so as to account for errors associated with glycogen analysis. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Glycogen Analysis

The pectoralis muscle and liver samples were homogenized in 8% perchloric acid (1g/4mL) and glycogen content was determined using methods described by Dreiling et al (1987). After homogenization, the samples were centrifuged at 14,000 rpm, 4°C for 30 minutes. One milliliter of the supernatant was transferred to a clean polypropylene tube and 2.0 mLs of petroleum ether was added to each sample and vortexed. The samples were centrifuged at 2000 rpm, 4°C for 15 minutes. Subsequently, 0.4mL of 8% perchloric acid and 2.6mL of iodine color reagent (1.3 mL of solution A in 100 mL of 67.8% saturated calcium chloride (anhydrous) solution; (solution A= 0.26g iodine + 2.6g potassium iodide dissolved in 10 mL of distilled water)) was added to a 10uL aliquot of the sample (bottom layer) in a disposable cuvette. All samples were read at a wavelength

of 460nm. The amount of glycogen present in a 10uL sample is determined by preparation of a known glycogen standard curve.

Hepatic glucose-6-phosphatase analysis

The liver samples taken at hatch and 7 d post-hatch were homogenized in a 0.25M sucrose solution (1g liver/10 mL) and centrifuged at 14,000 rpm for 10 minutes. The supernatant was diluted 1:4 with 0.25M sucrose solution. Three tubes were prepared for each experimental sample containing the following: 0.3mL of 0.1% histidine solution, 0.1 mL 0.25M sucrose solution, and 0.1mL of diluted sample. At 15 second intervals each tube was placed into a 37 °C water bath for a total incubation time of 10 minutes. A volume of 0.1mL of 0.4M glucose-6-phosphate solution was added to two tubes of the triplicate set prior to incubation, and the remaining tube of the triplicate served as a sample blank. After 10 minutes of incubation, 1mL of 10% TCA was added to each tube at 15 second intervals. To the sample blanks 0.1 mL of 0.4M glucose-6-phosphate solution was added to each tube. Subsequently, the inorganic phosphate levels were determined for each sample. A separate set of tubes were prepared for each sample in triplicate, plus an additional tube for a standard. Two milliliters of a 40% ferrous sulfate solution was added to each tube. In each corresponding tube, 1mL of the glucose-6-phosphatase (G6P) samples was added, with each sample blank receiving 1mL of 5% TCA. In the standard tube, 0.6mL of 5% TCA and 0.4mL of 1.25mM phosphate standard was added in 5% TCA. All the tubes were mixed and allowed to stand for at least 10 minutes (but less than 60 minutes) before reading optical density (OD) at 700nm. Milliunits of G6P/mL inorganic phosphate was calculated as follows: [(OD of “sample”)

– (OD of “blank”) X (500mM/OD of standard) X 1/10 X (4 dilution factor) X 10 X 1.8, where 1/10 refers to the 10 minute assay time, 10 refers to the 0.1mL sample size used, and 1.8 refers to the dilution due to glucose-6-phosphate. MilliUnits glucose-6-phosphatase (G6P)/ug protein was calculated as follows: (milliunits G6P/mL inorganic phosphate)/(ug protein/mL).

Procedure for Protein Determination

Protein was determined using the Lowry method described by the Protein Assay kit with Bovine Serum Albumin Assay Standard II using reagents #500-0007, #500-0115, #500-0114 and #500-0113 (BioRad, City, State).

Statistical Analysis

All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1996). Each bird served as an experimental unit for statistical analysis. Data from *in ovo* treatments A, B, C, and D were analyzed as a 2 X 2 factorial arrangement, with two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%). An additional statistical analysis was conducted to contrast the effects of the addition of dietary protein to the 0.1% HMB + 0.7% arginine *IOF* solution (Treatment E). These data were analyzed as a one-way ANOVA (SAS, 1996) comparing treatments D (0.1% HMB + 0.7% arginine in 0.4% saline), E (18% EWP + 0.1% HMB + 0.7% arginine in 0.4% saline) and C (Control). All data were sorted by age and treatment. Variables having different F-test were compared using the least-squares-means function in SAS (1996) and the treatment effects were considered significant at $P < 0.05$. All experiments were conducted with an equal frequency of variables within each treatment

RESULTS

The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%)

All *IOF* treatments resulted in significantly ($P < 0.05$) increased BW of poult by 3 to 4% in comparison to the controls, (Table 3.1), although there were no significant differences among *IOF* treatments. At 3 d post-hatch, poult of *in ovo* fed HMB and HMB + ARG had 4.2% and 5.1% greater BWs than the controls, respectively ($p < 0.05$), but BW of poult *in ovo* fed ARG were not significantly different from the other 3 treatments. The *IOF* treatment effects observed on BW at hatch diminished at by 7 d post-hatch. At day 10 and day 14, the BWs of *in ovo* fed poult of treatment HMB + ARG were 10.2% and 10.7% greater than the controls, respectively ($p < 0.05$, Table 3.1). Conversely, there were no differences in BWs of *in ovo* fed poult between treatments HMB, ARG or HMB + ARG at day 10 post-hatch, while the bodyweights of *in ovo* fed poult of treatment HMB + ARG were not different from *in ovo* fed poult of treatment HMB. There was no significant ARG X HMB effect observed on BW at hatch, 3, 7, 10 or 14 d post-hatch. Although there was a marginal positive effect of ARG on BW at hatch, there was a significant positive effect of HMB on BW through to 14 d of age, and this effect increased with age as indicated by a significant age X HMB effect on BW ($F = 2.57$, $p = 0.0397$).

Table 3.1 The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on the bodyweights of turkeys at hatch, 3, 7, 10 and 14 days post-hatch.¹

IOF treatment ²	Bodyweight (g)				
	Days of Age				
	Hatch	Day 3	Day 7	Day 10	Day 14
HMB	67.2±0.6 ^a	85.9±1.1 ^a	140±2.6 ^a	179.6±4.9 ^{ab}	252.2±7.9 ^{ab}
ARG	66.7±0.6 ^a	84.3±1.1 ^{ab}	134±2.4 ^a	172.6±4.6 ^{ab}	236.6±6.0 ^b
C(CONTROL)	64.5±0.8 ^b	82.0±1.4 ^b	135±2.8 ^a	164.4±4.0 ^b	230.2±10.0 ^b
HMB + ARG	67.5±0.6 ^a	86.4±1.1 ^a	140±2.4 ^a	182.6±5.0 ^a	257.7±6.2 ^a
Source of Variation	-----p-value-----				
ARG	0.06	0.23	0.95	0.27	0.47
HMB	0.01	0.01	0.02	0.01	0.01
ARG X HMB	0.17	0.44	0.84	0.61	0.91
SEM (DF) ³	0.324(267)	0.585(233)	1.24(170)	2.22(138)	3.53(141)

¹All data represents the mean value ± standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. ³ SEM (DF) = pooled standard error of the mean and (degrees of freedom).^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Breast muscle mass relative to BW of embryonic poult (25 days of incubation) was enhanced significantly within 48 hours of *in ovo* feeding of ARG (p<0.05, Table 3.2) in comparison to the control and other *in ovo* feeding treatments. By the time of hatch, this effect was diminished, and the relative breast muscle mass of poult *in ovo* fed HMB were significantly greater than the controls and ARG treatment groups (p<0.05 Table 3.2), while the breast muscle yield of poult *in ovo* fed treatments ARG, C and HMB + ARG were similar. There was no ARG X HMB interactive effect on breast muscle yield, rather there were significant additive effects of ARG and HMB on breast muscle

yield at hatch was ($p < 0.05$, Table 3.2). There were no significant treatment effects on relative breast muscle mass at 3, 7 or 14 d post-hatch.

Table 3.2 The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) on the relative pectoralis muscle weights of turkeys at 25 days of incubation and at hatch, 3, 7, and 14 days post-hatch.¹

	Pectoralis Muscle (% of Body Weight)				
	----- Days of Age -----				
IOF treatment ²	25E ³	Hatch	3	7	14
HMB	3.0 ^b	3.0 ^a	2.9 ^a	8. ^a	11.4 ^a
ARG	6.3 ^a	2.5 ^b	2.7 ^a	8.5 ^a	11.6 ^a
CONTROL	3.0 ^b	2.7 ^{ab}	3.0 ^a	8.7 ^a	11.8 ^a
HMB + ARG	3.1 ^b	2.7 ^{ab}	2.8 ^a	8.8 ^a	11.8 ^a
Source of Variation	----- p-value -----				
Arg	0.435	0.028	0.286	0.716	0.882
HMB	0.314	0.041	0.945	0.833	0.877
Arg X HMB	0.240	0.811	0.746	0.388	0.481
SEM (36) ⁴	0.080	0.032	0.107	0.020	0.229

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

² Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. ³25 days of incubation. ⁴SEM (36) = pooled standard error of the mean with 36 degrees of freedom. ^{a,b}Means within a column with different superscripts are significantly different ($P < 0.05$).

In ovo feeding of HMB significantly enhanced the relative liver mass at 25 days of incubation in comparison to the control and *in ovo* feeding treatments ($p < 0.05$, data not shown). By hatch, the relative liver mass of all *in ovo*-fed poult were significantly less than the controls ($p < 0.05$), but there were no significant treatment effects observed on relative liver mass at any of the other time points measured.

At hatch, poult *in ovo* fed treatments ARG, HMB and HMB + ARG had, respectively, a 84%, 78.7%, and 75% increase in total hepatic glycogen content at the day

of hatch when compared to the controls ($p < 0.05$, Table 3.3). While the hepatic glycogen status of *in ovo* fed poult of the HMB and HMB + ARG treatments were similar, they both were significantly greater than the controls, and significantly less than the hepatic glycogen status of *in ovo* fed poult of treatment ARG. Glycogen index, calculated as the sum of the total hepatic and muscle glycogen divided by body mass (mg/g), is a relative indicator of energy status to support metabolism and growth. *In ovo* fed poult had significantly greater glycogen index at the day of hatch in comparison to the controls (Table 3.5). By 3 d post-hatch, there were no significant differences in glycogen index between the treatments at any of the time points measured. When ARG and HMB were given in combination *in ovo*, there was a synergistic effect on total hepatic glycogen at the day of hatch ($p < 0.05$, Table 3.3), but glycogen status was not affected by *IOF* treatment at any subsequent time point measured. Additionally, poult *in ovo* fed treatments HMB, ARG, and HMB + ARG had significantly greater hepatic activity of glucose-6-phosphatase (G6P) activity. At 7 d post-hatch, there were no treatment effects on hepatic G6P activity (Table 3.6).

There were highly significant age effects on total hepatic glycogen and concentration, total pectoralis muscle glycogen and concentration, glycogen index, and hepatic glucose-6-phosphatase activity ($p < 0.001$). Conversely, there was no age X *in ovo* feeding treatment interaction effects on any of the physiological parameters measured.

Contrast between IOF of HMB with arginine versus HMB with arginine and 18% egg white protein (EWP)

Poult *in ovo* fed treatments HMB + ARG and EWP + HMB + ARG had 6.0%, 3.2%, and 8.9% greater BW than the controls at hatch, 3, and 10 d post-hatch, respectively ($p < 0.05$, Table 3.7). The BW of *in ovo* fed poult subjected to the HMB + ARG and EWP + HMB + ARG treatments were similar at hatch, 3, and 10 d post-hatch. Two weeks post-hatch, the BWs of poult *in ovo* fed HMB + ARG were significantly greater than the control treated poult, but not significantly greater than the BWs of poult *in ovo* fed EWP + HMB + ARG ($p < 0.05$, Table 3.7). There were no treatment effects on BWs of poult at 7 d post-hatch. Moreover, there were no differences in the relative breast yield or relative liver weights between the *in ovo* treatments C, HMB + ARG and HMB + ARG + EWP at day 25 of incubation, hatch, and day 3, 7 or 14 post-hatch (Table 3.8).

At day 25 of incubation, embryos subjected to *in ovo* feeding EWP + HMB + ARG had a significantly higher glycogen concentration than the control or HMB + ARG *in ovo* treatment ($p < 0.05$, Table 3.9). At hatch, the total and concentration of hepatic glycogen was significantly greater among poult *in ovo* fed HMB + ARG and EWP + HMB + ARG than the controls ($p < 0.05$, Table 3.9). While the hepatic glycogen stores of poult *in ovo* fed HMB + ARG were significantly greater those *in ovo* fed EWP + HMB + ARG at the day of hatch, this effect on hepatic glycogen was lost by 3 d post-hatch and subsequently. At day 7 post-hatch, poult *in ovo* fed EWP + HMB + ARG had significantly less hepatic glycogen reserves than the controls and those *in ovo* fed HMB +

ARG ($p < 0.05$, Table 3.9). *In ovo* feeding HMB + ARG or EWP + HMB + ARG had no affect on muscle glycogen content of poultls of any age (Table 3.10). In contrast, the glycogen index of poultls *in ovo* fed HMB + ARG or EWP + HMB + ARG was significantly greater than controls at hatch but not at any of the other time points measured. Hepatic glucose-6-phosphatase activity was greatly enhanced due to *in ovo* feeding of treatments HMB + ARG and EWP + HMB + ARG at the day of hatch but was diminished by 7 d post-hatch ($p < 0.05$, Table 3.12).

Table 3.3. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on hepatic glycogen concentration (mg/g) and total hepatic glycogen of turkeys at 25 days of incubation and at hatch, 3, 7, and 14 days post-hatch.¹

IOF treatment ²	Glycogen concentration (mg/g)					Total Glycogen (mg)				
	25E ³	Hatch	Day 3	Day 7	Day 14	25E ³	Hatch	Day 3	Day 7	Day 14
HMB	67.1 ^b	35.7 ^b	229.8 ^a	9.2 ^a	295.2 ^a	67.9 ^a	54.7 ^b	684.0 ^a	33.2 ^a	2229.5 ^a
ARG	89.4 ^{a,b}	49.3 ^a	228.1 ^a	9.5 ^a	231.3 ^a	80.2 ^a	77.0 ^a	661.2 ^a	39.0 ^a	1769.3 ^a
CONTROL	101.9 ^a	7.6 ^c	169.4 ^a	10.7 ^a	197.9 ^a	87.6 ^a	12.1 ^c	499.7 ^a	38.8 ^a	1262.4 ^a
HMB + ARG	78.9 ^{a,b}	30.6 ^b	190.7 ^a	15.5 ^a	132.8 ^a	66.0 ^a	48.5 ^b	534.3 ^a	51.6 ^a	987.4 ^a
Source of Variation	----- p-value -----									
Arg	0.978	0.021	0.732	0.354	0.345	0.715	0.024	0.951	0.377	0.500
HMB	0.080	0.541	0.688	0.412	0.993	0.186	0.574	0.764	0.737	0.865
Arg X HMB	0.340	0.004	0.093	0.177	0.155	0.826	0.007	0.111	0.384	0.114
SEM (36) ⁴	6.29	3.79	14.18	1.33	33.22	6.26	6.21	47.57	5.12	266.18

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³25 days of incubation.

⁴SEM (36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.4. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on pectoralis muscle glycogen concentration (mg/g) and total pectoralis muscle glycogen of turkeys at 25 days of incubation and at hatch, 3, 7, and 14 days post-hatch.¹

IOF treatment ²	Glycogen concentration (mg/g)					Total Glycogen (mg)				
	25E ³	Hatch	Day 3	Day 7	Day 14	25E ³	Hatch	Day 3	Day 7	Day 14
HMB	8.3 ^a	3.7 ^a	12.9 ^a	2.2 ^a	6.6 ^a	14.3 ^a	6.9 ^a	29.2 ^a	22.7 ^a	200.2 ^a
ARG	8.5 ^a	3.1 ^a	13.6 ^a	2.3 ^a	7.0 ^a	13.5 ^a	5.2 ^a	28.1 ^a	25.7 ^a	214.1 ^a
CONTROL	16.4 ^a	3.5 ^a	10.5 ^a	2.8 ^a	6.9 ^a	26.9 ^a	5.9 ^a	24.5 ^a	30.8 ^a	192.8 ^a
HMB + ARG	12.1 ^a	3.7 ^a	11.2 ^a	2.8 ^a	7.0 ^a	20.6 ^a	6.4 ^a	25.1 ^a	33.4 ^a	213.7 ^a
Source of Variation	----- p-value -----									
Arg	0.55	0.78	0.55	0.85	0.89	0.48	0.47	0.94	0.54	0.66
HMB	0.55	0.39	1.00	0.97	0.86	0.59	0.18	0.80	0.97	0.93
Arg X HMB	0.11	0.64	0.05	0.15	0.89	0.54	0.93	0.24	0.89	0.92
SEM (36) ⁴	1.73	0.226	0.594	0.176	0.573	2.44	0.418	1.64	2.26	19.30

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³25 days of incubation.

⁴SEM (36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.5. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on the glycogen index of turkeys at 25 days of incubation and at hatch, 3, 7, and 14 days post-hatch.¹

	25E ³	Hatch	Day 3	Day 7	Day 14
IOF treatment²	liver glycogen + muscle glycogen(mg)/ body mass (g)				
HMB	1.3 ^a	0.81 ^a	9.3 ^a	0.43 ^a	9.41 ^a
ARG	1.6 ^a	1.22 ^a	8.9 ^a	0.49 ^a	8.20 ^a
CONTROL	1.8 ^a	0.29 ^b	6.6 ^a	0.55 ^a	6.31 ^a
HMB + ARG	1.5 ^a	0.84 ^a	6.9 ^a	0.52 ^a	4.25 ^a
Source of Variation	-----p-value-----				
Arg	0.954	0.009	0.961	0.275	0.460
HMB	0.160	0.677	0.780	0.69	0.848
Arg X HMB	0.365	0.637	0.060	0.101	0.115
SEM (36) ⁴	0.114	0.086	0.615	0.271	1.08

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³25 days of incubation.

⁴SEM (36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.6. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on hepatic glucose-6-phosphatase activity of turkeys at hatch and 7 days post-hatch.¹

IOF treatment ²	(milliUnits Glucose-6-phosphatase/mL/ug protein)	
	Day Hatch	Day 7
HMB	43.3 ^a	14.0 ^b
ARG	41.0 ^a	16.6 ^{ab}
CONTROL	27.1 ^b	19.6 ^a
HMB + ARG	43.4 ^a	19.4 ^a
Source of Variation	-----p-value-----	
Arg	0.02	0.42
HMB	0.00	0.35
Arg X HMB	0.03	0.01
SEM (36) ³	1.50	0.737

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM (36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.7. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on the bodyweights of turkeys at hatch, 3, 7, 10, and 14 days post-hatch.¹

IOF treatment ²	Bodyweight				
	------(g)-----				
	Hatch	Day 3	Day 7	Day 10	Day 14
Control	64.5±0.74 ^b	82.0±1.3 ^b	134.5±2.8 ^a	164.4±6.6 ^b	230.2± 10.5 ^b
HMB + ARG	67.5±0.62 ^a	86.4±1.0 ^a	140.3±2.4 ^a	182.6±3.6 ^a	257.7±6.5 ^a
HMB + ARG + EWP	68.6±0.73 ^a	84.7±1.2 ^a	138.5±2.7 ^a	180.5±4.6 ^a	247.2±7.9 ^{ab}
P-value	0.0004	0.0309	0.2777	0.0546	0.0858
SEM (DF) ³	0.374(194)	0.672(164)	1.49(121)	2.59(87)	4.52(94)

¹All data represents the mean value ± standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM (DF) = pooled standard error and the (DF) degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.8. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on the relative pectoralis muscle mass of turkeys at 25 days of incubation, and at hatch, 3, 7, 10, and 14 days post-hatch.¹

	25E	Hatch	Day 3	Day7	Day 14
	----- (% of Body Weight) -----				
Control	3.0 ^a	2.7 ^a	3.0 ^a	8.7 ^a	11.8 ^a
HMB + ARG	3.1 ^a	2.7 ^a	2.8 ^a	8.8 ^a	11.8 ^a
HMB + ARG + EWP	2.7 ^a	2.4 ^a	2.6 ^a	8.9 ^a	12.1 ^a
P-value	0.5172	0.2643	0.1572	0.9711	0.9169
SEM (27) ⁴	0.106	0.076	0.083	0.255	0.336

¹All data represents the mean value (% of bodyweight) \pm standard error of 10 sample birds per treatment.

² Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³25 days of incubation.

⁴SEM (27) = pooled standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.9. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on hepatic glycogen concentration and total hepatic glycogen of turkeys at 25 days of incubation, and at hatch, 3, 7 and 14 days post-hatch.¹

IOF treatment ²	Glycogen concentration (mg/g)					Total Glycogen (mg)				
	25E ³	Hatch	Day 3	Day 7	Day 14	25E ³	Hatch	Day 3	Day 7	Day 14
Control	101.9 ^b	7.6 ^c	169.4 ^a	10.7 ^a	197.9 ^a	87.6 ^a	12.1 ^c	499.7 ^a	38.8 ^a	1262.4 ^a
HMB + ARG	78.9 ^b	30.6 ^a	190.7 ^a	15.5 ^a	132.8 ^a	66.1 ^a	48.5 ^a	534.3 ^a	51.6 ^a	987.4 ^a
HMB + ARG + EWP	135.2 ^a	20.0 ^b	153.0 ^a	6.8 ^b	223.3 ^a	98.1 ^a	31.5 ^b	418.3 ^a	28.3 ^b	1356 ^a
P-value	0.764	0.0002	0.685	0.075	0.625	0.480	0.691	0.811	0.377	0.501
SEM (27) ³	5.51	1.96	17.60	1.48	40.68	6.28	3.22	51.97	5.31	280.9

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³25 days of incubation.

⁴SEM (27) = pooled standard error of the mean with 27 degrees of freedom

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.10. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on pectoralis muscle glycogen concentration and total pectoralis muscle glycogen of turkeys at 25 days of incubation, and at hatch, 3, 7 14 days post-hatch.¹

IOF treatment ²	Glycogen concentration (mg/g)					Total Glycogen (mg)				
	25E ³	Hatch	Day 3	Day 7	Day 14	25E ³	Hatch	Day 3	Day 7	Day 14
Control	16.4 ^a	3.5 ^a	10.5 ^a	2.8 ^a	6.9 ^a	26.9 ^a	6.9 ^a	24.5 ^a	30.8 ^a	192.8 ^a
HMB + ARG	12.1 ^a	3.7 ^a	11.2 ^a	2.8 ^a	7.0 ^a	20.6 ^a	6.9 ^a	25.0 ^a	33.4 ^a	213.7 ^a
HMB + ARG + EWP	12.3 ^a	3.4 ^a	12.7 ^a	3.3 ^a	8.3 ^a	13.0 ^a	6.9 ^a	27.0 ^a	44.9 ^a	249.8 ^a
P-value	0.764	0.840	0.388	0.589	0.624	0.480	0.691	0.83	0.40	0.53
SEM(27) ³	2.48	0.263	0.655	0.248	0.661	3.54	0.476	1.65	4.32	19.75

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³25 days of incubation.

⁴SEM (27) = pooled standard error of the mean with 27 degrees of freedom

^{a,b}Means within a column with different superscripts are significantly different (P<.05)

Table 3.11. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein the glycogen index of turkeys at 25 days of incubation, and at hatch, 3, 7 14 days post-hatch.¹

	25E ³	Hatch	Day 3	Day 7	Day 14
IOF treatment²	total liver glycogen + total muscle glycogen(mg)/ body mass (g)				
Control	1.83 ^a	0.29 ^c	6.6 ^a	1.8 ^a	6.3 ^a
HMB + ARG	1.51 ^a	0.84 ^a	6.9 ^a	1.5 ^a	4.3 ^a
HMB + ARG + EWP	2.43 ^a	0.58 ^b	5.4 ^a	2.4 ^a	6.5 ^a
P-value	0.122	0.005	0.648	0.437	0.703
SEM (27) ³	0.114	0.086	0.615	0.049	1.08

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³25 days of incubation.

⁴SEM(27) = pooled standard error of the mean with 27 degrees of freedom

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 3.12. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on hepatic glucose-6-phosphatase activity of turkeys at hatch though 14 days post-hatch.¹

IOF treatment ²	Hepatic Glucose-6-Phosphatase Activity	
	(milliunits/mL/ug protein)	
	Hatch	14 d Post-Hatch
Control	27.1 ^b	19.6 ^a
HMB + ARG	43.4 ^a	19.4 ^a
HMB + ARG + EWP	31.8 ^b	19.8 ^a
P-value	0.0003	0.985
SEM(27) ³	1.50	0.850

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM (27) = pooled standard error of the mean with 27 degrees of freedom

^{a,b}Means within a column with different superscripts are significantly different (P<.05)

DISCUSSION

The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%)

Though the specific pharmacological mechanisms of action of HMB and arginine are not known, it is known that they elicit their effects indirectly by a cascade of events. Glucose and arginine are potent insulin secretagogues. Additionally, pharmacological doses of arginine have been shown to enhance plasma IGF-1 *in vitro* (Chevalley et al (1998). Other studies have demonstrated that parental administration of IGF-I increases jejunal glucose absorption in adult rats (Zhang et al., 1995), while oral IGF-I administration increases jejunal uptake of the non-hydrolysable form of glucose, 3-O-methylglucose in neonatal pigs (Alexander and Carey, 1999). Studies by Lane et al (2002) also demonstrated that rat pups given a milk replacer supplemented with IGF-I had increased jejunal uptake of glucose above the controls. The increased intestinal uptake of glucose, would subsequently lead to increased uptake and storage of glucose as glycogen in the liver and muscles (by the action of insulin). Thus, it may be hypothesized that arginine may modulate carbohydrate metabolism indirectly through the action of insulin and IGF-1.

Any changes in the physiological parameters measured may be an indirect response to *in ovo* feeding of arginine and a direct response to enhanced plasma IGF levels. Hence, the response to *in ovo* feeding of arginine would be delayed from the onset of nutrient administration. Consequently, there were no changes in the physiological parameters measured just 48 hours after *in ovo* feeding of any of the

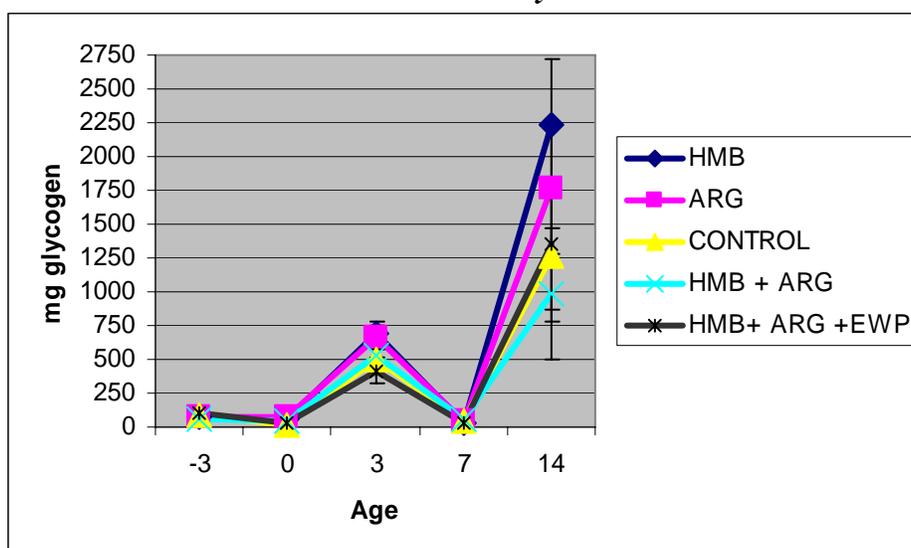
treatments. At hatch, however, the concentration and the total hepatic glycogen of *in ovo* fed poult of HMB and/or arginine were significantly enhanced. *In ovo* fed poult had a higher gluconeogenic activity at hatch, than the controls, as indicated by hepatic glucose-6-phosphatase activity. Thus, *in ovo* fed poult had a greater capacity to convert lipids of the yolk stores and dietary proteins into glucose, which is stored in the liver and muscles as glycogen. While there was no statistical difference in hepatic glycogen status among the *in ovo* fed poult at 3 d post-hatch, poult *in ovo* fed HMB or ARG had an approximately 26% greater glycogen reserves than the controls, while those *in ovo* fed HMB + ARG had 11% greater glycogen reserves than the controls.

All poult experienced a precipitous decline in hepatic glycogen at 7 d post-hatch (Figure 3.1). At this stage of development, poult are no longer relying upon a high rate of gluconeogenesis for glucose production. By 7 d post-hatch, the activity glucose-6-phosphatase had declined to half the activity observed at the day of hatch, indicating that poult acquire much of the glucose they need from consuming the external diet. This metabolic shift in glucose metabolism is marked by a significant decline in hepatic glycogen. At 14 d post-hatch, poult subjected to *IOF* treatments HMB, ARG, Control and HMB + ARG had 70, 40, 32, and 19 times greater total hepatic glycogen contents than those sample poult at 7 d post-hatch, respectively (Figure 3.1).

Muscle glycogen reserves were not affected by *in ovo* feeding of HMB and /or arginine, thus rejecting my initial hypothesis that *in ovo* feeding of arginine and/or HMB would enhance muscle glycogen reserves. The hormone insulin augments the uptake of glucose from the blood by the muscles and other tissues (Langslow et al., 1970). A rise

in plasma insulin occurs after the intake of dietary carbohydrates. Once within the muscle, glucose is stored as glycogen. Muscle glycogen can readily be accessed for energy to fuel working muscles. Unlike the liver, muscle glycogen is only available within the muscles and cannot be released into the circulation. The data from this experiment suggests that *in ovo* feeding of non-carbohydrate nutrients, such as HMB and arginine, do not elicit the release of insulin; thus, most of the glucose produced from hepatic gluconeogenesis is stored as glycogen in the liver and not in the muscles.

Figure 3.1. The temporal effects of *in ovo* feeding (IOF²) of arginine and β -hydroxy- β -methylbutyrate (HMB) on total hepatic glycogen status from pre-hatch to 14 days post-hatch in turkeys.¹



¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

² Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. The CONTROL treatment were not injected. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. Treatment HMB + ARG + EWP *in ovo* feeding solution contained 0.1% HMB and 0.7% arginine and 18% egg white protein in 0.4% saline.

Poults of the experimental and control groups followed the same temporal changes in total hepatic glycogen depletion and repletion (Figure 3.1) over the course of

two weeks. Upon hatching all poult had markedly reduced hepatic stores, which were replenished after 3 days of eating an external diet. Again, hepatic glycogen stores were markedly diminished at 7 d post-hatch, and replenished by 14 d post-hatch. While poult *in ovo* fed HMB, ARG, and HMB + ARG followed the same linear trend as the controls, they had greater hepatic glycogen content than the controls through 14 d post-hatch.

Contrast between IOF of HMB with arginine versus HMB with arginine and 18% egg white protein (EWP)

The concentration of hepatic glycogen (mg/g) was significantly enhanced 24 hours after *in ovo* feeding of HMB + ARG + EWP in comparison to the controls and treatment HMB + ARG. This effect was not due to differences in the relative liver weights among the treatment groups at 25 days of incubation. Thus, poult *in ovo* fed HMB + ARG + EWP had a slower rate of hepatic glycogen depletion than the other two treatment groups. Dietary HMB and arginine independently enhanced hepatic glycogen content, whereas the addition of egg white protein had a direct effect on hepatic glycogen within 48 hours of nutrient administration. Poult *in ovo* fed HMB + ARG + EWP used the dietary protein present in the *in ovo* nutrient solution for immediate energy needed for rapidly growing tissues. Conversely, by the day of hatch poult *in ovo* fed HMB + ARG had significantly greater glycogen concentrations and total glycogen content and than those subjected to the *in ovo* HMB + ARG + EWP and control treatments, while the hepatic glycogen content of poult *in ovo* fed HMB + ARG and HMB + ARG + EWP were both significantly greater than that of the controls. Therefore the effects of *in ovo* feeding of protein were immediate and enhanced hepatic glycogen by day 25 of

incubation, while the effects of *in ovo* feeding of HMB + ARG were delayed and seen at hatch. Evidently, poult *in ovo* fed HMB + ARG and HMB + ARG + EWP had more available energy as glycogen per gram of bodyweight (g) than the controls at the day of hatch. With more available energy, these *in ovo* fed poult had greater energy supplies needed to fuel more rapid growth resulting in greater bodyweights at hatch through 14 d post-hatch.

Previous studies (Romanoff, 1967; Donaldson et al, 1992a, 1992b, 1993; Christensen et. al., 1992, 2000, 2001, 2003) have demonstrated the importance of gluconeogenesis in the carbohydrate metabolism of the avian embryo and neonate. The research reported herein demonstrates the importance of protein substrates for hepatic glucose production. Donaldson et al. (1992a), demonstrated that administration of alanine immediately upon hatch, significantly enhanced hepatic glycogen stores as did carbohydrates, whereas, carbohydrates inhibited gluconeogenesis. Thus the inclusion of protein in the HMB + ARG *IOF* solution improved hepatic glycogen status via enhanced gluconeogenesis.

The activity of hepatic glucose-6-phosphatase at the day of hatch was significantly greater in poult *in ovo* fed HMB + arginine . Glucose-6-phosphatase is one of the key gluconeogenic enzymes required for the conversion of proteins and amino acids into glucose, the primary fuel needed for energy. Thus, the poult *in ovo* fed HMB + ARG had a greater hepatic gluconeogenic potential than the other two treatment groups. Therefore, these poult were more capable of utilizing dietary proteins for

energy. Hepatic glucose-6-phosphatase activity had dropped considerably by 7 d post-hatch, regardless of treatment.

Hepatic gluconeogenesis is the primary mechanism of glucose production in the avian embryo and neonate (Romanoff, 1967). The avian embryo develops in virtually carbohydrate-free environment, thus it must rely upon gluconeogenesis for the production of glucose, the primary fuel for growth and development. Developmentally, the gluconeogenic pathways are highly active and decrease after hatching when the chicks are fed a high carbohydrate diet. Earlier studies have demonstrated that the hepatic gluconeogenic enzymes, fructose-1,6-diphosphatase and glucose-6-phosphatase decreased from the 18th day of incubation to about 20 days after hatching and then remained more or less constant up to 6 months of age in chickens (Thind et. al, 1966). Nelson et al. (1966) and Wallace and Newsholme (1967) also found fructose-1,6-diphosphatase and glucose-6-phosphatase to be less active in adult birds than in embryos. The activity of glucognogenic enzymes increases as the chick embryo developments and it reaches a maximum level of activity at around the time of hatching (Okuno et al., 1964; Felicioli etl., 1967; Sheid and Hirschberg, 1967) and declines during the first weeks post-hatch. Thus our experimental data parallels with the finding of these earlier experiments.

Enhanced glycogen stores may provide the critical energy needed for more rapid early growth and development of the avian embryo and neonate (Donaldson et al., 1992a, 1992b, 1993, Christensen et al., 2000). During the first week post-hatch, the initiation of feed intake is often delayed in young poults. Lipids from yolk stores, albumin and body

proteins become substrates needed for gluconeogenesis (Romanoff, 1967). As a result, energy reserves are depleted). Depletion of energy reserves may lead to increased mortality and stunted growth (Donaldson et al., 1992b). *In ovo* feeding of HMB and arginine or protein may serve as a tool to enhance post-hatch growth by enhancing glycogen reserves prior to hatch, and thus have greater energy reserves and a greater potential to grow and develop more rapidly after hatch.

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Chapter 4

Intestinal Adaptation of the Jejunal Brush Border Enzymes in Neonatal Turkey Poults to In Ovo Feeding of β -Hydroxy- β -methyl-butryate (HMB), Arginine, Protein or Carbohydrate

ABSTRACT

In-ovo feeding (IOF), injecting nutrients into the amnion prior to internal pipping, may enhance early growth performance by enhancing the activity of the brush border enzymes and subsequent nutrient uptake, thus providing the energy needed for metabolic activity. The first experiment studied the effect of *in-ovo* feeding solutions containing egg white protein (EWP), HMB and carbohydrates (S) on jejunal leucine aminopeptidase (LAP) and sucrase-isomaltase activities of Hybrid® poults. The second experiment studied the effect of *in-ovo* feeding solutions containing arginine (ARG), HMB and EWP on jejunal leucine aminopeptidase (LAP) and sucrase-isomaltase activities of Hybrid® poults. At 23 d of incubation, 100 eggs per treatment group were *in ovo* fed 1.5 ml of a .4% saline solution containing 4 nutritional treatments consisting of a two by two factorial arrangement. All poults were fed *ad libitum* within 24 hours after hatch. In the first experiment, jejunal LAP activity was depressed at hatch in poults *in ovo* fed EWP, in comparison to the other treatments ($P < 0.05$). *In ovo* feeding of S enhanced jejunal maltase and LAP activities at the 7-d post-hatch over the controls ($P < 0.05$). In experiment two *in ovo* feeding of ARG + HMB enhanced sucrase and maltase activity at the 25-day of incubation and at 14-d post-hatch, over the controls ($P < 0.05$). Jejunal sucrase and maltase activities were 3-fold greater in poults *in ovo* fed ARG + HMB than

all other treatments at 14-d post-hatch ($P < 0.05$). Jejunal sucrase activity was enhanced at hatch and 7-d post-hatch in poult *in ovo* fed ARG over all other treatments ($P < 0.05$); while jejunal maltase activity was enhanced at hatch by *in ovo* feeding ARG and ARG + HMB over the controls and HMB ($P < 0.05$). Jejunal LAP activity was greater in *ovo* fed poult of ARG + HMB over the controls and other treatments at 25-d of incubation, hatch, 3-d and 14-d post-hatch ($P < 0.05$). *In ovo* feeding of ARG + HMB + EWP enhanced jejunal sucrase and maltase activities at the 7-d and 14-d post-hatch, respectively over the controls and ARG + HMB treatment ($P < 0.05$). Jejunal LAP activity was enhanced by *in ovo* feeding of ARG + HMB + EWP over the controls and ARG + HMB treatment at hatch, 3-d and 14-d post-hatch ($P < 0.05$). This study demonstrates that administration of dietary proteins (EWP) and carbohydrates, or amino acids or their metabolites, HMB, *in ovo* may enhance the intestinal digestion, which may lead to increased uptake of nutrients from an exogenous diet in turkeys.

INTRODUCTION

The gastrointestinal tract (GIT) presents the first barrier to metabolism of dietary nutrients and is the primary supply organ of the body responsible for hydrolyzing, transporting and absorbing nutrients. The GIT supplies the body with the energy and nutrients to meet the metabolic requirements for maintenance, activity, growth and development. Therefore, it is of critical importance that the GIT functions optimally throughout all developmental stages of life.

Preocial birds hatch with an immature GIT. During the first seventy-two hours post-hatch, the GIT tract undergoes rapid morphological, biochemical and cellular development in order to better digest and absorb orally consumed nutrients (Uni et. al, 1996, 1998, 1999, 2000, 2001, 2003a; Noy et. al., 2001a). It has been hypothesized that the digestive and absorptive capacity of the gut may limit the energy supply needed for the growing chick (Kirkwood, 1983; Lilja 1983; Kirkwood and Prescott 1984 Konarzewski et al. 1989); due to a low absorptive and digestive capacity. Thus, young hatchlings experience mal-absorption of nutrients during this window of time in which the gut is reaching functional maturity. Overcoming these obstacles within the first seventy-two hours post-hatch are critical in the development and survivability of young hatchlings. Young hatchlings, which already have limited energy reserves, may develop a negative energy balance due to an inability to adequately absorb orally consumed nutrients due to limited digestive and absorptive capacity. Intestinal development may be a major limitation of neonatal growth and survival in part because the brush border enzymes have different developmental timetables that may influence digestion in post-hatch birds (Uni et. al, 1998, 2000; Ferraris, 2001). Consequently, the brush border enzymes of the intestine may play a rate-limiting role in providing substrates for growth.

The brush border membrane disaccharidases, the enzymes responsible for the hydrolytic cleavage of the dietary carbohydrates, are present in low concentrations in the chick embryo at 12 days of incubation and they increase marked during the last few days of incubation when jejunal villi elongate and increase in the number (Bell, 1971; Sell et.

al. 1989). Siddons (1969) and Matsushita (1985) found that the activity of the disaccharidases, maltase and sucrase in the chick increased rapidly from days 19 to 21 days of incubation until 21 days after hatch before reaching a plateau. More recent studies by Uni et al. (2000, 2003) have demonstrated that maltase, aminopeptidase (AP), SGLT-1 and ATPase activities begin to increase at 19-d of incubation and increased further at the day of hatch. The newly hatched chick therefore possesses marked hydrolytic activity due to the intestinal sucrase-isomaltase complex (Siddons, 1970). The activity of the sucrase-isomaltase complex in the intestine of the young chick ensures the rapid digestion of dietary carbohydrate originating from grain starches and utilized as dietary energy upon the initiation of feed intake.

The development and activity of the intestinal brush border enzymes (sucrase-isomaltase, peptidases, responsible for the cleavage of dietary proteins into peptides and free amino acids) are dependent upon age and diet. Lei and Slinger (1970) observed an increase in sucrase activity of 2-week old chicks fed a high starch, low fat diet as compared with chicks fed a low starch, high fat diet. Rodriguez and Rodriguez (1982) reported that the specific activities of maltase and sucrase of chick intestines increased in proportion to increases in the dietary concentration (30% *versus* 60% dry matter) of cane molasses. Similarly, Sell et al. (1989) found significant effects of feeding diets differing in carbohydrate and fat content on jejunal disaccharidase activities of turkeys.

Conversely, the absence of food resulted in a decrease in the intestinal brush border enzyme and nutrient transporter activity (Ferraris and Diamond, 1989). Under normal hatchery practices, the avian neonate may be denied access to feed and water for

up to 48 to 72 hours post-hatch (Moran and Reinhart, 1980). This delayed access to feed may be a hindrance to enteric development. Studies have shown that orally consumed nutrients help accelerate enteric development, function and maturation (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Studies by Geyra et al. (2001a) have shown that delaying access to feed up to 48 hours after hatching adversely affected the GIT due to a reduction in the intestinal surface area, number of cells/crypt, and the percent of proliferating cells, particularly in the duodenum and jejunum. Additionally, Geyra et al. (2002) demonstrated that the expression of the transcriptional factors CdxA and CdxB needed for expression of intestinal genes responsible for intestinal digestion and absorption (sucrase-isomaltase) was depressed by <48 hours of starvation in comparison to fed chicks post-hatch. Delayed access to feed for 48 hours post-hatch also resulted in increased intestinal intracellular mucins, which might have been due to impaired mucin secretion or enhanced mucin production. Changes in mucin dynamics may affect the absorptive, digestive and protective functions of the gut (Uni et.al., 2003b; Smirnov et al., 2004). Therefore, denied or delayed access to feed may developmentally delay post-hatch enteric maturation and limit subsequent growth.

A novel method of supplementing the *in ovo* nutrition of oviparous species is described as “*in ovo* feeding” within the US Patent (6,592,878) of Uni and Ferket (2003) involves the administration of exogenous nutrients into the amnion of the developing embryo of turkeys at about 23 days of incubation, respectively. Because the late term embryo orally consumes the amniotic fluid prior to pipping of the air cell, this *in ovo* feeding technology is a potential way to present exogenous nutrients to the enteric tissues

and stimulate their development for absorption and utilization. Thus, *in ovo* feeding may serve as a tool to overcome the constraints of post-hatch growth by expediting GIT maturation prior to hatch so that the hatchlings are more capable to digest and absorb nutrients upon the initiation of feed intake. Thus, the compensatory changes conducive to the digestion and absorption of an external diet occur prior to hatch and may prevent post-hatch mal-absorption and digestion of nutrients. Tako et al. (2004) demonstrated that *in ovo* feeding of carbohydrates and/or HMB, a leucine catabolite, increased intestinal villus width and surface area in comparison to the controls in broiler chicks at hatch. Chicks *in ovo* fed HMB had an increase of the villus surface area of 45% greater than the controls, while *in ovo* feeding of carbohydrate alone or in combination with HMB resulted in a 33% increase in the villus surface area in comparison to the controls at 3 days post-hatch. Jejunal sucrase-isomaltase activity was higher in embryo chicks *in ovo* fed either carbohydrates or HMB alone or in combination over the controls at 19 days of incubation (Tako et al., 2004). Consequently, bodyweights of chicks *in ovo* fed either carbohydrates or HMB alone were significantly greater than the controls at hatch through 10 days post-hatch. Additionally, preliminary studies by Uni and Ferket (2004) demonstrated that HMB fed *in ovo* not only enhanced hepatic glycogen stores, but significantly increased the villus surface area in comparison to the controls.

Dietary supplementation of β -hydroxy- β -methylbutyrate (HMB), a leucine catabolite has been shown to have many positive physiological benefits (Peterson et al., 1999a, 1999b; Nissen et al., 1996; Flakoll et al., 2004; Nissen et al, 1997; Nissen et al., 1994). In humans, dietary HMB supplementation has been shown to decrease exercise-

induced proteolysis, significantly increase fat-free mass and to increase the amount of weight lifted during resistance training (Nissen et al., 1996). Other studies have shown that HMB *in vitro* stimulated macrophage proliferation and function in the macrophage chicken cell line, MQ-NCSU with the phagocytic potential of the MQ-NCSU being 31.7% greater than the controls (Peterson et al., 1999a).

Studies by Flakoll et al., (2004) demonstrated that 23 elderly women given a supplementation of 2g HMB, 5g arginine and 1.5g lysine daily had significantly greater limb circumference, leg strength, and handgrip strength in comparison to the placebo group. In addition, the experimental group experienced a 20% increase in protein synthesis opposed to the placebo group. Experiments by Budford and Koch (2004) indicated that supplementation with glycine, arginine and α -keto-isocaproic acid (leucine catabolite) enhanced anaerobic cycling performance of male cyclists. Chevalley et al. (1998) demonstrated that pharmacological doses of arginine *in vitro* enhanced collagen and bone formation by stimulating IGF-I production in osteoblasts. Thus HMB and the amino acid, arginine were identified as *in ovo* feeding components which may indirectly enhance the intestinal function and development, due to the enhancement of IGF-I levels.

It has been demonstrated that IGF-II and insulin are involved in the mechanisms governing the differentiation of the intestinal epithelium, while IGF-I is mostly associated with crypt cell proliferation (Jehle et al., 1999). Nutrient absorption and digestion are a direct result of the functional and morphological development of the GIT (Baranylova and Holman, 1976, Sell et al., 1991; Akiba and Murakami, 1995; Yamauchi et al., 1996; Uni, 1999). Thus IGF's have an indirect role on nutrient digestion and absorption.

Studies by Lane et al. (2002) demonstrated that IGF-I and IGF-II enhanced the uptake of glucose, in immature rats with concomitant increases in serum glucose levels. Thus *in ovo* feeding of arginine may enhance plasma IGF-I and IGF-II levels, which may indirectly enhance the activity of the nutrient transporters and the amount of energy available for rapid growth and development.

We hypothesize that *in ovo* feeding will increase the GIT capacity to absorb and digest nutrients at hatch by up-regulating the activity of brush border enzymes responsible for digestion of carbohydrates (sucrase and maltase) and proteins (leucine aminopeptidase) prior to hatching. Our goal is to elucidate the effects of *in ovo* feeding solutions containing combinations of egg white protein, carbohydrates, β -hydroxy- β -methylbutyrate (HMB), and arginine (ARG) on the activity of the fore mentioned intestinal brush border enzymes. Egg white protein (EWP) was chosen as a test nutrient because of its predominance in the amniotic fluid.

The turkey was chosen as an animal model due to its economic importance within the poultry industry. Additionally, the turkey embryo develops in a closed environment, independent of maternal influences and is thus an ideal animal system in which to study the effects of exogenous nutrients on embryonic growth and development.

MATERIALS AND METHODS

Incubation and In Ovo Feeding (IOF)

Two experiments were conducted in which viable Hybrid® turkey eggs embryos were obtained at 19d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). At 21 d of

incubation 500 eggs were evenly distributed among 4-5 gram weight categories ranging from 65 to 85 g per egg. These eggs were then evenly distributed among five treatment groups of 100 eggs each, such that the weight distribution profile among five treatment groups was identical. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was incised using a 23 gauge needle and 2.0mL (experiment one) and 1.5mL (experiment two) of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15mm. The area of shell around the injection hole was disinfected with an ethyl alcohol-laden swab, sealed with cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as aseptically as possible, such that the *in ovo* feeding treatment solutions contained the following in experiment one: 18% egg white protein in 0.9% saline (EWP); 18% EWP + 0.1% HMB in 0.9% saline (EWP + HMB), 0.1% HMB in 0.9% saline (HMB) or 20% Dextrin + 3% Maltose in 0.9% saline (S). The controls (C) were not injected, but they were subjected to the same handling procedures as the *in ovo* feeding treatment groups. The *in ovo* feeding solutions for the second experiment contained the following: 0.1% HMB in 0.4% saline (HMB); 0.7% arginine in 0.4% saline (ARG), 0.1% HMB + 0.7% ARG in 0.4% saline (HMB + ARG) or 18% EWP + 0.1% HMB + 0.7% ARG in 0.4% saline (EWP + HMB + ARG). The controls (C) were not injected but they were subjected to the same handling procedures as the *in ovo* feeding treatment groups. The HMB used in this study was calcium salt, kindly provided by Metabolic Technologies, Inc (Ames, IA). The egg white protein (egg whites from chicken #E0500), corn dextrin (Type I

#D2006), maltose monohydrate (#M5885) and free base L-arginine was purchased from Sigma (St. Louis, MO).

Animal husbandry

Upon hatching, each poult was identified by neck tag. Hatchability rate of viable eggs exceeded 95% in experiment 1 and 96% in experiment 2, and did not differ significantly among treatment groups. About 12 poult were randomly assigned to each of 7 replicate pens per treatment. All poult were housed in a total confinement building with supplemental heat from propane-fired heaters to maintain about 27°C. Each floor pen was bedded with soft pine wood shavings, and equipped with automatic nipple drinkers and manual self-feeders, and a supplemental incandescent heat lamp to a spot brooding temperature of 40°C. Each pen of poult was given *ad libitum* access to a typical turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat) that meets or exceeds the National Research Council (1994) nutrient requirements for turkeys. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University

Tissue sampling brush border enzyme analysis

At time of sampling, 10 birds per treatment were euthanized by cervical dislocation and within 2 minutes the jejunum was dissected from the end of the duodenal loop to the Meckel's diverticulum and flushed with ice cold saline 0.9% and placed on ice until freeze-storage at -20°C for future analysis. Jejuna samples were obtained at hatch and 7 d post-hatch in experiment 1, and at 25 d of incubation, hatch, 3 d, 7 d and 14 d post-

hatch in experiment 2. Jejunal samples were analyzed for brush border membrane activity of sucrase, maltase, and leucine aminopeptidase (LAP) enzymes. The jejunal samples were homogenized in ice cold 0.9% saline (5mg/5mL tissue) using a polytron homogenizer. The supernatant from each sample was diluted (1:50 for analysis of sucrase and maltase activity, 1:75 dilution for LAP analysis), and then 25 μ L of it was pipetted in triplicate to a 96 well plate. Subsequently 25uL of substrate, 50mM maltose solution for the maltase assay or 56 mM sucrose solution for the sucrase assay was added to the wells in duplicate. Twenty-five microliters of distilled water was added to a separate corresponding well to serve as the tissue blank and subsequently incubated at 37°C for 30 minutes. At the end of this incubation period, 200 uL of color reagent was added to all the wells. [One purpurogalin glucose oxidase (PGO) capsule (Sigma # 510-6, St. Louis, MO) was dissolved in 100mL of distilled water in an amber bottle yielding an enzyme solution. Then 50mg o-dianisidine dihydrochloride (Sigma #510-50) was dissolved in 20mL of distilled water to produce the color reagent. The enzyme-color reagent was prepared by combining 100mL of the enzyme solution with 1.6mL of the color reagent.] Each plate was then incubated at 37°C for 30 minutes and read using a microplate plate reader at a wavelength of 450nm. The quantity of substrate hydrolyzed was determined by colorimetric analysis. Unknown values of maltase and sucrase activity of the diluted samples were calculated by extrapolation from known values of the glucose standard curve as follows:

umoles of substrate hydrolyzed per hour per milliliter = [(mean OD sample-sample blank)-(y intercept standard (std) curve)]/slope std curve * dilution factor * 0.222.

The factor of 0.222 was determined mathematically by converting 25ul to 1mL, which was a factor of 40. This value was subsequently multiplied by a factor of 2 to calculate substrate hydrolyzed per hour. Subsequently the value was divided by 180 (the molecular weight of glucose) to calculate micromoles (μ moles) of substrate hydrolyzed per hour, and multiplied by of 2, to account for the two glucose molecules produced from maltose. Subsequently, the jejunal leucine aminopeptidase activity was determined using methods described by Goldberg et al (1959).

Procedure for Protein Determination

Protein was determined using the Lowry method as described by BioRad® Protein Assay kit with Bovine Serum Albumin Assay Standard II BioRad ®#500-0007, BioRad® #500-0115, BioRad® #500-0114 and BioRad® #500-0113 reagents.

Statistical Analysis

All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1996). Each bird served as an experimental unit for statistical analysis. Because highly significant age effects were observed, the treatment effects evaluated by neonatal age (i.e. hatch, 3d and 7d of age). In experiment one, data from *in ovo* treatments A, B, C, and D were analyzed as a 2X2 factorial arrangement, with two levels of egg white protein (0% and 18%) and two levels of HMB (0% and 0.1%). Variables having different F-test were compared using least-squares-means function in SAS (1996) and the treatment

effects were considered significant at $P < 0.05$. An additional analysis was conducted to contrast the effects of dietary carbohydrates versus dietary protein on intestinal maltase and LAP activity. In experiment two, data from *in ovo* treatments A (HMB), B (ARG), C, and D (HMB + ARG) were analyzed as a 2 X 2 factorial arrangement, with two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%). Variables having different F-test were compared using least-squares-means function in SAS (1996) and the treatment effects were considered significant at $P < 0.05$. An additional analysis was conducted to contrast the inclusion of dietary protein in the HMB + ARG *in ovo* feeding solution on intestinal maltase, sucrase and LAP activity.

RESULTS

Experiment 1:

There were no significant differences observed in jejunal maltase activity between the IOF treatment groups at hatch or 7-d post-hatch (Table 4.1). Jejunal LAP activity was significantly depressed at hatch in poult *in ovo* fed EWP in comparison to the remaining IOF treatments ($p < 0.05$, Table 4.1). A significant EWP X HMB interaction effect was observed on jejunal LAP activity ($p < 0.05$, Table 4.1), revealing that in IOF solutions containing EWP, HMB increased jejunal LAP activity at hatch, but when EWP was excluded from the IOF solution, HMB depressed jejunal LAP activity hatch. Conversely, *in ovo* feeding of carbohydrates (S) significantly enhanced jejunal maltase ($p < 0.05$, Table 4.2) and LAP ($p < 0.05$, Table 4.2) activities at 7-day post-hatch in comparison to the controls and poult *in ovo* fed EWP.

Experiment 2:

Significant treatment effects were observed on sucrase and maltase activity, but these effects were not consistent at the different time points (Table 4.3). At 25 d of incubation, HMB significantly ($p < .05$) increased sucrase activity; especially when in combination with ARG as revealed by a significant HMB X ARG interaction effect. This interaction effect was no longer observed at hatch, rather HMB depressed sucrase activity ($p < .5$) whereas ARG enhanced sucrase activity ($p < .01$). There were no treatment effects on sucrase activity at 3 d post-hatch. At 7 d post-hatch HMB apparently depressed sucrase activity when combined with ARG, but the converse was observed at 14 d post-hatch. Similar treatment and developmental age effects were observed in maltase activity (Table 4.4) as in sucrase activity, with the exception at 3 d post-hatch when HMB alone depressed maltase activity. Evidently, HMB and ARG had synergistic effects on brush border enzyme activity, which was absent when ARG or HMB were *in ovo* fed alone. Jejunal sucrase and maltase activities were 3-fold greater in pouts *in ovo* fed ARG + HMB than the jejunal activity of poult of the other *in ovo* feeding treatments at 14 d post-hatch (Table 4.3 and Table 4.4, respectively).

Table 4.1 The effects of *in ovo* feeding (IOF) of two levels of β -hydroxy- β -methyl butyrate (HMB) (0% and 0.1%) and two levels of egg white protein (0% and 18%) on the jejunal maltase and LAP activities of turkeys at hatch and 7 days post-hatch (Experiment 1).¹

	Jejunal Maltase Activity		Jejunal LAP Activity	
	umole maltose/min/ug protein		(units/min/ug protein)	
IOF treatment ²	Hatch	Day 7	Hatch	Day 7
A (Protein)	365.4 ^a	296.9 ^a	65.7 ^b	46.3 ^a
B (protein + HMB)	453.9 ^a	312.5 ^a	115.9 ^a	46.8 ^a
C (control)	425.1 ^a	350.1 ^a	122.8 ^a	41.0 ^a
D (HMB)	433.3 ^a	360.5 ^a	109.3 ^a	39.1 ^a
Source of Variation	-----p-value-----			
Protein	0.570	0.229	0.091	0.350
HMB	0.163	0.755	0.216	0.912
Protein* HMB	0.245	0.950	0.036	0.865
SEM (36) ³	33.50	41.30	14.40	6.80

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment B *in ovo* feeding solution contained 18% egg white protein + .1% HMB in .9% NaCl saline. Treatment C is the non-injected control. Treatment D *in ovo* feeding solution contained .1% HMB in .9% NaCl saline.

³SEM(36) = pooled standard error of the mean with 36 degrees of freedom

^{a,b}Means within a column with different superscript are significantly different (P<.05).

Table 4.2. The effects of *in ovo* feeding (IOF) of egg white protein versus carbohydrate on the on the jejunal maltase and LAP activities of turkeys at hatch and 7 days post-hatch (Experiment 1).¹

	Jejunal Maltase Activity		Jejunal LAP Activity	
	umole maltose/min/ug protein		(units/min/ug protein)	
IOF treatment ²	Hatch	Day 7	Hatch	Day 7
A (protein)	365.4 ^a	296.9 ^b	65.7 ^b	46.4 ^b
C (control)	425.1 ^a	350.1 ^b	122.8 ^a	41.0 ^b
S (SUGAR)	441.2 ^a	518.4 ^a	92.2 ^b	93.3 ^a
Source of Variation	-----p-value-----			
Treatment (27) ³	33.0	40.8	13.7	8.7

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment A *in ovo* feeding solution contained 18% egg white protein in .9% NaCl saline. Treatment C is the non-injected control. Treatment S contained 20% dextrin + 3% maltose in .9% NaCl saline.

³SEM(27) = standard error of the mean with 27 degrees of freedom

^{a,b}Means within a column with different superscript are significantly different (P<.05).

Jejunal LAP activity was significantly greater in poult *in ovo* fed ARG + HMB than among poults in the other treatment groups at 25-day of incubation, hatch, 3 d and 14 d post-hatch (p<0.05, Table 4.5). This response was evident from the highly significant ARG X HMB interaction effects observed. Conversely, neither HMB nor ARG alone had this stimulatory effect on jejunal LAP activity, with the exception at 3-day post-hatch. At 3-day post-hatch, poults *in ovo* fed ARG also had significantly enhanced jejunal LAP activity over the controls and other *in ovo* feeding treatments (p<0.05, Table 4.5).

Interestingly, poult *in ovo* fed ARG + HMB had significantly greater jejunal sucrase, maltase and LAP activities per centimeter length of the jejunum (cm) at 25-day of incubation and at 14 d post-hatch than those subjected to the other treatments ($p < 0.05$, Table 4.6, Table 4.7 and Table 4.8, respectively). This effect was absent at all other time points measured.

In ovo feeding a combination of EWP + ARG + HMB significantly enhanced jejunal sucrase and maltase activities at the 7-day and 14-day post-hatch over the controls ($p < 0.05$, Table 4.9 and Table 4.10, respectively). Moreover, *in ovo* feeding of ARG + HMB enhanced jejunal sucrase activity at 25-day of incubation and 14-day post-hatch (Table 4.9), while enhancing jejunal maltase activity at hatch and 14-day post-hatch (Table 4.10). Jejunal LAP activity of poult *in ovo* fed HMB + ARG was significantly enhanced over the controls and poult *in ovo* fed EWP + ARG + HMB at hatch, 3-day post-hatch ($p < 0.05$, Table 4.11). Also, jejunal LAP was significantly improved over the controls by *in ovo* feeding of either HMB + ARG or EWP + ARG + HMB at 14-day post-hatch ($p < 0.05$, Table 4.11).

Table 4.3 The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on jejunal sucrase activity at 25 d of incubation, hatch, and 3, 7 and 14 d post-hatch (Experiment 2).¹

Sucrase Activity (umole sucrose/hr/ug protein)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	319.1 ^b	55.4 ^b	28.6 ^a	321.0 ^{ab}	311.4 ^b
ARG	244.1 ^b	68.3 ^a	25.5 ^a	374.6 ^a	331.6 ^b
C (CON)	306.8 ^b	62.5 ^{ab}	28.6 ^a	312.3 ^b	342.9 ^b
HMB + ARG	548.4 ^a	60.1 ^b	28.9 ^a	269.0 ^b	1041.8 ^a
Source of Variation	-----p-value-----				
ARG	0.166	0.054	0.375	0.808	0.0001
HMB	0.011	0.006	0.282	0.035	0.0002
ARG X HMB	0.018	0.845	0.296	0.014	<0.0001
SEM (36) ³	28.62	1.32	0.790	10.83	41.54

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.4 The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on jejunal maltase activity at 25 d of incubation, hatch, and 3, 7, and 14 d post-hatch (Experiment 2).¹

Maltase Activity (umole maltose/hr/ug protein)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	88.8 ^b	31.5 ^b	14.9 ^b	100.0 ^b	187.9 ^b
ARG	134.5 ^b	39.8 ^a	21.7 ^a	164.7 ^a	190.5 ^b
Control	111.1 ^b	34.4 ^b	22.2 ^a	192.1 ^a	194.1 ^b
HMB + ARG	225.2 ^a	41.1 ^a	21.5 ^a	138.8 ^{ab}	616.2 ^a
Source of Variation	-----p-value-----				
ARG	0.002	<0.0001	0.004	0.780	0.003
HMB	0.166	0.606	0.0005	0.006	0.003
ARG X HMB	0.025	0.144	0.0009	0.110	0.002
SEM (36) ³	11.75	0.710	0.493	9.95	32.83

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).³

Table 4.5. The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on jejunal LAP activity at 25 d of incubation, hatch, and 3, 7, and 14 d post-hatch (Experiment 2).¹

LAP Activity (units/hr/ug protein)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	33.0 ^b	22.3 ^b	9.14 ^b	139.9 ^a	124.6 ^b
ARG	50.5 ^b	22.6 ^b	13.4 ^a	133.5 ^a	115.8 ^b
Control	50.3 ^b	22.9 ^b	12.1 ^b	131.8 ^a	117.7 ^b
HMB + ARG	94.1 ^a	25.5 ^a	14.0 ^a	124.9 ^a	399.5 ^a
Source of Variation	-----p-value-----				
ARG	0.001	0.004	<0.0001	0.261	0.001
HMB	0.145	0.071	0.062	0.972	0.0005
ARG X HMB	0.001	0.010	0.006	0.157	0.0008
SEM (36) ³	4.28	0.313	0.306	2.91	18.98

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.6 The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on sucrase activity per centimeter jejunal length at 25 d of incubation, hatch, and 3, 7, and 14 d post-hatch (Experiment 2).¹

Sucrase Activity (umole sucrose/hr/ug protein/cm)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	35.0 ^b	7.51 ^a	1.68 ^a	16.3 ^a	22.2 ^b
ARG	56.7 ^b	5.75 ^b	2.21 ^a	12.6 ^b	23.7 ^b
Control	54.5 ^b	6.71 ^{ab}	1.88 ^a	13.0 ^b	22.1 ^b
HMB + ARG	114.7 ^a	6.55 ^{ab}	1.88 ^a	11.3 ^b	66.5 ^a
Source of Variation	-----p-value-----				
ARG	0.0061	0.0525	0.1627	0.0141	<0.0001
HMB	0.1777	0.1043	0.2310	0.3523	0.0002
ARG X HMB	0.0091	0.9983	0.6834	0.0378	0.0002
SEM (36) ³	14.9	0.45	0.21	0.99	5.2

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.7 The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on maltase activity per centimeter jejunal length at 25 d of incubation, hatch, and 3, 7, and 14 d post-hatch (Experiment 2).¹

Maltase Activity (umole maltose/hr/ug protein/cm)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	19.0 ^b	4.33 ^{ab}	1.44 ^a	7.19 ^a	12.6 ^b
ARG	16.3 ^b	3.25 ^c	1.16 ^a	4.22 ^b	14.4 ^b
Control	20.5 ^b	3.66 ^{bc}	1.44 ^a	7.94 ^a	12.4 ^b
HMB + ARG	45.6 ^a	4.57 ^a	1.39 ^a	5.83 ^a	39.8 ^a
Source of Variation	-----p-value-----				
ARG	0.0905	0.7483	0.228	0.0084	0.0022
HMB	0.0382	0.0011	0.382	0.6328	0.0064
ARG X HMB	0.0223	0.2582	0.394	0.2001	0.0072
SEM (36) ³	6.80	0.31	0.130	0.89	4.40

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

² Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different ($P < .05$).

Table 4.8 The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%) on LAP activity per centimeter jejunal length at 25 d of incubation, hatch, 3, 7, and 14 d post-hatch (Experiment 2).¹

LAP Activity (units/hr/ug protein/cm)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
HMB	7.18 ^b	2.46 ^a	0.883 ^a	5.81 ^a	7.69 ^b
ARG	5.81 ^b	2.32 ^a	0.712 ^a	5.44 ^a	9.49 ^b
Control	9.24 ^b	2.43 ^a	0.784 ^a	5.48 ^a	7.64 ^b
HMB + ARG	17.6 ^a	2.80 ^a	0.905 ^a	5.26 ^a	25.3 ^a
Source of Variation	-----p-value-----				
ARG	0.0409	0.434	0.733	0.261	0.0001
HMB	0.0056	0.079	0.053	0.972	0.0012
ARG X HMB	0.0002	0.123	0.523	0.157	0.0013
SEM (36) ³	1.45	0.113	0.112	0.320	2.25

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.9. Effect of in ovo feeding (IOF) of arginine (ARG) and β -hydroxy- β -methyl butyrate (HMB) and protein on jejunal sucrase activity on turkeys at 25 d of incubation, hatch, 3, 7 and 14 d post-hatch (Experiment 2).¹

Sucrase Activity (umole sucrose/hr/ug protein)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
Control	306.8 ^b	62.5 ^a	28.6 ^a	312.3 ^{ab}	343 ^b
HMB + ARG	548.4 ^a	60.1 ^a	28.9 ^a	269.5 ^b	1042 ^a
EWP+ HMB + ARG	184.3 ^b	64.2 ^a	26.0 ^a	368.0 ^a	1186 ^a
Source of Variation	-----p-value-----				
Treatment	0.015	0.621	0.409	0.013	<0.0001
SEM (27) ³	45.36	1.70	0.938	12.59	63.14

¹All data represents the mean value of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM(27) = standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.10. Effect of in ovo feeding (IOF) of arginine and β -hydroxy- β -methyl butyrate (HMB) and protein on jejunal maltase activity on turkeys at 25 d of incubation, hatch, 3, 7, and 14 d post-hatch (Experiment 2).¹

Maltase Activity (umole maltose/hr/ug protein)					
IOF treatment²	Day 25	Hatch	Day 3	Day 7	Day 14
Control	111.1 ^a	34.37 ^b	22.2 ^a	192.1 ^a	194.1 ^b
HMB + ARG	225.2 ^a	41.17 ^a	21.5 ^a	138.8 ^b	616.2 ^a
EWP+ HMB + ARG	140.5 ^a	34.50 ^b	21.7 ^a	184.9 ^a	779.2 ^a
Source of Variation	-----p-value-----				
Treatment	0.303	0.002	0.860	0.029	0.0001
SEM (27) ³	28.98	0.816	0.555	8.27	48.92

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

² Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM(27) = standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 4.11. Effect of in ovo feeding (IOF) of arginine and β -hydrox- β -methyl butyrate (HMB) and protein on jejunal LAP activity on turkeys at 25 d of incubation, hatch, 3, 7, and 14 d post-hatch (Experiment 2).¹

LAP Activity (units/hr/ug protein)					
IOF treatment ²	Day 25	Hatch	Day 3	Day 7	Day 14
C (CON)	50.3 ^a	22.9 ^b	12.1 ^b	132 ^a	117.7 ^b
HMB + ARG	94.1 ^a	25.5 ^a	13.9 ^a	125 ^a	399.5 ^a
EWP+ HMB + ARG	50.6 ^a	22.5 ^b	12.5 ^b	123 ^a	454.7 ^a
Source of Variation	-----p-value-----				
Treatment	.145	0.0024	0.104	0.488	<0.0001
SEM (27) ³	9.53	0.338	0.361	3.10	27.55

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM(27) = standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

DISCUSSION

The first week post-hatch is very critical for young hatchlings because young poults undergo a metabolic and physiological transition from egg to feed nutrient source. During this critical post-hatch period, hatchlings have a limited digestive and absorptive capacity for carbohydrates and amino acids (Kirkwood, 1983; Lilja 1983; Kirkwood and Prescott 1984; Konarzewski et al. 1989, Uni et. al, 1996, 1998, 1999, 2000, 2001, 2003;

Noy et al., 2001a), and a low resistance to physiological stress and microbial challenge. Consequently, turkey poults may have high mortality rates and stunted growth, especially when confronted with environmental and microbiological challenges. Early poult mortality (EPM) in the commercial turkey industry ranges from 3 to 6% annually (Phelps, 1987).

Adaptation and improved development, digestive and absorptive function of the GI tract is imperative for improving the early survival of hatchlings. After hatch, the first meal is critical for the subsequent growth of the chicks (Noy and Sklan, 1998, 1999; Uni et al, 1998). Under commercial hatchery conditions, turkey embryos hatch over a 36-hour window and then the hatchlings are transported from the hatchery to the rearing site that may take an additional 24 hours or more. As a consequence, some birds may have access to feed and initiate feed intake over 50 hours after hatching (Moran and Reinhart, 1980). This protracted delay in feed intake initiation can adversely affect early growth (Misra, 1978; Hager and Beane, 1983; Wyatt et al, 1985; Nir and Levanon et al., 1993; Noy et al., 2001), muscle development (Mozdziak et al., 1997, 2002a-c; Halevy et al., 2000), and enteric development (Geyra et al., 2001a, 2002) and adaptation. The first meal stimulates the intestinal gene expression and activity of the nutrient transporters and brush border digestive enzymes (Geyra et al. 2001a, 2002).

In ovo feeding may circumvent this problem of early inanition by introducing exogenous nutrients into the amnion of the developing late term embryo. The embryo orally consumes the amnion and nutrients prior to pipping. These nutrients are presented to the enteric tissues and may stimulate gene expression and the activity of the digestive

brush border enzymes and nutrient transporters (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Therefore the neonate hatches with a greater capacity to digest and absorb incoming nutrients presented during the neonatal period.

The objective of the studies reported herein was to determine how select nutrients (egg white protein, dextrin and maltose, arginine, and HMB) administered by *in ovo* feeding affect the activity of intestinal brush border enzymes responsible for final digestion of carbohydrates and proteins.

In experiment 1, *in ovo* feeding of carbohydrates significantly enhanced jejunal LAP and maltase activities at one week post-hatch. *In ovo* feeding of HMB significantly depressed jejunal LAP activity at hatch, in comparison to the other treatments. *In ovo* feeding of HMB or protein alone did not enhance jejunal LAP or maltase activity at hatch or one week post-hatch.

In ovo feeding of ARG + HMB significantly enhanced sucrase, maltase and LAP brush border activity within 48 hours of nutrient administration. Additionally, *in ovo* fed poult of the ARG + HMB treatment group had increased sucrase, maltase and LAP activity at 14 d post-hatch. These results imply that *in ovo* feeding during the embryonic life may stimulate intestinal brush border enzymes for up to two weeks post-hatch. Also, the activity of the disaccharidases, sucrase and maltase were improved greatly by *in ovo* feeding of ARG + HMB + protein at 7 and 14 d post-hatch. The activity of the peptidase, LAP, was greatly enhanced by *in ovo* feeding of ARG + HMB + EWP only at 14 d post-hatch. *In ovo* feeding of HMB alone did not have to have a significant impact on the intestinal brush border activity of sucrase, maltase or LAP. *In ovo* feeding of ARG alone

enhanced maltase and sucrase activities at the day of hatch and LAP activity at 3 d post-hatch but not at the other time points measured.

Poults *in ovo* fed ARG + HMB had significantly greater bodyweights at hatch, 3,d, 10 d, and 14 d post-hatch (Foye Dissertation, Chapter 3), suggesting that these poults had a greater capacity for digestion and absorption. These results indicate that in addition to early access to feed, supplementation with additional dietary ARG + HMB may indirectly improve gut development and subsequent post-hatch growth in young hatchlings; thus suggesting that dietary HMB and arginine may serve as enteric modulators. Previous experimentation by Tako et al. (2004) and Uni and Ferket (2004) demonstrated that HMB may serve as an enteric modulator in which enhances the absorptive surface area and activity of the sucrase-isomaltase of the intestinal mucosa. The mechanisms of action responsible for the observed increase in activities of the brush border enzymes are not known.

Intestinal development and adaptation by age is has been shown to be modulated by anabolic hormones (Lane et al. 2002; Roffler et al, 2003). Food deprivation decreases plasma insulin-like growth factors (IGF-I), while the introduction of food increases plasma IGF-I. Insulin-like growth factors are responsible for the effects of growth hormone. IGF's enhance cellular development, muscle deposition, metabolism, and intestinal development (Lane et al. 2002; Roffler et al, 2003). Mammalian milk contains the peptides IGF-I and IGF-II, which affect multiple developmental processes including jejunal glucose uptake (Lane et al., 2002). Studies by Lane et al. (2002) have

demonstrated that when rat pups were reared on a milk replacement devoid of IGF's, jejunal glucose transport activity and gene expression were severely altered.

In ovo feeding of ARG + HMB may stimulate the production of IGF's prior to hatch, which may alter the activity and expression of the brush border enzymes and nutrient transporters. Thus *in ovo* feeding of ARG + HMB would enhance intestinal development and somatic growth indirectly via IGF's and may have effects that persist during the critical post-hatch period. In general *in ovo* feeding of HMB depressed activity of the brush border enzymes, while *in ovo* feeding of arginine alone had generally a stimulatory effect on the activity of the brush border enzymes. *In ovo* feeding of HMB and arginine in combination had a synergistic effect on the activity of the brush border enzymes, sucrase, maltase and LAP. Our lab will conduct future *in ovo* feeding studies to elucidate the effects of *in ovo* feeding of HMB and arginine on plasma IGF's.

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Chapter 5

Effect of In Ovo Feeding Solutions Containing Egg White Protein and β -hydroxy- β -methylbutyrate (HMB) on Jejunal Nutrient Transport Rates in turkey embryos and hatchlings.

ABSTRACT *In-ovo* feeding (IOF), injecting dietary components into the amnion prior to internal pipping, may enhance early growth performance by enhancing the activity of the brush border enzymes and nutrient transporters responsible for glucose uptake, SGLT-1 and for the alanine, B⁰⁺, thus providing supplemental energy needed for metabolic activity. To test this hypothesis, two identical trials were conducted with 400 viable Hybrid® eggs divided and injected on day 21 of incubation with 2.0 mL of the following four IOF solutions: 1) non-injected control (NC); 2) 0.9% saline injected control (PC); 3) 21% egg white protein (EWP) in 0.4% saline; and 4) 21% egg white protein (EWP) + 0.1% HMB in 0.9% saline. Jejunal glucose and alanine transport rates, maltase and leucine aminopeptidase (LAP) activities were determined at 23 and 25 d of incubation and at hatch. In a third trial, 300 viable Hybrid® eggs were divided into two treatments (150eggs/treatment) and injected on 23 d of incubation with 1.5 mL of the following two IOF solutions: 1) non-injected control (NC); and 2) 0.7% arginine + 0.1% HMB + 18% egg white protein (ARG + HMB + EWP) in 0.4% saline. Jejunal alanine transport rates were determined at hatch and 7 d post-hatch. All poults were fed *ad libitum* within 24 hours after hatch. In trials 1 and 2, there were no differences in observations between the NC and PC treatment groups. In trial one, jejunal glucose transport rates were enhanced by IOF of EWP + HMB over the NC and PC treatments at 25 d of incubation ($p < 0.05$).

Additionally, glucose transport rates were significantly enhanced by *IOF* of EWP alone or in combination with HMB over the NC and PC treatments groups at 25 d of incubation in trial two ($p < 0.05$). Conversely, jejunal glucose transport rates were depressed by *IOF* of EWP + HMB in comparison to the NC, PC and EWP *IOF* treatments at hatch in trial one ($p < 0.05$). In trial one, LAP activity was significantly enhanced by *IOF* of EWP or EWP + HMB over the controls ($p < 0.05$) at hatch. Jejunal maltase activity was unchanged at any of the time points measured in trial one. In trial 3 *IOF* of ARG + HMB + EWP significantly enhanced jejunal alanine transport rates at hatch and 7-d post-hatch, over the controls ($p < 0.05$). These studies demonstrate that administration of dietary proteins, or amino acids or their metabolites, HMB, *in ovo* may enhance the activity of jejunal LAP and amino acid and glucose transporters which may correlate to increased dietary protein digestion and uptake of amino acids and sugars in the embryonic turkey and hatchling.

INTRODUCTION

Glucose, the primary source of energy is vital for embryonic and neonatal growth in young hatchlings. Glucose is a substrate for the biosynthesis of macromolecules such as glycoproteins, proteoglycans, glycolipids needed for membrane integrity and nucleic acids. Therefore, glucose plays a primary role in cellular homeostasis and energy metabolism. Dietary carbohydrates provide glucose, the primary fuel needed for development, growth and maintenance, while dietary proteins provide, amino acids, the building blocks needed for tissues and proteins of the body.

The gastrointestinal tract (GIT) presents the first barrier to the digestion and absorption of dietary nutrients and is the primary supply organ of the body responsible for hydrolyzing, transporting and absorbing nutrients. The GIT supplies the body with the energy and nutrients needed to meet the metabolic requirements for maintenance, activity, growth, development, thermoregulation, and for the replenishment of energy reserves. Chick growth and development are dependent upon nutrient assimilation of the small intestine, which is a direct result of intestinal development, morphology and maturation (Baranylova and Holman, 1976; Sell et al., 1991; Akiba and Murakami, 1995; Yamauchi et al., 1996; Uni, 1999). Consequently, rapid and early maturation of the GIT post-hatch is important during the critical post-hatch period. Thus with rapid and early functional development of the intestine, the young chick can utilize dietary nutrients to grow optimally according to its genetic potential (Uni et al., 2003a; Uni and Ferket, 2004).

The late-term chick embryo has a limited capacity to digest and absorb nutrients as reflected by relatively low expression levels of the sucrase-isomaltase (SI), aminopeptidase (AP), ATPase and the sodium-glucose transporter (SGLT-1) in the small intestinal mucosa (Uni et al., 2003a). Intestinal absorption capacity increases close to hatch and continues to increase during the first few days posthatch (Uni et al., 1999; Sklan, 2001; Uni et al, 2003a). During the first seventy-two hours post-hatch, the GIT undergoes rapid morphological, biochemical and cellular development in order to better digest and absorb incoming nutrients from exogenous feed (Uni et. al, 1998, 2000, 2003a; Noy et. al., 2001a). It has been hypothesized that the digestive and absorptive capacity of

the gut may limit the energy supply needed for the growing chick (Kirkwood, 1983; Lilja 1983; Kirkwood and Prescott 1984; Konarzewski et al. 1989), due to a low absorptive and digestive capacity. Therefore, hatchlings may experience mal-absorption of dietary nutrients.

Delayed access to feed of up to 48 hours, is a common poultry practice that may adversely delay the development of the digestive and/or absorptive activity of the GIT (Moran and Reinhart, 1980). Studies by Geyra et al. (2001) have shown that greater 48 hours of delayed access to feed after hatching adversely affected the GIT due to a reduction in the intestinal surface area, number of cells/crypt, and the proportion of proliferating cells particularly in the duodenum and jejunum. Conversely, studies have shown that incoming nutrients aid in increasing enteric development, function and maturation (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Therefore, deprivation of feed may developmentally delay enteric maturation post-hatch. Thus, early access to feed may enhance GIT development, maturation, digestive and absorptive capacity by non-specific and specific mechanisms (Geyra et al., 2002).

GIT development, maturation and function are enhanced post-hatch specifically by the concentration and type of nutrients found within the lumen of the gut (Diamond et al., 1984; Ferraris and Diamond, 1989, Ferraris, 2001), and nonspecifically by the presence of food in the gut (Baranylova and Holman, 1976; Geyra et al., 2001a; Uni et al., 1998). Intestinal glucose transport by the Na⁺-coupled glucose co-transporter (SGLT-1) has been shown to be modulated *in vivo* (Hahn and Koldovsky, 1966; Ferraris and Diamond, 1989; Shirazi-Beechey et. al, 1991; Lescale-Matys et al., 1993) and *in vitro*

(Diamond et al., 1984; Ferraris and Diamond, 1989) with changes in dietary carbohydrate levels. Increasing carbohydrate content in the diet resulted in increasing rates of glucose transport in the small intestine (Diamond and Karasov, 1987; Karasov et al., 1987a; Solberg and Diamond, 1987; Buddington and Diamond, 1989; Ferraris and Diamond, 1989; Ferraris et al., 1992). Additional studies have demonstrated that intestinal amino acid transport activities were enhanced in response to increasing dietary proteins and/or amino acids (Scharer et al. 1967, 1971; Karasov et al., 1987; Torras-Llort et al., 1998).

A novel method of supplementing the *in ovo* nutrition of oviparous species is described as “*in ovo* feeding” within the US Patent (6,592,878) of Uni and Ferket (2003) involves the administration of exogenous nutrients into the amnion of the developing embryo of turkeys at about 23 days of incubation, respectively. Because the late term embryo orally consumes the amniotic fluid prior to pipping of the air cell, this *in ovo* feeding technology is an innovative means of presenting exogenous nutrients to the enteric tissues and stimulate their development for absorption and utilization. *In ovo* feeding may serve as a tool to overcome these constraints on post-hatch growth by expediting GIT maturation prior to hatch, thus chicks and poults hatch with a more mature functioning gut (Uni and Ferket, 2004). Thus, the compensatory changes conducive to the digestion and absorption of an external diet occur prior to hatch and may prevent post-hatch mal-absorption and digestion of nutrients by increasing the expression of brush border enzymes and nutrient transport mechanisms. Tako et al. (2004) demonstrated that *in ovo* feeding of carbohydrates and/or HMB increased intestinal villus width and surface area in comparison to the controls in broiler chicks at hatch. Chicks *in*

ovo fed HMB had an increase of the villus surface area of 45% greater than the controls, while *in ovo* feeding of carbohydrate alone or in combination with HMB resulted in a 33% increase in the villus surface area in comparison to the controls at 3 days post-hatch. Additionally, jejunal sucrase-isomaltase activity was higher in embryo chicks *in ovo* fed either carbohydrates or HMB alone or in combination over the controls at 19 days of incubation. Consequently, bodyweights of chicks *in ovo* fed either carbohydrates or HMB alone were significantly greater than the controls at hatch through 10 days post-hatch. Additionally, preliminary studies by Uni and Ferket (2004) demonstrated that HMB fed *in ovo* not only enhanced hepatic glycogen stores, but significantly increased the villus surface area in comparison to the controls at 19 days and 20 days of incubation and hatch.

Dietary supplementation of β -hydroxy- β -methylbutyrate (HMB), a leucine catbolite has been shown to have many positive physiological benefits (Peterson et al., 1999a, 1999b; Nissen et al., 1996; Flakoll et al., 2004; Nissen et al, 1997; Nissen et al., 1994). In humans, dietary HMB supplementation has been shown to decrease exercise-induced proteolysis, significantly increase fat-free mass and to increase the amount of weight lifted during resistance training (Nissen et al., 1996). Other studies have shown that HMB *in vitro* stimulated macrophage proliferation and function in the macrophage chicken cell line, MQ-NCSU with the phagocytic potential of the MQ-NCSU being 31.7% greater than the controls (Peterson et al., 1999a).

Studies by Flakoll et al., (2004) demonstrated that 23 elderly women given a supplementation of 2g HMB, 5g arginine and 1.5g lysine daily had significantly greater

limb circumference, leg strength, and handgrip strength in comparison to the placebo group. In addition, the HMB-treated group experienced a 20% increase in protein synthesis opposed to the placebo group. Experiments by Budford and Koch (2004) indicated that supplementation with glycine, arginine and α -keto-isocaproic acid (leucine catabolite) enhanced anaerobic cycling performance of male cyclists. Chevalley et al. (1998) demonstrated that pharmacological doses of arginine *in vitro* enhanced collagen and bone formation by stimulating IGF-I production in osteoblasts. Thus, HMB and the amino acid, arginine were identified as *in ovo* feeding components which may indirectly enhance the intestinal function and development, due to the enhancement of IGF-I levels.

It has been demonstrated that IGF-II and insulin are involved in the mechanisms governing the differentiation of the intestinal epithelium, while IGF-I is mostly associated with crypt cell proliferation (Jehle et al., 1999). Nutrient absorption and digestion are a direct result of the functional and morphological development of the GIT (Baranylova and Holman, 1976, Sell et al., 1991; Akiba and Murakami, 1995; Yamauchi et al., 1996; Uni, 1999). Thus, IGF's have an indirect role on nutrient digestion and absorption. Studies by Lane et al. (2002) demonstrated that IGF-I and IGF-II enhanced the uptake of glucose, in immature rats with concomitant increases in serum glucose levels. Thus, *in ovo* feeding of arginine may enhance plasma IGF-I and IGF-II levels, which may indirectly enhance the activity of the nutrient transporters and the amount of energy available for rapid growth and development.

The hypothesis of the study reported herein is that *in ovo* feeding of the late term turkey embryo will enhance the post-hatch digestive and absorptive capacity of the gut by

up-regulating the activity of the nutrient transporters (SGLT-1 and B⁰⁺ neutral amino acid carrier) and brush border digestive enzymes (maltase, leucine aminopeptidase (LAP)) during the peri-hatch period. Thus *in ovo* feeding of turkeys will increase nutrient uptake and utilization during the neonatal period, resulting in more rapid subsequent growth, weight and muscle gains and increased survivability. The objective of this study was to test the hypothesis that *in ovo* feeding saline solutions containing egg white protein, arginine, and β -hydroxy- β -methylbutyrate (HMB) on: 1) Activity of the brush border enzymes responsible for carbohydrate digestion, maltase and protein digestion, leucine aminopeptidase (LAP); 2) Activity of the jejunal sodium-glucose transporter (SGLT-1) and alanine (B⁰⁺) transporters. Egg white protein was chosen as the nutrient choice to its predominance in the albumin of the egg. Preliminary studies by Uni and Ferket demonstrated that HMB (a leucine metabolite) fed *in ovo* significantly enhanced villus surface area over the non-injected controls (Uni and Ferket, 2004). Therefore, our primary objective of this *in ovo* feeding experiment was to determine the ability of HMB to serve as an enteric modulator by enhancing the activity of the jejunal digestive and absorptive proteins.

The turkey was chosen as an animal model due to its economic importance within the poultry industry. Additionally, the turkey embryo develops in a closed environment, independent of maternal influences and is thus an ideal animal system in which to study the effects of exogenous nutrients on embryonic intestinal development and adaptation.

MATERIALS AND METHODS

Incubation and In Ovo Feeding (IOF)

Viable Nicholas[®] turkey embryos were obtained at 19d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). At 21 d of incubation 400 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65 g to 85 g per egg. These eggs were then evenly distributed among four treatment groups of 100 eggs each, such that the weight distribution profile among all 4 treatment groups was identical. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was then incised using a 23 gauge needle and 2.0 ml of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15 mm. The injection hole area was disinfected with an ethyl alcohol-laden swab, sealed with a cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as aseptically as possible so that the *in ovo* feeding solutions for trials one and two contained the following: Non-injected negative control (NC), Injected positive control (PC) 0.9% saline, 21% egg white protein (EWP) in 0.9% saline or 21% EWP + 0.1% HMB in 0.9% saline. In trial three, the same procedures were followed with 300 viable eggs (150 eggs/treatment) and were injected with 1.5 mL of the following treatments: Non-injected control (NC) or 18% EWP + 0.7% arginine + 0.1% HMB in 0.4% saline at 23 d of incubation. The HMB used in this study was the calcium salt kindly provided by

Metabolic Technologies, Inc. (Ames, IA). The HMB used in this study was calcium salt, kindly provided by Metabolic Technologies, Inc (Ames, IA). The egg white protein (egg whites from chicken #E0500), and free base L-arginine were purchased from Sigma (St. Louis, MO).

Animal Husbandry and Tissue Sampling

Jejunal tissue samples were obtained at 23 d, 25 d of incubation and at hatch in trials one and two. In trials one and two, a section of the jejunum was assayed immediately for glucose and alanine uptake, while a separate section was stored at 20 °C for future analysis of the jejunal brush border maltase and LAP activities. In trial three, jejunal tissue samples were taken at hatch and 7 d post-hatch and assayed immediately for glucose and alanine uptake. Hatchability of viable eggs for all treatment group was > 85% in trials one and two, and >95% for trial three. In trial three, poulters were randomly assigned to several pens per treatment and housed in a total confinement building at North Carolina State University. About 12 poulters were randomly assigned to 7 replicate pens per treatment. All the birds were housed in a total confinement building with supplemental heat from propane-fired heaters to maintain about 27°C. Each floor pen bedded with soft pine wood shavings, and equipped with automatic nipple drinkers, a manual self-feeder, and supplemental incandescent heat lamp to maintain a spot brooding temperature of about 40°C. Each pen of poulters were given *ad libitum* access to a typical turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat) that met or exceeded National Research Council (1994) nutrient requirements for turkeys immediately upon

hatching. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Jejunal Nutrient Uptake Analysis

Reagent preparation

On the day of the assay, a final concentration of 2.48mM glutamine and 0.496mM glutamine was added to an appropriate volume of glucose transport buffer (GTB) (1.25mL/100mL) [GTB prepared by dissolving 8.18g NaCl, 0.36g KCl, 0.37g CaCl₂, 0.16g KH₂PO₄, 0.30g MgSO₄, 5.96g HEPES in 1000 mL of distilled water, pH 7.4]. A total of 10mL of GTB was needed for each intestine plus 3-4 mL extra. Assay buffers were prepared with a substrate final concentration of 0.80 mM alanine or α -methylglucose and a volume of 75 μ L per 100mL of H³ labeled L- [2,3-H] alanine (alanine transport activity) or C¹⁴ methylglucose (glucose transport) and H³ methyl-L-glucose (glucose transport). H³ labeled alanine was used as a probe, to measure amino acid transport activity. C¹⁴ labeled methylglucose was used as a probe to measure active glucose transport, while H³ L-glucose measured glucose that was trapped and bound. Eight mL of assay buffer was needed for each intestine to be assayed plus 1-2 mL extra. Each intestine required four beakers. Three beakers were used for alanine and glucose transport assays performed at 37°C and one beaker was used for alanine transport assay at 4°C to measure non-active H³ alanine transport. Two sets of plastic beakers were numbered and placed in shaking racks. One set was labeled for assay buffer and the second set was for 2.5% Trichloroacetic Acid (TCA). Two mL of the appropriate

solution was transferred to each beaker. A third set of beakers, for the pre-assay buffer, was numbered and placed into shaking racks. One plastic beaker was needed per intestine. 2 mL of GTB with glutamine and butyrate was pipetted into the pre-assay beakers. A set of 5mL plastic centrifuge tubes were numbered and placed into test tube racks (4 tubes per intestine). Care was taken to ensure that the numbers on the tubes match the numbers on the assay and TCA beakers.

Assay procedure

After cervical dislocation, the mid- section of the jejunum was removed and cut into four 1mm rings. One millimeter jejunal rings were assayed in triplicate. Each ring (3 rings glucose; 4 rings alanine) was opened and placed in pre-assay buffer for immediate assay. The beakers containing pre-assay buffer with tissue and assay buffer were warmed in a water bath at 37°C for 10 minutes with gentle shaking. Each ring was transferred from the pre-assay buffer to assay buffer at timed intervals of 20-30 seconds with vigorous shaking for a 6 minutes (alanine) and 15 minutes (glucose). Each ring was then rinsed in 3 mL of ice-cold 30 mmol/L mannitol at intervals of 20-30 seconds. Each ring was transferred to TCA. The entire transfer was then repeated at 4°C using one piece of intestinal tissue from each poult, and was considered as an “ice value” for calculation of alanine. The TCA from each beaker was centrifuged in polystyrene tubes for 5 minutes at high speed. One milliliter of the supernatant and 10 mL of scintillation fluid was added to a scintillation vial and mixed. Specific activity of the buffer was

determined by counting 20uL aliquots of assay buffer and 980uL of TCA. All vials were counted for 5-10 minutes in a scintillation counter.

Assay calculations

The calibration factor was the triplicate counts per minute (CPM) average (10mL of scintillation fluid + 980 uL of TCA + 20uL of assay buffer) divided by 16 (# nmoles of isotope/20uL) X two (only half of TCA extracted). The count per minute for each jejunal ring was multiplied by the calibration factor to determine nmoles of alanine or α -methylglucose per sample. The values were divided by the assay times (6 minute alanine transport; 15 minutes glucose transport), which yielded the nmoles isotope/min/mm. To determine the concentration of alanine specifically transported, the values calculated from the 4°C procedure were subtracted from the values calculated from the 37°C procedure. Within each triplicate, values that were 85% or closer to one another were averaged.

Statistical Analysis

Two trials were conducted with the following *IOF* treatments were analyzed: Non-injected control, Injected control 0.9% saline, 21% egg white protein (EWP) in 0.9% saline or 21% EWP + 0.1% HMB in 0.9% saline. All data were analyzed using general linear models procedures for ANOVA in SAS® (1996). Each bird served as an experimental unit for statistical analysis. The data were analyzed in SAS® (1996) as a one-way ANOVA with treatments Non-injected control, Injected control 0.9% saline, 21% egg white protein (EWP) in 0.9% saline or 21% EWP + 0.1% HMB in 0.9% saline. All data were sorted by age and treatment. When ANOVA tests were significant

($P < 0.05$), the treatments were separated by least squares means (t-test). Trial three was conducted in which the following *IOF* treatments were analyzed: non-injected control and 18% egg white protein (EWP) + 0.7% arginine + 0.1% HMB in 0.4% saline. All data were analyzed in SAS®, 1996 as an unpaired t-test, significance ($P < 0.05$). All experiments were conducted with an equal frequency of variables within each treatment.

RESULTS

There was a 9- to 14-fold temporal increase in jejunal alanine transport rates between 23-d of incubation and hatch in trials one and two (Table 5.1 and Table 5.5, respectively). There were no differences in the jejunal transport rates between the non-injected controls, injected controls, 21% EWP or EWP + 0.1% HMB (EWP + HMB) treatments at days 23 or 25 of incubation in trials one and two. Additionally, jejunal transport rates remained unchanged between 23-d and 25-d of incubation in trials one and two. *In ovo* feeding of EWP + HMB significantly depressed jejunal transport activity at hatch in comparison to the other treatments ($p < 0.05$, Table 5.1); while jejunal transport rates at hatch were similar between the non-injected controls, injected controls and EWP treatments in trials one and two. In the third trial, poult that were *in ovo* fed 0.7% arginine (ARG) + 0.1% HMB + 18% egg white protein (ARG+HMB+EWP) had significantly greater jejunal alanine transport rates than the controls ($p < 0.05$, Figure 5.1) at hatch and 7-d post-hatch. There was a temporal 2.6-fold increase in jejunal alanine transport rates between hatch and 7 d post-hatch of poult of the ARG + HMB + EWP and non-injected treatments in trial three (Figure 5.1). Alanine transport rates were

significantly improved by *in ovo* feeding of ARG + HMB + EWP over the controls at hatch and 7 d post-hatch ($p < 0.05$, Figure 5.1).

Jejunal glucose transport rates in all treatments were increased over 20- to 40-fold between 23 d of incubation and hatch in trials one and two (Table 5.2 and Table 5.6, respectively). Jejunal glucose transport activity was significantly enhanced by *in ovo* feeding of EWP + HMB over the controls at 23 d and 25 d of incubation in trial one ($p < 0.05$, Table 5.2). In the second trial, jejunal glucose transport rates were enhanced only at 25-d of incubation by *in ovo* feeding of EWP and EWP + HMB, which had similar values that were significantly greater than the injected or non-injected controls ($p < 0.05$, Table 5.6). There was approximately a 1.5-fold increase in jejunal leucine aminopeptidase (LAP) activity of all treatments between 23 d and 25 d of incubation in trial one (Table 5.4). Subsequently, there was approximately a 1.5-fold increase in jejunal LAP activity of poultts *in ovo* fed either EWP or EWP + HMB at hatch, while jejunal LAP activity of poultts of the non-injected and injected controls remained similar to values seen at 25 d of incubation. Jejunal LAP activity was significantly enhanced over the controls at hatch by *in ovo* feeding of EWP or EWP + HMB ($p < 0.05$, Table 5.4), while the values were similar for the non-injected and saline-injected controls. There were no differences in jejunal LAP activity at any of the other time points measured in trial one. Jejunal maltase activity was not affected by *in ovo* feeding of any of the fore mentioned treatments at any of the time points measured in trial one.

Table 5.1 The effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal alanine uptake of turkeys at 23 and 25 d of incubation and at hatch (Trial 1) .¹

IOF Treatment	Day 23	Day 25	Day Hatch
	----- (nmoles alanine uptake/min/mm) -----		
Non-injected Control	0.130 ^a	0.150 ^a	1.43 ^a
Injected Control	0.120 ^a	0.153 ^a	1.41 ^a
EWP	0.114 ^a	0.135 ^a	1.55 ^a
EWP + HMB	0.100 ^a	0.161 ^a	1.16 ^b
Source of Variation	----- p-value -----		
Treatment	0.6809	0.4891	0.0467
SEM (76) ³	0.010	0.020	0.080

¹All data represents the mean of 20 birds per treatment.

²The treatments were Non-inj= non-injected control; Inj Cont=injected control; EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom.

^{ab}Means within a column with different superscript are significantly different (P<.05).

Table 5.2 Trial one-the effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal glucose uptake of turkey embryos and hatchlings .¹

IOF treatment²	Day 23	Day 25	Day Hatch
	-- (nmole α -methyl-glucose uptake/min/mm) ---		
Non-injected Control	0.0402 ^a	0.0212 ^b	2.55 ^{ab}
Injected Control	0.0683 ^a	0.0405 ^b	1.69 ^b
EWP	0.0343 ^a	0.0373 ^b	3.11 ^{ab}
EWP + HMB	0.0432 ^a	0.2050 ^a	4.32 ^a
Source of Variation	-----p-value-----		
Treatment	0.6926	<0.0001	0.0827
SEM(76) ³	0.210	0.010	0.690

¹All data represents the mean of 20 sample birds per treatment.

²The treatments were Non-inj = non-injected control; Inj Cont=injected control; EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom.

^{ab}Means within a column with different superscript are significantly different (P<.05).

Table 5.3 Trial one-the effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal maltase activity of turkey embryos and hatchlings .¹

IOF treatment ²	Day 23	Day 25	Day Hatch
	--- (umoles maltose/hr/ug protein) ---		
Non-Injected control	0.929 ^a	4.83 ^a	9.97 ^a
Injected Control	1.676 ^a	5.26 ^a	9.99 ^a
EWP	1.611 ^a	5.21 ^a	10.43 ^a
EWP + HMB	0.872 ^a	5.39 ^a	11.88 ^a
Source of Variation	-----p-value-----		
Treatment	0.6987	0.5467	0.4784
SEM(76) ³	0.410	0.430	1.30

¹All data represents the mean value \pm Standard Error. Each treatment represents a n=20.

²The treatments were Non-inj= non-injected control; Inj Cont=injected control; EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom.

^{ab}Means within a column with different superscript are significantly different (P<.05).

Table 5.4 Trial one-the effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal LAP activity of turkey embryos and hatchlings .¹

IOF treatment ²	Day 23	Day 25	Day Hatch
	----- (units LAP/hr/ug protein) -----		
Non-Injected Control	2.99 ^a	6.42 ^a	5.46 ^b
Injected Control	3.68 ^a	6.74 ^a	5.59 ^b
EWP	4.17 ^a	5.47 ^a	8.58 ^a
EWP + HMB	3.97 ^a	5.97 ^a	10.24 ^a
Source of Variation	-----p-value-----		
Treatment	0.7563	0.6754	0.0321
SEM(76) ³	0.690	0.570	0.820

¹All data represents the mean value \pm Standard Error. Each treatment represents a n=20.

²The treatments were Non-inj= non-injected control; Inj Cont=injected control; EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom

^{ab}Means within a column with different superscript are significantly different (P<.05).

Table 5.5 Trial two-the effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal alanine uptake of turkey embryos and hatchlings .¹

IOF Treatment	Day 23	Day 25	Day Hatch
	----- (nmole alanine uptake/min/mm) -----		
Non-injected Control	0.102 ^a	0.148 ^a	1.39 ^{ab}
Injected Control	0.108 ^a	0.144 ^a	1.38 ^{ab}
EWP	0.125 ^a	0.135 ^a	1.55 ^a
EWP + HMB	0.121 ^a	0.162 ^a	1.12 ^b
Source of Variation	-----p-value-----		
Treatment	0.830	0.884	0.151
SEM(76) ³	0.020	0.020	0.130

¹All data represents the mean value± Standard Error. Each treatment represents a n=20.

²The treatments were Non-inj= non-injected control; Inj Cont=injected control;

EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom

^{ab}Means within a column with different superscript are significantly different (P<.05).

Table 5.6 Trial two-the effects of in ovo feeding (IOF) of β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal glucose uptake of turkey embryos and hatchlings .¹

IOF treatment²	Day 23	Day 25	Day Hatch
	--- (nmole α -methyl-glucose uptake/min/mm) ---		
Non-injected Control	0.0551 ^b	0.101 ^b	4.147 ^a
Injected Control	0.0946 ^a	0.1169 ^b	4.381 ^a
EWP	0.0345 ^b	0.2405 ^a	5.265 ^a
EWP + HMB	0.0444 ^b	0.2455 ^a	4.945 ^a
Source of Variation	-----p-value-----		
Treatment	0.001	0.058	0.682
SEM(76) ³	0.010	0.050	0.720

¹All data represents the mean value± Standard Error. Each treatment represents a n=20.

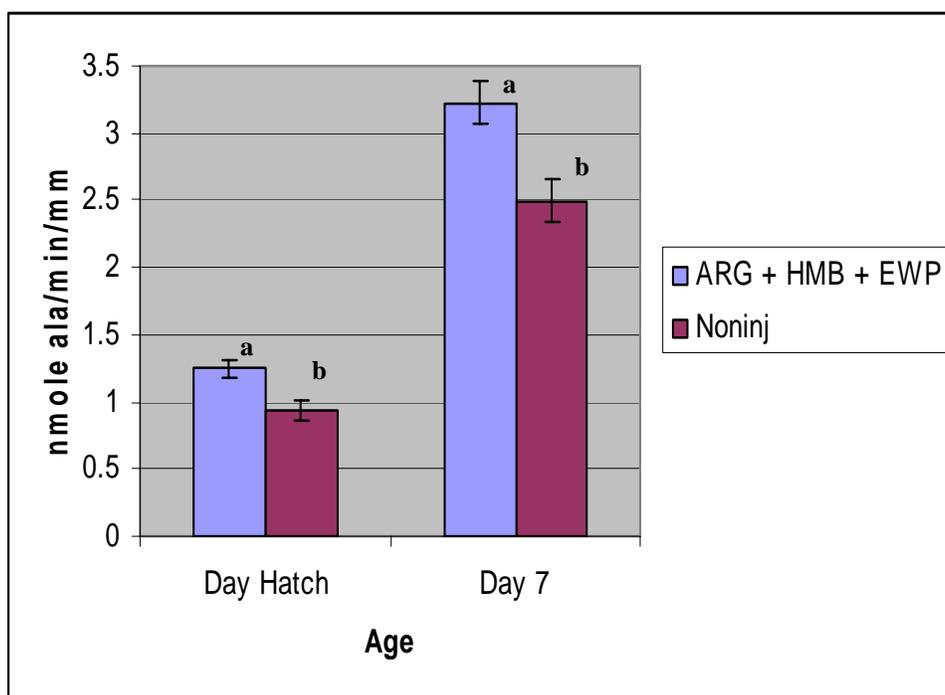
²The treatments were Non-inj= non-injected control; Inj Cont=injected control;

EWP=21% egg white protein; and EWP + HMB= 21% egg white protein + 0.1% HMB.

³SEM(76) = standard error of the mean with 76 degrees of freedom

^{ab}Means within a column with different superscript are significantly different (P<.05).

Figure 5.1 The effects of *in ovo* feeding (IOF²) of arginine and β -hydroxy- β -methylbutyrate (HMB) and egg white protein on the jejunal alanine uptake of turkeys at hatch and 7 days post-hatch (Trial 3).¹



¹All data represents the mean value \pm Standard Error. Each treatment represents a n=20.

^{ab}Values with different superscripts are significantly different $p < 0.05$

DISCUSSION

In ovo feeding of protein and/or HMB significantly enhanced LAP activity in embryonic intestine of turkey poults at hatch. Thus, embryonic intestinal peptidase activity is adaptive to diet and is not solely dependent upon age. Conversely, jejunal transport rates were depressed at hatch by *in ovo* feeding of EWP + HMB. Hence, intestinal neutral amino acid uptake rates may be independent of dietary stimulus and dependent upon age and maturation. There is a paucity of information regarding avian

embryonic LAP and amino acid uptake activity and expression. Limited studies have demonstrated intestinal transport activity of amino acids in chick embryos with increasing rates after hatch (Lerner, 1984). Uni et al. (2003) observed aminopeptidase expression to be detectable at 15 d of incubation and peaks at 19 d of incubation, and declining at hatch in chick embryo intestine (Uni et. al., 2003).

Jejunal glucose transport rates were enhanced by *in ovo* feeding of EWP + HMB (trial one) and EWP (trial two) at 23-d and 25-d of incubation, while declining at hatch. Thus, embryonic glucose transport activity may be adaptive non-specifically to diet and is not solely dependent upon age. In contrast, jejunal maltase (found within the sucrase-isomaltase (SI) complex) activity was not affected by *in ovo* feeding at any of the time points measured in the embryonic turkey. Previous studies have shown that sucrase-isomaltase is expressed in the late term chick embryo at 15 d of incubation and remains low until the peri-hatch period with a 3-fold increase (Sklan et al., 2003, Uni et al., 2003). In chicks, SGLT-1 expression was not detectable at 15 and 17 d of incubation, but was increased by 19 d of incubation, with small increases in expression until 7 d post-hatch (Sklan et. al., 2003, Uni et al., 2003). This low expression of SGLT-1 is consistent with other studies that have indicated that glucose uptake is low at hatching and increased gradually post-hatch (Sulistiyanto et. al., 1999; Sklan and Noy, 2000; Noy and Sklan, 2001). Therefore, it appears that SI and SGLT-1 expression follow differing ontogenetic programs. These data demonstrate that the programs regulating these systems involved in carbohydrate digestion are activated prior to exposure to dietary carbohydrates.

Changes in nutrient uptake for various nutrients occur independently of one another and independently of diet switches. Also, changes in nutrient uptake are not only dependent upon age, but can adapt rapidly and reversibly to diet (Obst and Diamond, 1992). The appearance of activity of intestinal enzymes and nutrient transporters are subjective to various timetables set by the internal developmental clock (Sulistiyanto et. al., 1999; Sklan and Noy, 2000; Noy and Sklan, 2001, Ferraris, 2001; Sklan et. al., 2003, Uni et al., 2003). Thus adaptation of intestinal brush border enzyme and nutrient transport activity is subjective to these pre-set developmental programs.

The regulation and ontogeny of sucrase-isomaltase (SI) is not simple. Studies by Yeh and Holt (1986) used intestinal isografts that had no contact with ingested food showed the timing of sucrase appearance in rodents to be independent of luminal contents. When these isografts were transplanted into animals 5 days younger than the donors, sucrase appeared earlier in the transplanted intestine than in the host. This evidence suggests that sucrase is controlled by an internal biological clock within the intestine itself. However, luminal contents can have a marked effect on the activity of sucrase, which is separate from this internal mechanism.

Chickens, like many other omnivorous vertebrates share a general trend of declining amino acid uptake and increasing glucose uptake with age, which correlates with the changes from an embryonic diet of proteins (albumin) and lipids to an external diet of mainly carbohydrate (Buddington and Diamond, 1989; Ferraris, 2001). Our data indicate that the late term turkey embryo has a greater capacity to transport neutral amino acids (alanine) and a very low capacity to transport glucose. The yolk and albumin of the

egg provide the energy and nutrients needed for the growth and development of the avian embryo. Thus, we suggest a greater emphasis on amino-acid uptake over sugar uptake, because the turkey embryo is dependent upon dietary protein (albumin) for rapid growth and can draw on yolk reserves to obtain energy. In this study, intestinal glucose uptake did not increase until hatching. Upon hatching, the internalized yolk reserves provide a source of energy, with yolk reserves being depleted during the first week post-hatch. Consequently, there is an increase in the activity and expression of the sodium glucose transporter post-hatch, with low levels present in the late term embryo (Sulistiyanto et. al., 1999; Sklan and Noy, 2000; Noy and Sklan, 2001; Sklan et. al., 2003, Uni et al., 2003). Thus, glucose uptake can keep pace with rapid growth surges post-hatch.

The period of postnatal growth is challenging for the rapidly growing vertebrates, such as precocial birds. Developmental programs of the body must maintain tissue maturation and progressive accretion of nutrients and energy. Additionally, limited energy must be differentially allocated to the various organs systems and whole animal. In precocial hatchlings, this presents an extreme challenge due to the need for locomotion and thermoregulation. Growth rates tend to be lower in the precocial birds than in similar sized altricial birds (Ricklefs 1983, Case 1978) because energy and nutrients get shunted away from growth and towards competing metabolic systems. Therefore *in ovo* feeding may serve as a tool to overcome these obstacles by providing supplemental nutrients, which may serve to enhance the development and functioning of the immature intestine of the avian embryo and neonate. With a more mature functioning gut, post-hatch poult

may better assimilate an external diet for more rapid subsequent growth and development (Uni and Ferket, 2004).

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Chapter 6

The Effects of In Ovo Feeding (IOF) of Beta-Hydroxy-Methylbutyrate (HMB) and Arginine with egg white protein on Plasma Insulin-like Growth Factors in post-hatch Turkeys

ABSTRACT Nutrition and nutritional status influence early growth and development, which are modulated by insulin-like growth factors (IGF's). An experiment was conducted to determine the effects of “*in ovo* feeding” (IOF), amniotic administration of exogenous nutrients at 23 d incubation on circulating IGF-I and IGF-II levels during the post-hatch period of turkeys. At 23 d of incubation, 100 viable Hybrid® eggs per treatment were injected with 1.5 ml solutions consisting of 4 nutritional treatments as a factorial arrangement of two levels of arginine (ARG 0% or 0.7%) and two levels of HMB (0% and .1%). The IOF treatments were as follows: 1) 0.1% HMB (HMB) in 0.4% saline; 2) 0.7% arginine (ARG) in 0.4% saline; 3) 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG); and 4) non-injected control. In addition, 100 Hybrid® eggs were *in ovo* fed 1.5mL saline containing 0.1% HMB + 0.7% arginine + 18% EWP as a contrast to the HMB + ARG and control treatments. All poultts were fed *ad libitum* within 24 hours after hatch. Heparinized blood samples were taken at hatch, 3 d, 7 d and 14 d post-hatch and plasma was analyzed for IGF-I and IGF-II levels using an acid-ethanol extraction method. At hatch, there was a significant effect of *IOF* of HMB and/or ARG on plasma IGF-I ($p<0.05$) and IGF-II ($p<0.05$) levels. *IOF* of HMB + ARG significantly enhanced plasma IGF-I over the controls at hatch ($p<0.05$). Poultts *in ovo* fed HMB + ARG had significantly greater plasma IGF-II levels than the HMB or ARG *in ovo*

treatments, but were similar to the controls at hatch. Plasma IGF-I levels were significantly enhanced by *IOF* of HMB + ARG + EWP over the controls and HMB + ARG *in ovo* treatment groups at 7 d post-hatch ($p < 0.05$).

INTRODUCTION

Nutritional status influences overall body and muscle growth and may do so directly or indirectly through its effect on regulatory factors, such as circulating insulin-like growth factor-I (IGF-I) (Kita et al., 1993; Cohick et. al., 1993; Bramheld, 1997; Beccavin et al., 2001; Roberson et. al., 2002). The normal growth of post-hatching birds requires growth hormone, produced from the pituitary gland (King and Scanes 1986). Circulating concentrations of growth hormone are low in chick embryos and begin to rise just prior to hatch. In chickens, circulating concentrations of growth hormone and IGF-I rise post-hatch, and peak during the early phase of post-hatch growth, and decline in the mid-growth phase of post-hatch development (King and Scanes 1986).

The effects of growth hormone are mediated by growth factors, IGF-I and IGF-II, produced locally within the tissues as well as being produced and released into the circulation by the liver. IGF-I and IGF-II stimulate proliferation and differentiation of a number of tissues in avian embryos. Studies by Chevalley et al. (1998) demonstrated that pharmacological doses of arginine *in vitro* enhanced collagen and bone formation by stimulating IGF-I production in osteoblasts. The IGF's stimulate overall growth of the early chick embryo and growth of specific tissues and organs. In addition to stimulating growth, growth hormone exerts both stimulatory and inhibitory effects on lipid metabolism (Campbell and Scanes 1985, 1987), modulates immune system development,

and alters thyroid hormone conversion increasing the plasma T₃ levels during early post-hatch life. Additional studies have demonstrated that IGF-II and myostatin (MSTN) are regulatory factors of muscle growth by regulating cell differentiation (Taylor et al., 2001; Langley et al., 2002; Rios et al., 2002; Joulia et. al., 2003) and proliferation (Rios et. al., 2001; Thomas et. al., 2000).

It has been demonstrated that IGF-II and insulin are involved in the mechanisms governing the differentiation of the intestinal epithelium, while IGF-I is mostly associated with crypt cell proliferation (Jehle et al., 1999). Nutrient absorption and digestion are a direct result of the functional and morphological development of the GIT (Baranylova and Holman, 1976, Sell et al., 1991; Akiba and Murakami, 1995; Yamauchi et al., 1996; Uni, 1999). Thus IGF's have an indirect role on nutrient digestion and absorption.

During the early post-hatch period, there are parallel increases in liver growth hormone receptor and IGF-I expression, suggesting that there is an increase in liver sensitivity to growth hormone along with the post-hatch rise in plasma rise in IGF1 (Burnside and Cogburn, 1992). Conversely, hepatic IGF1 receptor mRNA levels decrease between 1-4 weeks post-hatch in chickens (Armstrong and Hogg, 1992), suggesting that the liver becomes less sensitive to IGF-I within this period of high IGF1 production. During embryonic development, IGF-I steadily rise from day 6 to peak at day 15 of incubation, and then declines to low levels at hatch (Robcis et al., 1991). However, IGF1 mRNA is absent from the embryonic liver (Burnside and Cogburn, 1992), suggesting that the liver is not the source of embryonic IGF-I. The embryonic development of target tissues show marked tissue specific changes in IGF-I receptor expression: brain peak at day 6; heart

peak day 3; limb buds peak day 6 (Bassas et al., 1985, 1988). These differences in timing may play a critical role in the variable timing of differentiation of these tissues during development.

The first meal after hatch is critical for growth and development of young hatchlings. In standard hatchery practices hatchlings may be without food and water for up to 48 hours after hatch (Moran and Reinhart, 1980), which may cause delays in enteric development (Yamauchi et al 1996; Geyra et al. 2001, 2002) muscle (Mozdziak et al. 1997, 2002; Halevy et al., 2000), and body growth. Under fasting conditions, circulating IGF-I and IGF-II levels are reduced as well as hepatic IGF-I mRNA levels (Kita et al., 1993; Beccavin et al., 2001), which may have detrimental effects on the growth and development of the enteric, muscle and immune systems. Delaying access to feed for 24 hours after hatch increases mortality rates and stunted growth of broilers, which persisted until market age (Vieira and Moran, 1999). Additionally, studies have shown that birds denied access to feed did not have compensatory growth equal to the birds that were fed early (Misra, 1978; Hager and Beane, 1983; Wyatt et al., 1985; Nir and Levanon et. al, 1993; Noy et al., 2001b).

New innovations by Uni and Ferket (US Patent # US 6,592,878 B2, Jul 2003, Uni and Ferket, 2004) involve the administration of exogenous critical nutrients by day 23 of incubation into the amnion of developing turkey and chick embryos. The late term embryo orally consumes the amniotic fluid prior to hatching and therefore the “*in ovo*” administered nutrients are presented to the enteric tissues for digestion and absorption and then can be utilized or stored as energy. Consequently, *in ovo* feeding is

fundamentally feeding the embryo an external diet prior to hatch (Uni and Ferket, 2004) and thus may serve to increase the levels of circulating IGFs prior to hatch. Because IGFs are the direct mediators of growth, we hypothesize that *in ovo* feeding will serve as a tool to overcome constraints on growth due to denied access to feed immediately after hatch by enhancing the concentrations of circulating insulin-like growth factors. Elevated levels of IGFs may directly improve the growth and development of the gut, muscles, and body during the post-hatch period. Thus, *in ovo* feeding of dietary proteins, amino acids, or their metabolites is hypothesized to enhance circulating IGF levels post-hatch.

In humans, dietary HMB supplementation has been shown to decrease exercise-induced proteolysis, significantly increase fat-free mass and to increase the amount of weight lifted during resistance training (Nissen et al., 1996). Additionally, studies by Foye et al. (Dissertation) have demonstrated that *in ovo* feeding of a combination of HMB and arginine enhanced the bodyweights (Chapter 3) and activity of the brush border enzymes, sucrase, maltase and leucine aminopeptidase (Chapter 4). Thus, HMB and arginine were identified as *in ovo* feeding components, which may indirectly enhance bodyweights, the relative breast weight and intestinal morphology and functionality, due to the enhancement of plasma IGF-I levels.

Our objective was to elucidate the effects of *in ovo* feeding of dietary protein (egg white protein), amino acids (arginine) and β -hydroxy- β -methylbutyrate (HMB) on plasma IGF-I and IGF-II levels at hatch, 3 d, 7 d and 14 d post-hatch. Egg white protein was chosen as the nutrient choice to its predominance in the albumin of the egg. Preliminary

studies by Uni and Ferket (2004) demonstrated that HMB (a leucine metabolite) fed *in ovo* significantly enhanced intestinal villus surface area, muscle deposition, bodyweight and hepatic glycogen in comparison to conventional hatchlings. In addition, studies by Tako et al., 2004 have demonstrated that *in ovo* feeding of HMB increased the villus surface area due to increased cell proliferation and villus height. Therefore, HMB may serve as an enteric modulator and may influence plasma IGF levels. One of the objectives of this *in ovo* feeding experiment was to determine if HMB may serve as a modulator of plasma IGF levels. Recent studies (Cremades et al., 2004) have suggested that dietary arginine may enhance the insulin-like growth factor system. Our primary objective was to determine the effects of dietary arginine *in ovo* on plasma IGF levels.

The turkey was chosen as an animal model due to its economic importance within the poultry industry. Additionally, the turkey embryo develops in a closed environment, independent of maternal influences and is thus an ideal animal system to study the effects of exogenous nutrients on embryonic growth and development.

MATERIALS AND METHODS

Incubation and In Ovo Feeding (IOF)

Viable Hybrid® turkey eggs were obtained at 19d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). At 21 d of incubation, 500 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65 g to 85 g per egg. These eggs were then evenly distributed among five treatment groups of 100 eggs,

each with an identical weight distribution profile among all 5 treatment groups. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was incised using a 23 gauge needle and 1.5 ml of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15 mm. The injection site was disinfected with an ethyl alcohol-laden swab, sealed with a cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as aseptically as possible such that the *in ovo* feeding treatment solutions contained the following: A) 0.1% HMB in 0.4% saline; B) 0.7% arginine (ARG) in 0.4% saline; D) 0.1% HMB + 0.7% arginine (ARG) in 0.4% saline; and E) 0.1% HMB + 0.7% arginine (ARG) + 18% egg white protein (EWP) in 0.4% saline (HMB + ARG + EWP). The controls (C) were not injected, but they were subjected to the same handling procedures as the *in ovo* feeding treatment groups. Preliminary experimentation was conducted in our laboratory indicating that *in ovo* injection of 2.0 mL of 0.9% saline did not affect embryo and poult bodyweights, breast yield or glycogen status. The HMB used in this study was the calcium salt, kindly provided by Metabolic Technologies, Inc. (Ames, IA). Free-base L-arginine and egg white protein (egg whites from chicken catalog # E0500) was purchased from Sigma (St. Louis, MO).

Animal husbandry and sampling

Upon hatching, each poult was identified by neck tag and the body weights recorded at hatch, 3 d, 7 d, 10 d and 14 d post-hatch. Hatchability rate of viable eggs exceeded 95% and did not differ significantly among treatment groups. About 12 poults were randomly assigned to 7 replicate pens per treatment. All the poults were housed in

a total confinement building with supplemental heat from propane-fired heaters to maintain about 27°C. Each floor pen was bedded with soft pine wood shavings, and equipped with automatic nipple drinkers, and manual self-feeder, and a supplemental incandescent heat lamp to a spot brooding temperature of 40°C. Each pen of poults was given *ad libitum* access to a typical turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat) that met or exceeded National Research Council (1994) nutritional requirements for turkeys. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University. Blood samples were collected by cardiac puncture or wing vein from 10 poults per treatment in lithium heparinized syringes from each treatment at hatch, day 3, 7 and 14 post-hatch.

Plasma IGF-I and IGF-II Analysis

Blood samples were spun by centrifugation to collect the plasma. Plasma IGF-I and IGF-II measurements were performed using acid-ethanol extraction as described by McMurtry et. al., (1994, 1998) at the Animal Nutrition Laboratory U.S., Department of Agriculture, Beltsville Maryland. The intra-assay coefficient of variation was 3.1% and 4.2% for IGF-I and IGF-II assays, respectively.

Statistical Analysis

All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1996). Each bird served as an experimental unit for statistical analysis. Data from *in ovo* treatments A, B, C, and D were analyzed as a 2 X 2 factorial arrangement, with two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%). An additional statistical analysis was conducted to contrast the effects of the

addition of dietary protein to the 0.1% HMB + 0.7% arginine IOF solution (Treatment E). These data were analyzed as a one-way ANOVA (SAS, 1996) comparing treatments D (0.1% HMB + 0.7% arginine in 0.4% saline), E (0.1% HMB + 0.7% ARG + 18% egg white protein (EWP) in 0.4% saline) and C (non-injected Control). All data were sorted by age and treatment. Variables having different F-test were compared using the least-squares-means function in SAS (1996) and the treatment effects were considered significant at $P < 0.05$. All experiments were conducted with an equal frequency of variables within each treatment

RESULTS

At hatch, there was a significant effect of *IOF* of HMB and/or ARG on plasma IGF-I ($p < 0.05$ Table 6.1) and IGF-II ($p < 0.05$ Table 6.1.) levels. This effect was mainly due to the interaction of HMB X ARG on plasma IGF-I ($p = 0.0417$) and IGF-II ($p = 0.0072$) levels. *IOF* of HMB + ARG significantly enhanced plasma insulin-like growth factor-I over the controls at hatch ($p < 0.05$, Table 6.1). IGF-II circulating levels were similar for the *in ovo* fed poult of HMB + ARG and controls, while the HMB + ARG *in ovo* fed treatments were significantly greater than poult *in ovo* fed either HMB or ARG at hatch ($p < 0.05$, Table 6.1).

At 3 d post-hatch, there was a significant effect of *IOF* of HMB and/or ARG on circulating IGF-I ($p < 0.05$, Table 6.1) levels. This effect was mainly due to the HMB X ARG interaction effect on plasma IGF-I ($p = 0.0406$) levels. At 3 d post-hatch, there were no significant differences in the IGF-I levels of poult *in ovo* fed HMB + ARG, controls, and ARG treatments. At 3-d posthatch, poult *IOF* HMB + ARG had significantly

higher IGF-I levels than poult IOF HMB alone ($p < 0.05$, Table 6.1). There was no effect of IOF on plasma IGF-II levels at 3-d post-hatch.

At 7 d post-hatch, there was a significant effect of IOF of HMB and/or ARG on plasma IGF-II ($p = 0.0009$, Table 6.1) levels, with IGF-I levels approaching significance ($p = 0.0754$). This effect was mainly due to the interaction of HMB X ARG on plasma IGF-II ($p < 0.0001$, Table 6.1) levels. At 7 d post-hatch, the IGF-I circulating levels of poult of the control, HMB + ARG, and HMB *in ovo* fed treatments were not significantly different. While plasma IGF-I poult *in ovo* fed HMB and ARG were similar, only poult *in ovo* fed ARG and the controls were significantly different at 7-d post-hatch ($p < 0.05$, Table 6.1). Poult *in ovo* fed ARG had depressed IGF-I plasma levels at 7 d post-hatch in comparison to the controls and HMB + ARG groups ($p < 0.05$, Table 6.1). Poult *in ovo* fed HMB + ARG and the controls had plasma IGF-II levels that were similar, and were significantly greater than poult *in ovo* fed HMB and ARG at 7 d post-hatch ($p < 0.05$, Table 6.1). Additionally, plasma IGF-II levels were similar for poult *in ovo* fed either HMB or ARG alone at 7 d post-hatch. At 14 d post-hatch, there were no significant interactions or experimental differences between treatments, in circulating IGF-I or IGF-II levels between the controls, HMB, HMB + ARG, and ARG treatments (Table 6.1).

Additional analysis was conducted to determine the effect of the inclusion of egg white protein (EWP) to the HMB + ARG IOF solutions to determine if there would be enhancements in circulating IGFs. Comparisons were made between circulating IGF-I and IGF-II levels of the non-injected controls, HMB + ARG and HMB + ARG + EWP treatment groups. Poult *in ovo* fed HMB + ARG had significantly enhanced plasma

levels of IGF-I and IGF-II over the HMB + ARG + EWP treatment groups at hatch ($p < 0.05$, Table 6.2). Plasma IGF-I levels of HMB + ARG treatment group were significantly enhanced over the controls and HMB + ARG + EWP *in ovo* fed groups at hatch ($p < 0.05$, Table 6.2). Plasma IGF-II levels of poult of the controls and HMB + ARG treatments were similar, with only plasma IGF-II levels of treatments HMB + ARG and HMB + ARG + EWP being significantly different at hatch ($p < 0.05$, Table 6.2). Poults *in ovo* fed HMB + ARG + EWP had significantly depressed plasma IGF-II levels in comparison to HMB + ARG *in ovo* treatment ($p < 0.05$ Table 6.2). This effect was lost by 3 d post-hatch, and there were no differences in plasma levels of IGF-I or IGF-II between the controls and poults *in ovo* fed HMB + ARG or HMB + ARG + EWP (Table 6.2). Plasma IGF-I levels were significantly enhanced by *in ovo* feeding of HMB + ARG + EWP over the controls and HMB + ARG *in ovo* treatment group at 7 d post-hatch ($p < 0.05$, Table 6.2). Plasma IGF-II levels of poults *in ovo* fed HMB + ARG + EWP were significantly enhanced over poults *in ovo* fed HMB + ARG at 7 d post-hatch, but were similar to the controls ($p < 0.05$, Table 6.2). At 14 d post-hatch, there were no differences in circulating IGF-I or IGF-II levels between the controls, HMB + ARG, or HMB + ARG + EWP *IOF* treatments (Table 6.2).

DISCUSSION

The insulin-like growth factors (IGF-I and II) are structurally related proteins with multiple effects on growth, development and metabolism in mammals (Rotwein 1991, Jones and Clemmons 1995). The roles of IGFs in avian growth and development are not as well understood as in mammals. In addition, more is known about the function of

IGF-I than IGF-II in birds (McMurtry et al., 1997). Early studies (Huybrechts et al., 1989; McMurtry et al., 1998) have demonstrated much higher levels of circulating IGF-II than IGF-I during embryonic development in turkeys and chickens. It has been hypothesized (McMurtry et al., 1997, 1998) that elevated levels of IGF-II during embryonic development may be important for embryogenesis. It has been demonstrated that IGF-II is important in mammalian fetal development (Stewart and Rotwein, 1996). In this study, plasma IGF-I and IGF-II levels were not measured during embryonic development, due to the minute quantities of blood collected during incubation.

McMurtry et al (1998) demonstrated a similar pattern of circulating IGF-II levels post-hatch in broiler chickens, while the circulating levels of IGF-II in the turkey remained fairly uniform during the post-hatch period. Conversely, our results showed a precipitous decline in plasma IGF-II levels by approximately half from the day of hatch to 3 d post-hatch, with levels increasing at 14 d post-hatch in turkeys. Although there is a paucity of information about the function and levels of IGF-II in turkeys, it is very unlikely that IGF-II levels are related to growth in the domestic bird. Exogenous administration of IGF-II to broiler chickens had no effect on somatic growth (Spencer et al., 1996). Scanes et al (1989) reported that IGF-II levels are unrelated to growth rates in either dwarf or high growth rate lines of chicken. Other studies have suggested that IGF-II maybe involved in the regulation of lipid metabolism in birds (Spencer et al., 1995).

Previous studies have demonstrated that feeding enhances plasma IGF-I levels, while feed deprivation causes depression in plasma IGF-I levels (Vasilatos-Younken and Scanes, 1991; Morishita et al., 1993; Kita et al., 1996; McMurtry et al., 1998). Our

research confirms these findings. Plasma IGF-I levels were significantly enhanced over the controls only at hatch by *IOF*. *IOF* solutions including HMB + ARG enhanced circulating levels of IGF-I over conventional poult, whereas *IOF* of HMB or ARG alone did not at hatch. This effect had diminished during the early post-hatch period, and plasma IGF-I and IGF-II levels were the same as conventional poult.

Studies by McMurtry et al. (1998) demonstrated that feeding a high protein diet (21% crude protein) enhanced plasma IGF-I levels, relative to a low protein diet (12% crude protein), while plasma IGF-II levels were unaffected by variations in dietary protein in broiler chickens. Our results confirm these findings, circulating IGF-II levels were not enhanced in comparison to the controls by the inclusion of egg white protein to the *IOF* solutions at hatch, 3 d or 14 d post-hatch. Our results also paralleled the findings of McMurtry et al (1998), in that the inclusion of dietary protein resulted in significantly enhanced plasma IGF-I levels over the controls. This effect was only seen at 7 d post-hatch and was absent at all other time points measured. Conversely at hatch, poult *in ovo* fed the HMB + ARG treatment had plasma IGF-I levels that were significantly greater than the controls and the HMB + ARG + egg white protein treatment group.

While the effects of *IOF* of a combination of HMB, ARG, with or without protein may be transitory, they may provide the regulatory stimulus needed to fuel more rapid growth and development during the early critical post-hatch period. Previous studies by presented in this Dissertation have demonstrated that *in ovo* feeding of HMB + ARG enhanced jejunal brush border maltase, sucrase, leucine aminopeptidase (Chapter 4) and glucose transport activities (Chapter 5), total hepatic glycogen and bodyweights (Chapter

2 and Chapter 3) at hatch. These data suggests that these improvements maybe due to enhanced IGF-I levels at hatch which may accelerate enteric development, growth and function and ultimately growth. While IGFs are expected to be involved in the very rapid growth rates seen in the domestic fowl, the aspect of growth regulated in part by IGFs has yet to be identified.

Table 6.1. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on the plasma IGF-I and IGF-II of turkeys at hatch, and 3, 7, and 14 days post-hatch¹.

	IGF-I				IGF-I			
	Hatch	Day 3	Day 7	Day 14	Hatch	Day 3	Day 7	Day 14
IOF treatment²	-----ng/mL-----							
HMB	15.57 ^b	11.24 ^b	11.76 ^{ab}	18.23 ^a	47.47 ^b	23.65 ^a	24.98 ^b	56.93 ^a
ARG	17.41 ^b	11.27 ^{ab}	10.5 ^b	16.75 ^a	55.34 ^b	21.85 ^a	23.54 ^b	53.1 ^a
CONTROL	18.1 ^b	12.89 ^{ab}	12.57 ^a	17.3 ^a	64.1 ^{ab}	31.89 ^a	40.31 ^a	56.38 ^a
HMB+ARG	26.56 ^a	14.52 ^a	12.54 ^a	18.0 ^a	80.75 ^a	32.89 ^a	37.45 ^a	61.41 ^a
Source of Variation	-----p-value-----							
ARG	0.0706	0.3128	0.8075	0.879	0.1043	0.6478	0.5119	0.9464
HMB	0.2389	0.5884	0.0348	0.6483	0.5542	0.8225	0.828	0.581
ARG X HMB	0.0417	0.0406	0.0138	0.941	0.0072	0.097	<0.001	0.4299
Treatment	0.0344	0.1311	0.0754	0.9703	0.0179	0.3586	0.0009	0.8127
SEM(36) ³	2.79	1.00	0.60	2.40	7.49	4.20	3.20	6.10

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Table 6.2. The contrast of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on the plasma IGF-I and IGF-II of turkeys at hatch, and 3, 7, and 14 days post-hatch.¹

	IGF-I				IGF-II			
	Day Hatch	Day 3	Day 7	Day 14	Day Hatch	Day 3	Day 7	Day 14
IOF treatment²	----- (ng/mL) -----							
CONTROL	18.1 ^b	12.89 ^a	12.57 ^b	17.30 ^a	64.1 ^{ab}	31.89 ^a	40.31 ^{ab}	56.38 ^a
HMB+ARG	26.6 ^a	14.52 ^a	12.54 ^b	18.04 ^a	80.8 ^a	32.89 ^a	37.46 ^b	61.41 ^a
HMB+ARG+EWP	15.7 ^b	14.96 ^a	15.64 ^a	19.86 ^a	55.3 ^a	39.86 ^a	50.46 ^a	59.71 ^a
Source Variation	-----p-value-----							
treatment	0.0403	0.5199	0.0007	0.6768	0.0624	0.3412	0.095	0.8848
SEM(27) ³	3.10	1.30	0.60	2.90	7.50	4.10	4.40	8.10

¹All data represents the mean value \pm standard error of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM(27) = standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

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Chapter 7

The Effects of In Ovo Feeding of Arginine (ARG) and/or β -hydroxy- β -methylbutyrate (HMB) with Egg White Protein on Jejunal Gene Expression in the turkey Embryo and hatchling

ABSTRACT Intestinal genes are specifically expressed to produce brush border membrane proteins that digest and absorb the dietary nutrients. Increased density of intestinal nutrient transporters and brush border membrane proteins in response to dietary stimulus is advantageous due to increased nutrient absorption and digestion, which may be correlated to increased expression of the absorptive and digestive related intestinal genes. Increased nutrient absorption and digestion provides the energy and nutrients needed to meet the metabolic needs and to fuel more rapid subsequent growth. One experiment was conducted to determine the effects of *in ovo* feeding (IOF) arginine (ARG) and/or HMB with egg white protein (EW) on intestinal gene expression of the digestion/absorption related genes sodium glucose transporter (SGLT-1), peptide transporter (Pept-1), sucrase-isomaltase (SI), and aminopeptidase (AP) of Hybrid® poult. At 23 d of incubation, 100 eggs per treatment were each injected with 1.5 ml of the following: 0.1% HMB in 0.4% saline; 0.7% arginine (ARG) in 0.4% saline; 0.1% HMB + ARG in 0.4% saline; 18% egg white (EW) protein + HMB + ARG in 0.4% saline solution. The controls were non-injected and subjected to standard hatchery procedures. The data were analyzed as a factorial arrangement with two levels of ARG (ARG 0% or 0.7%) and two levels of HMB (0% and .1%). Additionally, the data were analyzed as 1-way ANOVA contrasting treatments HMB + ARG, EW + HMB + ARG and the non-injected controls. All poult were fed *ad libitum* within 24 hours after hatch.

There was an HMB X ARG interaction effect on jejunal Pept-1, SGLT-1 and SI expression levels at hatch, 3d, and 7 d ($p < 0.05$). The relative jejunal Pept-1 gene expression was higher at 25 d of incubation in poult *in ovo* fed HMB than the controls and poult *in ovo* fed ARG alone or HMB + ARG ($p < 0.05$). At hatch, relative Pept-1, SGLT-1 and SI jejunal gene expression was significantly greater by *in ovo* feeding of HMB alone in comparison to the controls, ARG alone and HMB + ARG ($p < 0.05$). Relative jejunal SI expression levels were significantly enhanced by *in ovo* feeding of HMB + ARG over the controls, HMB and ARG *in ovo* feeding groups at 3 d post-hatch ($p < 0.05$). A one-way ANOVA was conducted to compare jejunal gene expression levels in the controls, HMB + ARG and 18% egg white protein (EW) + HMB + ARG *in ovo* treatments. The relative jejunal SGLT-1 expression levels of poult *in ovo* fed HMB + ARG and EW + HMB + ARG were similar with both having significantly greater relative jejunal SGLT-1 expression than the controls at 25 d of incubation ($p < 0.05$). At 3 d post-hatch, only poult *in ovo* fed EW + HMB + ARG had significantly greater relative jejunal Pept-1 expression levels than the controls ($p < 0.05$). The relative jejunal SGLT-1 and SI expression levels of poult *in ovo* fed HMB + ARG and EW + HMB + ARG were significantly greater than the controls at 3 d post-hatch ($p < 0.05$). The relative jejunal SGLT-1 and SI expression levels of poult *in ovo* fed EW + HMB + ARG was significantly greater than the relative jejunal SGLT-1 and SI expression levels of the controls at 14 d post-hatch ($p < 0.05$). Thus, *in ovo* feeding may enhance early growth by improving intestinal function and development, which may provide the nutrients and energy needed for more rapid growth during the post-hatch period.

INTRODUCTION

At hatch, young poult and chicks transition from a *in ovo* diet of lipids and protein to an external diet composed primarily of carbohydrate (Noble and Ogunyemi, 1989; Noy and Sklan, 1998). Thus, the gastrointestinal tract (GIT) in neonatal poultry must readily adapt to the compensatory changes necessary to meet the metabolic demands of rapid growing. Intestinal development maybe a rate determining step in growth; the brush border enzymes have different developmental timetables that may influence digestion in post-hatch birds (Ferraris, 2001; Uni et al., 2003a; Uni and Ferket, 2004)). Thus the brush border enzymes of the intestine may play a rate limiting role in providing substrates for growth, as the GIT is of the primary supply organs of the body that it must function optimally very early in life to ensure survival. Consumed and digested nutrients are utilized to meet the metabolic requirements needed for more rapid growth and development in young poult (Uni et al., 2003; Uni and Ferket, 2004).

Many of the digestion/absorption-related genes begin to show increased levels of expression just prior to hatching. In the chick embryo, maltase, aminopeptidase (AP), sodium-glucose co-transporter (SGLT-1) and ATPase activities begins to increase at 19-d of incubation and increases further at the day of hatch (Uni et al., 2003a). These intestinal transcripts are first detected at 15-d of incubation. The relative expression (gene expression/ β -Actin gene expression) of these genes was low at 15 and 17 days of incubation, but increases 9 to 25 fold by day 19 of incubation, and declining in gains by the day of hatch (Uni et al., 2003a).

During the first week post-hatch, the small intestine undergoes rapid morphological changes (Uni et al., 2000; Geyra et al., 2001b; Uni et al., 2003) and steadily increases the capacity to digest and absorb nutrients (Uni et al., 1998, 1999, 2000; 2003a; Noy et. al., 2001a; Sklan, 2001). It has been hypothesized that the digestive and absorptive capacity of the gut may limit the energy supply needed for the growing chick (Kirkwood, 1983; Lilja 1983; Kirkwood and Prescott 1984; Konarzewski et al. 1989) due to a low absorptive and digestive capacity. Therefore, hatchlings may experience mal-absorption of dietary nutrients. The GIT is the major supply organ of the body, which provides the nutrients and energy needed for growth, development, thermoregulation, maintenance, and for the replenishment of energy reserves. Therefore rapid and early maturation of these tissues post-hatch is of paramount importance during the critical post-hatch period.

Additionally, under normal hatchery practices, the avian neonate may not have access to feed and water for up to 48 hours post-hatch (Moran and Reinhart, 1980), which may be hinder to enteric development. In contrast, orally consumed nutrients can enhance enteric development, function and maturation (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Thus incoming dietary nutrients may initiate the necessary cellular mechanisms responsible for enhanced digestion/absorption of carbohydrates and proteins.

Studies by Geyra et al. (2001a) have shown that up to 48 hours of delayed access to feed after hatching adversely affected the GIT due to a reduction in the intestinal surface area, number of cells/crypt, and the % of proliferating cells in particularly the

duodenum and jejunum. Additionally, Geyra et al. (2002) demonstrated that the intestinal expression of the transcriptional factors, CdxA and CdxB needed for the up-regulation of the absorption/digestion-related genes was depressed by 48 hours of starvation in comparison to fed chicks post-hatch. As a result, depressed enteric maturation and function may lead to reduced uptake of metabolizable energy and nutrients, limiting post-hatch growth.

New innovations by Uni and Ferket (Patent # US 6,592,878 B2, Jul 2003, Uni and Ferket, 2004) aim to circumvent these limitations on growth, by administering exogenous nutrients at day 23 of incubation into the amnion of developing turkey and chick embryos. Because, the late term embryo orally consumes the amniotic fluid prior to hatching, the “*in ovo*” administered nutrients are presented to the enteric tissues for digestion and absorption (Uni and Ferket, 2004). *In ovo* feeding is fundamentally feeding the embryo an external diet prior to hatch, and helps to overcome the constraints on post-hatch development and growth of domestic poultry.

Experimentation by Tako et al. (2004) demonstrated that *in ovo* feeding of carbohydrates and/or HMB increased intestinal villus width and surface area in comparison to the controls in broiler chicks at hatch. Chicks *in ovo* fed HMB had 45% greater villus surface area than the controls, while these *in ovo* fed carbohydrate alone or in combination with HMB had a 33% greater villus surface area than the controls at 3 days post-hatch (Tako et al., 2004). Additionally, jejunal sucrase-isomaltase activity was higher in embryos chicks *in ovo* fed either carbohydrates or HMB alone or in combination than the controls at 19 days of incubation. Also, bodyweights of chicks *in*

ovo fed either carbohydrates or HMB alone were significantly greater than the controls at hatch through 10 days post-hatch (Tako et al., 2004).

“*In ovo* feeding” may enhance weight and muscle growth by increasing the expression of the absorption/digestion-related genes, resulting in better digestion and nutrient uptake. Studies by Uni and Ferket (2005) demonstrated that *in ovo* feeding enhances GIT maturation prior to hatch thus chicks and poults hatch with a more mature functioning gut. Secondly, *in ovo* feeding may prevent post-hatch mal-absorption of nutrients by causing the compensatory changes conducive to an external diet to occur prior to hatching (Tako et al., 2004). Thus, the expression and activity of the carbohydrate and protein digestive and absorptive systems are up-regulated during the peri-hatch period and hatchlings can adequately digest, absorb and utilize nutrients from feed.

Numerous studies have demonstrated the up-regulation in the expression and activity of intestinal absorption/digestion-related genes in response to diet in adult animals (Buddington et al., 1991; Caviedes-Vidal et al., 2000; Erickson et al., 1995; Ferraris 2001; Ferraris et al 1988, 1997; Jiang et al., 2001; Matsushita 1985). However, there is a paucity of studies which identify the effects of nutrient administration “*in ovo*” on intestinal gene expression. The objective of this experiment was to determine how dietary factors given by *in ovo* feeding can alter the expression of genes in the intestinal epithelium of the jejunum during development in turkeys. The avian egg serves as an adequate animal model for experimentation with intestinal nutrient-gene interactions due to the closed environment and known nutrient content. Therefore nutrient manipulations

in the egg of the developing avian embryo may be correlative to intestinal gene expression. Conversely, the mammalian embryo develops in an environment with constant maternal influences and therefore it is more difficult to identify the inducers of intestinal cellular change.

MATERIALS AND METHODS

Incubation and In Ovo Feeding (IOF)

Viable Hybrid® turkey eggs were obtained at 19d of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°C). At 21 d of incubation 500 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65 g to 85 g per egg. These eggs were then evenly distributed among five treatment groups of 100 eggs each, such that the weight distribution profile among all 5 treatment groups was identical. At 23 d of incubation, each egg was candled to identify the location of the amnion. A hole was incised using a 23 gauge needle and 1.5 ml of *in ovo* feeding solution injected into the amnion using a 23 gauge needle to a depth of about 15 mm. The injection hole area was disinfected with an ethyl alcohol-laden swab, sealed with a cellophane tape, and transferred to hatching baskets. The *in ovo* feeding solutions were prepared as aseptically as possible, such that the *in ovo* feeding treatment solutions contained the following: A) 0.1% HMB in 0.4% saline; B) 0.7% ARG in 0.4% saline; D) 0.1% HMB + 0.7% ARG in 0.4% saline; and E) 18% egg white protein (EWP) + 0.1% HMB + 0.7% ARG in 0.4% saline. The controls (C) were not injected with a solution, but they were subjected to the

same handling procedures as the *in ovo* feeding treatment groups. Preliminary experimentation conducted in our laboratory indicated that *in ovo* injection of 2.0 mL of 0.9% saline did not affect embryo and poult bodyweights, breast yield or glycogen status. The HMB used in this study was the calcium salt, kindly provided by Metabolic Technologies, Inc. (Ames, IA). Free-base L-arginine and egg white protein (egg whites from chicken catalog # E0500) was purchased from Sigma (St. Louis, MO).

Animal husbandry

Upon hatching, each poult was identified by neck tag and the body weights recorded at hatch, 3 d, 7 d, 10 d and 14 d post-hatch. Hatch of viable eggs exceeded 95% and did not differ significantly among treatment groups. Poults were randomly assigned to four rooms of approximately two hundred square feet each at Dearstyne Avian Research Facility, North Carolina State University. Twenty-five poults from each treatment were randomly assigned to each of four rooms. Each room was equipped with manual self-feeders and drinkers. The concrete floor was bedded with wood shavings and supplemental heat was provided to a spot brooding temperature of 40°C. Poults were given a turkey starter diet (2935 kcal/kg, 27.5% protein, and 5.6% fat), which met or exceed the National Research Council (1994) requirements for turkeys. At hatch 3 d, 7 d and 10 d post-hatch, 10 poults were randomly selected for sampling from each treatment (≈ 2 poults/room/treatment). The 10 poults per treatment were euthanized by cervical dislocation and jejunal samples were flash frozen in liquid nitrogen. The samples were stored in nuclease-free microfuge tubes at -80°C for later analysis. All experimental

protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

RNA Extraction and cDNA Production

Total RNA was isolated from the jejunal segment using TRIS® reagent 2mL/200mg of according to the manufacturer's protocol (MRC Molecular Research Center, Cincinnati, OH, USA catalog #TR 118). Subsequently, DNAase was removed using methods described by Ambion® (Austin, Texas, USA) using the DNA-free (cat #1906) system. Total RNA quality was determined by gel electrophoresis. Complementary DNA (cDNA) synthesis was performed with extracted total DNase-treated RNA from the jejuna according to procedures detailed by Bio-Rad iScript™ cDNA synthesis kit® catalog #170-8891, running each sample in a thermocycler at 5 min at 25° C, 30 min at 42°C, 5 min at 85°C and held at 4°C.

Real-Time Polymerase Chain Reaction

A primary cDNA standard was prepared by pooling 5 uL of cDNA from all experimental treatments for each sampling age. Working cDNA standards were prepared using the following dilutions with DEPC-treated water: 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 of the pooled cDNA primary standard. Experimental cDNA samples were diluted 1:10 with DEPC-treated water. Subsequently, the experimental and standards were run in triplicate using procedures detailed by Bio-Rad (Hercules, CA USA) iQ SYBR® Green super mix catalog #170-8882. A standard curve was prepared for each 96 well plate with a correlation coefficient of >0.9. The reactions were performed according to the following

temperatures and cycles: cycle 1 for 3 min at 95°C, cycle 2 for 15 seconds at 95°C for 50 repeats, cycle 3 for 1 min at 60°C for two steps and no repeats, cycle 4 at 1 min for 95°C with one repeat, cycle 5 for 1 min at 55°C for one repeat and one step, cycle 6 for 10 seconds at 55°C for 100 repeats and one step.

Specific Primer Design

Anti-sense- and Sense-specific primers were designed to amplify the intestinal genes of interest using software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The custom primers were manufactured by Invitrogen® (Carlsbad, CA USA) and were as follows:

Gallus gallus (chicken) Intestinal peptide transporter cDNA (cPEPT1) accession number AY029615, (Chen et al. 2002)

cPEPT-1 primers, annealing temperature 58°C
Sense 5' CAG GGA TCG AGA TGG ACA CT 3'
Anti-sense 5' CAC TTG CAA AAG AGC AGC AC 3'

Gallus gallus (chicken) Intestinal sodium glucose co-transporter cDNA (cSGLT-1) 1 accession number AJ236903 (Gal-Garber,O et. al, 2000a)

cSGLT-1 primers, annealing temperature 57°C
Sense 5' CAT CTT CCG AGA TGC TGT CA 3'
Anti-sense 5' AAT TCG GCT GAT CAT TCC AG 3'

Gallus gallus (chicken) Intestinal brush border enzyme aminopeptidase (AP) cDNA(cAP) accession number cAP Y17105 (Gal-Garber,O. and Uni,Z, 2000b)

cAP primers, annealing temperature 54°C
Sense 5' TGG AAT GAC CTG TGG TTG AA 3'
Antisense 5' GCA ATG GAG TCG AAG ACC TC3'

Gallus gallus (chicken) Intestinal brush border enzyme sucrose-isomaltase cDNA (cSI) accession number Y08960 (Uni,Z,)

cSI primers, annealing temperature 52.7°C
Sense 5' TAC GGC CAT CAA ACA TCC TT 3'
Anti-sense 5' TAT GCT GGC ATT GCT GGT AG 3'

Gallus gallus (chicken) β -actin cDNA (c β -actin) as an internal control accession number XM_139732. c β -Actin primers, annealing temperature 57°C
 Sense 5' CTT CAC CAA CAT GGC TGA GA 3'
 Anti-sense 5' AAG GAA GGC TGG AAA AGA GC3'

Calculations

Relative jejunal gene expression was calculated as the Δ Ct (cycle threshold) of the experimental samples by subtracting the Ct average of the experimental samples in duplicates minus the Ct value of the experimental blank/ β -actin gene expression. The $\Delta\Delta$ Ct value was calculated using the following equation: $(Ct_x - Ct_R)_{control} - (Ct_x - Ct_R)_{test}$, where Ct_x = gene of interest and Ct_R = endogenous reference gene (β -actin).

Statistical Analysis

All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1996). Each bird served as an experimental unit for statistical analysis. Data from *in ovo* treatments 0.1% HMB, 0.7% ARG, 0.1% HMB + 0.7% ARG and the non-injected controls were analyzed as a 2 X 2 factorial arrangement, with two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%). An additional statistical analysis was conducted to contrast the effects of the addition of 18 % dietary protein (EW) to the 0.1% HMB + 0.7% ARG IOF solution. These data were analyzed as a one-way ANOVA (SAS, 1996) comparing treatments 1% HMB + 0.7% ARG in 0.4% saline (HMB + ARG), 18% EWP + 0.1% HMB + 0.7% ARG in 0.4% saline (EW + HMB + ARG) and the controls. All data were sorted by age and treatment. Variables having different F-test were compared using the least-squares-means function in SAS (1996) and the treatment

effects were considered significant at $P < 0.05$. All experiments were conducted with an equal frequency of variables within each treatment

RESULTS

The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%)

At 25 d of incubation, the relative gene expression of jejunal Pept-1 in poult *in ovo* fed HMB was greater than poult *in ovo* fed ARG + HMB, but not different from the controls or poult *in ovo* fed ARG alone ($p < 0.05$, Table 7.1). Relative gene expression levels of jejunal SGLT-1 was similar between the controls and poult *in ovo* fed HMB alone or HMB + ARG at 25-d of incubation with a main effect of HMB. Conversely, poult *in ovo* fed ARG alone had significantly depressed relative jejunal SGLT-1 expression levels in comparison to poult *in ovo* fed HMB at 25-d of incubation ($p < 0.05$, Table 7.2). Jejunal relative AP expression levels were similar for the controls and poult *in ovo* fed HMB or HMB + arginine at 25-d of incubation, while poult *in ovo* fed ARG alone had significantly depressed AP relative expression levels in comparison to poult *in ovo* fed HMB alone at 25-d of incubation with main effects of ARG ($p < 0.05$, Table 7.4). Jejunal sucrase-isomaltase (SI) relative gene expression was significantly depressed in poult *in ovo* fed ARG alone, in comparison to the controls, HMB and HMB + ARG treatments at 25-d of incubation with independent and main effects of ARG and HMB ($p < 0.05$, Table 7.3).

At hatch, relative Pept-1, SGLT-1 and SI jejunal gene expression was significantly enhanced by *in ovo* feeding of HMB alone in comparison to the controls,

ARG alone and HMB + ARG *in ovo* fed poult with interactive effects of ARG and HMB ($p < 0.05$, Table 7.1, 7.2, 7.3, respectively). Relative jejunal Pept-1, SGLT-1 and SI expression levels were similar in the controls and poult *in ovo* fed ARG at hatch. Relative jejunal AP expression was significantly suppressed in the HMB + ARG *in ovo* fed poult in comparison to the controls and poult *in ovo* fed HMB or ARG alone at hatch ($p < 0.05$, Table 7.4).

At 3 days post-hatch there were highly significant ARG X HMB effects on the genes of interest (Table 7.1, 7.2, 7.3, and 7.4, $p < 0.0003$). Relative jejunal Pept-1 and AP expression levels were similar between the controls and poult *in ovo* fed HMB + ARG at 3-d post-hatch, while the relative jejunal Pept-1 and AP expression levels of the controls and HMB + ARG treatment were significantly greater than poult *in ovo* fed ARG or HMB alone ($p < 0.0003$, Table 7.1, 7.4). Relative expression of jejunal SGLT-1 was similar between the controls and poult *in ovo* fed HMB and HMB + ARG at 3 d post-hatch. Additionally, poult of the controls, HMB and ARG treatments had relative jejunal SGLT-1 expression levels that were similar at 3 d post-hatch. While the SGLT-1 expression levels of poult of the controls, HMB and HMB + ARG treatment groups were similar, relative jejunal SGLT-1 expression levels were only significantly different between the ARG and HMB + ARG *in ovo* feeding groups, with poult of the HMB + ARG *in ovo* feeding group having enhanced relative jejunal SGIT-1 expression over the ARG *in ovo* fed group at 3 d post-hatch ($p < 0.05$, Table 7.2). Relative jejunal SI expression levels were significantly enhanced by *in ovo* feeding of HMB + ARG over the controls, HMB and ARG *in ovo* feeding groups at 3 d post-hatch ($p < 0.05$, Table 7.3).

The relative jejunal SI expression levels of the controls were significantly greater than the relative jejunal SI expression levels in poult *in ovo* fed HMB or ARG separately, which were similar at 3 d post-hatch ($p < 0.05$, Table 7.3).

At 7 days post-hatch, there were significant ARG X HMB effects on jejunal SGLT-1 and SI expression levels (Figure 7.2 and 7.3, respectively, $p < 0.05$). There were no significant differences in the relative jejunal Pept-1 or AP expression levels between the controls or *in ovo* feeding treatments (HMB, ARG, HMB + ARG) at 7 d or 14 d post-hatch. Poult *in ovo* fed HMB or ARG alone had similar relative jejunal SGLT-1 expression levels which were significantly greater than the controls or poult *in ovo* fed HMB + ARG at 7 d post-hatch ($p < 0.05$, Table 7.2). Poult *in ovo* fed HMB + ARG at significantly depressed relative jejunal SI expression levels in comparison to the controls or poult *in ovo* fed HMB or ARG alone at 7 d post-hatch ($p < 0.05$, Table 7.3).

At 14 d post-hatch, the controls and poult *in ovo* fed HMB or ARG had similar relative jejunal SGLT-1 expression levels, which were both significantly greater than the relative jejunal SGLT-1 expression levels in poult *in ovo* fed HMB + ARG ($p < 0.05$, Table 7.2). Conversely, the controls and poult *in ovo* fed HMB + ARG and ARG alone had relative jejunal SI expression levels that were similar, which were significantly greater than relative jejunal SI expression levels of poult *in ovo* fed HMB alone at 14 d post-hatch ($p < 0.05$, Table 7.3). At 14 days post-hatch there were no main and independent or interactive effects of ARG and HMB for any of the genes of interest.'

TABLE 7.1. The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) on the on relative jejunal PEPT-1 gene expression of turkeys at 25 days of incubation (25E), hatch and 3, 7 and 14 days post-hatch.¹

IOF Treatment ²	% of IOF		(Δ Ct= Ct Pept-1 gene-Ct sample blank)				
	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.39 ^{ab}	1.22 ^b	1.99 ^b	1.11 ^a	1.37 ^a
HMB	0.1	0	1.36 ^b	1.09 ^c	2.12 ^a	1.12 ^a	1.27 ^a
ARG	0	0.7	1.42 ^{ab}	1.21 ^b	2.18 ^a	1.08 ^a	1.30 ^a
HMB+ARG	0.1	0.7	1.47 ^a	1.42 ^a	1.88 ^b	1.16 ^a	1.30 ^a
Source of Variation			-----p-value-----				
ARG			0.093	0.0006	0.589	0.825	0.609
HMB			0.775	0.378	0.134	0.172	0.993
ARG X HMB			0.315	0.0003	0.0002	0.364	0.979
SEM(36) ³			0.040	0.040	0.050	0.040	0.060

¹ All data represents the mean of 10 sample birds per treatment.

² Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³ SEM(36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b} Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.2. The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) on the on relative jejunal sodium glucose transporter (SGLT-1) gene expression of turkeys at 25 days of incubation (25E), hatch and 3, 7 and 14 days post-hatch.¹

IOF treatment ²	% of IOF		(Δ Ct= Ct SGLT-1 gene-Ct sample blank)				
	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.41 ^{ab}	1.13 ^b	1.25 ^b	0.981 ^a	1.15 ^b
HMB	0.1	0	1.33 ^b	1.02 ^c	1.28 ^b	0.954 ^b	1.16 ^b
ARG	0	0.7	1.44 ^a	1.13 ^b	1.30 ^b	0.913 ^b	1.16 ^b
HMB+ARG	0.1	0.7	1.37 ^b	1.36 ^a	1.82 ^a	1.02 ^a	1.33 ^a
Source of Variation			----- p-value-----				
ARG			0.424	<0.0001	0.512	0.949	0.079
HMB			0.057	0.050	0.261	0.097	0.075
ARG X HMB			0.979	<0.0001	0.058	0.007	0.131
SEM(36) ³			0.040	0.030	0.040	0.020	0.050

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.3 The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) on the on relative jejunal sucrase-isomaltase (SI) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch.¹

IOF treatment ²	% of IOF		(Δ Ct= Ct SI gene-Ct sample blank)				
	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.56 ^b	1.52 ^b	1.76 ^b	1.35 ^b	1.76 ^b
HMB	0.1	0	1.50 ^b	1.36 ^c	1.94 ^a	1.37 ^b	1.94 ^a
ARG	0	0.7	1.71 ^a	1.63 ^b	1.97 ^a	1.32 ^b	1.72 ^b
HMB+ARG	0.1	0.7	1.59 ^b	2.04 ^a	1.45 ^c	1.49 ^a	1.75 ^b
Source of Variation			-----p-value-----				
ARG			0.0011	<0.0001	0.0001	0.193	0.091
HMB			0.0103	0.0149	<0.0001	0.007	0.135
ARG X HMB			0.3722	<0.0001	<0.0001	0.036	0.265
SEM(36) ³			0.030	0.050	0.030	0.030	0.070

¹All data represents the mean of 10 sample birds per treatment.

² Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.4. The effects of *in ovo* feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) on the on relative jejunal aminopeptidase (AP) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7, and 14 days post-hatch.¹

IOF treatment ²	% of IOF		$(\Delta Ct = Ct \text{ AP gene} - Ct \text{ sample blank})$				
	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.74 ^b	1.46 ^b	1.61 ^a	1.51 ^a	1.65 ^a
HMB	0.1	0	1.70 ^b	1.41 ^c	1.74 ^a	1.49 ^a	1.70 ^a
ARG	0	0.7	1.86 ^a	1.52 ^b	1.75 ^a	1.44 ^a	1.63 ^a
HMB+ARG	0.1	0.7	1.75 ^b	1.74 ^a	1.55 ^b	1.51 ^a	1.74 ^a
Source of Variation			-----p-value-----				
ARG			0.037	0.0007	0.548	0.393	0.804
HMB			0.091	0.1116	0.336	0.374	0.248
ARG X HMB			0.403	0.0125	0.0003	0.158	0.659
SEM(36) ³			0.040	0.050	0.040	0.030	0.070

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline.

³SEM(36) = pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

Contrast between IOF of HMB with arginine versus HMB with arginine and 18% egg white protein (EW)

A one-way ANOVA was conducted to compare jejunal gene expression levels in the controls, HMB + ARG and 18% egg white protein (EW) + HMB + ARG *in ovo* treatments (Table 7.5). At 25 d of incubation, there were no significant differences in the relative jejunal Pept-1, SI and AP expression levels between the controls or HMB + ARG, EW + HMB + ARG treatment groups. Conversely, the relative jejunal SGLT-1 expression levels of poults *in ovo* fed HMB + ARG and EW + HMB + ARG were similar

with both having significantly greater relative jejunal SGLT-1 expression over the controls at 25 d of incubation ($p < 0.05$, Table 7.5).

In ovo feeding of EW + HMB + ARG or HMB + ARG did not enhance the relative jejunal Pept-1, SGLT-1, SI or AP expression levels over the controls at hatch. At 3 d post-hatch, only poult *in ovo* fed EW + HMB + ARG had significantly enhanced relative jejunal Pept-1 expression levels over the controls ($p < 0.05$, Table 7.5). The relative jejunal SGLT-1 and SI expression levels of poult *in ovo* fed HMB + ARG and EW + HMB + ARG were significantly greater than the controls at 3 d post-hatch ($p < 0.05$, Table 7.5). There were no significant differences in the relative jejunal AP expression between the controls, HMB + ARG and EW + HMB + ARG *in ovo* treatments at 3 d post-hatch. At 7 d post-hatch, poult *in ovo* fed HMB + ARG and EW + HMB + ARG did not have significantly greater jejunal Pept-1, SGLT-1, SI and AP gene expression levels in comparison to the controls. The relative jejunal SGLT-1 and SI expression levels of poult *in ovo* fed EW + HMB + ARG was significantly greater than the relative jejunal SGLT-1 and SI expression levels of the controls at 14 d post-hatch ($p < 0.05$, Table 7.5). Conversely, the relative jejunal AP expression levels of the controls and poult *in ovo* fed EW + HMB + ARG were similar, with both treatments having relative jejunal AP expression levels that were significantly greater than the controls at 14 d post-hatch ($p < 0.05$, Table 7.5). At 14 d post-hatch, there were no significant differences in the relative jejunal Pept-1 gene expression levels between the treatments (controls, HMB + ARG, EW + HMB + ARG).

TABLE 7.5. The contrast of in ovo feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) and protein on the relative jejunal gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch.¹

	SGLT-1				
	(ΔCt= Ct SGLT-1 gene-Ct sample blank)				
IOF treatment²	25E	Hatch	Day 3	Day 7	Day 14
Control	1.41 ^a	1.13 ^b	1.25 ^a	0.981 ^b	1.15 ^b
HMB+ARG	1.37 ^{ab}	1.36 ^a	1.18 ^a	1.02 ^a	1.33 ^a
EW+HMB+ARG	1.29 ^b	1.34 ^a	1.15 ^a	1.02 ^a	1.28 ^a
p-value	0.099	0.0001	0.231	0.089	0.027
SEM(27) ³	0.030	0.040	0.040	0.020	0.050
	PEPT-1				
	(ΔCt= Ct Pept-1 gene-Ct sample blank)				
Control	1.39 ^a	1.22 ^b	1.99 ^a	1.11 ^b	1.39 ^a
HMB+ARG	1.47 ^a	1.42 ^a	1.88 ^{ab}	1.16 ^a	1.47 ^a
EW+HMB+ARG	1.38 ^a	1.35 ^{ab}	1.80 ^b	1.14 ^{ab}	1.28 ^a
p-value	0.212	0.0278	0.072	0.050	0.928
SEM(27) ³	0.040	0.050	0.050	0.020	0.050
	SI				
	(ΔCt= Ct SI gene-Ct sample blank)				
Control	1.56 ^a	1.52 ^b	1.76 ^a	1.35 ^b	1.76 ^a
HMB+ARG	1.59 ^a	2.04 ^a	1.45 ^b	1.49 ^a	1.75 ^a
EW+HMB+ARG	1.50 ^a	2.00 ^a	1.44 ^b	1.50 ^a	1.53 ^b
p-value	0.249	<0.0001	<0.0001	<0.0001	0.018
SEM(27) ³	0.030	0.040	0.030	0.030	0.060
	AP				
	(ΔCt= Ct AP gene-Ct sample blank)				
Control	1.74 ^a	1.46 ^b	1.61 ^a	1.51 ^a	1.66 ^a
HMB+ARG	1.75 ^a	1.74 ^a	1.55 ^a	1.51 ^a	1.74 ^a
EW+HMB+ARG	1.71 ^a	1.73 ^a	1.51 ^a	1.53 ^a	1.61 ^a
p-value	0.744	0.0001	0.208	0.013	0.238
SEM(27) ³	0.030	0.040	0.040	0.010	0.050

¹All data represents the mean of 10 sample birds per treatment.

²Treatments= Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP).

³SEM(27) = pooled standard error of the mean with 27 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.6. *The effects of in ovo feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) and protein on the on relative ($\Delta\Delta Ct$) jejunal sucrase-isomaltase (SI) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch.¹*

IOF treatment ²	$\Delta\Delta Ct$ ³				
	25E	Hatch	Day 3	Day 7	Day 14
HMB	-1.70 ^b	-19.3 ^b	-1.99 ^b	0.77 ^a	0.62 ^{ab}
ARG	0.94 ^a	-19.4 ^b	-0.190 ^b	-0.34 ^{ab}	-1.80 ^b
HMB+ARG	0.24 ^a	-7.62 ^a	5.54 ^a	-2.15 ^b	0.48 ^{ab}
EW+HMB+ARG	0.99 ^a	-7.65 ^a	5.72 ^a	-2.25 ^b	3.10 ^a
Source of Variation	-----p-value-----				
p-value	0.0043	<0.0001	<0.0001	0.014	0.065
SEM(36) ⁴	0.600	0.600	0.600	0.700	1.20

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. Treatment EW + HMB + ARG *in ovo* feeding solution contained 18% egg white protein + .1% HMB + .7% arginine in 0.4% saline.

³ $(Ct_x - Ct_R)_{control} - (Ct_x - Ct_R)_{test}$,

⁴SEM(36)= pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.7. The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) and protein on the on relative ($\Delta\Delta Ct$) jejunal sodium glucose transporter (SGLT-1) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch.¹

IOF treatment ²	$\Delta\Delta Ct^3$				
	25E	Hatch	Dy 3	Dy 7	Dy 14
HMB	0.13 ^a	-0.01 ^b	-0.25 ^a	1.80 ^a	-0.45 ^{ab}
ARG	0.16 ^a	1.84 ^a	-0.20 ^a	0.62 ^{ab}	-0.18 ^a
HMB+ARG	1.28 ^a	-3.64 ^c	0.99 ^a	-0.76 ^b	-3.37 ^b
EW+HMB+ARG	1.96 ^a	-3.44 ^c	1.78 ^a	-0.56 ^b	-3.20 ^b
Source of Variation	-----p-value-----				
p-value	0.528	<0.0001	0.556	0.032	0.069
SEM(36) ⁴	0.800	0.600	1.20	0.700	1.10

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. Treatment EW + HMB + ARG *in ovo* feeding solution contained 18% egg white protein + .1% HMB + .7% arginine in 0.4% saline.

³ $(Ct_x - Ct_R)_{control} - (Ct_x - Ct_R)_{test}$.

⁴SEM(36)= pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.8. *The effects of in ovo feeding (IOF) of arginine and β -hydrox- β -methylbutyrate (HMB) and protein on the on relative ($\Delta\Delta Ct$) jejunal Pept-1 gene expression of turkeys at 25 days of incubation (25E), hatch, 3,7 and 14 days post-hatch.¹*

IOF treatment ²	$\Delta\Delta Ct$ ³				
	25E	Hatch	Day 3	Day 7	Day 14
HMB	-0.04 ^a	0.34 ^{ab}	-1.57 ^b	0.93 ^a	-0.91 ^a
ARG	0.47 ^a	2.22 ^a	-0.83 ^{ab}	-0.23 ^a	0.11 ^a
HMB+ARG	-0.99 ^a	-2.81 ^c	1.11 ^{ab}	-0.90 ^a	-0.70 ^a
EW+HMB+ARG	0.21 ^a	-1.81 ^{bc}	2.54 ^a	-0.50 ^a	-1.24 ^a
Source of Variation	-----p-value-----				
p-value	0.651	0.011	0.105	0.347	0.917
SEM(36) ⁴	0.800	1.10	1.20	0.700	1.40

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. Treatment EW + HMB + ARG *in ovo* feeding solution contained 18% egg white protein + .1% HMB + .7% arginine in 0.4% saline.

³ $(Ct_x - Ct_R)_{control} - (Ct_x - Ct_R)_{test}$,

⁴SEM(36)= pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

TABLE 7.9. The effects of *in ovo* feeding (IOF) of arginine and β -hydroxy- β -methylbutyrate (HMB) and protein on the on relative ($\Delta\Delta Ct$) jejunal aminopeptidase (AP) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch.¹

IOF treatment ²	$\Delta\Delta Ct^3$				
	25E	Hatch	Day 3	Day 7	Day 14
HMB	-0.84 ^a	-0.58 ^a	-1.33 ^b	1.23 ^a	0.18 ^a
ARG	0.43 ^a	0.43 ^a	-1.24 ^b	0.49 ^a	0.19 ^a
HMB+ARG	0.32 ^a	-3.57 ^b	0.64 ^{ab}	0.83 ^a	-1.52 ^a
EW+HMB+ARG	0.92 ^a	-3.60 ^b	1.43 ^a	0.52 ^a	-0.73 ^a
Source of Variation	-----p-value-----				
p-value	0.415	0.017	0.119	0.616	0.598
SEM(36) ⁴	0.700	1.10	0.900	0.400	1.00

¹All data represents the mean of 10 sample birds per treatment.

²Treatment HMB *in ovo* feeding solution contained 0.1% in .4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in .4% saline. Treatment HMB + ARG *in ovo* feeding solution contained .1% HMB + 0.7% arginine in .4% saline. Treatment EW + HMB + ARG *in ovo* feeding solution contained 18% egg white protein + .1% HMB + .7% arginine in 0.4% saline.

³ $(Ct_x - Ct_R)_{control} - (Ct_x - Ct_R)_{test}$,

⁴SEM(36)= pooled standard error of the mean with 36 degrees of freedom.

^{a,b}Means within a column with different superscripts are significantly different (P<.05).

At 25 d of incubation, all *in ovo* fed poults (except the HMB *in ovo* fed poults) had enhanced relative ($\Delta\Delta Ct$) jejunal SI gene expression over the experimental and internal controls (Table 7.6). At hatch, all *in ovo* fed poults had depressed relative ($\Delta\Delta Ct$) jejunal SI, expression in comparison to the experimental and internal controls (Table 7.6). Poults *in ovo* fed either HMB + ARG or EW + HMB + ARG had enhanced relative ($\Delta\Delta Ct$) jejunal SI expression levels over the experimental and internal controls at 3 d and 14 d post-hatch (Table 7.6). At one week post-hatch, only poults *in ovo* fed HMB had enhanced relative ($\Delta\Delta Ct$) jejunal SI expression levels over the controls (Table 7.6). The

relative ($\Delta\Delta\text{Ct}$) jejunal SI expression levels were improved in poults *in ovo* fed HMB, HMB + ARG, and EW + HMB + ARG over the controls at 14 d post-hatch, while poults *in ovo* fed ARG had depressed relative ($\Delta\Delta\text{Ct}$) jejunal SI levels below the controls (Table 7.6).

At 25 d of incubation, all *in ovo* fed poults had enhanced relative ($\Delta\Delta\text{Ct}$) jejunal SGLT-1 gene expression levels above the experimental and internal controls (Table 7.7). By the time of hatch, only poults *in ovo* fed ARG had enhanced relative ($\Delta\Delta\text{Ct}$) jejunal SGLT-1 expression levels above the experimental and internal controls (Table 7.7). At 3 d posthatch, poults *in ovo* fed HMB + ARG and EW + HMB + ARG had increased relative ($\Delta\Delta\text{Ct}$) jejunal SGLT-1 expression levels above the experimental and internal controls (Table 7.7). Conversely, poults *in ovo* fed HMB and ARG alone had greater relative ($\Delta\Delta\text{Ct}$) jejunal SGLT-1 expression levels than the experimental and internal controls at 7 d post-hatch. Relative jejunal SGLT-1 gene expression levels were depressed below the controls at 14 d post-hatch in all *in ovo* fed poults (Table 7.7).

In ovo feeding of ARG enhanced the relative ($\Delta\Delta\text{Ct}$) jejunal Pept-1 gene expression levels above the controls at 25 d of incubation, hatch and 14 d post-hatch (Table 7.8). Conversely, ARG *in ovo* fed poults had depressed relative ($\Delta\Delta\text{Ct}$) jejunal Pept-1 gene expression below the controls at 3 d and 7 d post-hatch (Table 7.8). *In ovo* feeding of HMB enhanced jejunal Pept-1 gene expression levels above the controls only at hatch and 7 d post-hatch. *In ovo* feeding a combination of HMB + ARG only enhanced the relative ($\Delta\Delta\text{Ct}$) jejunal Pept-1 gene expression levels above the controls at 3 d post-hatch, while *in ovo* feeding of EW + HMB + ARG enhanced the relative ($\Delta\Delta\text{Ct}$)

jejunal Pept-1 gene expression levels above the controls at 25 d of incubation and 3 d post-hatch (Table 7.8).

Poult *in ovo* fed HMB alone had enhanced relative ($\Delta\Delta\text{Ct}$) jejunal AP gene expression above the controls at 7 d and 14 d post-hatch (Table 7.9). *In ovo* feeding of ARG alone enhanced the relative ($\Delta\Delta\text{Ct}$) jejunal AP gene expression levels above the controls at all time points measured; with the exception of 3 d post-hatch (Table 7.9). *In ovo* feeding of HMB + ARG and EW + HMB + ARG enhanced the relative ($\Delta\Delta\text{Ct}$) jejunal AP gene expression above the controls at all of the time points measured with the exception of hatch and 14 d post-hatch (Table 7.9).

DISCUSSION

Additional analysis was completed to contrast the effects of the inclusion of egg white protein to the HMB + ARG IOF solution with the controls and the HMB + ARG treatment. Inclusion of egg white protein in the HMB + ARG IOF solution enhanced the relative jejunal SGLT-1 gene expression levels at over the controls at 25 days of incubation, the relative jejunal Pept-1 and SGLT-1 gene expression levels over the controls at 3 d post-hatch, and the relative jejunal SGLT-1 and SI gene expression levels over the controls at 14 d post-hatch (Table 7.4). While, inclusion of egg white protein enhanced the relative jejunal gene expression of the genes of interest above the controls, the relative jejunal gene expression levels of IOF treatments HMB + ARG and HMB + ARG + egg white protein were similar at all time points measured. Thus, the inclusion

egg white protein to the IOF solution did not additionally enhance jejunal gene expression levels.

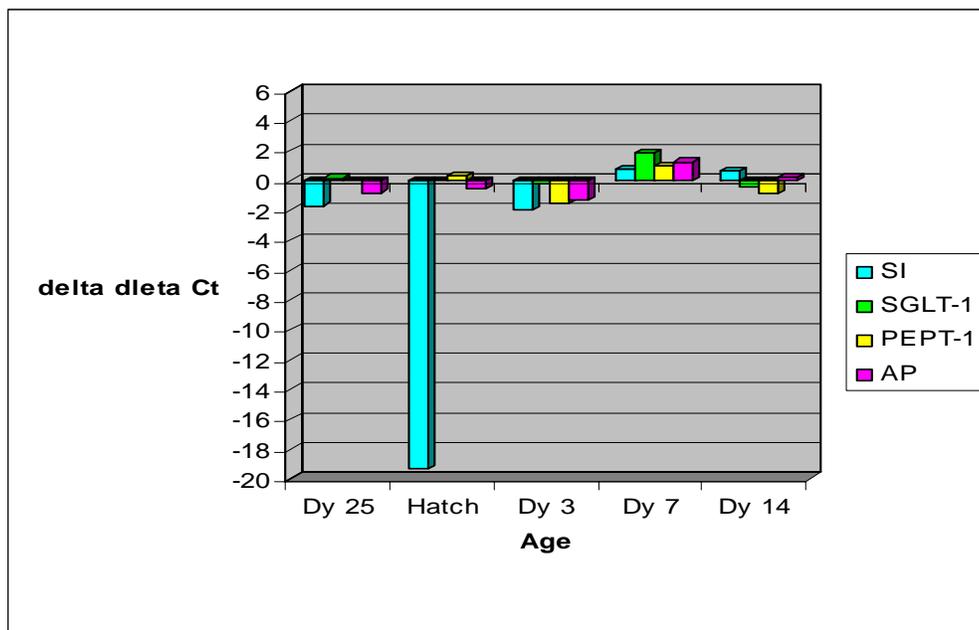
Relative jejunal expression (ΔCt) was normalized with the β -actin expression levels. β -actin was chosen as the housekeeping gene for all experimental analysis, based upon the assumption that β -actin is equally expressed in all tissues consistently throughout development. Conversely, recent literature has indicated that most housekeeping genes, such as β -actin and GAPDH, do not behave perfectly as an internal control and that their expression levels may fluctuate with experimental treatment. In this experiment, β -actin expression levels changed upon treatment (data not shown) with treatment of the IOF solutions at the time points measured and may have produced an artifact in the relative jejunal expression levels of the genes of interest. Additionally, the biological expression levels (ΔCt) are interpreted as two fold the ΔCt values. Thus, while there were numerical statistical differences in the relative jejunal gene expression levels between the treatments and controls, there were no biological differences in the jejunal gene expression for the genes of interest at the time points measured.

Subsequently, the relative gene expression was calculated as the $\Delta\Delta Ct$, in which the relative intestinal gene expression levels of the experimental samples were normalized using the expression levels of the experimental controls and the housekeeping gene β -actin (Figures 7.1, 7.2, 7.3, and 7.4, 7.5, 7.6, 7.7, 7.8). This provided better visualization of the up-regulation or down-regulation of the genes of interest relative to the experimental and internal controls. The internal controls β -actin levels and the experimental non-injected controls were the baseline and the values above or below

the baseline value (zero) was interpreted as enhanced or suppressed gene expression, respectively. The data were represented graphically by each *in ovo* feeding treatment (Figures 7.1, 7.2, 7.3, and 7.4) and by gene of interest (Figure 7.5, 7.6, 7.7, 7.8).

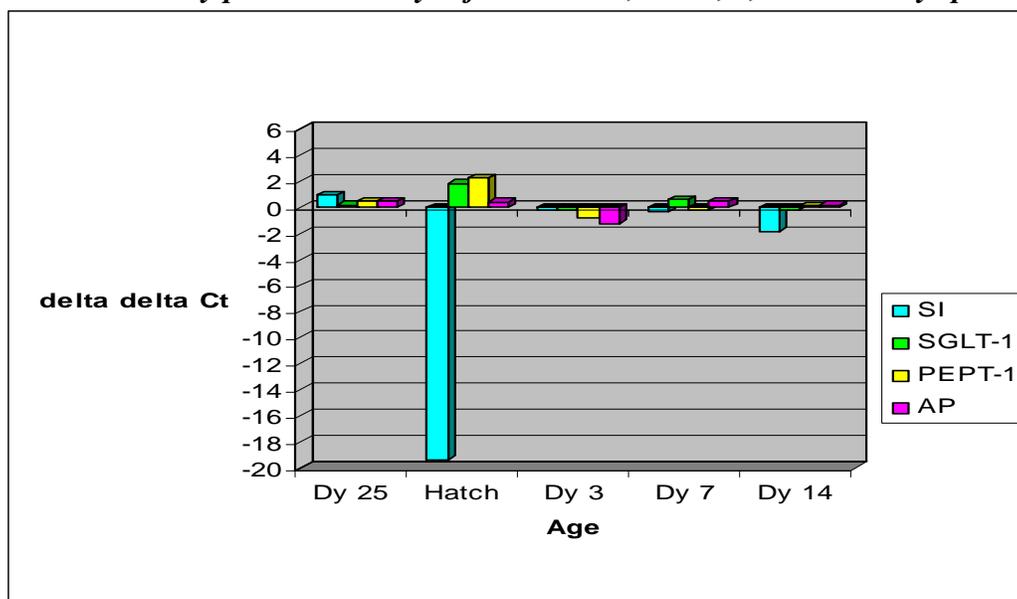
Comparison of each *in ovo* feeding treatment independently revealed that IOF of ARG alone enhanced the relative expression of all genes of interest with the exception of SI over the controls at hatch and 7 days post-hatch, and Pept-1 at 7 days post-hatch (Figure 7.2). IOF of HMB + ARG (Figure 7.3) and HMB + ARG + egg white protein (Figure 7.4) enhanced the relative gene expression of all genes of interest above the controls at 25E and 3 d post-hatch, with the exception of Pept-1 at 25 E. Also, IOF of HMB + ARG (Figure 7.3) and EW + HMB + ARG (Figure 7.4) enhanced relative jejunal AP expression levels at 7 days post-hatch and SI expression levels at 14 days post-hatch above the controls. IOF HMB alone only enhanced the relative gene expression of the genes of interest above the controls at 7 days post-hatch and only SI expression levels at 14 days post-hatch (Figure 7.1). IOF of ARG alone (Figure 7.2) enhanced all genes of interest at 25E and hatch (with the exception of SI at hatch), and SGLT-1 and AP expression levels at 7 days post-hatch.

FIGURE 7.1. *The effects of IOF of 0.1% HMB on the relative ($\Delta\Delta C_t$) jejunal gene expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days post-hatch¹.*



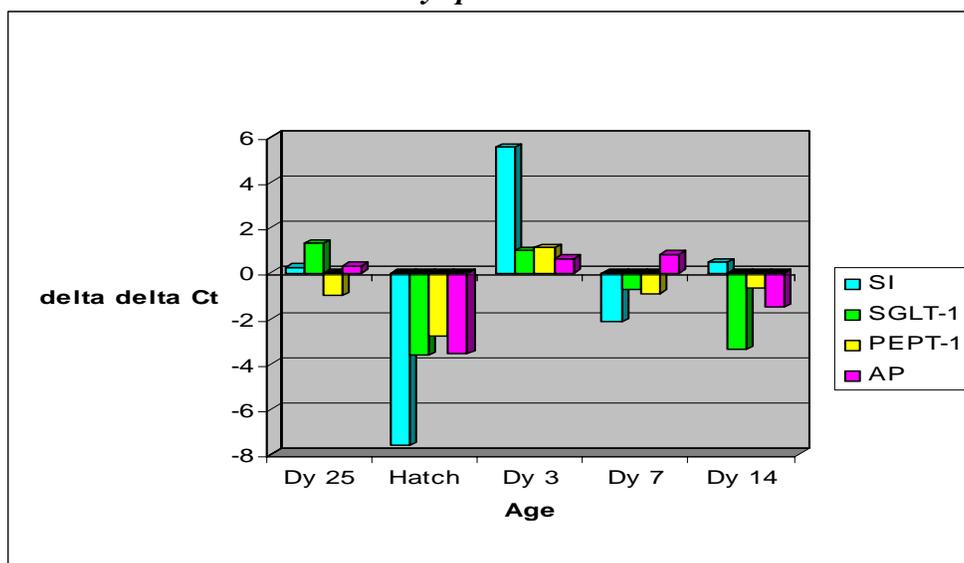
¹All data represents the mean value of 10 samples. Genes of Interest include SI=Sucrase-isomaltase, SGLT-1=Sodium Glucose Transporter, PEPT-1= Peptide Transporter, AP=Aminopeptidase

FIGURE 7.2. *The Effects of IOF of 0.7% arginine on relative ($\Delta\Delta C_t$) jejunal gene expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹*



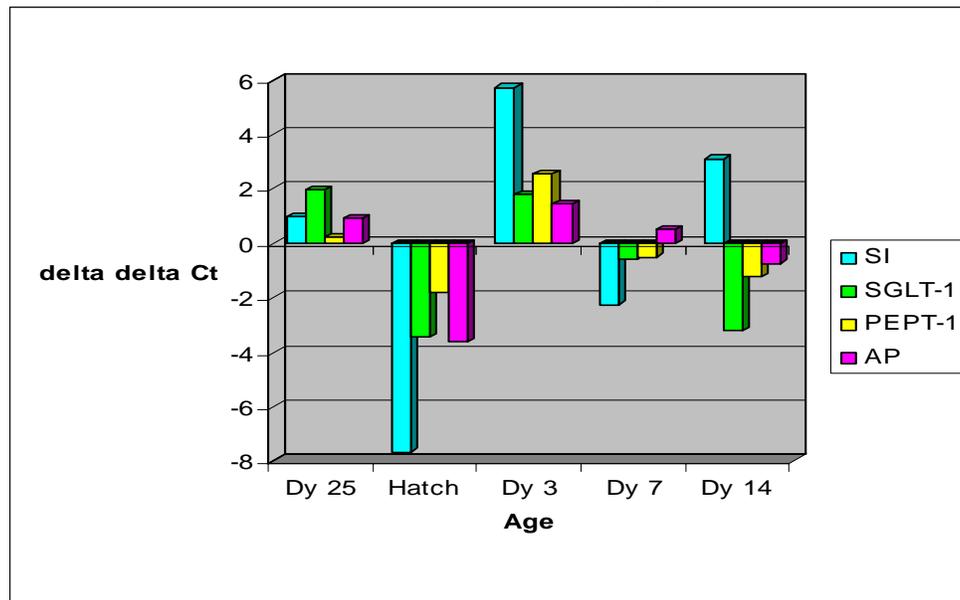
¹All data represents the mean value of 10 samples. Genes of Interest include SI=Sucrase-isomaltase, SGLT-1=Sodium Glucose Transporter, PEPT-1= Peptide Transporter, AP=Amino-peptidase

FIGURE 7.3. *The Effects of IOF of 0.1% HMB and 0.7% arginine on relative ($\Delta\Delta C_t$) jejunal gene expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹*



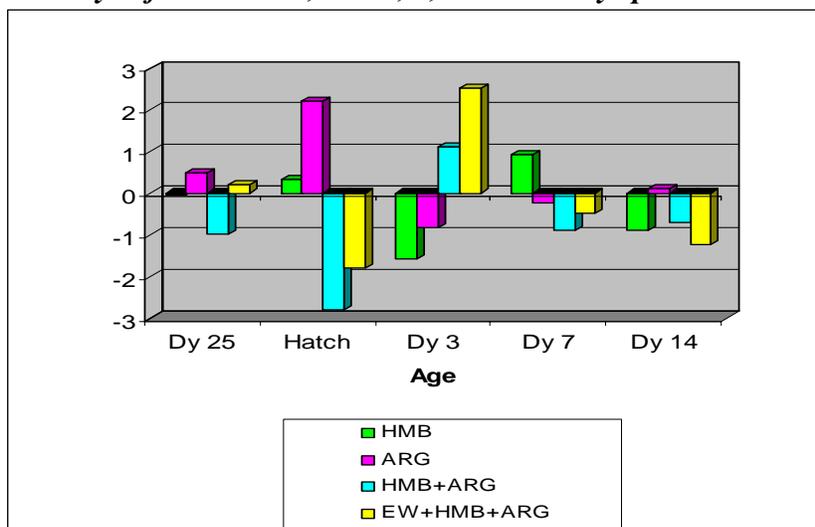
¹All data represents the mean value of 10 samples Genes of Interest include SI=Sucrase-isomaltase, SGLT-1=Sodium Glucose Transporter, PEPT-1= Peptide Transporter, AP=Aminopeptidase

FIGURE 7.4 The Effects of IOF of 0.1% HMB and 0.7% arginine and 18% egg white protein on relative ($\Delta\Delta C_t$) jejunal gene expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹



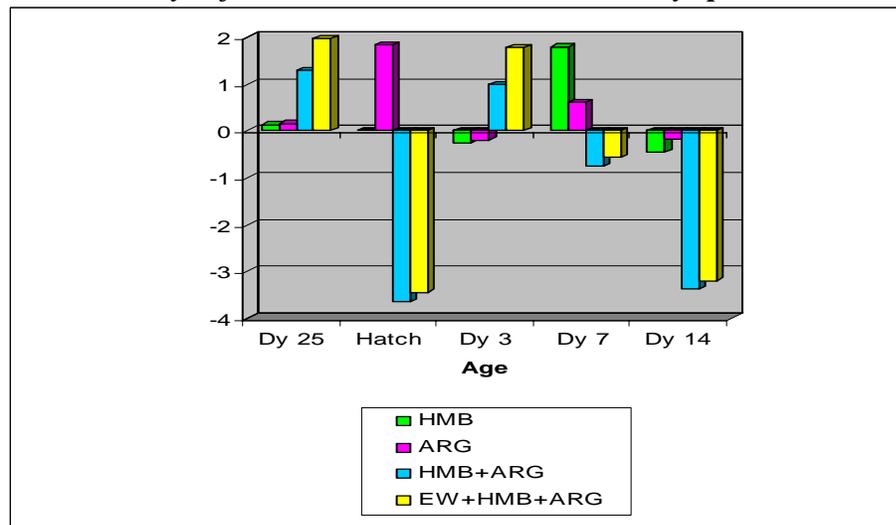
¹All data represents the mean value of 10 samples. Genes of Interest include SI=Sucrase-isomaltase, SGLT-1=Sodium Glucose Transporter, PEPT-1= Peptide Transporter, AP=Aminopeptidase

FIGURE 7.5 The Effects of IOF arginine and/or HMB with egg white protein on relative ($\Delta\Delta C_t$) peptide transporter (*Pept-1*) jejunal expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹



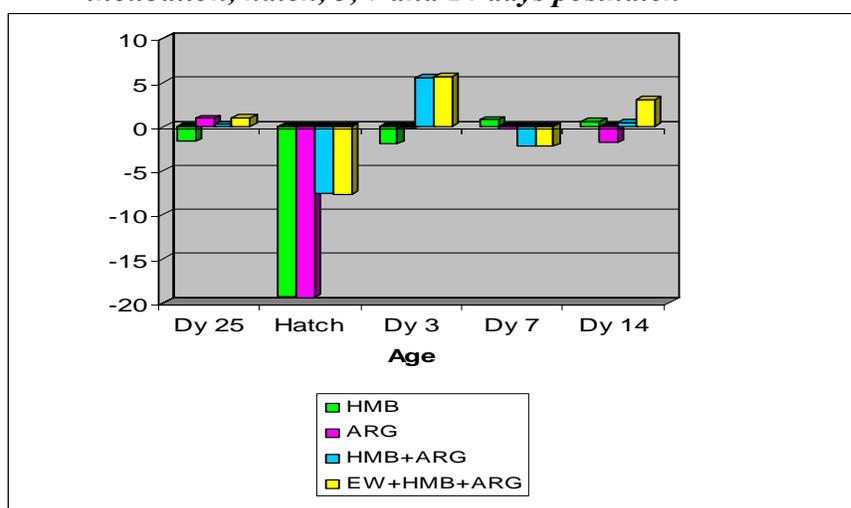
¹All data represents the mean value of 10 samples. Treatments HMB=0.1% HMB in 0.4% saline, ARG=0.7% arginine in 0.4% saline, HMB + ARG=0.1% HMB & 0.7% arginine in 0.4% saline, EW + HMB + ARG= 18% egg white protein & 0.1% HMB + 0.7% arginine in 0.4% saline

FIGURE 7.6. The Effects of IOF arginine and/or HMB with egg white protein on relative ($\Delta\Delta C_t$) sodium glucose transporter (SGLT-1) jejunal expression in turkey poult at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹



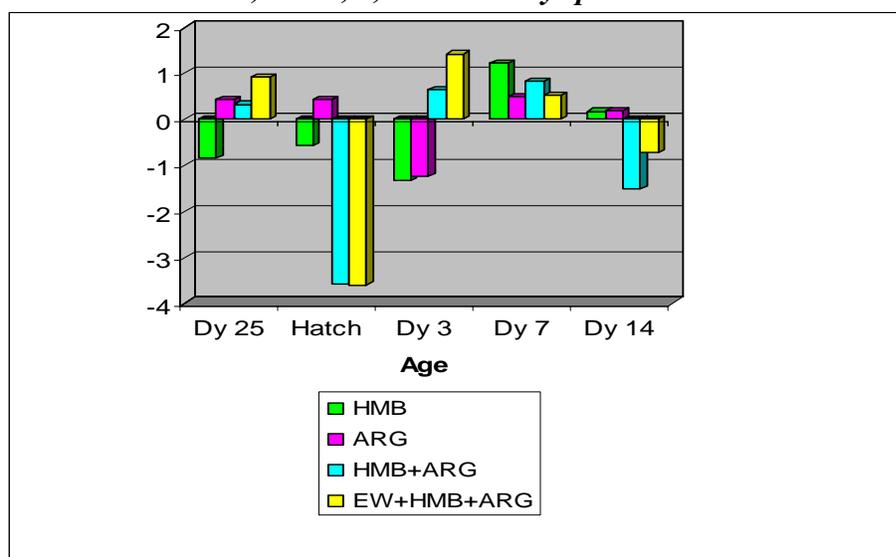
¹All data represents the mean value of 10 samples. Treatments HMB=0.1% HMB in 0.4% saline, ARG=0.7% arginine in 0.4% saline, HMB + ARG=0.1% HMB & 0.7% arginine in 0.4% saline, EW + HMB + ARG= 18% egg white protein & 0.1% HMB + 0.7% arginine in 0.4% saline

FIGURE 7.7. The Effects of IOF arginine and/or HMB with egg white protein on relative ($\Delta\Delta C$) sucrase-isomaltase (SI) jejunal expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹



¹All data represents the mean value of 10 samples. Treatments HMB=0.1% HMB in 0.4% saline, ARG=0.7% arginine in 0.4% saline, HMB + ARG=0.1% HMB & 0.7% arginine in 0.4% saline, EW + HMB + ARG= 18% egg white protein & 0.1% HMB + 0.7% arginine in 0.4% saline

FIGURE 7.8. *The Effects of IOF arginine and/or HMB with egg white protein on relative ($\Delta\Delta C$) aminopeptidase (AP) jejunal expression in turkey poults at 25 days of incubation, hatch, 3, 7 and 14 days posthatch¹*



¹All data represents the mean value of 10 samples. Treatments HMB=0.1% HMB in 0.4% saline, ARG=0.7% arginine in 0.4% saline, HMB + ARG=0.1% HMB & 0.7% arginine in 0.4% saline, EW + HMB + ARG= 18% egg white protein & 0.1% HMB + 0.7% arginine in 0.4% saline

Subsequently, each gene of interest was graphically represented for better visualization of how each gene responded to each IOF treatment relative to the controls and age of the bird. IOF of EW + HMB + ARG and ARG alone greatly enhanced Pept-1 (Figure 7.5), SGLT-1 (Figure 7.6), SI (Figure 7.7) and AP (Figure 7.8) expression levels at 25E, with the exception of IOF of ARG on SGLT-1 expression levels. Also, IOF of HMB + ARG and EW + HMB + ARG greatly enhanced the relative jejunal Pept-1, SGLT-1, SI and AP expression levels at 3 days post-hatch. The relative expression

($\Delta\Delta C_t$) of the genes of interest (Figure 7.5, 7.6, 7.7, 7.8) revealed that jejunal Pept-1 (7.5) and SI (Figure 7.7) expression levels were enhanced relative to the controls by IOF of EW + HMB + ARG at 25E and 3 days post-hatch, IOF of ARG at 25E and hatch for Pept-1. Also, IOF of HMB enhanced relative jejunal Pept-1 and SI levels greater than the controls at hatch and 7 days post-hatch. Relative expression ($\Delta\Delta C_t$) of SGLT-1 (Figure 7.6) was enhanced relative to the controls by IOF of HMB + ARG and EW + HMB + ARG at 25E and 3 days post-hatch, by IOF of ARG at hatch and 7 days post-hatch and by IOF of HMB at 7 days post-hatch. All of the IOF treatments enhanced the relative jejunal AP expression levels above the controls at 25E and 7 days post-hatch (Figure 7.8), with the exception of the HMB IOF treatment at 25E. At 14 days post-hatch, the relative jejunal Pept-1 (Figure 7.5) and SGLT-1 (Figure 7.6) expression levels were depressed below the controls by all IOF treatments. Conversely, all IOF treatments except ARG enhanced relative SI jejunal expression levels above the controls at 14 days post-hatch (Figure 7.7), while IOF of HMB and ARG enhanced relative jejunal AP expression levels above the controls at 14 days post-hatch (Figure 7.8).

Our experimental data imply that in IOF of ARG and/or HMB with protein may enhance expression of the digestion/absorption related genes through two weeks post-hatch. These data also imply that the SGLT-1, SI, AP and Pept-1 jejunal genes respond independently to *IOF* and not in concert. The improvements in the relative gene expression were intermittent between hatch and 14 d post-hatch. Thus, additional studies must be conducted to determine the specific regulatory mechanisms involved in the regulation of the digestion/absorption related genes of the intestine.

The intestinal epithelial tissue carrying out absorption is in a continuous state of renewal (Uni et al., 1998, 2000, 2003a). Enterocytes produced in the intestinal crypt migrate to the tips of the villi where they are shed into the intestinal lumen (Cheng and Leblond, 1974, Uni et al., 2000), leading to complete cell renewal every 2-3 days in rodents (Ferraris and Diamond, 1993), 3-4 days in ovine (Attaix and Meslin, 1991), 5-6 days in human (Traber, 1990), and 2-3 days in chicken (Uni et al., 2003). During this migration process the enterocytes acquire differentiated functions specifically needed for digestion, absorption and mucin secretion (Imondi et al., 1969; Ferraris et al., 1992b; Weiser 1973; Traber et al, 1992; Meddings et al., 1990; Thomson et al., 1994; Uni et al., 2000, 2003; Geyra et al., 2001).

Intestinal genes are specifically expressed to produce the proteins of the brush border membrane that digest and absorb the diverse nutrient molecules of the diet (Ferraris, 2001). Intestinal gene expression may be dependent upon age (developmental stage) and the luminal presence of nutrient molecules. Intestinal phenotype is determined by pre-wired developmental patterns, which predict the appearance of specific sets of digestive and absorptive proteins within the gut correlative with age (Traber, 1997).

The adult intestinal phenotype is established following a series of developmental transitions defined by gene expression of various specific sets of genes in each cell (Traber, 1997). Many of the epithelial genes of the intestine express preprogrammed timetables, with their expression levels being influenced by dietary components within the lumen (Traber, 1997). A change in secondary phenotype in response to dietary stimulus is advantageous due to increased nutrient absorption and digestion, which may

be correlated to increased expression of the absorptive and digestive related intestinal genes. Increased nutrient absorption and digestion provides the energy and nutrients needed to meet the metabolic needs and to fuel more rapid subsequent growth.

IOF may serve as a tool to enhance the expression of the digestion/absorption-related intestinal genes during embryonic development. Therefore, young hatchlings have an increased capacity to digest and absorb incoming nutrients from an external diet, which may persist through two weeks post-hatch.

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Chapter 8

Dissertation Summary

“The effects of in ovo feeding on glycogen status, intestinal development and function and growth in turkey poults”

Glycogen Status and Growth

The liver and muscles are the primary storage sites for glycogen. Upon consumption of carbohydrates insulin facilitates the uptake of liberated glucose into the muscles. Once within the muscles or liver, glucose is either utilized for energy or stored as a polymer, glycogen. Muscles may store up to $\frac{3}{4}$ of the body's glycogen reserves due to its predominance in the body relative to the liver.

Unlike the liver, skeletal muscle requires the action of insulin for the uptake of glucose from the blood. Insulin release occurs with the consumption of arginine- or carbohydrate-rich meals and the resultant rise in blood sugar. Our studies demonstrate that muscle glycogen was not enhanced by *in ovo* feeding of protein, but it was enhanced by *in ovo* feeding of carbohydrates (Figure 8.1). *In ovo* feeding of carbohydrates most likely resulted in the release of insulin and the uptake and storage of glucose in the form of glycogen in the muscles (Figure 8.1). In contrast to liver, skeletal muscle lacks the gluconeogenic enzymes needed for the conversion of proteins and amino acids into glucose and therefore would have been unable to use protein fed *in ovo* for enhancement of muscle glycogen stores (Figure 8.1). In these studies *in ovo* feeding of dietary protein targeted hepatic glycogen enhancement by taking advantage of high hepatic

gluconeogenic rates in the avian neonate; whereas *in ovo* feeding of dietary sugars enhanced muscle glycogen. Thus overall body glycogen status measured as glycogen index ((total muscle glycogen + total liver glycogen)/ bodyweight) was most improved by *in ovo* feeding of carbohydrate due to the action of insulin which targeted the muscles for glycogen deposition. An enhancement of liver or muscle glycogen ultimately improves bodyweights by fueling more rapid subsequent post-hatch growth. Bodyweights were improved by *in ovo* feeding of HMB, protein and carbohydrates at hatch and one-week post-hatch.

In our first experiment (Chapter 2) *in ovo* feeding of HMB enhanced total muscle glycogen reserves at hatch and 7 d post-hatch. While the mechanism of action of HMB have not been identified, these data suggest that *in ovo* feeding of HMB stimulated the release of insulin, which may have resulted in increased uptake of endogenously produced glucose from the liver and the formation of glycogen within the muscles. Hence, energy (glycogen) is re-partitioned from the liver to muscles stores; suggesting that HMB had an indirect, hormone-mediated affect on total muscle glycogen. Thus, HMB may inhibit hepatic gluconeogenesis indirectly due to the action of insulin. From our initial findings in experiment one (Chapter 2), we hypothesized that *in ovo* feeding of insulin secretagogues, such as carbohydrates, HMB and arginine (ARG), would enhance muscle glycogen and glycogen index due to the action of insulin. Contrary to our previous findings, *in ovo* feeding of HMB did not significantly enhance muscle glycogen stores greater than the controls. *In ovo* feeding of HMB and/or ARG with or without protein enhanced hepatic glycogen reserves at hatch and was absent at all time points

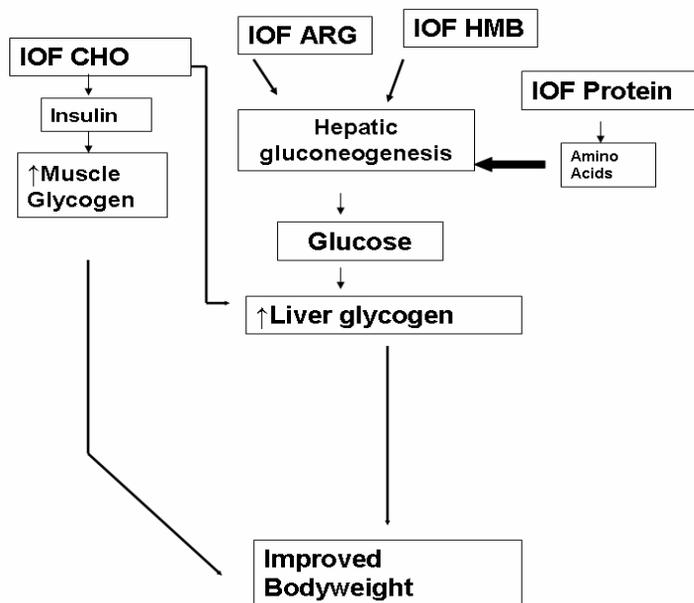
measured, while muscle glycogen reserves were not enhanced by *in ovo* feeding of ARG and/or HMB with or without protein at any of the time points measured. Also, in experiment two, poult *in ovo* fed HMB and ARG had glycogen indices that were numerically greater than but were not statistically greater than the controls. Nonetheless, marginal enhancement of glycogen reserves may have been correlated to improved weight gains seen through the first two weeks of post-hatch life. Weight gains were consistent in experiments one and two. *In ovo* feeding of proteins, carbohydrates, HMB or ARG improved the body weights of poult during the early post-hatch period (Figure 8.1). These results parallel the findings of Uni and Ferket (2003), who observed turkey poult *in ovo* fed 2mL of 24% egg white protein had a 4-8% increase in bodyweights through 24 days post-hatch over the controls (Patent # US 6,592,878 B2).

Several studies have demonstrated the importance of gluconeogenesis in carbohydrate metabolism and survival of the avian embryo and neonate (Donaldson et al, 1992, 1993; Christensen et. al., 1992, 2000, 2001, 2003). Hepatic gluconeogenesis is the primary mechanism responsible for glucose production in the avian embryo and neonate (Romanoff, 1967). In experiment one (Chapter 2), *in ovo* feeding of egg white protein enhanced hepatic glycogen reserves (Figure 8.1). Thus, suggesting that *in ovo* feeding of gluconeogenic precursors, such as proteins and/or amino acids or metabolites would enhance the hepatic activity of the gluconeogenic enzymes, such as glucose-6-phosphatase an indicator of hepatic gluconeogenic activity. Our initial hypothesis was validated by our second *in ovo* feeding trial in which poult were fed *in ovo* HMB and/or ARG with or without protein had much greater hepatic glucose-6-phosphatase activity

than the controls at hatch. This observation agrees with numerous studies demonstrating high gluconeogenic activity at hatch (Okuno et al., 1964; Felicioli et al., 1967; Sheid and Hirschberg, 1967). This effect was lost by one-week post-hatch, which parallels with previous reports that have demonstrated a metabolic shift in energy metabolism at one-week post-hatch. After poults have consumed feed for about 1 week, they no longer rely upon gluconeogenesis as the primary source of glucose (Romanoff, 1967; Donaldson et al., 1992, 1993; Christensen et al., 1992, 2000, 2001, 2003).

HMB, a leucine metabolite has been demonstrated to have physiological benefits in steers (Van Koeveering et al., (1994), broilers (Nissen et al., 1994; Fuller et al., 1994; Uni and Ferket, 2004; Tako et al 2004) and humans (Flakoll et al., 2004). Our results did not parallel these observations. While the percent breast yields were numerically improved by *in ovo* feeding of HMB, there was no statistical difference from controls at 7 days post-hatch in experiment one.

Figure 8.1. *The summation of the effects of in ovo feeding (IOF) of protein, β -hydroxy- β -methylbutyrate (HMB), arginine (ARG) and carbohydrates (CHO) on glycogen status and growth in turkey poults in experiments one and two.*



Jejunal Function and Expression

Adaptation and improved digestive and absorptive function of the GI tract is imperative for improving the early survival of hatchlings. After hatch, the first meal is critical for the subsequent growth of the chicks (Noy and Sklan, 1998, 1999; Uni et al, 1998). Under commercial hatchery conditions, turkey embryos hatch over a 36-hour window and then the hatchlings are transported from the hatchery to the rearing site that may take an additional 24 hours or more. As a consequence, some birds may have access

to feed and initiate feed intake over 50 hours after hatching (Moran and Reinhart, 1980). This protracted delay in feed intake initiation can adversely affect early growth (Misra, 1978; Hager and Beane, 1983; Wyatt et al, 1985; Nir and Levanon et al., 1993; Noy et al., 2001a), muscle development (Mozdziak et al., 1997, 2002a-c; Halevy et al., 2000), and enteric development (Geyra et al., 2001a, 2002) and adaptation. The first meal stimulates the intestinal gene expression and activity of the nutrient transporters and brush border digestive enzymes (Geyra et al. 2001a, 2002).

In ovo feeding may circumvent this problem of early inanition by introducing exogenous nutrients into the amnion of the developing late term embryo. The embryo orally consumes the amnion and nutrients prior to pipping. These nutrients are presented to the enteric tissues and may stimulate gene expression and the activity of the digestive brush border enzymes and nutrient transporters (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Therefore the neonate hatches with a greater capacity to digest and absorb ingested nutrients consumed during the neonatal period. Previous experimentation by Tako et al. (2004) and Uni and Ferket (2004) demonstrated that HMB may serve as an enteric modulator in which enhances the absorptive surface area and activity of the brush border enzyme, sucrase-isomaltase of the intestinal mucosa. Additionally, numerous studies have demonstrated that dietary carbohydrates and proteins enhance the activity and expression of their digestion/absorption related proteins in the intestine (Diamond et al., 1984; Diamond and Karasov, 1987; Karasov and Debnam, 1987; Ferraris and Diamond, 1997; Ferraris et al., 1992a; Ferraris et al., 1992b). Therefore, we hypothesized that *in ovo* feeding of HMB and other potential enteric

modulators and dietary nutrients (carbohydrates, proteins) would enhance enzymatic activity and expression of the jejunal brush border membrane enzymes and nutrient transporters (Figure 8.2).

We demonstrated that *in ovo* feeding of carbohydrates enhanced the activity of the brush border enzymes sucrase-isomaltase (SI) and leucine aminopeptidase (LAP) at 7 days post-hatch (Chapter 4). Also, in a separate experiment, we demonstrated that *in ovo* feeding of egg white protein with or without protein enhanced jejunal leucine aminopeptidase activity at hatch, while *in ovo* feeding of egg white protein + HMB enhanced jejunal glucose transport activity at 25E and hatch (Chapter 5). Generally, *in ovo* feeding ARG + HMB or ARG alone, greatly enhanced jejunal brush border activity of LAP, sucrase, and maltase at hatch, 3 days and 14 days post-hatch (Chapter 4). Additionally, the carbohydrate related digestion/absorption related genes, SGLT-1 (sodium glucose co-transporter), SI and the protein related digestion/absorption related genes jejunal Pept-1 (peptide transporter), AP (aminopeptidase) were up-regulated relative to the controls by *in ovo* feeding of egg white protein + HMB + ARG, HMB + ARG, and ARG alone, particularly at 25E and 3 days post-hatch (Chapter 7). Thus, we validated our initial hypothesis by demonstrating that *in ovo* feeding of HMB, ARG and protein enhances jejunal brush border membrane enzymatic activity (Figure 8.2).

In ovo feeding of HMB and ARG expedites GIT maturation before hatch; thus chicks and poults hatch with a more mature gut, which may prevent post-hatch mal-absorption of nutrients by causing the compensatory changes conducive to an external diet to occur prior to hatching. Consequently, the expression and function of digestion

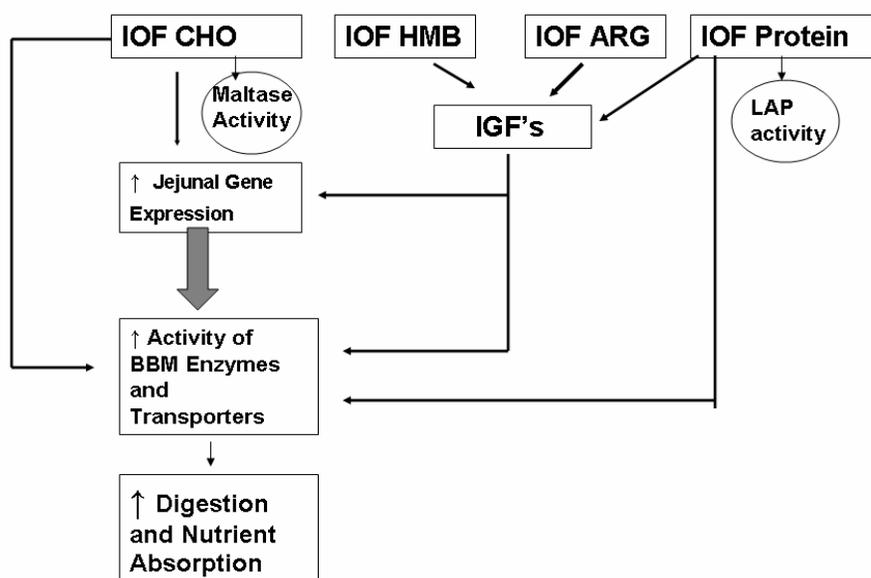
and nutrient absorption at hatch are enhanced such that dietary carbohydrates and protein consumed by the neonatal poult is better utilized. As one of the primary supply organs, optimal functioning of the gastrointestinal tract (GIT) is critical for post-hatch survival. Incoming nutrients are utilized to meet the metabolic requirements needed for rapid growth and development in young poult. Additionally, young poult are transitioning from a lipid-based *in ovo* diet to a carbohydrate-based external diet during the early post-hatch period.

Intestinal development and adaptation by age is has been shown to be modulated by anabolic hormones (Lane et al. 2002; Roffler et al, 2003). Food deprivation decreases plasma insulin-like growth factors (IGF-I), while the introduction of food increases plasma IGF-I. IGF's enhance cellular development, muscle deposition, and intestinal development, and stimulates metabolism (Lane et al. 2002; Roffler et al, 2003). Mammalian milk contains the peptides IGF-I and IGF-II, which affect multiple developmental processes, including jejunal glucose uptake (Lane et al., 2002). Studies by Lane et al. (2002) have demonstrated that when rat pups were reared on a milk replacement devoid of IGF's, jejunal glucose transport activity and gene expression were severely altered. Thus *in ovo* feeding may enhance intestinal functioning of the digestive enzymes and nutrient transporters indirectly by the action of insulin like growth factors.

Based upon our findings and previous reports (Lane et al., 2002; Roffler et al., 2003) we hypothesize that *in ovo* feeding of ARG and/or HMB with or without protein stimulates the production of IGF's, which may alter the activity and expression of the brush border enzymes and nutrient transporters (Figure 8.2). Thus, *in ovo* feeding of

ARG + HMB would enhance intestinal development and somatic growth indirectly *via* IGF's and may have effects that persist during the critical post-hatch period.

Figure 8.2 The summation of the effects of *in ovo* feeding (IOF) of protein, β -hydroxy- β -methylbutyrate (HMB), arginine (ARG) and carbohydrates (CHO) on jejunal brush border and nutrient transporters activity and expression in turkey poults in experiments one and two.



Our experiments demonstrate that *in ovo* feeding of HMB + ARG significantly enhanced plasma IGF-I levels at hatch and *in ovo* feeding of egg white protein (EW) + HMB + ARG significantly enhanced plasma IGF-II levels at 7 days post-hatch in comparison to the controls (Chapter 6). These observations support our initial hypothesis that *in ovo* feeding may indirectly enhance intestinal function and development due to

enhanced plasma insulin like growth factors (Figure 8.2). Therefore *in ovo* feeding may serve as a tool to overcome the level one constraints (limited availability of food resources) level two constraints (the ability to utilize available resources), and level three constraints (re-allocation of energy to tissue maturation and compromised growth) (Ricklefs et al. 1969; 1979). Our results imply that *in ovo* feeding a) enhances and spares the body's glycogen reserves, b) enhances the development and functioning of the gut, and c) provides enough energy for optimal somatic growth and organ maturation.

Level three constraints on growth may be overcome indirectly by *in ovo* feeding. *In ovo* feeding enhances glycogen reserves and spares residual yolk and body protein (muscle) stores. Additionally, *in ovo* feeding enhances the development of the GIT and its function and capacity during embryonic and post-hatch development. Therefore, poults have an increased capacity for diet digestion and nutrient uptake, which can be used for growth and metabolism or stored as energy. Thus, *in ovo* fed hatchlings may have enough energy to support both growth and tissue and organ development, so overall growth is not compromised. Adequate energy may be allocated for the development and maturation of the GIT, immune system, muscular and skeletal systems. For example, poults may not be immuno-compromised due to an immature immune system, and they may be more resistant to microbial challenge during the early growth. Thus, "*in ovo* feeding" may improve hatchability, survivability and reduce morbidity of commercial turkeys during the early post-hatch period.

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