ABSTRACT

Title of Document: INTEGRATING MACRONUTRIENT METABOLISM IN DEVELOPING CHICKEN EMBRYOS

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The objective of this thesis research was to determine the pathways of glucose metabolism and utilization in small and large egg embryos during the latter half of development, and whether *in ovo* nutrient supplementation alters glucose use. A further objective was to determine the contribution of glutamate, glutamine and glycerol to glucose, glycogen and non essential amino acid (NEAA) synthesis during embryo development. *In ovo* stable isotope ([U-^{13}C]glucose, [U-^{13}C]glutamate, [U-^{13}C]glutamine and [U-^{13}C]glycerol) injection approaches were developed along with mass isotopomer distribution analysis of metabolic intermediates and end-products to acquire a metabolic phenotype of the fluxes and partition of these substrates through central pathways. Embryos developing in small and large eggs maintained similar rates of glucose metabolism. Thus, glucose entry and utilization gradually increased from day 12 to 18 embryonic. By embryonic day 20, gluconeogenesis accounted for >80% of glucose entry, a part (65%) of which was represented by glucose carbon

recycling. Glutamate and glutamine were not found to be significant gluconeogenic precursors in day 19 embryos. However, catabolism of these amino acids contributed to ~25% of proline flux in the liver. By contrast, there was significant [M+3] ^{13}C-isotopomer abundance in blood glucose and in liver and muscle glycogen when [U-^{13}C]glycerol was injected *in ovo*. These observations clearly confirmed that glycerol derived from triacylglycerides is a significant precursor for glucose and glycogen synthesis. *In ovo* supplementation on day 9 embryonic of glucose and/or amino acids (5 non-essential amino acids) did not alter gluconoegenesis. However, these supplemental treatments significantly reduced catabolism of glucose via glycolysis. ^{13}C-Mass isotopomer abundances of most substrates differed when each was individually compared in blood and in the various tissues, indicating differences in substrate utilization between tissues. In summary, this thesis research has provided new information on the degree and pathways of nutrient (glucose, glycerol, amino acids) use by the developing embryo and the rapid adjustments in the activity of networks of enzymes involved in non-essential amino acid, glucose and glycogen metabolism to support embryo survival. Most importantly, this work has systematically evaluated the potential substrates that the embryo utilizes for glucose synthesis, in particular, the significant role of glycerol.

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INTRODUCTION

During the first week post hatch, large losses in poultry productivity occur due to high mortality rates, reduced weight gain and low feed efficiency ratios (Fanguy *et al*., 1980; Hager and Beane, 1983). Two of the most important factors determining optimal development of late term embryos and growth of chicks are: 1) nutrient availability and reserves *in ovo* and 2) the metabolic transitions *in ovo* just prior to hatch. This doctoral dissertation investigated the metabolic transition in macronutrient utilization in chicken embryos during the last half of incubation (day 12 to 20).

Chicken embryos develop in a 'fixed' nutrient environment from the time of laying to the time of hatching. On a dry matter basis, the egg contains 45% lipids, 47% protein and less than 3% carbohydrates (Romanoff and Romanoff, 1967). The metabolic machinery of the developing embryo is geared towards amino acid and lipid metabolism for tissue protein synthesis and energy production. However, towards the later stages of incubation (~ day 18), the embryo begins to adjust its metabolism for utilization of exogenous carbohydrates and proteins derived from feed sources post hatch. This metabolic transition involves a progressive increase in the use of glucose carbon as an energy source and also gradual up-regulation of the lipogenic machinery in the liver. The degree to which the gastrointestinal tract (GIT) is developed, the content of the residual yolk at hatch and energy reserves (glycogen stores, subcutaneous fat depots) are vital in aiding the metabolic shift from *in ovo* to post hatch development. Various post hatch management practices, for example delayed access to feed and water, also negatively impacts this transition process (Fanguy *et al*., 1980).

Previous research would suggest that the enzyme and transport systems for carbohydrate metabolism (disaccharidases, Na^+- D-glucose co transporter) are well developed at the time of hatch (Chotinsky *et al*., 2001). By contrast, carbohydrate absorption from the GIT does not begin to increase until 2-3 days post hatch (Noy and Sklan, 1999a; 2001). Furthermore, *in ovo* delivery of nutrients (glucose, amino acids, and peptides) has been shown to have a positive effect on body weight and GIT maturation and on nutrient reserves at hatch (Coles *et al.*, 1999, 2003; Ohta *et al*., 1999; Tako *et al*., 2004). Similarly, chicks hatched from larger eggs perform better and have higher glycogen stores and subcutaneous fat depots than those hatched from smaller eggs (Uni *et al*., 2005; Speake *et al*., 1998). These observations suggest that nutrient availability *in ovo* may be 'limiting' and this in turn could lead to delays in the metabolic transition for digestion and metabolism of exogenous feed nutrients.

Overall Aim of Research:

The overall aim of this research project was to define the macronutrients utilized in central pathways of metabolism during the latter half (day 12 to 20) of chicken embryonic development and to determine the substrate fluxes in response to *in ovo* supplementation of nutrients.

Research Hypothesis:

We hypothesized that chicks from small eggs perform more poorly compared to chicks from larger eggs because of their limited supplies of specific macronutrients during embryonic development.

As a consequence, the nutrient deficient environment of the smaller eggs results in a competition for three-carbon substrates for energy and tissue protein synthesis (e.g. non essential amino acid synthesis). Thus, we expected higher rates of gluconeogenesis and an increased flux of amino acid carbon through the Krebs cycle in the embryonic chicks from small eggs. In turn, this will limit the availability of amino acids for tissue growth, glycogen synthesis and for synthesis of other vital substrates (e.g. nucleic acids).

It is further hypothesized that supplementation of nutrients *in ovo* will alleviate competition for three-carbon substrates and spare glucose and/or amino acid carbon for glycogen synthesis and conserve yolk lipids.

Despite the vast amount of data on the profiles of gene expression during embryogenesis, there is a paucity of information on the types and fluxes of nutrients through metabolic pathways that are the end-product (metabolic phenotype) of changes in gene expression. Further, with more and more evidence supporting the concepts of whole animal integrative metabolism and that metabolic control is distributed across related pathways (Veech and Fell, 1996, Fell, 1998), the determination of macronutrient requirements can only be defined once we have a thorough knowledge of the composition of substrates contributing to the metabolic phenotype of the animal at different physiological states.

In recent years, several groups, including our laboratory, have used universally labeled stable isotope carbon tracers (e.g. [U -^{13}C]glucose, [U -^{13}C]amino acids) as specific and general metabolic probes which has allowed the central and intersecting pathways of metabolism to be defined at the tissue (cellular) level (Tserng and Kalhan, 1983, Katz *et al*., 1989, Wykes *et al*., 1998). Employing this approach, it has proven

possible to determine the proportional contribution of specific macronutrients to major metabolic pathways such as gluconeogenesis, Krebs cycle fluxes, oxidation via acetyl-CoA and non-essential amino acid and fatty acid synthesis. In this approach, ^{13}C-mass isotopomer distribution analysis is conducted on primary and secondary substrates of the central pathways, namely the Krebs cycle, gluconeogenesis and fatty acid metabolism. Thus, glucose, amino acid and fatty acid metabolism can be described in detail to provide information on the fluxes and metabolic organization *in vivo*. In consequence, the ability to predict changes in nutrient utilization by metabolic pathways in response to changes in nutrient supply (e.g. *in ovo* injection) and genetic selection will be possible so that the production potential of animals can be optimized.

Specific Experimental Objectives

1. ***Experiment 1***: The objective was to determine the composition of macronutrient fluxes through the central pathways of metabolism in developing chick embryos (day 12 to 18 of incubation) using [U -^{13}C]glucose tracer. This study tested the hypothesis that small eggs are nutrient limited compared to large eggs and thus small eggs compensate by having a higher rate of gluconeogenesis. Further, this study determined the relative contribution of glucose carbon towards non-essential amino acid synthesis (NEAA) and Krebs cycle metabolism from day 12 to 20 of incubation (Chapter 2).
2. ***Experiment 2***: The objective was to determine the influence of *in ovo* nutrient supplementation (glucose, amino acids) on partitioning of nutrient fluxes through central metabolic pathways using a [U -^{13}C]glucose tracer. This study tested the hypothesis that *in ovo* supplementation of glucose and/or amino acids increases

glucose synthesis and spares glucose from catabolism for energy and NEAA synthesis (Chapter 3).

3. ***Experiment 3***: The objective was to determine the contributions of glutamate and glutamine to gluconeogenesis, NEAA synthesis and energy metabolism in late term (day 19) chicken embryos using [U-^{13}C]glutamate and [U-^{13}C]glutamine tracers. This study tested the hypothesis that the contributions of glutamate and glutamine to gluconeogenesis and NEAA synthesis will be reduced in small versus large egg embryos because of the central roles these two amino acids serve in nitrogen exchanges and nucleic acid synthesis (Chapter 4).
4. ***Experiment 4***: The objective was to determine the contribution of glycerol to gluconeogenesis and NEAA synthesis in developing chicken embryos using [U -^{13}C]glycerol tracer. This study tested the hypothesis that glycerol is the major precursor for gluconeogenesis and that a larger proportion of glucose synthesis derives from glycerol in small compared to large egg embryos (Chapter 5).

CHAPTER 1: LITERATURE REVIEW

The Avian Egg

The avian egg is a package of all essential nutrients required for development of the embryo into a viable chick. An egg weighing 60 g comprises, on average, 6.5 g of shell (11%), 18.5 g yolk (31%) and 35 g of albumen (58%) by weight. The egg yolk accounts for 99% of the lipids and 60% of the protein present in an egg. However, eggs contain very little carbohydrate (<3%), 70% of which is in the albumen. Further, ~56% of the carbohydrate present in albumen is protein-bound (Romanoff and Romanoff, 1967). The nutrient distribution in yolk and albumen is summarized in **Table 1**. The proportion of yolk to albumen varies with breed and age of the flock, egg size, maternal nutrition etc. For example, broiler breeders had a higher yolk: albumen ratio (0.515) compared to that of commercial layers (0.401; Harms and Hussein, 1993). Yolk to albumen ratio was also found to be higher in 55 week old hens (0.63) compared to 36 week old hens (0.47; Applegate and Lilburn, 1996). The nutritional and metabolic implications of an altered yolk: albumen ratio (Peebles *et al*., 2000; Vieira and Moran, 1998) on embryonic growth and development is not clear.

Dynamics of Avian Embryo Development

Subsequent to the establishment of the embryo during the first third of incubation, a series of synchronized events occurs within the egg until hatch. The incubation temperature and humidity have a profound effect on the evolving developmental patterns during the course of incubation and also on egg weight loss due to evaporation. Thus, lower the humidity, higher the weight loss during incubation (Murray, 1925). With 45% lipids, 47% protein and less than 3% carbohydrates (on a dry matter basis) at incubation,

Table 1.1: Nutrient composition of an egg*

Nutrients (%)	Yolk (31%)	Albumen (58%)
Energy (kcal/100g)	352	48
Water	47.5	88.5
Protein	17.4	10.5
Carbohydrates	0.2	0.7 (50% bound)
Lipids	33	0.02
Cholesterol ester	0.8	-
Triglyceride	71.4	-
Free fatty acid	0.9	-
Free cholesterol	5.6	
Phosphoglyceride	20.7	
Inorganic elements	1.1	0.6

* Burley and Vadehra, 1989; Deeming and Ferguson, 1991.

the developing embryo must selectively utilize the available nutrients for gluconeogenesis and energy metabolism. After a period of initial anaerobic glycolysis at the start of incubation, the embryo switches to fatty acid oxidation for energy. This occurs after an active chorio-allantoic membrane is established and also with access to oxygen.

During the first two weeks of incubation, embryonic tissues especially liver accrete proteins at a rapid rate whereas lipid content of the tissues does not increase until day 12-13 of incubation (Romanoff and Romanoff, 1967). Thus, amino acids are probably the predominant metabolic substrate utilized during the initial stages of development. During the first week of incubation, due to an inflow of water from albumen, wet weight of yolk increases steadily with a concomitant decrease in wet weight of albumen. The loss of solids from albumen follows a gradual course with the rate increasing around day 13 of incubation corresponding to its flow to the amniotic sac. This mixture is orally consumed by the embryo resulting in partial absorption of proteins by the enterocytes from this mixture. The albumen-amniotic fluid mixture also enters the yolk sac and this process continues until pipping begins (Moran, 2007).

Solid loss from yolk appears to occur in two phases with the first phase occurring around day 5 of incubation, corresponding to an increase in solid content of sub embryonic space (Romanoff and Romanoff, 1967). The second phase corresponds to utilization of yolk lipids for energy production from embryonic day 15, which has been predicted to supply 90% of the energy requirements of late term embryo (Turro *et al*., 1994).

Nutrient Metabolism in Developing Chicken Embryo

Glucose Metabolism

Glucose available in the egg at incubation plays an important role in the initiation of embryonic development and further as an energy substrate via anaerobic catabolism (Moran, 2007). This results in production of lactic acid, which may serve as a gluconeogenic precursor. Plasma glucose can be detected as early as day 4 of incubation and increases steadily across incubation. Plasma glucose concentrations increase from 6 – 8 mM early during embryonic development to 10 – 12 mM at two to three weeks post hatch (Hazelwood, 1971).

The low amounts of glucose in the egg at the start of incubation and the subsequent steady increase in blood glucose concentration implies that gluconeogenic pathways are active from early in embryo development. Gluconeogenic enzymes, (viz. pyruvate carboxylase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase) increase in activity, reaching a peak by embryonic day 17-20 and decreasing thereafter (Pearce, 1977). Gluconeogenic activity in the embryo versus the hatchling appears to be regulated by localization of the rate limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK), which can be expressed in the mitochondria or cytosol, or both. Thus during embryonic development, cytosolic form of PEPCK may predominate with the contribution of Krebs cycle intermediates (lipids, amino acids) higher towards de novo gluconeogenesis (Brady *et al*., 1979). Glucose synthesis primarily occurs in liver which can produce glucose both by gluconeogenesis and glycogenolysis. The contribution of the kidney to gluconeogenesis ranges from 5-50% (Corssmit *et al*., 2001) and is significant during periods of nutrient deprivation or prolonged fasting. A distinct distribution of

PEPCK isoforms was also observed in the liver and the kidney of day old chicks with the mitochondrial form predominating in the liver and the cytosolic form in the kidney (Watford *et al*., 1981).

Glucose can be metabolized through glycolysis, pentose phosphate pathway, UDPG glycogen synthetic pathway or uronic acid pathway. The relative significance of these pathways varies with stage of incubation, with uronic acid pathway being active during first week of incubation and glycolytic pathway later during incubation. In general, glycolysis and the activities of glycolytic enzymes increase with embryonic development thus preparing the embryonic metabolism to utilize a high carbohydrate diet post hatch (Pearce, 1977). Goodridge, 1968 demonstrated that glucose oxidation in embryonic (day 16 to hatch) liver slices were low. However the oxidation rates were found to be 20 times greater in liver slices from 4-wk old chicks.

Pentose phosphate pathway is not known to play any major role in degradation of glucose during embryonic development except during the initial stages of incubation. Pentose phosphate pathway may be more important in providing pentose units towards nucleic acid biosynthesis, while most of the NADPH supplies come from malic enzyme activity (Hazelwood, 1971).

The role of other carbohydrates in the intermediary metabolism of the embryo is not well known. Even though fructose, galactose, xylose and arabinose are known to be absorbed from intestines of chicken, the relative amounts in comparison with glucose are very low.

Tissue Glycogen Storage. Hepatic glycogen starts accumulating early (day 6) during embryonic development (by the uronic acid pathway), peaks by day 12, declines

to 50% by day 13 and then increases to over 400% by day 18 (Hazelwood, 1971). The small drop at day 12 is attributed to the increase in activity of thyroid hormones and/or epinephrine. This glycogen store has been proposed to be a vital source of energy for the hatchling and by the first day of life heart and liver glycogen drops to 40% and 16% respectively of pre hatch levels (Freeman, 1969). Glycogen stores are especially important at hatch to provide glucose for energy through anaerobic glycolysis as the amount of oxygen available at this stage is limited until the onset of pulmonary respiration (Moran, 2007). Various authors have also reported a positive correlation between glycogen reserves in various tissues and body weight at hatch (John *et al*., 1988; Christensen *et al*., 1999; Christensen *et al*., 2001), the reason being attributed to sparing catabolism of muscle protein for energy (Uni *et al*., 2005).

Amino acid Metabolism

The embryo utilizes amino acids from the egg contents for tissue accretion at a much higher rate during the latter half of incubation. Even with a high rate of amino acid accretion by the embryo during the final stages of incubation, a significant amount of both essential and NEAA remain unused in the egg by day 19 of incubation (**Figure 1.1**). However, during the final stages of incubation, concentrations of certain amino acids in the egg (e.g. Glycine, Proline; **Figure 1.2**) do not seem sufficient to support embryonic development (Ohta *et al*., 1999). Thus synthesis of these amino acids must occur from other substrates, the potential candidates being glutamate and glutamine in the case of proline.

Apart from protein synthesis, amino acids can be synthetic precursors for gluconeogenesis. This has been demonstrated in various species (Yeung and Oliver,

1967; Mallette *et al*., 1969; Heitmann *et al*., 1973) with glutamate, glutamine, alanine and aspartate being the major contributors followed by glycine, serine and threonine (Lobley, 1992). Amino acids also play a variety of roles in different tissues which include: Nitrogen shuttling between tissues, Ammonia metabolism, Nucleotide synthesis and Substrates for energy metabolism. However our knowledge of embryonic amino acid utilization for different purposes is very limited.

Lipid Metabolism

During embryonic development, the respiratory quotient stays around 0.7 confirming the fact that 90% of energy requirement of the embryos is derived from oxidation of yolk lipids (Sato *et al*., 2006). During the latter half of incubation, lipids are preferentially absorbed from the yolk contents, as evidenced by a decrease in percentage of lipids in the yolk (65% on day 13, 44% on day 21; Deeming and Ferguson, 1991). This preferential fatty acid utilization could be sparing protein and carbohydrate use for biosynthetic purposes at the same time providing twice the energy per unit weight for metabolism. Lipid modifications in the yolk sac membrane could also provide glycerol moieties as a potential gluconeogenic precursor.

During initial stages of incubation, the yolk sac membrane and the embryo take up only relatively small amount of yolk lipids. However, during the latter stages of incubation, (after day 13) uptake of yolk contents into the embryo increases significantly (**Figure 1.3**). In fact, lipid uptake by the embryonic liver has been shown to increase significantly between day 15 and 18 of incubation, accounting for a 15% increase in liver weight (Peebles *et al*., 1999). This is visually evident due to the yellowish, greasy texture of the liver just prior to hatch and in day old chicks.

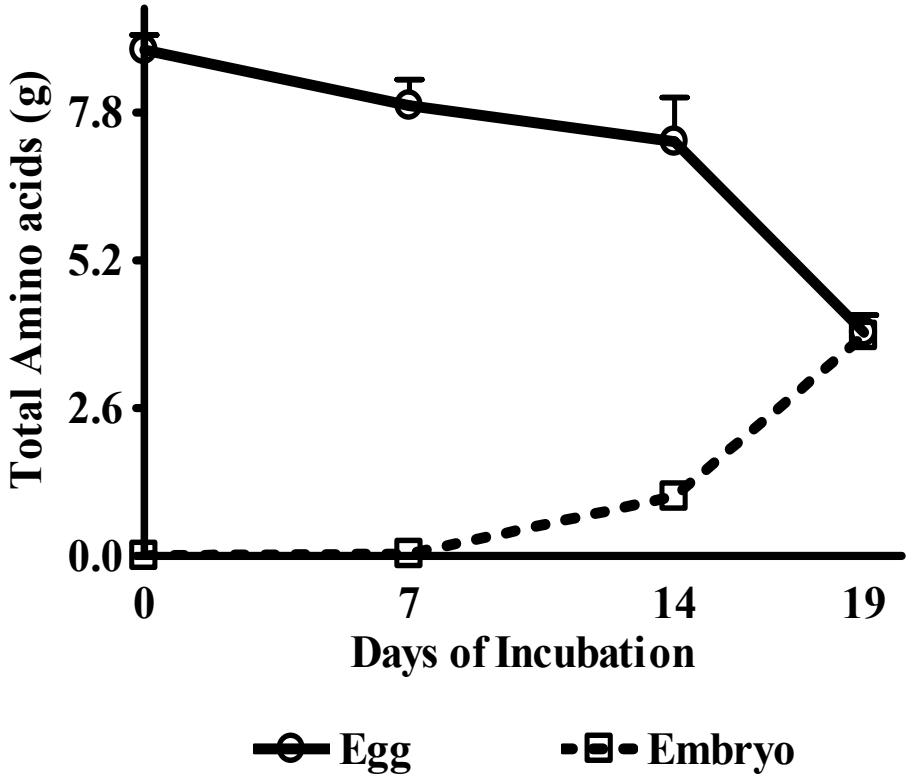

Figure 1.1: Amino acid content in the egg and the developing chicken embryo at different stages of incubation (Adapted from Ohta *et al*., 1999).

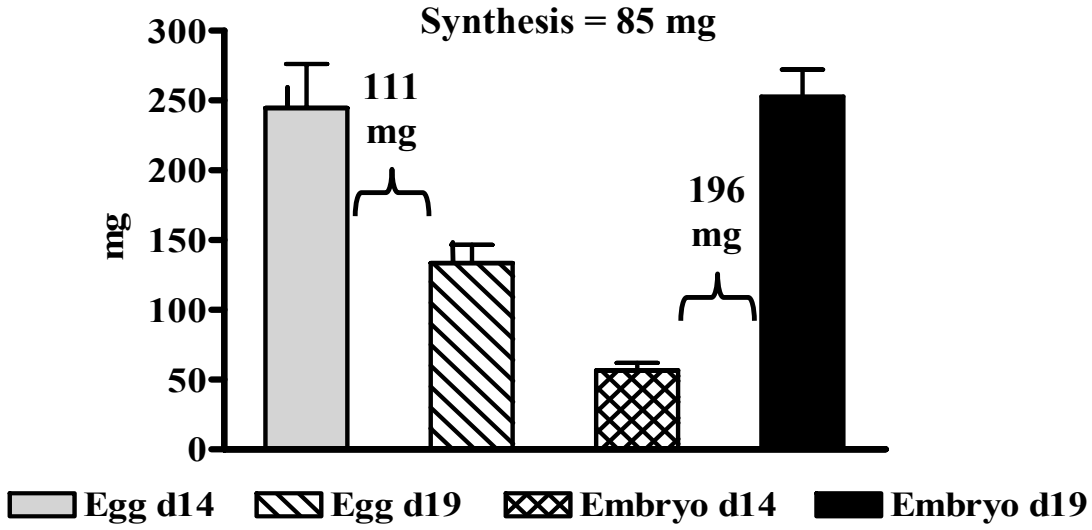

Figure 1.2: Proline utilization by the developing chicken embryo between day 14 and day 19 of incubation (Adapted from Ohta *et al*., 1999).

Intact lipid droplets are engulfed by the yolk sac membrane by phagocytosis. Within the yolk sac membrane there is extensive hydrolysis and re-esterification of lipid droplets before assimilation by the embryo. Once the lipid enters the embryo, it is broken down to free fatty acids by the action of lipoprotein lipase. Lipoprotein lipase expression is highest in adipose tissue followed by heart and muscle and very low in liver and brain. During the latter half of incubation, subcutaneous fat depots start appearing and it is estimated that 25 % of yolk lipids are stored in the subcutaneous adipose tissue of the embryo. These provide a source of energy for hatching. Large egg embryos are found to accumulate more subcutaneous fat compared to small egg embryos (Speake *et al*., 1998).

As the embryo develops in a high fat environment, lipogenesis is very low. This rapidly changes upon consumption of dietary nutrients, at which point the activity of lipogenic enzymes in the liver increase rapidly (Pearce, 1971). Contrary to mammalian fatty acid synthesis, which takes place in both liver and adipose tissue, avian liver accounts for 90 – 95% of *de novo* fatty acid synthesis.

Nutrient Metabolism in the Post hatch Chick

Newly hatched chicks are fed a starter diet with greater than 30% carbohydrates. Upon consumption of this high carbohydrate diet, the activities of glucogenic and glycogenic pathways begin to decline. This is in contrast to the mammalian neonates where gluconeogenesis and glycogen synthesis peak after birth. In newborn chicks, excess glucose is thus rapidly incorporated into fatty acids in the liver and this increase during the first 6-7 days after hatching. This is concomitant with an increase in activities of lipogenic enzymes, acetyl-Co A carboxylase, fatty acid synthetase, ATP citrate lyase and malate dehydrogenase (Pearce, 1977). Numerous factors, including residual yolk

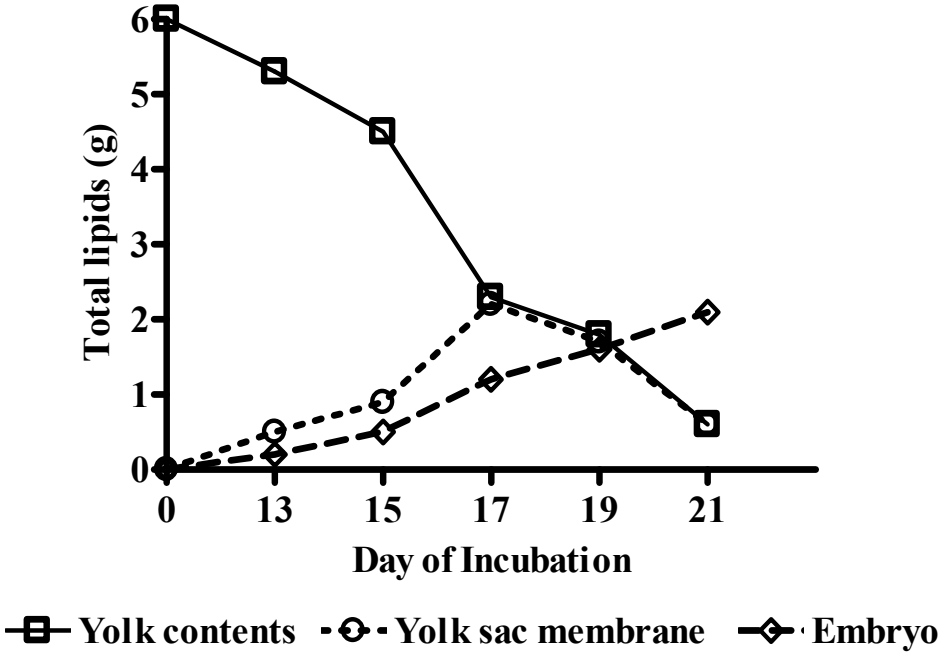

Figure 1.3: Transfer of yolk contents to the yolk sac membrane and to the developing chicken embryo with advancing stages of incubation (Adapted from Deeming and Ferguson, 1991).

content, levels of pancreatic and intestinal enzymes, maturity of the GIT, nutrient transporters, exogenous nutrient intake and digestibility affect early growth rate of hatchlings.

Residual Yolk

The residual yolk contains 50% lipids and accounts for 20 – 30 % of the chick's body weight at hatch (Noy *et al.*, 1996). It is considered it to be the primary energy source for the neonatal chicks, with 90% being utilized within the first two days of hatch. The residual yolk is utilized via two routes: 1) transfer to the bloodstream via endocytosis of lipid droplets and 2) transfer to the intestines via the yolk stalk (Noy and Sklan, 1998). The presence of feed nutrients in the GIT promotes the transfer of the residual yolk to the intestinal lumen. By contrast, in fasted chicks, the residual yolk is not transferred to the intestinal lumen (Noy and Sklan, 2001). In addition to acting as an energy source, the residual yolk is suggested to have other functions, including transfer of maternal antibodies to the newborn and maturation of GIT. Therefore, utilization of yolk components as an energy source in fasted chicks may in turn be compromising the development of the immune system and digestive tract.

Enteric Development

Enteric development during the perinatal period (embryonic day 18 to post hatch day 4-5) is one of the most investigated aspects of nutrient utilization in poultry. Most of the research has involved characterization of intestinal morphology, measurements of specific activities and mRNA expression of the enzymes involved in carbohydrate digestion. Sklan *et al*. (2003) observed sucrase-isomaltase mRNA expression by

embryonic day 15, which peaked at day 19 and decreased considerably at hatch before again slowly increasing in expression post hatch. Concurrently, a parallel pattern of Na^+-D-glucose cotransporter (SGLT-1) mRNA expression was observed beginning at embryonic day 19 (Uni *et al*., 2003). The authors suggested that SGLT-1 expression immediately post hatch may limit the uptake of glucose from the small intestines. In general, disaccharidase (maltase, lactase etc) activities peaked by embryonic day 18 and decreased after hatch (Chotinsky *et al*., 2001). Furthermore, the activity of trypsin, amylase and lipase increased only upon ingestion of exogenous feed and did not change in starved chicks (Sklan and Noy, 2000). Rapid physical (length, surface area) and morphological changes (enterocyte proliferation and migration) occur during the perinatal period and have been suggested by various authors to be more limiting than the enzyme systems (Nitsan *et al.*, 1991; Ritz *et al*., 1995).

Post hatch Nutrient Uptake

Maximum utilization of nutrients post hatch depends on a variety of factors including the nutrient reserves available to the hatchling (e.g. glycogen, residual yolk), access to feed, maturity of GIT, and nutrient content of the diet. Sulistiyanto *et al*. (1999) observed an increase in metabolizability with dextrin, starch and casein from day 1 to 10 post hatch and concluded energy utilization to be age dependent. This age dependency could be contributed to the immature pancreatic and brush border enzyme systems and/or lack of sodium for the co-transporters (Noy and Sklan, 1999). Further, glucose and methionine absorption increased only 24-48 h post hatch and absorption rates of methionine, glucose and oleic acid were higher in fed hatchlings compared to fasted ones

(Noy and Sklan, 1999a; 2001). Oleic acid absorption is elevated in day-old hatchlings and does not change with age. High proline to glucose uptake ratios was also observed in chicks immediately post hatch, which may suggest the importance of amino acids as metabolic fuels (Obst and Diamond; 1992).

Hormonal Regulation of Embryo Metabolism

The endocrine system starts developing very early during incubation, with hormone producing cells in pituitary, hypothalamus and pancreas appearing between 3-8 days of incubation. There is a close association between hormone levels and the changing metabolic patterns throughout incubation.

Plasma insulin increases from 130pg/ml at day 10 to 389 pg/ml at 16 days of incubation. During the later stages of embryogenesis the insulin levels showed two peaks with a peak of 460 pg/ml on day 17 and a second peak of 488 pg/ml at hatch (Lu *et al*., 2007). Insulin reduces glycogenolysis and incorporates glucose molecules into glycogen. Hyperaminoacidemia or leucine, isoleucine, lysine, phenylalanine or arginine perfusion into the liver increases insulin release in various species suggesting glucose and amino acids promote insulin release in identical ways (Floyd *et al*., 1966; Hohlweg *et al*., 1999).

Glucagon levels increase from 59 pg/ml at 10 days of incubation to 428 pg/ml at hatch (Lu *et al*., 2007). At around 13-14 day of incubation glycogen levels drop suddenly and again increase rapidly by day 18 of incubation. This sudden drop is thought to be due to the sudden increase in the circulating levels of glucagon and/or epinephrine and also due to release of pituitary thyroid stimulating hormone and its target hormone, thyroxine (Hazelwood, 1971). Glucagon decreases hepatic glycogen levels by activating the

enzyme glycogen phosphorylase. In adipose tissue, glucagon mobilizes glycerol and fatty acids, and this action is potentiated by insulin.

The insulin to glucagon ratio from 14 to 17 days of incubation ranged from 1.7 to 2.2, which is significantly greater than normal insulin to glucagon ratio in the post-hatch chicks (1.2-1.7). Thus insulin seems to be an important promoter of chick embryo growth by promoting protein deposition during the rapid growth period (Lu *et al*., 2007).

Catecholamines can be detected as early as 9 days of incubation, and the levels increase progressively for the next 12 days. Noradrenaline is the dominant partner during incubation whereas the ratio predominantly changes to adrenaline at hatch and in post hatch chicks implying its primary role in metabolic functions like glycogenolysis. Even though adrenaline has the same metabolic effects as that of glucagon, it is less potent in chicken compared to rats.

Corticosteroids increase lipogenesis and gluconeogenesis in the liver and also increase glycogen deposition. Circulating levels of corticosteroids can be detected from day 9 *in ovo* with plasma glucocorticoid showing two distinct peaks, one at day 14 (contains both cortisol and corticosterone) and one immediately before hatch which contains principally corticosterone (Jenkins and Porter, 2004).

The thyroid hormone triiodothyronine increased significantly at pipping and hatch after remaining relatively unchanged during incubation. Thyroxine, after a gradual increase up to day 16, showed a rapid increase up to day 19 (Lu *et al*., 2007).

Substrate Selection for Metabolism

Selection of a substrate for a metabolic process depends on its availability, localization and compartmentalization of the pathway enzymes in the tissue of interest,

the physiological status, etc. Depending on these factors, a wide variety of metabolites can act as substrates for a synthetic or degradative pathway. Further, in the fed state, an active GIT can alter the profile of the metabolites appearing in the portal blood from the luminal side. For example, most of aspartate, glutamate etc in the diet is oxidized by the gut tissues. These amino acids have to be *de novo* synthesized for utilization by peripheral tissues (Stoll *et al*., 1999; El-Kadi *et al*., 2006).

With respect to gluconeogenesis, lactate, gluconeogenic amino acids and triglyceride glycerol are all potential substrates for gluconeogenesis. The relative contributions of these substrates at different stages of embryonic growth are not well defined. However, lactate concentrations are high at the start of incubation and prior to hatch due to anaerobic glycolysis (Moran, 2007), thus making it a good candidate to donate carbons for glucose synthesis at these stages. Glutamate and glutamine are central to intermediary metabolism and can donate their carbons towards gluconeogenesis, non-essential amino acid synthesis and other functions (e.g. glutathione synthesis, Krebs cycle metabolism etc). They together comprise ~14% of egg protein (1.2 g, Ohta *et al*., 1999). Further, the role of glutamine and glutamate in the proliferation and differentiation of intestinal mucosa and as an oxidative fuel is widely acknowledged (Windmueller and Spaeth, 1974; Wu, 1998; Reeds and Burrin, 2001).

Substrate preference also varies with the tissue concerned. Brady *et al.*, (1979) found lactate to be the greatest contributor to gluconeogenesis in isolated chicken hepatocytes, followed by pyruvate, dihydroxyacetone, glyceraldehydes and fructose. In kidney, however, the major metabolic substrates were found to be amino acids and glycerol (Watford *et al*., 1981, Magnuson *et al*., 2003). As a further illustration of tissue

substrate preference; branched chain amino acids which can constitute up to 50% of the non-dispensable amino acids in the diet do not undergo extensive degradation in the liver and are extensively oxidized by the peripheral tissues (muscle; Harper *et al*., 1984).

Factors Affecting Macronutrient Utilization

The avian embryo is solely dependent on the macronutrient content of the egg to meet its metabolic requirements. A variety of factors contribute to the relative utilization of these macronutrients for energy and tissue synthesis. These include egg size and composition, breeder age, nutrient supplementation, maternal nutrition, turning of eggs during incubation, etc. A clear understanding of how these factors affect the metabolic organization and regulation should provide a framework for predicting growth responses when dietary nutrient supply and composition are altered.

Egg size, Composition and Breeder Age

Egg size has a positive effect on body weight at hatch and subsequent growth rate of hatchlings; however this correlation decreases with increasing chick age (Applegate *et al*., 1999; Applegate and Lilburn, 1999). Small eggs have a greater proportion of albumen than yolk and large eggs have more yolk than albumen (Vieira and Moran, 1998). Further, yolk deposition in the egg increases with breeder age (Peebles *et al*., 2000). Lourens *et al*., (2006) found a larger proportion of the egg's energy content left in the residual yolk sac of embryos from large eggs and concluded that this is due to the surplus nutrient milieu of large eggs.

Nutrient Supplementation

In ovo administration of nutrients has been found to accelerate embryonic enteric development and increase body weight at hatch (Coles *et al.*, 1999, 2003; Ohta *et al*., 1999; Tako *et al*., 2004). It has been hypothesized that the reason for the improvements in chick growth relates to the greater maturity of the GIT at hatch which, in turn, supports higher rates of nutrient absorption. Uni et al. (2005) found significantly higher glycogen reserves, increased weight at hatch and increased breast muscle size following *in ovo* supplementation on day 17.5 of incubation with a solution containing maltose, sucrose, dextrin, and β-hydroxy-β-methylbutyrate. They attributed the improved energy status of *in ovo* fed embryos to reduced muscle protein mobilization for gluconeogenisis. *In ovo* injection of an amino acid mixture increased the crude protein content of the day 19 embryos (Ohta *et al*., 1999). However, *in ovo* administration of amino acids, carbohydrates or peptides gives very similar growth responses. Thus, these external nutrients may be altering fluxes through metabolic pathways, thereby alleviating competition for three carbon units and sparing essential nutrients (amino acids or glucose) for tissue synthesis.

Integration of Macronutrient Metabolism

The central pathways of carbohydrate, amino acid and lipid metabolism intersect with great regularity with resulting exchange of carbon skeletons (**Figure 1.4**). Thus it is apparent that there is extensive nutrient flux through connected pathways that ultimately determines the availability of a nutrient for tissue needs. Not surprisingly, many of these pathways have a high degree of metabolic flexibility that allows the cell, tissue and animal to alter the choice of substrates depending upon the prevailing nutrient

environment (e.g. fed *vs.* fasted) or stage of development (e.g. *in ovo vs*. post hatch). An example of the flexibility of metabolic pathways is demonstrated by the fact that 2-day-old chicks, when starved for 48 hours appear to conserve glucose carbon by lowering carbon flux through pyruvate dehydrogenase and, instead increasing the oxidation of fatty and amino acids (Sunny *et al*., 2004). Embryonic metabolic machinery undergoes a transition from utilizing amino acids and fat for gluconeogenesis to utilizing dietary carbohydrates and proteins for lipogenesis post hatch (Pearce, 1977, Noy and Sklan, 1997).

A further point which needs consideration in this regard is that nutrient needs of the whole animal are an integration of the metabolic needs of different tissues and organ systems. In that sense, individual tissue substrate utilization through central pathways of metabolism has to be considered. The rate of flux of a particular nutrient through these pathways may differ between tissues depending on nutrient availability, developmental stages or the physiological stages. In post hatch chicks, PEPCK is predominantly mitochondrial in liver. However in kidneys, a significant portion of PEPCK is found in cytosol and this portion can go up from 21% in fed chicks to 40-50% in fasted chicks. This difference in the PEPCK isoforms allows the liver and kidney to utilize different substrates, lactate and amino acids respectively for gluconeogenesis (Watford et al., 1981). Nuclear magnetic resonance experiments with $^{2}H_2O$ and [^{13}C]propionate showed a 66% gluconeogenic flux from phosphoenolpyruvate in mice lacking hepatic PEPCK (Magnuson *et al*., 2003). This further substantiates the flexibility of tissues in maintaining glucose homeostasis.

More and more scientific evidence is being generated suggesting that 'true' rate limiting steps in metabolic networks may be seldom present. Metabolic control analysis suggests that the control of flux is most likely distributed across numerous enzymes and thus altering a single enzyme does not affect the flux of a metabolite even though it may affect its concentrations (Veech and Fell, 1996, Fell, 1998). Thus we have to look at a pathway as a whole to understand its nutrient or genetic regulation.

Metabolic Investigations

Various approaches have been used to investigate substrate utilization and net fluxes through different pathways of metabolism at the tissue or whole animal level. These include use of stable isotopes ($^{2}H_2O$, ^{13}C-lactate, 2-$^{13}C_1$-glycerol, U-$^{13}C_6$-glucose etc), radio isotopes (3-^{14}C-lactate, 3-^{14}C-alanine, 2-^{14}C-acetate), liver biopsy, splanchnic catheterization and arterial-venous concentration difference and ^{13}C-NMR (Nuclear magnetic resonance). Even though these methods have provided a wealth of information in recent years, they have limitations. The following are some of the limitations of the methods mentioned above;

1. Radioisotopes, in addition to being radioactive provide only a measure of total radioactivity compared to stable isotopes which provide the specific activity of the chemical mass which is a direct measure of its enrichment (Rennie, 1999).
2. Most of the compounds labeled at one or two specific carbons or hydrogens (e.g. 2-$^{13}C_1$-glycerol, 1, 2-$^{13}C_2$-glucose) may only provide information about a single pathway or metabolite, whereas uniformly labeled compounds provide a profile or metabolic signature of multiple pathways (Bequette *et al*., 2006).

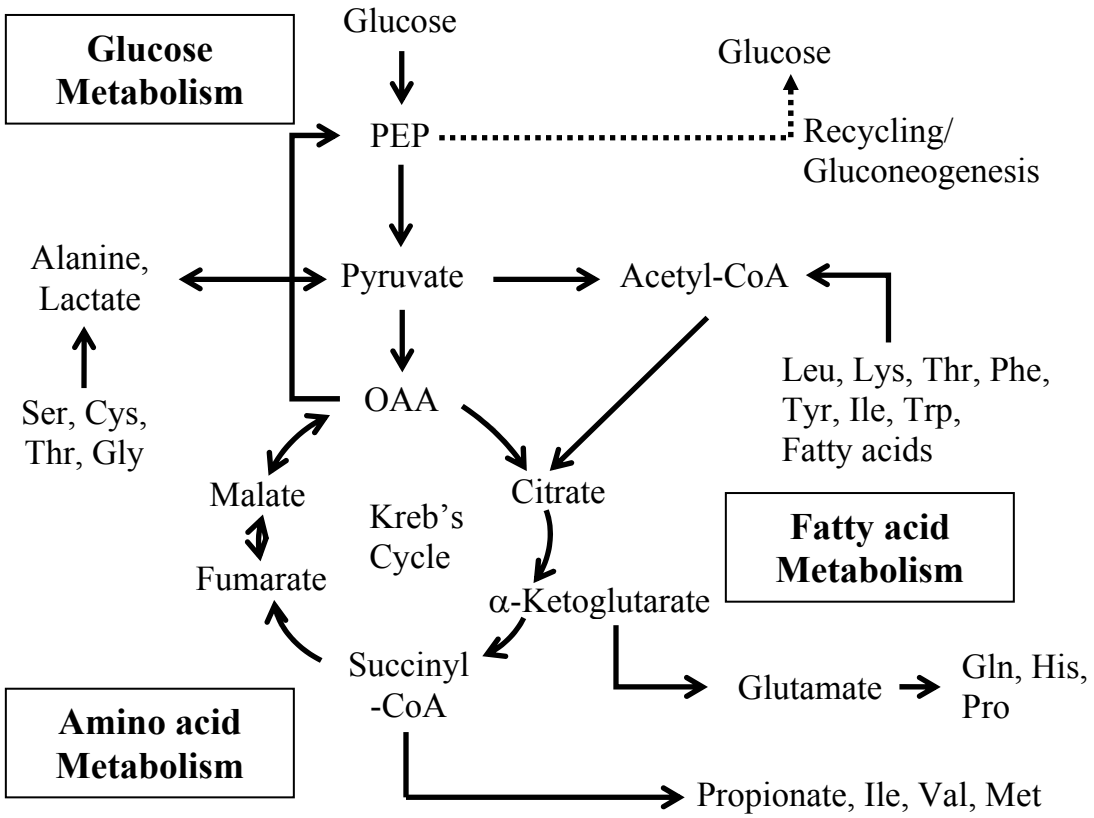

Figure 1.4: Integration of Macronutrient metabolism through central pathways of metabolism including glycolysis, Krebs cycle and fatty acid oxidation.

3. Measuring glucose turnover with $^{2}H_2$ tracers will result in an over estimate due to futile cycling, where as ^{13}C tracers give an under estimate due to recycling (Kalhan,1996).

4. NMR, with its numerous advantages in determining the detailed structural information of a compound, is however plagued by low sensitivity and high operating costs.

Mass Isotopomer Distribution Analysis with Stable Isotopes

Investigation of metabolic pathways and measuring nutrient fluxes using stable isotopes has contributed immensely to our knowledge of intermediary metabolism. Advances in mass spectrometry have increased the precision and sensitivity with which these measurements can be made. Some of the advantages of using stable isotopes in metabolism research are: a. non radioactive (safe to use in infants and pregnancy) b. with sufficient enrichment, stable isotopes can be traced for a long time as they do not decay over time c. samples can be stored indefinitely d. multiple labels can be used simultaneously to measure pathway fluxes e. relatively easy to determine the position of the label (Reeds *et al*., 1997; Coggan, 1999).

Coupled with mass isotopomer distribution analysis (MIDA), stable isotopes have been used to investigate a wide range of nutrient metabolic pathways in a variety of species and have provided a wealth of information (Brunengraber *et al*., 1997, Bequette *et al*., 2006). However, a major assumption when applying MIDA is that the precursor originates from a single pool, which is not always the case. Thus, the method has its limitations in that it provides an underestimate of the contribution of the precursor to the product as other substrates can also contribute to the same cause (Landau, 1999).

To define macronutrient needs of the developing chicken embryo, we employed a novel *in ovo* [U-^{13}C] tracer approach. [U-^{13}C] labeled compounds (e.g. glucose, amino

acids, and glycerol) were administered by injection into developing eggs and before conducting ^{13}C-MIDA analysis. [U-^{13}C] labeled compounds have a definite advantage over other stable isotopes labeled at one or two positions in that, upon catabolism, labeled ^{13}C-skeletons distribute amongst several pathways, thereby providing a 'snap shot' of the metabolic profile of a particular tissue and the activity of the pathways in that tissue (Bequette *et al*., 2006).

To demonstrate the above point, we took [U-^{13}C] Glucose as an example. [U-^{13}C] Glucose has been used in a number of studies to quantify glucose and Krebs cycle metabolism (Tserng and Kalhan, 1983, Katz *et al*., 1989, Wykes *et al*., 1998). [U-^{13}C] Glucose (M+6) is heavier by six mass units, and yet it is indistinguishable from unlabeled glucose and metabolized through and by the same pathways as unlabeled glucose (M). Metabolism of U-^{13}C glucose through glycolysis and Krebs's cycle produces specific isotopomer patterns (M+1, M+2, M+3 ….) in intermediates along the pathway. The relative labeling patterns of two intermediates can be used for deriving relative contributions of metabolites towards energy metabolism based on the precursor to product relationship.

[U-^{13}C] Glucose, when given in diet or introduced *in ovo*, undergoes metabolism through glycolysis to yield pyruvate (M+3; three mass units heavier). The probability of two uniformly labeled pyruvate combining together to form U-^{13}C glucose is considered negligible. This pyruvate can either enter Krebs's cycle as acetyl CoA (M+2) or form oxaloacetate (M+3) by carboxylation. The latter gives rise to phosphoenol pyruvate (M+3) which is then re-incorporated into glucose (M+3). The ratio of M+3 glucose and M+6 glucose thus provides a minimum estimate of glucose recycling. This value is an

under estimate due to dilutions from unlabeled carbon sources at pyruvate and oxaloacetate and also due to the presence of oxaloacetate-fumarate substrate cycle (Reeds *et al*., 1997). This approach has been extended to investigate the organization of Krebs's cycle by MIDA (**Figure 1.5**). This is accomplished by monitoring the isotopomer patterns in Krebs's cycle intermediates or their transamination partners (Katz *et al*., 1989; Wykes *et al*., 1998). The underlying assumption is equilibrium between pyruvate (3 carbon pools) and alanine, α-ketoglutarate and glutamate and also oxaloacetate and aspartate.

Simultaneously with determining the labeling of the amino acids, we can determine the isotopomer patterns in Krebs cycle intermediates viz. citrate, α-ketoglutarate, succinate, fumarate, malate and oxaloacetate. Measurement of both citric acid cycle intermediates and their transamination partners provides a good comparison of the nutrient fluxes and insights into compartmentalization of these metabolites. The relative labeling patterns of lower isotopomers, for example M+1, 2, 3 in glutamate after injection of a [U-^{13}C] Glucose tracer also provide us with an indication of the activity of Krebs cycle. Thus a higher amount of labeling of M+1, 2, 3 in glutamate indicates a higher recycling of the tracer carbon and thus a higher activity of Krebs cycle (Reeds et al., 1997).

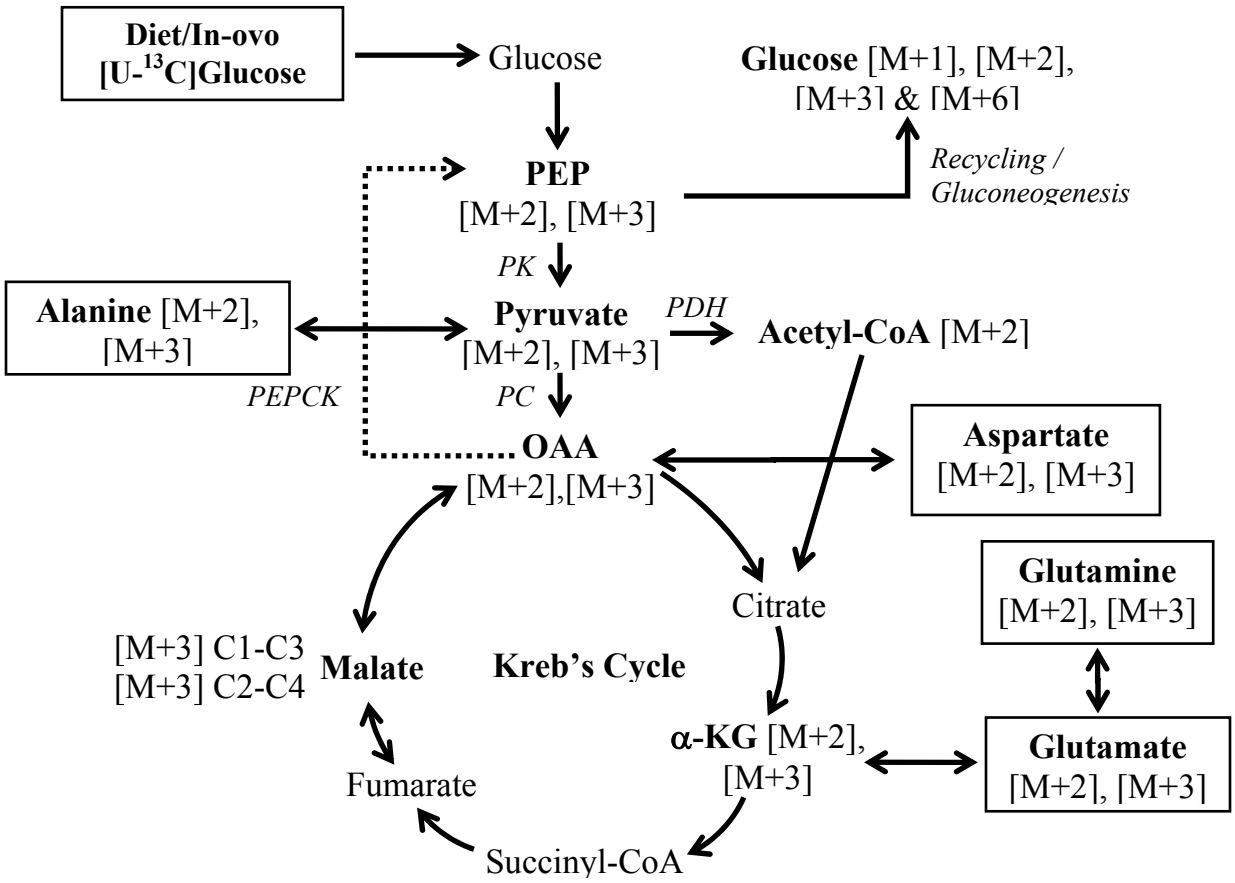

Figure 1.5: Mass isotopomer distribution patterns in gluconeogenic and Krebs cycle metabolic pathways after *in ovo* injection of [U-^{13}C] glucose tracer. M+1, M+2 etc represents the isotopomers of each metabolite which are heavier by 1, 2, etc mass units respectively.

Summary

From the available literature it is clear that macronutrient (amino acids, glucose and fatty acids) metabolism during embryonic development follows complex patterns of substrate utilization that continually must adapt throughout the incubation period. It is also clear that various external (e.g. environmental temperature and humidity) and internal (e.g. nutrient availability, hormonal changes) factors have a profound effect on nutrient uptake by and growth of the embryo. However, very little information exists in the literature to answer the following questions, **1)**. What are the major metabolic substrates for gluconeogenesis and NEAA synthesis during embryonic development? **2)**. How do the nutrient fluxes vary and adapt at these different stages of development to satisfy specific metabolic and tissue needs? This type of information is particularly important, considering that the embryo undergoes a metabolic transition from lipid (yolk) and protein (albumin) use during embryo development to carbohydrate (starch) and protein consumption and use post hatch. Research in various species has provided evidence regarding the integrative nature of substrate metabolism and also the flexibility of the metabolic pathways during various physiological states. Thus, knowledge of the patterns of substrate use will improve our understanding of the overall nutrient needs of the embryo, and by extension, the required nutrient composition of the egg contents at lay. In this connection, it may be possible to formulate diets for laying broilers that optimizes the composition of the egg contents for embryo growth and survival at hatch.

Based on the available literature, a general hypothesis that the nutrient deficient environment of the smaller eggs results in a competition for three-carbon substrates for energy and tissue protein synthesis (e.g. non essential amino acid synthesis) was

developed. Furthermore, it was hypothesized that small egg embryos will have higher rates of gluconeogenesis and an increased flux of amino acid carbon through the Krebs cycle thus limiting the availability of amino acids for tissue growth and glycogen synthesis. Thus, the embryo may possess the ability to sense changes in its nutrient environment and adjust the relative flux and choice of nutrients through various pathways of metabolism to achieve a balance between the anabolic and catabolic processes and maintain whole body homeostasis. This hypothesis was tested in this dissertation with four experiments using a novel *in ovo* stable isotope injection approach. This approach allowed us to determine the relative contributions of various classes of macronutrients to various pathways of metabolism (gluconeogenesis, glycogen synthesis and NEAA synthesis).

CHAPTER 2: EXPERIMENT 1

GLUCONEOGENESIS AND GLUCOSE CARBON UTILIZATION IN EMBRYOS FROM SMALL AND LARGE CHICKEN EGGS[1, 2]

[1]Funded by a Maryland Agricultural Experiment Station grant to Brian J. Bequette.
[2] Presented in part at the annual meetings of the Federation of American Societies for Experimental Biology: April 28–May 2, 2007, Washington DC, USA [Sunny NE, Adamany J, Bequette BJ. 2007. Gluconeogenesis and carbon utilization in embryos from small and large chicken eggs.]

Abstract

The objective of this study was to quantify glucose metabolism and the contribution of glucose to non-essential amino acid synthesis during day 12 to 18 of embryonic development in small and large egg embryos. [U-^{13}C]Glucose (15 mg in 75 μL water) was injected into the chorio-allantioic fluid of small (mean ± standard deviation, 51.1 ± 3.46g) and large (65 ± 4.35g) eggs for three consecutive days prior to tissue and blood collection on day 12, 14, 16 and 18 of embryonic development (5 eggs/age group). Blood and liver were analyzed by gas chromatography mass spectrometry for ^{13}C-mass isotopomer distribution in glucose, non-essential amino acids and Krebs cycle intermediates. On days 16 and 18, the weight of embryos from small eggs was less than that for large eggs (17.1 and 20.5 g vs. 23.4 and 26.8 g; $P<0.05$). For both small and large embryos, rates of glucose entry (0.16 to 0.92 g/d), glucose carbon recycling (7 to 33%) and gluconeogenesis (21 to 68%) increased ($P < 0.05$) from day 12 to 18. These rates were significantly different between small and large embryos only on days 12 ($P < 0.05$) and 14 ($P < 0.1$) embryonic. From day 12 to 18 for both sizes of eggs, glucose made a small but increasing ($P < 0.05$) contribution to liver alanine (5 to 20%), aspartate (0.2 to 6%) and glutamate (0.9 to 13%) synthesis and to Krebs cycle intermediate fluxes. In conclusion, small egg embryos, despite their smaller size, maintained similar rates of gluconeogenesis compared to large egg embryos. Furthermore, the gradual increase in metabolic utilization of glucose during incubation suggests that chick embryos may be adapting their metabolism for post hatch carbohydrate utilization.

Key words: *In ovo*, Chicken, Embryo, Glucose, Amino acid, Metabolism

Introduction

The avian embryo develops in a macronutrient environment that is comprised of mainly lipids and proteins and with very little carbohydrates (<3%; Romanoff and Romanoff, 1967). This low carbohydrate content of the egg would necessitate high rates of gluconeogenesis by the developing embryo and this is consistent with the observation that the activity of key gluconeogenic enzymes increases with embryonic age (Pearce 1971). To date, however, there have been no direct measures of the quantitative aspects of glucose metabolism and utilization during chicken embryonic development. Furthermore, because there is a high degree of connectivity between gluconeogenesis/ glycolysis and the Krebs cycle, glucose carbon would be predicted to be a significant contributor to non-essential amino acid (NEEA) synthesis, as has previously been observed in the neonatal humans (Miller *et al*., 1996).

To date, it is clear that during chicken embryonic development glucose is not a significant oxidative substrate as compared to the post hatch chick where glucose oxidation is 20-fold greater (Goodridge, 1968). However, the embryonic metabolic machinery undergoes a transition during the later stages of incubation adapting from *in ovo* lipid metabolism to carbohydrate metabolism post hatch. Thus with increasing *in ovo* availability of glucose via gluconeogenesis, the contribution of glucose carbon to NEAA synthesis and energy metabolism also increases with embryo development.

Egg size and the albumen: yolk ratio has been shown to correlate with body weight at hatch and growth rate in poults and chicks (Applegate *et al*., 1999; Applegate and Lilburn, 1999; Lourens *et al*., 2006). Lourens *et al*., (2006) concluded that the differences in body weight at hatch of chicks from small vs. large eggs is due to the

surplus supply of nutrients in the large eggs and the size of the residual yolk sac at hatch. Consistent with this view is the demonstration that *in ovo* administration of nutrients increased the glycogen content of the liver, accelerated embryonic enteric development and increased body weight at hatch (Coles *et al.*, 1999, 2003; Ohta *et al*., 1999; Tako *et al*., 2004). Uni *et al*. (2005) hypothesized that the basis for these responses was that the administered glucose and amino acids reduced the catabolism of albumin derived amino acids for gluconeogenesis, thus sparing them for muscle and tissue protein synthesis. Furthermore, Speake *et al*., (1998) observed an increase in subcutaneous fat in large egg embryos compared to their smaller counterparts, and suggested that these larger fat stores could provide an easily available energy source during the first 1 to 2 days post hatch when feed intake is limited.

The main objective of this study was to quantify glucose metabolism by small and large chicken embryos during the latter period (days 12 to 18) of incubation. A secondary objective was to determine the contribution of glucose carbon to NEAA synthesis and Krebs cycle metabolism. We hypothesized that the macronutrient environment of the egg may be limiting for optimal embryonic metabolism and development. Thus, we predicted that small egg embryos would not be able to maintain the high rates of gluconeogenesis required for normal metabolism and glycogen synthesis compared to embryos from large eggs. To test this hypothesis, we developed and employed a novel stable isotope ([U-^{13}C] glucose) approach *in ovo* to acquire quantitative rates of glucose metabolism and the contribution of glucose to NEAA synthesis in small compared to large egg embryos between days 12 and 18 of incubation.

Materials and Methods

Egg Incubation and Experimental Protocol

Fertilized eggs were obtained from Perdue Farms, Inc.(Salisbury, MD) from two broiler flocks of different ages, one flock 30 weeks old (small eggs; mean ± standard deviation, 51.1 ± 3.46g; n = 40) and the other 40 weeks old (large eggs; 65 ± 4.35g; n = 40). Small and large eggs were incubated at standard temperature (37° C) and relative humidity (65%). On day 9 of incubation, all the eggs were candled for viable embryos. The minimum sample size required to detect a significant treatment effect with standard alpha (0.05) and power (80%) was determined to be five based on measured standard deviations of various response variables from a similar study from our lab (Sunny *et al*., 2004). In order to attain a minimum sample size of 5 at each sampling time point and to account for potential hatchability losses (35%) due to *in ovo* injection procedures, 8 viable eggs were randomly selected as a group. Four groups each from small and large eggs were incubated for collection on days 12, 14, 16 and 18 (blocks) of incubation.

In ovo [U-^{13}C]Glucose Tracer Validation

A pilot study was conducted to determine the time-course for enrichments of glucose and NEAA to reach isotopic (e.g. M+2, M+3, M+6) and isotopomer (e.g. M+2: M+3) steady-states following *in ovo* injection of [U-^{13}C] glucose. [U-^{13}C] glucose (15 mg in 75 µL of sterile water) was injected into the chorio-allantoic fluid of 4 eggs of the similar weight starting on day 14 of incubation. The air space end of the egg was sterilized with 70% ethanol before piercing the egg shell. A 25 gauge needle was used to inject the [U-^{13}C] glucose solution, which was deposited 2-3 mm beneath the egg shell

membrane into the chorio-allantoic fluid. The tracer was assumed to mix uniformly with the fluid compartment to which it was injected and absorbed by the developing embryo through the extensive chorio-allantoic capillary network. After 1, 2, 3 and 4 days of administering the [U-^{13}C] glucose, one egg was removed and sampled for blood glucose and liver NEAA isotopomer enrichments.

In ovo [U-^{13}C]Glucose Injection

One group each from small and large eggs was randomly selected on day 9, 11, 13 or 15 for injection of [U-^{13}C] glucose (99 atom percent ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, MA). Based on the pilot study, it was determined that isotopic and isotopomer steady state is achieved after three consecutive days of administering [U-^{13}C] glucose. Thus, in the main experiment, each egg was administered [U-^{13}C] glucose (15 mg in 75 µL of sterile water) into the chorio-allantoic fluid for three consecutive days before sample collection on the fourth day.

Sample Collection and Analysis

Following [U-^{13}C] glucose injection for three days, each group of eggs was dissected on the fourth day to collect blood and tissue samples. Thus, a group each of small and large eggs was dissected on days 12, 14, 16 and 18 of incubation. After removing the egg shell surrounding the air cell, the egg shell membrane was carefully peeled off to expose the extra embryonic membranes. Whole egg contents were then carefully transferred to a petri-dish, taking care that the vitelline vessels (artery and vein) were on the top and clearly visible. Embryos were blood sampled by making a small nick on the vitelline vessels and blood was withdrawn into a glass pasture pipette with a

rubber bulb. Blood was then transferred into a 2 ml tube and frozen immediately at -20ºC for later analysis. Liver, intestine and muscle tissues were dissected, rinsed with ice-cold normal saline to remove excess blood and other debris, and transferred into 2 ml plastic tubes for storage at -80ºC.

Amino Acid Enrichments

For all blood (100 μL) and tissue (50 mg) samples, NEAA were isolated by cation-exchange (AG 50W-X8 resin, 100-200 mesh; Bio-Rad Laboratories, Hercules, CA) and amino acids eluted from the resin with 2 volumes of 2 *M* NH_4OH followed by one volume of water. The eluate was freeze dried, reconstituted in 250 μL of double distilled water, dried under a stream of N_2 gas, and amino acids converted to their heptafluoro-butyryl isobutyl derivatives (MacKenzie and Tenaschuk, 1979ab) prior to separation by GC (Heliflex® AT™-Amino acid, 25 m × 0.53 mm × 1.20 μm, Alltech). Selected ion monitoring was done by gas chromatography-mass spectrometry (GC-MS; 5973N Mass Selective Detector coupled to a 6890 Series GC System, Agilent, Palo Alto, CA) under methane negative chemical ionization conditions. The following ions of mass-to-charge (m/z) were monitored: Alanine 321 – 324, Aspartate 421 – 425, Glycine 307 – 309, Serine 533 – 536, Proline 347 – 352, Glutamine 361 – 366, Glutamate 435 – 440 and Arginine 778 – 784.

Glucose Enrichments

For determination of blood glucose enrichments, samples (100 μL) were acidified with ice-cold 15% sulpho salicylic acid (w/v) and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The elute containing free glucose was collected after

passing the supernatant over 0.5g of cation exchange resin. The solution was concentrated by freeze drying and analysed by GC-MS for glucose enrichment after forming the di-O-isopropylidene acetate derivative of glucose. After separation using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas, selective ion monitoring of ions with m/z 287 – 292 was performed with MS under electrical ionization mode (Hannestad and Lundblad, 1997).

Glucose and Amino Acid Concentrations

For determination of glucose and amino acid concentrations in embryonic blood, isotope dilution with mass spectrometry was employed (Calder *et al*., 1999). To a known weight (0.1 g) of blood was added an equivalent known weight of a solution containing 400 mg hydrolyzed [U-^{13}C]algae protein powder (99 atom % ^{13}C; Martek Biosciences Corp., Columbia, MD), 0.874 μmol [indole-D_5]tryptophan, 1.18 μmol [methyl-D_3]methionine, 1.94 μmol [U-^{13}C]glutamate, 5.42 μmol [U-^{13}C]glutamine, 2.15 μmol [U-^{13}C]arginine and 53.6 μmol [U-^{13}C; 1, 2, 3, 4, 5, 6, 6- D_7]glucose and the samples frozen at -20° C. Subsequently, these samples were processed for measurement of amino acid concentrations by forming the tertiary-butyldimethylsilyl derivative prior to GC-MS under electron ionization conditions (El-Kadi et al., 2006) using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas. Calibration curves were generated from gravimetric mixtures of labeled and unlabeled amino acids. For all the NEAA that had become ^{13}C-labelled, a correction was made to account for isotopomer (M+1, M+2 and M+3) abundances.

For determining the glucose concentrations, blood samples were processed and GC-MS performed as discussed above for glucose enrichments with m/z 287 and 300.

Enrichment of Krebs Cycle Intermediates in Liver

After deproteinising the liver (100mg) with 1.5mL of 15% sulpho-salicylic acid (w/v), 5 mmol of freshly prepared hydroxylamine hydrochloride (1 mL) was added to the supernatant and neutralized with 2 mol/L potassium hydroxide. These samples were then sonicated (15 min) and allowed to react at 65º C for 1 h. The solution was then titrated to pH < 2 with hydrochloric acid (6 mol/L), saturated with sodium chloride and Krebs cycle acids extracted twice with 3 ml ethyl acetate. The organic phase was blown down under a stream of N_2 and the tertiary butyldimethylsilyl derivative formed prior to separation on a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1µm Hewlett-Packard; helium as carrier gas) and selective ion monitoring by MS under electrical ionization mode (Des Rosiers *et al*., 1994). Ions with m/z were monitored: lactate 261 – 264, pyruvate 274 – 277, succinate 289 – 293, malate 419 – 423, oxaloacetate 432 – 436 and ketoglutarate 446 – 451.

Calculations

The normalized crude ion abundances for glucose, amino acids and Krebs cycle intermediates were corrected for the natural abundance of stable isotopes present in the original molecule and that contributed by the derivative ^{13}C using the matrix approach (Fernandez *et al*., 1996). Natural isotopomer distributions in unlabelled glucose and amino acids were quantified from blood samples taken from embryos that had not received the isotopic tracer. Corrected enrichments are reported as moles of tracer (M+*n*) per 100 moles of tracee (M+0) for the calculations described below.

Apparent glucose entry was calculated as:

(93/ [M+6]glucose) × [U-^{13}C] glucose injected (g/d) (1)

where 93 is the isotopic purity of the [U-^{13}C] glucose tracer.

Glucose carbon recycling was calculated as described previously by Pascual *et al*. (1997).

$$([M+1] + [M+2] \times 2 + [M+3] \times 3)/([M+1] + [M+2] \times 2 + [M+3] \times 3 + [M+6] \times 6) \quad (2)$$

where [M+*n*] is moles of tracer per 100 moles of tracee for blood glucose.

The minimum estimate of gluconeogenesis was calculated as:

$$([M+1] + [M+2] + [M+3]) / ([M+1] + [M+2] + [M+3] + [M+6]) \quad (3)$$

where [M+*n*] is moles of tracer per 100 moles of tracee for blood glucose.

Catabolism of glucose and glucose carbon recycling leads to the synthesis of [M+3] and [M+6]glucose. Thus, metabolism of these 2 glucose isotopomers leads to the synthesis of [M+3]pyruvate isotopomer, and subsequently [M+3]pyruvate, [M+3]alanine, [M+3]oxaloacetate and [M+3]aspartate. Because these [M+3] isotopomers can only arise from catabolism of [M+3] and [M+6]glucose, product:precursor relationship can be caluated to determine the contribution of glucose to alanine and aspartate fluxes as:

$$[M+3]\text{alanine or asparate} / ([M+6]\text{glucose} + 0.5 \times [M+3]\text{glucose}) \quad (4, 5)$$

The assumption applied here is that the intracellular labeling of the amino acid arises only by synthesis from extracellular (blood) glucose and not by uptake of the labeled NEAA from blood (Pascual *et al*., 1998).

Furthermore, the contribution of glucose to glutamate flux can be calculated as:

$$2 \times [M+3]\text{glutamate} / ([M+6]\text{glucose} + 0.5 \times [M+3]\text{glucose}) \quad (6)$$

Here, [M+3]glutamate is multiplied by a factor of two because the [M+3] glutamate isotopomer enrichment is 50% lower as a consequence of the equilibrium reaction

between oxaloacetate and fumarate and the subsequent loss of half the [M+3] isotopomer enrichment between citrate and α-ketoglutarate (Pascual *et al*., 1998).

Statistical Analysis

After verifying the assumptions of normality and homogeneity of variance, results were analyzed by ANOVA using mixed procedure of SAS (version 8.0, SAS Institute, Inc. Cary, NC) with small and large eggs as treatment groups and days of incubation as the blocking factor. Treatment means were compared by Tukey-Kramer multiple comparison test. Data are presented as least square means ± SEM and the differences are considered significant at $P < 0.05$ while $P < 0.1$ was considered a trend.

Results

Embryo Weights

On days 12 and 14 of incubation, embryonic weights (**Table 2.1**) of the small and large eggs did not differ. From day 16 onwards, however, embryos from large eggs were heavier than those from small eggs ($P < 0.05$). On day18 of incubation, embryos from large eggs were 14.7% heavier than those from small eggs.

Glucose Metabolism

Figure 2.1 shows the time-course of enrichments of [M+3] and [M+6] glucose isotopomers in embryonic blood after 1, 2, 3 or 4 days of daily injection of [U-^{13}C] glucose into incubating eggs. By day 3, glucose isotopic and isotopomer steady state had been attained. Thus, in the main study, glucose tracer was administered for 3 consecutive days prior to blood and tissue sampling.

On day 12 of incubation, blood [M+6] glucose enrichments were higher ($P < 0.05$) compared to those on days 14, 16 and 18 (**Figure 2.2**). Furthermore, on day 12, [M+6] glucose enrichment was higher ($P < 0.05$) in the blood of embryos from large compared to small eggs. Enrichments of [M+1], [M+2] and [M+3] glucose isotopomers were not different between small and large egg embryos. However, there was a significant increase ($P < 0.05$) in the enrichment of these lower mass isotopomers between days 12 to 18 of incubation.

Glucose metabolism in small and large e**gg** embryos is summarized in **Table 2.1**. Blood glucose concentrations increased ($P < 0.5$) from day 12 to 16 of incubation, and these did not differ between small and large egg embryos. Small egg embryos had a higher rate of glucose entry ($P < 0.5$), glucose carbon recycling ($P < 0.1$) and gluconeogenesis ($P < 0.1$) on day 12 and 14 of incubation compared to large egg embryos. From day 16 of incubation onwards, small and large egg embryos had similar rates of glucose entry, glucose carbon recycling and gluconeogenesis.

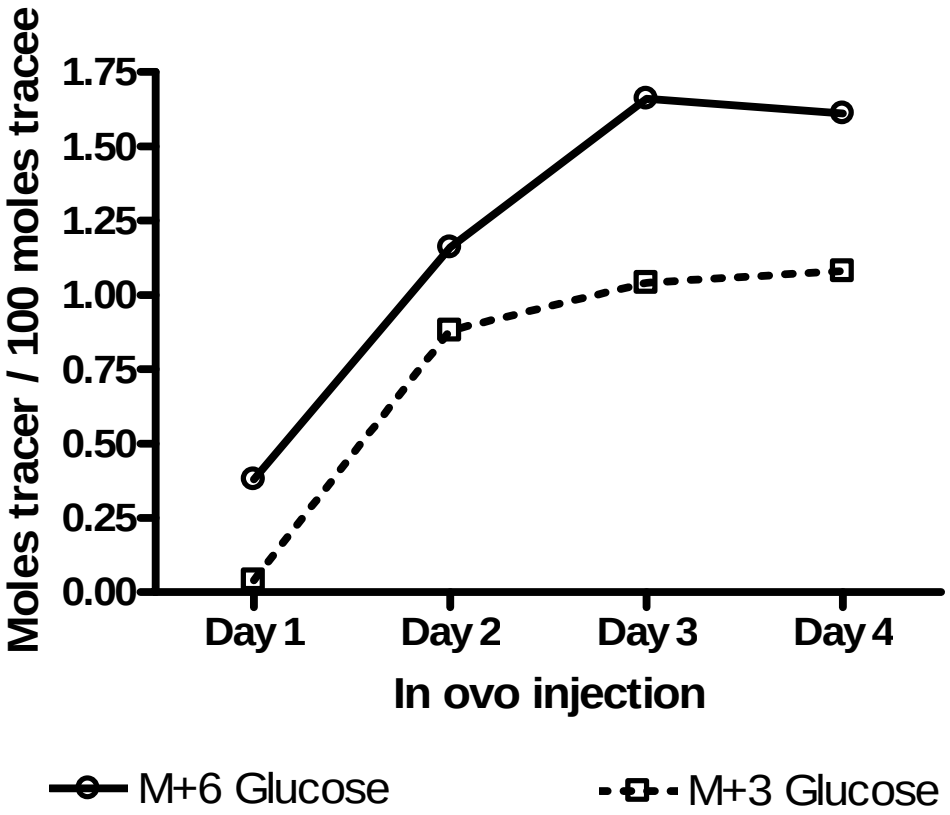

Figure 2.1: Enrichments of M+3 and M+6 isotopomers during 4 days of [U-^{13}C] glucose injection for validating the tracer approach. Glucose isotopomers were in metabolic steady state by three days of tracer injection.

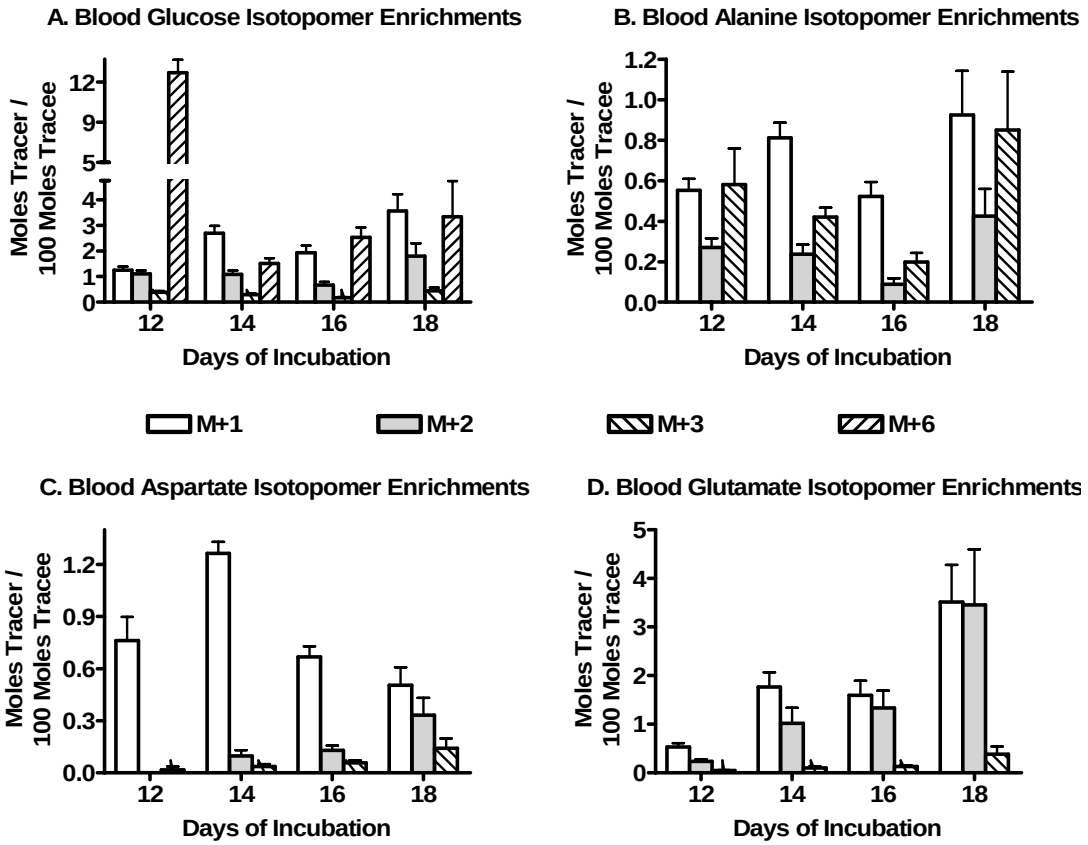

Figure 2.2: Enrichments of **A**. Glucose isotopomers, **B**. Alanine isotopomers, **C**. Aspartate isotopomers and **D**. Glutamate isotopomers in blood of small and large chicken embryos during 12 to 18 days of incubation after 3 days of [U-^{13}C]glucose injection. Enrichments are expressed as moles of tracer per 100 moles or tracee. Each bar represents an average of five embryos

Table 2.1: Embryonic weights and metabolism of glucose in small and large embryos during day 12 to 18 of development[1].

	Egg type[2]	Day of Incubation[3]					Day of Incubation effect	Egg type Effect[4]
		12	14	16	18	SEM		
Embryo weights (g)	**S**	5.78^{d}	10.53^{c}	17.07^{b}	23.36^{a}	0.388	< 0.001	<0.001
	L	5.99^{d}	12.35^{c}	20.45^{b}	26.80^{a}	0.768		
Blood Glucose Concentration (mg/dL)	**S**	84.6^{b}	.	112.6^{a}	107.8^{a}	3.77	< 0.001	NS
	L	90.2^{b}	.	118.4^{a}	111.4^{a}	3.36		
Glucose entry (g/d)	**S**	0.211^{b}	1.29^{a}	0.522^{b}	0.884^{ab}	0.222	< 0.001	NS
	L	0.077^{b}	0.812^{a}	0.699^{a}	0.823^{a}	0.188		
Glucose carbon recycling (%)	**S**	11.1^{c}	45.7^{a}	21.4^{bc}	30.5^{ab}	5.36	< 0.001	NS
	L	3.8^{c}	34.5^{a}	14.8^{b}	36.6^{a}	3.62		
Fractional Gluconeogenesis (%)	**S**	30.0^{c}	77.7^{a}	54.9^{b}	66.9^{ab}	4.77	< 0.001	0.011
	L	12.2^{c}	68.9^{a}	47.6^{b}	68.9^{a}	3.37		

[1]Values are means of five embryos
[2]**S** = small egg embryos**, L** = large egg embryos
[3]Means with different superscripts are significantly different from each other
[4]NS = non-significant

Contribution of Glucose to NEAA Synthesis

The enrichments of M+1, M+2 and M+3 isotopomers of alanine, aspartate and glutamate in blood and liver free pools did not differ between small and large egg embryos at any stage of incubation. However it was of interest that the enrichments of most of these isotopomers were higher in the blood and liver of small embryos on day 18. Further there were significant differences ($P < 0.5$) in the enrichments of these isotopomers of all the three amino acids in blood (**Figure 2.2**) and liver with increasing days of incubation. Thus in the blood, a greater proportion of alanine (5 to 20%), aspartate (0.2 to 6%) and glutamate (0.9 to 13%) was derived from glucose carbon between day 12 to 18 of embryonic development (**Figure 2.3**) with a similar scenario in the liver.

Enrichments of Krebs Cycle Intermediates in Liver

Due to limited amounts of tissue samples, the enrichments of M+1, M+2 and M+3 in lactate, pyruvate and α-keto glutarate were measured in livers from day 16 and 18 embryos (**Appendix 1E**). Lactate, pyruvate and α-keto glutarate isotopomers were more highly enriched ($P < 0.05$) on day 18 except for M+3 α-keto glutarate which was not significantly different. In general there were no differences between small and large embryos in the enrichments of M+1, M+2 and M+3 in lactate, pyruvate and α-keto glutarate.

Figure 2.3: Contribution of glucose to alanine (Ala), aspartate (Asp) and glutamate (Glu) in chicken embryos form days 12 to 18 of embryonic development

Amino Acid Concentrations

Amino acid concentrations in the embryonic blood are presented in **Table 2.2** for the NEAA and **Table 2.3** for essential amino acids. There was a significant effect with days of incubation ($P < 0.05$) on the concentrations of all the amino acids measured except tyrosine. A significant difference ($P < 0.05$) was observed between small and large egg embryos for arginine, glutamine, glycine, proline, isoleucine, leucine, threonine, tyrosine and valine, with embryonic blood from large egg embryos having higher concentrations of these amino acids.

Table 2.2: Blood non-essential amino acid concentrations (μmol/L) in small and large embryos during day 12 to 18 of incubation[1]

Amino acid[2]	Egg Type[3]	Day of Incubation[4]				SEM	Period effect[5]	Treatment Effect[5]
		12	14	16	18			
Ala	**S**	282.4^{b}	293.3^{b}	391.5^{a}	280.3^{b}	24.08	0.005	NS
	L	350.6^{a}	232.1^{b}	322.5^{a}	324.7^{a}	29.69		
Asp	**S**	132.1^{a}	83.2^{b}	123.0^{a}	58.6^{b}	13.85	< 0.001	NS
	L	120.0^{a}	56.6^{c}	85.1^{b}	79.4^{b}	6.46		
Glu	**S**	435.0^{a}	155.2^{b}	176.5^{b}	150.3^{b}	28.83	< 0.001	NS
	L	392.8^{a}	153.9^{c}	202.0^{b}	161.0^{c}	11.48		
Gln	**S**	1103.1^{a}	770.7^{b}	1021.1^{a}	943.8^{ab}	74.63	< 0.001	< 0.001
	L	1354.3^{a}	1143.4^{b}	1501.2^{a}	1468.2^{a}	61.65		
Gly	**S**	743.3^{b}	949.3^{a}	767.9^{b}	408.9^{c}	43.02	< 0.001	< 0.001
	L	733.6^{c}	971.0^{b}	1179.7^{a}	595.8^{c}	55.69		
Pro	**S**	241.2^{b}	258.4^{b}	342.2^{a}	375.5^{a}	24.38	< 0.001	< 0.001
	L	269.5^{c}	307.9^{c}	405.5^{b}	520.4^{a}	29.23		
Ser	**S**	630.0^{ab}	533.1^{b}	587.8^{ab}	699.6^{a}	42.19	< 0.001	NS
	L	644.2^{b}	435.3^{d}	574.7^{c}	734.5^{a}	23.23		

[1]Values are means of 5 to 10 embryos
[2]Ala = alanine, Asp = aspartate, Glu = glutamate, Gln = glutamine, Gly = glycine, Pro = proline, Ser = serine
[3]**S** = small egg embryos**, L** = large egg embryos
[4]Means with different superscripts are significantly different from each other
[5]NS = non-significant

Table 2.3: Blood essential amino acid concentrations (μmol/L) in small and large embryos during day 12 to 18 of incubation[1]

Amino Acid[2]	Egg Type[3]	Day of Incubation[4]				SEM	Period effect[5]	Treatment Effect[5]
		12	14	16	18			
Arg	**S**	358.4^{ab}	315.0^{ab}	367.9^{a}	287.3^{b}	23.37	< 0.001	< 0.001
	L	346.3^{b}	350.7^{b}	491.4^{a}	382.3^{b}	24.08		
Ile	**S**	283.4^{a}	279.2^{b}	256.1^{ab}	179.7^{c}	15.33	< 0.001	< 0.001
	L	282.1^{b}	230.5^{b}	388.7^{a}	297.9^{b}	18.47		
Leu	**S**	277.8^{a}	228.0^{b}	282.2^{a}	188.7^{b}	19.62	< 0.001	< 0.001
	L	299.2^{c}	326.4^{bc}	425.1^{a}	359.2^{b}	17.70		
Lys	**S**	460.1^{b}	399.0^{b}	844.6^{a}	226.3^{c}	40.98	< 0.001	NS
	L	457.0^{b}	482.4^{b}	944.2^{a}	267.2^{c}	45.67		
Met	**S**	86.4^{a}	68.4^{b}	83.5^{ab}	90.5^{a}	6.82	< 0.001	NS
	L	67.7^{b}	58.8^{b}	67.5^{b}	119.7^{a}	6.53		
Phe	**S**	119.1^{ab}	98.9^{b}	126.3^{a}	120.3^{ab}	9.40	0.007	NS
	L	123.0^{a}	103.2^{b}	117.9^{ab}	132.0^{a}	5.58		
Thr	**S**	427.1^{a}	414.7^{a}	361.5^{a}	464.4^{a}	36.55	0.010	0.010
	L	324.3^{b}	525.8^{a}	574.7^{a}	577.7^{a}	44.03		
Trp	**S**	153.7^{a}	126.1^{b}	126.2^{b}	106.7^{b}	8.33	0.008	NS
	L	130.8^{a}	129.3^{a}	133.5^{a}	130.9^{a}	2.39		
Tyr	**S**	412.1^{a}	371.2^{ab}	296.6^{bc}	246.3^{c}	38.31	NS	< 0.001
	L	370.6^{b}	467.9^{a}	406.0^{ab}	433.7^{ab}	26.96		
Val	**S**	458.5^{a}	356.7^{b}	363.7^{b}	423.0^{ab}	23.16	< 0.001	< 0.001
	L	442.5^{b}	435.9^{b}	478.4^{b}	603.4^{a}	25.45		

[1]Values are means of 5 to 10 embryos
[2]Arg = arginine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine
[3] **S** = small egg embryos**, L** = large egg embryos
[4] Means with different superscripts are significantly different from each other
[5] NS = non-significant

Discussion

The primary goal of this study was to characterize adaptations in glucose metabolism during the latter half of chicken embryonic development using a new *in ovo* [U-^{13}C]glucose stable isotope injection approach. [U-^{13}C]glucose was selected as the metabolic tracer as it distributes its carbon skeleton through central metabolic pathways including glycolysis and Krebs cycle metabolism thus allowing simultaneous quantification of glucose and amino acid metabolism (Bequette *et al*., 2006). Assuming that a 60g egg has a minimum of 250mg of free glucose (Romanoff and Romanoff, 1967), the 15mg [U-^{13}C]glucose injected per day *in ovo* in this study was only 6% of the available glucose in the egg and thus would not have accounted for any significant metabolic effects due to *in ovo* tracer.

Glucose Metabolism

Plasma glucose can be detected as early as 4 days of incubation with the embryonic gluconeogenic enzymes increasing to hatch with a corresponding increase in plasma glucose from 60 mg/dL early during embryonic development to 150 mg/dL at hatch (Hazelwood, 1971). Consistent with the evolving metabolism of the embryo, glucose metabolism differed considerably between days of incubation in this study. Glucose entry, glucose carbon recycling and gluconeogenesis were at the highest on day 14 of incubation, decreased from day 14 to 16 and remained unchanged on day 16 and 18. These changes in glucose metabolism observed in this study correlate well with the hormonal changes during embryonic development. Around day 13-14, hypophyseal and adrenocortical activity develops together with an increase in the release of epinephrine and a concomitant release of glycogen from the liver (Hazelwood 1971; Jenkins and

Porter, 2004). Around the same time the insulin: glucagon ratio starts increasing together with high circulating corticosterone, favoring deposition of glucose into glycogen (Lu *et al*., 2007). Further, these changes are associated with an increase in the blood glucose concentrations, as activities of gluconeogenic enzymes increase and attain maxima by day 16 to 17 of embryonic development and thereafter decrease towards hatch (Pearce, 1977). Thus an influx of glucose from glycogen breakdown in liver and the rapid increase in activities of gluconeogenic enzymes may be responsible for the high rates of glucose metabolism observed on day 14 in this study. Further, with the negative feedback exerted by corticosterone on hypophyseal and adrenocortical activity (Jenkins and Porter, 2004), glucose metabolism stabilizes over day 16 and 18.

Glucose to NEAA Synthesis

As glucose is a major energy substrate in various species and distributes its carbon via glycolysis and Krebs cycle metabolism, it can be a significant contributor to NEAA synthesis (Miller *et al*., 1996; Pascual *et al*., 1998). In this study, glucose carbon was not a major source for NEAA synthesis. Only 5-20% of alanine, 0.2-6% of aspartate and 0.9-13% glutamate flux derived from glucose carbon during day 12 to 18 of incubation. Further, no significant enrichments could be detected in serine, glycine, proline and arginine. On day 19 of embryonic development, the egg has more than one third of the total amino acid content still available for the embryo (Ohta *et al*., 1999). Thus synthesis of these amino acids may not be a priority for the late term embryo even with high rates of utilization for protein synthesis.

Despite the fact that only a small proportion of glucose carbon was recovered in the NEAA, the isotopomer profile (M+1, M+2, M+3) of alanine, aspartate and glutamate

in blood and liver showed a progressive increase in incorporation of glucose carbon with embryonic development (**Figure 2**). This is particularly true for the M+1 and M+2 of glutamate in the blood and liver with the moles tracer per 100 moles tracee increasing from 0.53 to 3.5 for M+1, 0.23 to 3.5 for M+2 in blood and 0.92 to 1.9 for M+1, 0.44 to 1.3 for M+2 in liver from day 12 to 18 of embryo development. Even though glucose can contribute to glutamate synthesis via oxaloacetate and acetyl co-A, the M+2 signal in glutamate arises predominantly due to the decarboxylation of the 3-carbon end product (pyruvate) of glycolysis by pyruvate dehydrogenase enzyme to form acetyl co-A. Thus the ratio of M+2 / M+3 of glutamate is a crude indicator of the activity of the flux of glucose carbon into Krebs cycle via pyruvate dehydrogenase (Pascual *et al*., 1998). In this study, this ratio showed a significant increase in blood (4.9 to 11.3) and liver (2.6 to 7.1) from 12 to 18 days of embryonic development. Similarly a higher day 18 enrichment profile was observed with the M+1 to M+3 isotopomers of α-keto glutarate, the corresponding Krebs cycle intermediate of glutamate compared to day 16. Goodridge, (1968) had observed higher rates of $^{14}CO_2$ production from [U-^{14}C]glucose in liver slices with increasing age of the embryo. Together with the data from this study, this indicates a progressive increase in the flow of glucose carbon through glycolysis and Krebs cycle metabolism with embryonic development. This is also evidence of a shift in metabolism where the embryo is evolving towards utilizing a predominantly carbohydrate rich diet post hatch from a lipid rich *in ovo* environment. The possibility that, higher incorporation of glucose carbon into amino acids and Krebs cycle intermediates is related to the altered or increased availability of the substrate, (glucose or three carbon units) needs further investigation.

Small and Large Embryos

In general, the small and large egg embryos maintained similar rates of gluconeogenesis and NEAA synthesis in spite of the fact that significant differences in embryonic weights were observed on day 16 and 18 of incubation. In this study the amino acid concentrations of glycine, glutamine, valine, leucine, isoleucine, threonine, arginine and tyrosine were lower ($P < 0.05$) in the blood of small embryos on day 16 and 18 and those of proline, aspartate and tryptophan were lower ($P < 0.05$) on day 18 of incubation. Thus the low embryonic weights of the small embryos could be partially explained by the lower availability of these amino acids for tissue protein synthesis. Further, the similar rates of gluconeogenesis maintained by small and large eggs in this study would be consistent with the observation of Lourens *et al*., 2006 that small and large embryos are equally efficient in energy transfer from the egg and that the differences in embryonic weights are due to the differences in available nutrients *in ovo*. Thus *in ovo* nutrient supplementation could be alleviating this nutrient limitation by increasing nutrient availability, thus increasing glycogen stores in the liver or accelerating the development of the gut. A further factor attributing to the differences in embryonic weights between small and large eggs could be the differences in yolk composition and the glycerol availability for glucose synthesis which is beyond the scope of discussion in this paper.

In conclusion, glucose metabolism changed with stages of development consistent with the evolving metabolic and hormonal patterns. However, despite differences in embryonic weight, glucose metabolism and the contribution of glucose carbon to NEAA synthesis did not differ between small and large embryos from day 12 to 18 of

incubation. Lastly, although there was a progressive increase in the incorporation of glucose carbon into NEAA, glucose was not the major source of carbon skeletons for NEAA synthesis in developing chicken embryos.

CHAPTER 3: EXPERIMENT 2

GLUCONEOGENESIS AND GUCOSE CARBON UTILIZATION IN CHICKEN EMBRYOS SUPPLEMENTED IN OVO WITH GLUCOSE AND AMINO ACIDS[1, 2]

[1]Funded by a Maryland Agricultural Experiment Station grant to Brian J. Bequette.
[2] Presented in part at ADSA PSA AMPA ASAS joint annual meeting, 2007. San Antonio, TX, USA [Sunny NE, Adamany J, Owens SL, Bequette BJ. 2007. Gluconeogenesis and carbon utilization in day 20 chicken embryos supplemented in ovo with glucose and amino acids]

Abstract

The aim of this study was to quantify gluconeogenesis and glucose carbon utilization on day 12 and 20 of embryonic development in small vs. large embryos supplemented *in ovo* glucose and/or amino acids. Groups of small (n = 5 to 9; mean ± standard deviation, 55.3 ± 0.98g) and large (n = 5 to 9; 66.8 ± 1.18g) eggs were randomly dosed into the chorio-allantoic fluid (250µL) on day 9 of embryonic development with either sterile water (**C**), glucose (100mg; **G**), an amino acid mixture (75 mg; **AA**) or a mixture of glucose and amino acids (50mg + 37.5mg; **G+AA**). Beginning day 9 or 17, each egg was given a daily dose of [U-^{13}C]glucose (15 mg/d in 75 µL sterile water) into the chorio-allantoic fluid followed by tissue and blood collection on day 12 and 20 respectively. Blood and tissues were analyzed by mass spectrometry for ^{13}C-mass isotopomer distribution in glucose, alanine, aspartate and glutamate. Embryonic weights on day 12 and 20 were lower ($P < 0.001$) for the small vs. large eggs, and *in ovo* nutrient treatments did not affect weights in either group. *In ovo* nutrients did not affect glucose metabolism; however, glucose entry (0.4 ± 0.06g/d vs. 0.8 ± 0.11g/d), glucose carbon recycling (16.9 ± 2.25% vs. 61.4 ± 2.12%) and gluconeogenesis (37.9 ± 3.60% vs. 84.6 ± 1.32%) increased ($P < 0.05$) from day 12 to day 20. The contribution of glucose carbon to alanine was greater ($P < 0.001$) for **C** (42.7%) and **G** (39.1%) compared to **AA** (28.2%) and **G+AA** (15.5%) treatments. Glucose flux to alanine was lowest ($P < 0.05$) in the liver when compared to other tissues. However, glucose flux to aspartate and glutamate were higher ($P < 0.05$) in liver compared to other tissues. Thus, despite differences in embryo weights, absolute rates of gluconeogenesis were similar for the small and large eggs. Further, fluxes of glucose to amino acids were different between blood and tissues

suggesting that the metabolic profile in blood is not always a true indicator of tissue nutrient needs.

Key words: *In ovo*, Chicken, Embryo, Glucose, Amino acid, Metabolism

Introduction

The final stages of chicken embryonic development (day 18-21) are characterized by rapid transition of the embryonic metabolic machinery, which maintains high rates of gluconeogenesis *in ovo* from amino acids and/or triglyceride-glycerol to maintain high rates of lipogenesis post hatch from dietary carbohydrates and amino acids (Pearce, 1977). At the same time, the embryo rapidly accumulates glycogen reserves in liver and muscle and also builds up subcutaneous fat as energy reserves for hatching (Hazelwood, 1971). Further, this phase is also characterized by rapid development of organ systems, especially the intestines thus rapidly depleting *in ovo* lipid and amino acid resources. A positive correlation has been noticed between glycogen reserves in various tissues and body weight at hatch (Christensen et al., 1999, 2001; John et al., 1988). Further, small egg embryos have lower subcutaneous fat depots compared to their larger counterparts (Speake *et al*., 1998). These observations raise the question; how does *in ovo* macronutrient availability affect the metabolic partitioning of substrates for various biosynthetic purposes?

In ovo administration of nutrients have been found to accelerate embryonic enteric development and increase body weight at hatch (Coles *et al.*, 1999, 2003; Ohta *et al*., 1999; Tako *et al*., 2004). Uni et al. (2005) found significantly higher glycogen reserves, increased weight at hatch and increased breast muscle size following *in ovo* supplementation on day 17.5 of incubation, a solution containing maltose, sucrose,

dextrin, and β-hydroxy-β-methylbutyrate into the amniotic fluid. Even though the metabolic basis for these responses is not clear, the authors attributed the improved energy status of *in ovo* fed embryos to reduced muscle protein mobilization for gluconeogenisis. In another study, *in ovo* injection of an amino acid mixture increased the crude protein content of day 19 embryos (Ohta *et al*., 1999). I*n ovo* administration of amino acids, carbohydrates or peptides may be altering fluxes through metabolic pathways, thereby alleviating competition for three carbon units for gluconeogenesis and sparing essential nutrients (amino acids or glucose) for tissue synthesis.

The aim of this study was to quantify glucose metabolism in small and large egg embryos (day 12 and 20) supplemented *in-ovo* with glucose and/or amino acids. We hypothesized that *in ovo* supplementation of glucose and/or amino acids will alleviate the competition for three-carbon units for gluconeogenesis, thus sparing amino acids for net tissue synthesis. Eggs supplemented *in ovo* with glucose and/or amino acids were hypothesized to maintain higher rates of gluconeogenesis. A further objective of this study was to characterize the flow of glucose carbon in to non-essential amino acids (NEAA) in day 20 embryos in response to *in ovo* glucose and/or amino acid supplementation. *In ovo* [U-^{13}C]glucose tracer injection coupled with mass isotopomer distribution analysis (MIDA) of blood glucose and tissue NEAA isotopomers were used to investigate the metabolic basis of the *in ovo* nutrient treatment responses.

Materials and Methods

Egg Incubation and Experimental Protocol

The experimental protocol was approved by the Animal Care and Use Committee of the University of Maryland. Fertilized, small (mean ± standard deviation, 55.3 ± 0.98g; n = 80) and large (66.8 ± 1.18g; n = 80) broiler eggs were obtained from Perdue Farms, Inc., Salisbury, MD from the same broiler flock (40 weeks of age) and incubated at 37°C temperature and 65% relative humidity. On day 9 of incubation, eggs were candled for viable embryos and randomly grouped into eight groups (n = 9) each of small and large eggs. Two groups each from small and large eggs were randomly allotted one of the 4 treatments (two sets/ treatment/ group) viz. sterile water (250µL, **C**), glucose (100mg in 250µL, **G**), an amino acid mixture (75mg in 200µL, **AA; Table 1**) or a mixture of glucose and amino acids (37.5mg **AA** plus 50mg **G** in 250 µL, **G+AA**). All the eggs were injected *in ovo* into the chorio-allantoic fluid, according to their respective treatments on day 9 of incubation.

In ovo [U-^{13}C]Glucose Injection

One group from each treatment was randomly selected from the small and large egg groups to receive an injection of a solution (75 µL) containing 15 mg [U-^{13}C]glucose (99 atom percent ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, MA) on day 9, daily for three days before blood and tissue collection on day 12 of incubation. The remaining treatment groups from small and large eggs were administered [U-^{13}C]glucose, as described above from day 17 of incubation for the next three days before blood and tissue collection on day 20.

The site of *in ovo* treatment and tracer injection was wiped sterile with 70% ethanol and the egg shell pierced to insert a 25 gauge needle. The treatments and [U-^{13}C]glucose tracer solution was deposited from the air cell side into the chorio-allantoic fluid for eggs sampled on day 12. However, as the embryos were larger in size during the last week of incubation, [U-^{13}C]glucose solution was deposited from the narrow end for the eggs sampled on day 20 to avoid injection of the tracer into the embryonic tissues. The tracer was assumed to mix uniformly with the fluid compartment to which it was injected and absorbed by the developing embryo through the extensive chorio-allantoic capillary network.

Sample Collection and Analysis

Following [U-^{13}C] glucose injection for three days, the egg shell around the air cell was removed and the egg shell membrane was carefully peeled off exposing the extra embryonic membranes on the forth day. Whole egg contents were then carefully transferred to a petri-dish taking care that the vitelline vessels (artery and vein) were on the top and clearly visible. Embryos were bled by making a nick across the vitelline vessels and blood was drawn using a glass pasture pipette with a rubber stopper, transferred into a 2 ml tube and frozen at immediately at -20ºC for later analysis. Liver, intestine, muscle and kidney tissues were harvested, rinsed with cold normal saline to wash off excess blood and other debris, transferred into 2 ml plastic tubes and immediately frozen at -80ºC.

Table 3.1: Composition of the amino acid mixture injected *in ovo* into small and large eggs on day 9 of embryonic development[1].

Amino acid	Amount (g)
Alanine	7.5
Arginine	5.3
Aspartic acid	3.0
Asparagine	2.0
Glutamate	3.5
Glutamine	3.5
Proline	5.2

[1]The amino acid mixture was dissolved in 100 mL of double distilled water, pH adjusted to 7.4 and 250μL of the solution containing 75mg of amino acids was injected into each egg.

Glucose Enrichments

For determination of blood glucose enrichments, samples (100 μL) were acidified with ice-cold 15% sulpho-salicylic acid (w/v) and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The elute containing free glucose was collected after passing the supernatant over 0.5g of cation exchange resin. The solution was concentrated by freeze drying and analysed by GC-MS for glucose enrichment after forming the di-O-isopropylidene acetate derivative of glucose. After separation using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas, selective ion monitoring of ions with mass-to-charge (m/z) 287 – 292 was performed with MS under electrical ionization mode (Hannestad and Lundblad, 1997).

Amino acid Enrichments

For all blood (100 μL) and tissue (50 mg) samples, NEAA were isolated by cation-exchange (AG 50W-X8 resin, 100-200 mesh; Bio-Rad Laboratories, Hercules, CA) and amino acids eluted from the resin with 2 volumes of 2 *M* NH_4OH followed by one volume of water. The eluate was freeze dried, reconstituted in 250 μL of double distilled water, dried under a stream of N_2 gas, and amino acids converted to their heptafluoro-butyryl isobutyl derivatives (MacKenzie and Tenaschuk, 1979ab) prior to separation by GC (Heliflex® AT™-Amino acid, 25 m × 0.53 mm × 1.20 μm, Alltech). Selected ion monitoring was done by gas chromatography-mass spectrometry (GC-MS; 5973N Mass Selective Detector coupled to a 6890 Series GC System, Agilent, Palo Alto, CA) under methane negative chemical ionization conditions. The following ions of m/z were monitored: Alanine 321 – 324, Aspartate 421 – 425, Glycine 307 – 309, Serine 533

– 536, Proline 347 – 352, Glutamine 361 – 366, Glutamate 435 – 440 and Arginine 778 – 784.

Glucose and Amino acid Concentrations

For determination of glucose and amino acid concentrations in embryonic blood, isotope dilution with mass spectrometry was employed (Calder *et al*., 1999). To a known weight (0.1 g) of blood was added an equivalent known weight of a solution containing 400 mg hydrolyzed [U-^{13}C]algae protein powder (99 atom % ^{13}C; Martek Biosciences Corp., Columbia, MD), 0.874 μmol [indole-D_5]tryptophan, 1.18 μmol [methyl-D_3]methionine, 1.94 μmol [U-^{13}C]glutamate, 5.42 μmol [U-^{13}C]glutamine, 2.15 μmol [U-^{13}C]arginine and 53.6 μmol [U-^{13}C; 1, 2, 3, 4, 5, 6, 6- D_7]glucose and the samples frozen at -20° C. Subsequently, these samples were processed for measurement of amino acid concentrations by forming the tertiary-butyldimethylsilyl derivative prior to GC-MS under electron ionization conditions (El-Kadi et al., 2006) using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas. Calibration curves were generated from gravimetric mixtures of labeled and unlabeled amino acids. For all the NEAA that had become ^{13}C-labelled, a correction was made to account for isotopomer (M+1, M+2 and M+3) abundances.

For determining the glucose concentrations, blood samples were processed and GC-MS performed as discussed above for glucose enrichments with m/z 287 and 300.

Calculations

The normalized crude ion abundances for glucose, amino acids and Krebs cycle intermediates were corrected for the natural abundance of stable isotopes present in the

original molecule and that contributed by the derivative ^{13}C using the matrix approach (Fernandez *et al*., 1996). Natural isotopomer distributions in unlabelled glucose and amino acids were quantified from blood samples taken from embryos that had not received the isotopic tracer. Corrected enrichments are reported as moles of tracer (M+*n*) per 100 moles of tracee (M+0) for the calculations described below.

Apparent glucose entry was calculated as:

$$(93/\ [M+6]\text{glucose}) \times [U\text{-}^{13}C]\ \text{glucose injected (g/d)} \quad (1)$$

where 93 is the isotopic purity of the [U-^{13}C] glucose tracer.

Glucose carbon recycling was calculated as described previously by Pascual *et al*. (1997).

$$([M+1] + [M+2] \times 2 + [M+3] \times 3) / ([M+1] + [M+2] \times 2 + [M+3] \times 3 + [M+6] \times 6) \quad (2)$$

where [M+*n*] is moles of tracer per 100 moles of tracee for blood glucose.

The minimum estimate of gluconeogenesis was calculated as

$$([M+1] + [M+2] + [M+3]) / ([M+1] + [M+2] + [M+3] + [M+6]) \quad (3)$$

where [M+*n*] is moles of tracer per 100 moles of tracee for blood glucose.

Catabolism of glucose and glucose carbon recycling leads to the synthesis of [M+3] and [M+6]glucose. Thus, metabolism of these 2 glucose isotopomers leads to the synthesis of [M+3]pyruvate isotopomer, and subsequently [M+3]pyruvate, [M+3]alanine, [M+3]oxaloacetate and [M+3]aspartate. Because these [M+3] isotopomers can only arise from catabolism of [M+3] and [M+6]glucose, product:precursor relationship can be caluated todetermine the contribution of glucose to alanine and aspartate fluxes as:

$$[M+3]\text{alanine or asparate} / ([M+6]\text{glucose} + 0.5 \times [M+3]\text{glucose}) \quad (4, 5)$$

The assumption applied here is that the intracellular labeling of the amino acid arises only by synthesis from extracellular (blood) glucose and not by uptake of the labeled NEAA from blood (Pascual *et al*., 1998).

Furthermore, the contribution of glucose to glutamate flux can be calculated as:

$$2 \times [M+3]\text{glutamate} / ([M+6]\text{glucose} + 0.5 \times [M+3]\text{glucose}) \quad (6)$$

Here, [M+3]glutamate is multiplied by a factor of two because the [M+3] glutamate isotopomer enrichment is 50% lower as a consequence of the equilibrium reaction between oxaloacetate and fumarate and the subsequent loss of half the [M+3] isotopomer enrichment between citrate and α-ketoglutarate (Pascual *et al*., 1998).

Statistical Analysis

After verifying for assumptions of normality and homogeneity of variance, results were analyzed by ANOVA using mixed procedure of SAS (version 8.0, SAS Institute, Inc. Cary, NC). Data was analyzed with the four *in ovo* nutrient supplements as treatment groups and small and large eggs as the blocking factor. Treatment means were compared by Tukey-Kramer multiple comparison test. Blood and tissue means were compared to each other using a *t*-test. Results are presented as least-squares means ± SEM and the differences are considered significant at $P \leq 0.05$ while $P \leq 0.1$ is considered a trend.

Results

Embryonic weights were different ($P < 0.001$) between small and large embryos both on day 12 (5.2 ± 0.09g verses 6.3 ± 0.09g) and 20 (31.9 ± 0.27g verses 37.0 ± 0.26g) of embryo development. However, *in-ovo* nutrient treatments did not affect embryonic weights on day 12 or 20 (**Table 3.2 and 3.3**).

Glucose Metabolism

The enrichment of [U-^{13}C]glucose isotopomers in blood of day 12 and day 20 chicken embryos following *in ovo* nutrient supplementation is presented in **appendix 2A and 2B** respectively. In general the [U-^{13}C]glucose (M+6) enrichment in the blood of day 12 embryos was higher ($P < 0.05$) than those of day 20 embryos. The metabolism of glucose was more than two fold higher ($P < 0.05$) in day 20 embryos compared to that of day 12 embryos (**Table 3.2 and 3.3**). Thus the entry of glucose increased from 0.39g /d in day 12 embryos to 0.79g /d in day 20 embryos with a corresponding increase in glucose carbon recycling (16.9% to 60.8%) and fractional gluconeogenesis (37.8% to 84.2%).

For both day 12 and day 20 embryos, the metabolism of glucose was similar between the **C** group and the nutrient supplemented groups. However, on day 12, small and large embryos showed differences in glucose entry rates ($P = 0.016$) and glucose carbon recycling ($P = 0.048$) while on day 20, small and large embryos maintained similar rates of glucose metabolism.

Blood glucose concentrations also increased ($P < 0.05$) form 123.9mg /dL on day 12 to 153.7mg /dL on day 20 of development. Blood glucose concentrations between *in ovo* nutrient treatment groups were different for both small ($P = 0.005$) and large ($P = 0.003$) egg embryos on day 12. However glucose concentrations between *in ovo* treatment groups on did not show any statistical significance on day 20 of incubation.

Table 3.2: Embryonic weights and metabolism of glucose in small and large embryos on day 12 of development after *in ovo* nutrient supplementation[1]

	Egg type[2]	*In ovo* nutrient treatments[3]					*In ovo* nutrient Effect[4]	Egg type Effect[4]
		C	**G**	**AA**	**G+AA**	**SEM**		
Embryo weights (g)	**S**	5.27^{a}	5.29^{a}	4.96^{a}	5.44^{a}	0.199	NS	< 0.001
	L	6.39^{a}	6.22^{a}	6.42^{a}	6.28^{a}	0.151	NS	
Blood Glucose Concentration (mg/dL)	**S**	117.9^{b}	124.0^{b}	114.2^{b}	139.8^{a}	4.60	0.005	NS
	L	114.0^{c}	128.6^{ab}	120.3^{bc}	132.9^{a}	3.44	0.003	
Glucose entry (g/d)	**S**	0.45^{a}	0.35^{ab}	0.12^{c}	0.22^{bc}	0.065	0.014	0.016
	L	0.43^{a}	0.59^{a}	0.31^{a}	0.51^{a}	0.150	NS	
Glucose carbon recycling (%)	**S**	9.8^{b}	18.9^{a}	12.1^{ab}	13.5^{ab}	2.56	NS	0.048
	L	15.3^{a}	19.3^{a}	27.0^{a}	19.0^{a}	5.76	NS	
Fractional Gluconeogenesis (%)	**S**	24.8^{b}	44.0^{a}	31.3^{ab}	34.4^{ab}	4.93	NS	NS
	L	34.5^{a}	42.1^{a}	52.1^{a}	39.4^{a}	8.60	NS	

[1]Values are means of five embryos
[2]**S** = small egg embryos**, L** = large egg embryos
[3]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other
[4]NS = non-significant

Table 3.3: Embryonic weights and metabolism of glucose in small and large embryos on day 20 of development after *in ovo* nutrient supplementation[1]

	Egg type[2]	*In ovo* nutrient treatments[3]					*In ovo* nutrient Effect[4]	Egg type Effect[4]
		C	**G**	**AA**	**G+AA**	**SEM**		
Embryo weights (g)	**S**	31.4[a]	32.6[a]	31.5[a]	32.5[a]	0.630	NS	< 0.001
	L	36.7[a]	37.5[a]	37.5[a]	37.9[a]	0.551	NS	
Blood Glucose Concentration (mg/dL)	**S**	158.4[a]	160.4[a]	151.8[ab]	144.9[b]	4.12	0.050	NS
	L	148.0[a]	154.8[a]	158.7[a]	152.9[a]	5.54	NS	
Glucose entry (g/d)	**S**	0.82[a]	0.88[a]	0.82[a]	0.68[a]	0.233	NS	NS
	L	0.81[a]	0.67[a]	0.79[a]	0.81[a]	0.173	NS	
Glucose carbon recycling (%)	**S**	54.9[a]	61.3[a]	62.2[a]	58.0[a]	4.83	NS	NS
	L	68.4[a]	63.9[ab]	65.1[ab]	57.2[b]	3.61	NS	
Fractional Gluconeogenesis (%)	**S**	80.1[a]	84.7[a]	85.1[a]	82.6[a]	3.16	NS	NS
	L	88.8[a]	85.7[ab]	86.8[ab]	82.2[b]	2.18	NS	

[1]Values are means of five embryos
[2]**S** = small egg embryos**, L** = large egg embryos
[3]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other
[4]NS = non-significant

Contribution of Glucose to Alanine, Aspartate and Glutamate

The enrichments of alanine, aspartate and glutamate isotopomers (M+1, M+2, M+3) in blood and tissues of day 20 embryos after *in ovo* nutrient supplementation, is presented in **appendices 2C, 2D and 2E**. In general, the enrichments of isotopomers in all the three amino acids were similar between small and large embryos in the blood and tissues measured. However with *in ovo* nutrient treatments, differences in isotopomer enrichments ($P \leq 0.1$) were observed for alanine in blood, liver and kidney; for aspartate in liver, intestine and kidney; and for glutamate in blood, liver and kidney. Further, with the **C** treatment and all the three *in ovo* nutrient treatments the isotopomer enrichments of all the three amino acids showed significant differences ($P \leq 0.05$) between the different pools sampled (blood and tissues).

The main results from the isotopomer profile of amino acids are presented below. For the **C**, **G** and **AA** treatments, [M+3] aspartate enrichment in liver and kidney was similar and significantly higher ($P < 0.05$) than blood, intestine and muscle (**Appendix 2D**). For the G+AA treatment, [M+3] aspartate enrichment in the kidney was lower compared to the liver. With all the treatments, [M+3] glutamate in the liver was higher ($P < 0.001$) than the [M+3] glutamate enrichments in other tissues and blood. Further, among the *in ovo* nutrient treatments, **G** injection resulted in the highest ($P < 0.001$) [M+3] aspartate enrichment in liver (**Appendix 2E**).

The proportional contributions of glucose to alanine, aspartate and glutamate are presented in **Table 3.4**. The calculation for the contribution of glucose carbon to amino acids is based on the precursor to product relationship of the [M+3] amino acid isotopomer in blood or tissues and the M+3 and M+6 isotopomers of blood glucose. The

cumulative errors (e.g. due to tracer injection techniques, blood sampling etc) in measurement of the enrichment of blood glucose isotopomers is higher compared to the errors in the measurement of amino acid enrichments. Further, the isotopomer enrichments of the amino acids were similar between small and large egg embryos. Thus the proportional contributions are presented as a combined average of the values from both small and large egg embryos.

The contribution of glucose to alanine was different with *in ovo* nutrient supplementation only in blood ($P < 0.05$) and liver ($P = 0.02$), whereas significant differences in the contribution of glucose to glutamate was detected only in the liver ($P < 0.05$). No differences were detected in the contribution of glucose to aspartate with nutrient supplementation. Significant differences ($P < 0.05$) however existed for the contribution of glucose to amino acids between blood and the different tissues sampled. Thus, the average contribution of glucose to alanine in kidney (38%) was greater than that in the liver (20%) with a lower proportion of aspartate (11% and 8%) and glutamate (14% and 9%) deriving from glucose carbon in liver and kidney respectively (**Table 3.4**).

Table 3.4: Contribution of glucose to alanine, aspartate and glutamate in blood and tissues in day 20 chicken embryos supplemented with *in ovo* nutrients[1].

	***In ovo* nutrient treatments[2]**	**Blood**	**Liver**	**Intestine**	**Muscle**	**Kidney**	**SEM**	**Blood and tissue effect[3]**
Glucose to alanine (%)	**C**	42.7	25.1	43.6	43.1	34.1	5.97	NS
	G	39.1	13.4	32.5	29.6	44.7	3.29	< 0.001
	AA	28.2	24.7	33.0	37.1	41.9	3.47	0.006
	G+AA	15.5	16.9	29.7	34.9	29.8	4.24	0.005
	Treatment effect	< 0.001	0.020	NS	NS	NS		
Glucose to aspartate (%)	**C**	1.7	10.7	5.0	4.8	8.9	1.34	< 0.001
	G	2.2	8.6	4.2	2.4	7.0	0.85	< 0.001
	AA	2.0	12.6	5.1	4.2	9.5	1.28	< 0.001
	G+AA	1.7	12.1	5.2	3.8	5.3	1.00	< 0.001
	Treatment effect	NS	NS	NS	NS	NS		
Glucose to glutamate (%)	**C**	13.3	25.6	7.9	10.9	15.5	3.13	0.002
	G	12.0	36.8	6.7	7.9	7.2	2.09	< 0.001
	AA	11.8	15.9	7.6	2.1	7.8	2.16	NS
	G+AA	6.7	10.2	4.4	8.4	7.0	1.65	NS
	Treatment effect	NS	< 0.001	NS	NS	NS		

[1]Values are means both small and large embryos
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids
[3] NS = non-significant

Amino acid Concentrations

Concentrations of non essential and essential amino acids in day 12 embryonic blood are presented in **Tables 3.5** and **3.6** and those in day 20 embryonic blood are presented in **Tables 3.7** and **3.8**. The concentrations of the amino acids present in the *in ovo* nutrient mixture were significantly higher ($P < 0.05$) on day 12 in the **AA** and **G+AA** treatment groups compared to **C** and **G** treatments. However on day 20, this effect was not present and the concentrations between treatments were similar for most of the amino acids in the injection mixture. The branched chain amino acids leucine, isoleucine and valine showed significant treatment effects on day 12 ($P < 0.05$) with the concentrations of these amino acids higher than the **C** for the **G** and **G+AA** treatments. On day 20 the concentrations of the three branched chain amino acids were higher ($P < 0.05$) for all the nutrient treated groups compared to the **C**. *In ovo* nutrient supplementation, in general did not affect the concentrations of any other essential amino acids.

Table 3.5: Blood non-essential amino acid concentrations (μmol/L) in small and large embryos on day 12 of incubation following *in ovo* nutrient supplementation[1]

Amino acid[2]	Egg Type[3]	*In ovo* nutrient treatments[4]				SEM	*In ovo* nutrient Effect[5]	Egg type Effect[5]
		C	**G**	**AA**	**G+AA**			
Ala	**S**	281.2^{c}	278.9^{c}	1006.2^{a}	611.4^{b}	55.52	< 0.001	0.008
	L	293.7^{b}	323.9^{b}	630.5^{a}	547.2^{a}	43.55	< 0.001	
Asp	**S**	118.9^{c}	116.8^{c}	369.9^{a}	277.2^{b}	14.4	< 0.001	< 0.001
	L	122.6^{b}	92.2^{b}	265.3^{a}	226.6^{a}	14.72	< 0.001	
Glu	**S**	266.1^{d}	328.4^{c}	578.5^{a}	505.2^{b}	21.95	< 0.001	< 0.001
	L	255.4^{b}	258.0^{b}	434.5^{a}	411.5^{a}	20.23	< 0.001	
Gln	**S**	1608.7^{b}	1716.5^{b}	1797.9^{ab}	2015.5^{a}	79.34	0.012	NS
	L	1414.1^{c}	1814.1^{b}	2049.5^{a}	1934.8^{ab}	74.35	< 0.001	
Gly	**S**	759.9^{b}	896.3^{a}	670.6^{b}	973.0^{a}	41.54	< 0.001	NS
	L	722.0^{b}	798.8^{ab}	879.0^{a}	853.3^{ab}	45.10	NS	
Pro	**S**	249.8^{c}	275.4^{c}	616.5^{a}	391.4^{b}	36.56	< 0.001	0.047
	L	240.3^{c}	311.1^{bc}	413.4^{a}	373.1^{ab}	31.10	0.002	
Ser	**S**	690.9^{a}	727.8^{a}	524.6^{b}	730.6^{a}	46.85	0.019	NS
	L	689.7^{a}	663.8^{a}	491.7^{b}	641.9^{a}	32.17	< 0.001	

1Values are means of 5 to 10 embryos
[2]Ala = alanine, Asp = aspartate, Glu = glutamate, Gln = glutamine, Gly = glycine, Pro = proline, Ser = serine
[3]**S** = small egg embryos**, L** = large egg embryos
[4]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other
[5]NS = non-significant

Table 3.6: Blood essential amino acid concentrations (µmol/L) in small and large embryos on day 12 of incubation following *in ovo* nutrient supplementation[1]

Amino Acid[2]	Egg Type[3]	*In ovo* nutrient treatments[4]				SEM	*In ovo* nutrient Effect[5]	Egg type Effect[5]
		C	**G**	**AA**	**G+AA**			
Arg	**S**	375.1^{c}	378.2^{c}	623.0^{a}	480.4^{b}	26.33	< 0.001	NS
	L	355.2^{c}	408.4^{bc}	455.9^{ab}	507.8^{a}	23.10	0.001	
Ile	**S**	275.1^{c}	318.1^{b}	280.6^{bc}	358.4^{a}	13.23	< 0.001	NS
	L	262.3^{b}	338.9^{a}	349.2^{a}	350.4^{a}	12.73	< 0.001	
Leu	**S**	261.9^{c}	295.8^{b}	284.8^{bc}	364.6^{a}	11.86	< 0.001	NS
	L	241.5^{b}	338.3^{a}	330.2^{a}	359.8^{a}	13.42	< 0.001	
Lys	**S**	459.5^{a}	449.1^{a}	382.0^{a}	411.2^{a}	32.35	NS	NS
	L	437.1^{ab}	429.2^{ab}	361.9^{b}	511.1^{a}	31.52	0.035	
Met	**S**	74.5^{a}	80.0^{a}	81.4^{a}	89.6^{a}	5.97	NS	NS
	L	84.5^{a}	82.3^{a}	87.7^{a}	78.0^{a}	5.01	NS	
Phe	**S**	120.9^{b}	127.8^{ab}	129.2^{ab}	143.6^{a}	6.32	NS	NS
	L	120.4^{a}	132.0^{a}	134.9^{a}	135.3^{a}	8.28	NS	
Thr	**S**	347.2^{ab}	390.8^{a}	316.5^{b}	367.6^{ab}	20.63	NS	0.007
	L	379.1^{a}	401.3^{a}	448.6^{a}	417.3^{a}	31.50	NS	
Trp	**S**	127.3^{b}	133.3^{ab}	140.1^{ab}	148.5^{a}	7.01	NS	NS
	L	147.1^{a}	139.9^{a}	140.1^{a}	145.2^{a}	8.48	NS	
Tyr	**S**	382.9^{a}	363.9^{ab}	322.2^{b}	386.8^{a}	16.95	NS	< 0.001
	L	409.7^{a}	466.2^{a}	424.8^{a}	425.4^{a}	23.21	NS	
Val	**S**	460.5^{b}	502.6^{b}	464.3^{b}	574.9^{a}	23.88	0.011	0.009
	L	459.8^{c}	546.9^{b}	603.9^{a}	559.0^{ab}	19.59	< 0.001	

[1]Values are means of 5 to 10 embryos
[2]Arg = arginine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine
[3] **S** = small egg embryos**, L** = large egg embryos
[4]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other
[5] NS = non-significant

Table 3.7: Blood non-essential amino acid concentrations (μmol/L) in small and large embryos on day 20 of incubation following *in ovo* nutrient supplementation[1]

Amino acid[2]	Egg Type[3]	*In ovo* nutrient treatments[4]				SEM	*In ovo* nutrient Effect[5]	Egg type Effect[5]
		C	**G**	**AA**	**G+AA**			
Ala	**S**	442.6^{a}	410.3^{ab}	442.4^{a}	331.4^{b}	34.69	NS	NS
	L	517.8^{a}	517.5^{a}	316.9^{b}	311.5^{b}	30.83	< 0.001	
Asp	**S**	148.4^{a}	114.2^{b}	121.6^{b}	132.8^{ab}	7.84	0.035	0.013
	L	137.6^{a}	93.0^{b}	99.1^{b}	120.6^{ab}	10.18	0.022	
Glu	**S**	252.1^{a}	218.4^{a}	218.4^{a}	220.7^{a}	14.45	NS	NS
	L	240.0^{a}	202.5^{a}	216.6^{a}	221.8^{a}	16.30	NS	
Gln	**S**	2251.0^{a}	2398.1^{a}	2575.0^{a}	2650.8^{a}	150.2	NS	NS
	L	1754.4^{b}	2058.3^{b}	2678.5^{a}	2598.6^{a}	146.3	< 0.001	
Gly	**S**	613.0^{b}	819.7^{a}	754.1^{ab}	736.3^{ab}	49.02	0.06	NS
	L	691.1^{a}	742.2^{a}	780.4^{a}	779.2^{a}	47.02	NS	
Pro	**S**	512.4^{a}	544.5^{a}	557.5^{a}	463.9^{a}	39.19	NS	NS
	L	547.2^{a}	573.6^{a}	533.2^{a}	490.4^{a}	48.08	NS	
Ser	**S**	909.1^{a}	932.4^{a}	838.1^{ab}	758.7^{b}	42.39	0.024	NS
	L	990.1^{a}	1019.0^{a}	725.8^{b}	750.2^{b}	49.63	< 0.001	

1Values are means of 5 to 10 embryos

[2]Ala = alanine, Asp = aspartate, Glu = glutamate, Gln = glutamine, Gly = glycine, Pro = proline, Ser = serine

[3]**S** = small egg embryos**, L** = large egg embryos

[4]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other

[5]NS = non-significant

Table 3.8: Blood essential amino acid concentrations (µmol/L) in small and large embryos on day 20 of incubation following *in ovo* nutrient supplementation[1]

Amino Acid[2]	Egg Type[3]	*In ovo* nutrient treatments[4]				SEM	*In ovo* nutrient Effect[5]	Egg type Effect[5]
		C	**G**	**AA**	**G+AA**			
Arg	**S**	351.4^{b}	369.9^{b}	537.5^{a}	453.2^{ab}	37.88	0.005	NS
	L	345.8^{b}	473.4^{ab}	532.5^{a}	436.2^{ab}	46.23	0.055	
Ile	**S**	193.5^{b}	288.6^{a}	311.9^{a}	290.3^{a}	16.38	< 0.001	NS
	L	212.6^{c}	231.6^{c}	364.7^{a}	302.6^{b}	20.58	< 0.001	
Leu	**S**	200.6^{b}	367.6^{a}	372.8^{a}	363.1^{a}	19.28	< 0.001	NS
	L	235.7^{b}	297.3^{b}	426.3^{a}	378.2^{a}	24.76	< 0.001	
Lys	**S**	227.2^{a}	299.1^{a}	248.1^{a}	243.1^{a}	29.97	NS	NS
	L	260.4^{a}	236.3^{a}	252.2^{a}	242.9^{a}	35.23	NS	
Met	**S**	93.7^{ab}	108.1^{a}	94.4^{ab}	84.8^{b}	6.53	NS	NS
	L	119.8^{a}	110.3^{ab}	81.2^{c}	90.7^{bc}	8.18	0.007	
Phe	**S**	147.1^{a}	132.4^{a}	143.0^{a}	113.3^{a}	11.31	NS	NS
	L	150.0^{a}	167.0^{a}	102.5^{b}	123.3^{b}	8.65	< 0.001	
Thr	**S**	511.1^{a}	498.6^{a}	505.4^{a}	453.4^{a}	48.60	NS	NS
	L	505.7^{a}	525.3^{a}	491.3^{a}	459.7^{a}	42.9	NS	
Trp	**S**	121.8^{ab}	126.0^{ab}	118.3^{b}	127.8^{a}	3.30	NS	NS
	L	115.7^{a}	124.6^{a}	129.3^{a}	123.2^{a}	5.91	NS	
Tyr	**S**	260.4^{a}	330.7^{a}	269.6^{a}	301.9^{a}	37.75	NS	0.015
	L	363.9^{a}	364.1^{a}	322.8^{a}	394.0^{a}	40.99	NS	
Val	**S**	470.8^{b}	628.1^{a}	642.0^{a}	621.0^{a}	24.90	< 0.001	NS
	L	469.4^{b}	535.0^{b}	722.8^{a}	650.9^{a}	37.42	< 0.001	

[1]Values are means of 5 to 10 embryos
[2]Arg = arginine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine
[3] **S** = small egg embryos**, L** = large egg embryos
[4]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids; Means with different superscripts are significantly different from each other
[5] NS = non-significant

Discussion

Lipids are the major substrates for energy metabolism in late term chicken embryos accounting for more than 90% of the energy needs of the embryo (Deeming and Ferguson, 1991; Sato *et al*., 2006). At the same time, the gluconeogenic and glycolytic enzyme activities also increase in the embryo (Pearce, 1977) increasing the availability of glucose via gluconeogenesis and subsequently providing an alternate carbon source for the central metabolic pathways as demonstrated in **Chapter 2** of this dissertation. The objective of this study was to obtain estimates of glucose metabolism on day 12 and 20 embryonic and transfer rates of glucose carbon to NEAA, in response to *in ovo* nutrient supplementation using an *in ovo* [U-^{13}C]glucose tracer injection approach.

As expected glucose availability was higher for day 20 embryos compared to their day 12 counterparts. This higher glucose availability was a result of a more than two fold increase (38% on day 12 vs. 84% on day 20) in new glucose synthesis (gluconeogenesis) from other *in ovo* carbon sources (e.g. amino acids, glycerol). Even though no comparable estimates of gluconeogenesis during chicken embryonic development are available in the literature, the rates of gluconeogenesis observed in this study on day 20 of embryonic development were similar or higher than those observed in fasting humans (Katz and Tayek, 1998) or rats fed a carbohydrate restricted diet (Pascual *et al*., 1997).

With increasing rates of glucose entry (0.39g/d to 0.79g/d), a higher proportion of glucose carbon was recycled back to glucose in day 20 embryos (61%) compared to day 12 (17%) embryos. This was evident in the higher enrichments of M+1, M+2 and M+3 isotopomers in blood glucose on day 20 of embryonic development. The glucose carbon recycling rates observed in day 20 embryos were higher than those observed in adult

chickens (46%; Belo *et al*., 1976), humans after 40 hrs of fast (36%; Katz and Tayek, 1998) and also higher than those observed in rats fed a low carbohydrate diet (50%; Pascual *et al*., 1997). Chicken embryos develop in a 'fixed' nutrient environment and thus the nutrient partitioning for gluconeogenesis and energy metabolism may be dependent on *in ovo* substrate availability (Lourens *et al*., 2006). Thus by maintaining a high rate of glucose carbon recycling, chicken embryos may be conserving glucose carbon and reutilizing it for essential metabolic functions (e.g. glycogen synthesis).

Based on the results from this study, the effect of *in ovo* nutrient supplementation on glucose metabolism remains inconclusive. The only significant treatment effects observed relative to the **C** were **a.** lower glucose entry with **AA** and **G+ AA** in **S** egg embryos on day 12, **b.** higher glucose carbon recycling and gluconeogenesis with **G** treatment in **S** embryos on day 12. Further, gluconeogenesis in day 12 embryos after **AA** and **G+AA** treatments were similar to the **C** group despite a 2 to 3 fold increase in blood amino acid concentrations of gluconeogenic amino acids (alanine, aspartate, glutamate and glutamine) with *in ovo* nutrient treatments. Thus these amino acids may not be major gluconeogenic precursors compared to other available substrates *in ovo*, for example glycerol. However on day 20, amino acid concentrations were higher than the **C** group only for arginine and the branch chain amino acids (leucine, isoleucine and valine). Further, metabolism of glucose was also similar between *in ovo* nutrient treatments on day 20 of embryonic development.

Even though glucose metabolism did not respond to *in ovo* nutrients, the flux of glucose carbon to alanine in the blood of day 20 chicken embryos responded to *in ovo* nutrient supplementation. The enrichment of alanine isotopomers in the blood was

lower with both **AA** and **G+AA** supplementation compared to the **C** and **G** treatments. Thus, when compared to the **C** and **G** treatments, the contribution of glucose to alanine in blood decreased by 31% and 62% with **AA** and **G+AA** supplementations respectively. Regression analysis suggested an interaction between **AA** and **G+AA** treatments as a possible reason for the difference between **AA** and **G+AA** treatment responses. One possible reason for the decrease in glucose carbon flux to alanine is that the **AA** and **G+AA** treatments reduce catabolism of glucose carbon via the glycolytic pathway thus sparing the glucose carbon for other metabolic purposes. This data thus provide a possible mechanism by which *in ovo* supplementation could alter the metabolic flux through central pathways of metabolism thus sparing glucose carbon and in turn resulting in improved energy status (e.g. higher liver glycogen) of the embryo.

The mass isotopomer distribution profile of alanine, aspartate and glutamate observed in the embryonic blood were different from those observed in the tissues. The mass isotopomer distribution profile of amino acids can be considered as the metabolic signature for each tissue, providing a snap shot of the metabolic activity and requirements of the tissue. Further more, these differences between blood and tissue profiles indicate that that conclusions based on blood values alone may not reflect whole body tissue metabolism.

Differences in nutrient fluxes were also observed between tissues. In general the flux of glucose carbon to alanine was ~ 40% lower in liver compared to intestine, muscle and kidney. The high rates of glucose carbon flux to alanine in intestine, muscle and kidney may be due to higher rates of glycolysis in these tissues even though only pyruvate enrichments will provide a complete picture. The contribution of glucose to

aspartate was highest in the liver (11%) followed by kidney (7.6%), intestine (5%) and muscle (4%). The higher contribution of glucose to aspartate is a reflection of a higher [M+3] aspartate enrichment and is in accordance with the gluconeogenic roles of the liver and the kidney. The higher appearance of [M+3] aspartate in these tissues could suggest a high activity of malate-aspartate shuttle with gluconeogenesis (Burelle *et al*., 2000) providing three carbon units for cytosolic form of phosphoenolpyruvate carboxykinase which is expressed in chicken embryos throughout incubation (Savon *et al*., 1993). Further, the contribution of glucose carbon to glutamate was also more than 2 fold higher in liver compared to other tissues. High labeling of aspartate and glutamate from glucose in liver is also consistent with the role of liver in Krebs cycle metabolism.

In this study, the availability of the nutrients *in ovo* was altered from day 9 with the objective of simulating an enriched nutrient environment before the onset of active glucose metabolism. This approach was different from previous studies (Tako *et al*., 2004; Uni *etal*., 2005) where nutrients were directly administered into the amniotic fluid, during the last 3-5 days of embryo development, after the onset of active gluconeogenesis, and thus stimulating active oral consumption. Further, the amount of carbohydrates injected *in ovo* was 2.5 fold higher in those studies compared to the amount of glucose injected in this study (100mg). Thus the metabolic and growth responses observed could vary with the type of nutrient injected, the amount of the nutrient injected, site of injection and the stage of embryo development.

In summary, embryos from small and large eggs maintained similar rates of gluconeogenesis despite significant differences in embryo weights. I*n ovo* nutrient supplementation had minimal effect on glucose metabolism. However, *in ovo* nutrients

altered nutrient fluxes through central metabolic pathways in embryonic blood even. Further, mass isotopomer analysis of amino acids in individual tissues provided a ‘snap shot’ of the metabolic activity of these tissues.

CHAPTER 4: EXPERIMENT 3

THE USE OF GLUTAMINE AND GLUTAMATE FOR GLUCONEOGENESIS AND NON-ESSENTIAL AMINO ACID SYNTHESIS IN LATE TERM CHICKEN EMBRYOS[1, 2]

[1]Funded by a Maryland Agricultural Experiment Station grant to Brian J. Bequette.
[2] Presented in part at the 2nd International Symposium on Energy and Protein Metabolism and Nutrition: September 9–13, 2007, Vichy, France. [Sunny NE, Adamany J, Bequette BJ. 2007. The use of glutamine and glutamate for gluconeogenesis and non essential amino acid synthesis in late term chicken embryos.]

Abstract

The objective of this study was to determine the contribution of glutamate (Glu) and glutamine (Gln) to the synthesis of glucose and proline, and to other non-essential amino acids (NEAA) on day 19 of embryonic development in small and large egg chicken embryos. Fertilized small (mean ± standard deviation, 53.1 ± 0.30g; n = 12) and large (69.7 ± 0.35g; n = 12) broiler eggs were incubated at 37° C and 65% relative humidity. Beginning on day 16 of incubation, half of the small and the large eggs were injected into the chorio-allantoic fluid either with [U-^{13}C]Glu or [U-^{13}C]Gln (3.5 mg in 90 μL H_2O) for three consecutive days prior to tissue and blood collection on day 19. Blood and tissues were analyzed by GC-MS for ^{13}C-mass isotopomer distribution in glucose and non-essential amino acids and Krebs cycle intermediates. Low isotopomer enrichments in blood glucose suggested minimal contribution of Glu and Gln to gluconeogenesis in day 19 chicken embryos. Following [U-^{13}C]Glu and [U-^{13}C]Gln injection, Glu and Gln [M+1], [M+2] and [M+3] isotopomer enrichments were relatively low in blood and tissues. Further, the enrichments of these isotopomers were minimal in aspartate and all the Krebs cycle intermediates. However the enrichment of [M+5] Glu and Gln was different ($P < 0.05$) between tissues with the lowest enrichment and thus the lowest tissue uptake observed in the liver and the highest uptake in the muscle. The contribution of Glu and Gln to proline flux differed ($P < 0.05$) between liver (24%) and other tissues (< 10%). In summary, small and large egg embryos maintained similar rates of Glu and Gln metabolism with minimal tissue synthesis of these amino acids. Further glutamate and glutamine carbon in the liver accounts for majority *in vivo* proline synthesis by these chicken embryos.

Key words: *In ovo*, Chicken, Embryo, Glucose, Glutamate, Glutamine

Introduction

Glutamate and Glutamine are located at a pivotal point in intermediary metabolism with their carbon and nitrogen contributing to a wide variety of biochemical pathways including gluconeogenesis, non-essential amino acid (NEAA) synthesis, Krebs cycle metabolism, glutathione synthesis and nucleic acid synthesis. In sheep, around 25 % of Glu and 20 % of Gln are converted to glucose accounting for ~ 12% of total glucose production (Heitmann and Bergman, 1978; 1981) with even higher contributions in rats (Kaloyianni and Freedland, 1990). Glu and Gln are extensively oxidised by the splanchnic tissues (~ 80%) in piglets, mice and humans (Windemueller & Spaeth, 1980; Matthews et al. 1993; Reeds et al. 1996) thus redistributing their carbon through central metabolic pathways to metabolic intermediates. However little is known about the metabolic roles of these two amino acids in the developing chicken embryo.

The average broiler egg (60 g) contains only 300 mg of available glucose, and thus relies on gluconeogenesis from amino acids or triglyceride-glycerol for glycogen synthesis and tissue metabolism. Late term embryos (day 18-20) maintain a high rate of glucose entry (0.8g/d; **Chapters 2 and 3**) and glycogen synthesis (10mg/g of liver; Uni *et al*., 2005) for hatching and energy metabolism. *In ovo,* Glu and Gln together comprise ~14% of chicken egg protein (1.2 g), and their availability in the egg contents is 2-fold greater than that which is accumulated by the embryo on day 19 (Ohta *et al*., 1999). Thus these amino acids are good candidates to donate their carbon skeleton for glucose synthesis through Krebs cycle and in consequence to the synthesis of NEAA. Further, previous data also indicates that there is a need for proline synthesis by the late term

chicken embryo as the amount of proline accreted between days 14 and 19 of embryonic development is 85mg higher than the proline utilized form the egg during the same period (Ohta *et al*., 1999). Even though one of the enzymes, pyrroline-5-carboxylase synthase, required for the conversion of Glu and Gln to proline is deficient in enterocytes (Wu *et al*., 1995), and with the avian species lacking a complete urea cycle, Glu and Gln are the only possible substrates for proline synthesis.

The objective of this study was to determine the contribution of Glu and Gln to synthesis of glucose and proline, and to other NEAA in day 19 chicken embryos. A further objective was to determine whether the metabolism of Glu and Gln towards these biochemical pathways is compromised in embryos from small, compared to large, eggs where *in ovo* nutrient availability (Lourens *et al*., 2006) and day 20 embryonic weights (31.8 vs. 36.8 g; **Chapter 3**) are lower. An *in ovo* [U-^{13}C]Glu and [U-^{13}C]Gln tracer injection approach was used to study the metabolism of these amino acids in day 19 chicken embryos.

Materials and Methods

Egg Incubation and Experimental Protocol

The experimental protocol was approved by the Animal Care and Use Committee of the University of Maryland. Fertilized, small (mean ± standard deviation, 53.1 ± 0.30g; n = 12) and large (69.7 ± 0.35g; n = 12) eggs were obtained from Perdue Farms, Inc., Salisbury, MD from a broiler flock of same age (40 wks old). All eggs were incubated at a standard temperature and relative humidity of 37°C and 65% respectively. On day 9 of incubation all the eggs were candled for viable embryos.

In ovo [U-^{13}C]Glu and [U-^{13}C]Gln Injection

On day 16 of incubation, half of the small and the large eggs were randomly assigned to an injection group of either [U-^{13}C]Glu or [U-^{13}C]Gln (99 atom percent ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, MA). These stable isotope tracers were injected into the chorio-allantoic fluid (3.5 mg in 90 μL H_2O) from day 16 for three consecutive days prior to blood and tissue (liver, intestines, muscle, kidney) collection on day 19. Before tracer injection, the broad end of the egg was wiped sterile with 70% ethanol and the egg shell pierced to insert a 25 gauge needle. The stable isotope solution was deposited 5-6 mm beneath the egg shell. The tracer was assumed to mix uniformly with the fluid compartment beneath the egg shell membrane and absorbed continuously by the developing embryo through the extensive chorio-allantoic capillary network.

Sample Collection and Analysis

Following [U-^{13}C]Glu or [U-^{13}C]Gln injection for three days, each group of eggs was dissected on the forth day to collect blood and tissue samples. After removing the egg shell around the air shell, the egg shell membrane was carefully peeled off exposing the extra embryonic membranes. Whole egg contents were then carefully transferred to a petri-dish taking care that the vitelline vessels (artery and vein) were on the top and clearly visible. Embryos were bled by making a nick across the vitelline vessels and blood was drawn using a glass pasture pipette with a rubber stopper, transferred into a 2 ml tube and frozen at immediately at -20ºC for later analysis. Liver, intestine, muscle and kidneys were harvested, rinsed with cold normal saline to wash off excess blood and other debris, transferred into 2 ml plastic tubes and immediately frozen at -80ºC.

Glucose Enrichments

For determination of blood glucose enrichments, samples (100 μL) were acidified with ice-cold 15% sulpho-salicylic acid (w/v) and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The elute containing free glucose was collected after passing the supernatant over 0.5g of cation exchange resin. The solution was concentrated by freeze drying and analysed by GC-MS for glucose enrichment after forming the di-O-isopropylidene acetate derivative of glucose. After separation using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas, selective ion monitoring of ions with mass-to-charge (m/z) 287 – 292 was performed with MS under electrical ionization mode (Hannestad and Lundblad, 1997).

Amino acid Enrichments

For all blood and tissue samples, acidic amino acids, Glu and aspartate (Asp) were separated from all other AA using sequential anion and cation exchange resins (AG 1-X8 and AG 50W-X8 resins respectively, 100-200 mesh; Bio-Rad Laboratories, Hercules, CA). After equilibrating the anion exchange resin (1g) with 2mL of 0.1 moles/L hydrochloric acid, the de-proteinsed sample (pH adjusted to ~ 9.5) was poured onto the resin. The flow through from the anion resin, containing all amino acids except Glu and Asp was stored briefly. After rinsing the resin with 40-50 mL of double distilled water, Glu and Asp was eluted from the anion resin by a strong acid (one moles/L hydrochloric acid) followed by one mL of double distilled water. The fraction containing all the other amino acids was applied onto a cation-exchange resin and the resin rinsed with 2 mL of double distilled water. Amino acids were eluted with 2 volumes of 2

moles/L ammonium hydroxide followed by 1 volume of water. Both amino acid fractions were freeze dried, reconstituted in 250 µL of double distilled water, dried under N_2 gas, and converted to heptafluoro-butyryl isobutyl amino acids (MacKenzie and Tenaschuk, 1979ab) prior to separation by GC (Heliflex® AT™-Amino acid, 25 m × 0.53 mm × 1.20 µm, Alltech). Selected ion monitoring was done by gas chromatography-mass spectrometry (GC-MS; 5973N Mass Selective Detector coupled to a 6890 Series GC System, Agilent, Palo Alto, CA) under methane negative chemical ionization conditions. The ions (mass-to-charge) monitored were Alanine 321 – 324, Aspartate 421-425, Glycine 307 – 309, Serine 533 – 536, Proline 347 -352, Glutamine 361 - 366, Glutamate 435 - 440, Arginine 778 – 784.

Enrichment of Krebs Cycle Intermediates

After deproteinising the liver (100mg) with 1.5mL of 15% sulpho-salicylic acid (w/v), 5 mmol of freshly prepared hydroxylamine hydrochloride (1 mL) was added to the supernatant and neutralized with 2 mol/L potassium hydroxide. These samples were then sonicated (15 min) and allowed to react at 65º C for 1 h. The solution was then titrated to pH < 2 with hydrochloric acid (6 mol/L), saturated with sodium chloride and Krebs cycle acids extracted twice with 3 ml ethyl acetate. The organic phase was blown down under a stream of N_2 and the tertiary butyldimethylsilyl derivative formed prior to separation on a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1µm Hewlett-Packard; helium as carrier gas) and selective ion monitoring by MS under electrical ionization mode (Des Rosiers *et al*., 1994). Ions with m/z monitored were: lactate 261 – 264, pyruvate 274 – 277, succinate 289 – 293, malate 419 – 423, oxaloacetate 432 – 436 and ketoglutarate 446 – 451.

Calculations

The normalized crude ion abundances for glucose, amino acids and Krebs cycle intermediates were corrected for the natural abundance of stable isotopes present in the original molecule and that contributed by the derivative ^{13}C using the matrix approach (Fernandez *et al*., 1996). Natural isotopomer distributions in unlabelled glucose and amino acids were quantified from blood samples taken from embryos that had not received the isotopic tracer. Corrected enrichments are reported as moles of tracer (M+*n*) per 100 moles of tracee (M+0) for the calculations. The contribution of Glu and Gln to glucose and non-essential AA synthesis was determined by precursor-product relationships. Here, the ratio of M+*n* (where *n* is the number of carbon atoms in the metabolite) in the product over the precursor was used to calculate the flux to the product.

Statistical Analysis

After verifying for assumptions of normality and homogeneity of variance, results were analyzed by ANOVA using mixed procedure of SAS (version 8.0, SAS Institute, Inc. Cary, NC) with small and large eggs as treatment groups and blood, liver, intestine, muscle and kidney as blocks. Differences between [U-^{13}C]Glu and [U-^{13}C]Gln injected groups were compared using a *t*-test. Mean comparisons were done by Tukey-Kramer multiple comparison tests. Results are presented as least-squares means ± SEM and the differences are considered significant at $P < 0.05$ while $P < 0.1$ is considered a trend.

Results

In both [U-^{13}C]Glu and [U-^{13}C]Gln injected groups, the embryonic weights from the small eggs (28.7 ± 0.41g) were lower ($P < 0.05$) when compared to embryos from the large eggs (34.7 ± 0.82g).

No significant enrichments (moles tracer per 100 moles tracee) could be detected in blood glucose isotopomers with either *in ovo* [U-^{13}C]Glu or [U-^{13}C]Gln. Thus, despite our expectations, there was little contribution of either Glu or Gln to blood glucose synthesis in the small or large day 19 embryos.

The enrichments of Glu and Gln isotopomers (M+1, M+2, M+3 and M+5) with *in ovo* [U-^{13}C]Glu or [U-^{13}C]Gln is presented in **Table 4.1**. There were no differences in enrichment of either the Glu [M+5] or Gln [M+5] isotopomers within blood and any tissues when comparing small and large egg embryos. However, for each of these amino acids their isotopomer enrichments differed ($P \leq 0.05$) between blood, liver, intestine, muscle and kidney pools. Because there were no differences between small and large egg embryos in their metabolism of Glu and Gln towards glucose and NEAA synthesis, all values are reported are means of small and large egg embryos together. Thus, with [U-^{13}C]Glu as tracer, tissue enrichment of Glu [M+5] was highest in the intestine (0.32) and lowest in the liver (0.18) and Gln [M+5] was highest in the intestine and kidney (0.35) and lowest in liver (0.19). Similarly with [U-^{13}C]Gln as tracer, tissue enrichment of Glu [M+5] was highest in the muscle (0.72) and lowest in the liver (0.15) and Gln [M+5] was highest in the muscle (1.1) and lowest in liver (0.21). Blood Gln contributed to 8.5, 28.5, 43.9 and 22.8% of intracellular Gln flux in liver, intestine, muscle and kidney respectively ($P \leq 0.05$). The corresponding contributions to the tissues from blood Glu

was not calculated due to the high variation in the blood enrichments of Glu after *in ovo* administration of [U-^{13}C]Glu.

The contribution of Glu and Gln to alanine and proline fluxes is presented in **Table 4.2**. Alanine and proline flux from Glu and proline flux from Gln were different ($P < 0.05$) between the different pools sampled. Alanine flux from Glu varied from 23.5% in kidney to 9.5% in the muscle. Glu (26.7%) and Gln (22.2%) both made a larger contribution to intracellular proline flux in the liver compared to other tissues. Neither Glu nor Gln contributed to the synthesis of aspartate and arginine in the blood or any of the tissues.

Table 4.1: Glutamate and Glutamine enrichments in blood and tissues of day 19 chicken embryos after administration of either [U-^{13}C]Glu or [U-^{13}C]Gln tracer *in ovo.*

	Sampled pool[1]					SEM	P ≤
	Blood[2]	Liver	Intestine	Muscle	Kidney		
[U-^{13}C]Glu tracer							
Glu [M+1]	0.497^{d}	0.717^{c}	0.877^{b}	0.796^{bc}	1.02^{a}	0.050	< 0.001
Glu [M+2]	0.092^{b}	0.153^{b}	0.207^{b}	0.134^{b}	0.548^{a}	0.109	0.048
Glu [M+3]	0.171^{a}	0.05^{b}	0.09^{b}	0.05^{b}	0.08^{b}	0.030	0.022
Glu [M+5]	7.79^{a}	0.175^{b}	0.321^{b}	0.283^{b}	0.176^{b}	0.773	< 0.001
[U-^{13}C]Glu tracer							
Gln [M+1]	-	0.778^{a}	0.805^{a}	0.697^{a}	0.779^{a}	0.043	NS
Gln [M+2]	-	0.153^{a}	0.192^{a}	0.237^{a}	0.256^{a}	0.043	NS
Gln [M+3]	-	0.062^{c}	0.145^{ab}	0.091^{bc}	0.199^{a}	0.027	0.004
Gln [M+5]	-	0.191^{b}	0.345^{a}	0.268^{ab}	0.346^{a}	0.047	0.071
[U-^{13}C]Gln tracer							
Glu [M+1]	0.647^{c}	0.680^{c}	0.904^{b}	0.722^{c}	1.05^{a}	0.042	< 0.001
Glu [M+2]	0.104^{d}	0.158^{bc}	0.207^{a}	0.153^{c}	0.188^{ab}	0.011	< 0.001
Glu [M+3]	0.130^{a}	0.04^{c}	0.120^{ab}	0.070^{bc}	0.09^{ab}	0.018	0.006
Glu [M+5]	2.60^{a}	0.153^{b}	0.479^{b}	0.722^{b}	0.333^{b}	0.316	< 0.001
[U-^{13}C]Gln tracer							
Gln [M+1]	0.805^{b}	0.930^{ab}	0.990^{a}	0.794^{b}	1.073^{a}	0.051	< 0.001
Gln [M+2]	0.112^{c}	0.115^{c}	0.160^{b}	0.173^{b}	0.251^{a}	0.014	< 0.001
Gln [M+3]	0.07^{b}	0.205^{a}	0.196^{a}	0.191^{a}	0.211^{a}	0.015	< 0.001
Gln [M+5]	2.45^{a}	0.209^{c}	0.697^{bc}	1.08^{b}	0.558^{bc}	0.220	< 0.001

[1]Means with different superscripts are significantly different from each other
[2]Blood Gln enrichments with [U-^{13}C]Glu tracer are not reported

Table 4.2: Contribution of Glutamate and Glutamine to blood and tissue alanine and proline fluxes in day 19 chicken embryos.

Proportion of intracellular flux from Glu or Gln (%)[1]	Sampled pool[3]					SEM	P ≤[4]
	Blood[2]	Liver	Intestii	Muscle	Kidney		
Ala flux from Glu	< 5[c]	12.7[b]	12.9[b]	9.5[bc]	23.5[a]	2.47	< 0.001
Ala flux from Gln	7.6[a]	12.2[a]	10.4[a]	6.3[a]	10.2[a]	2.12	NS
Pro flux from Glu	-	26.7[a]	5.6[b]	6.2[b]	9.2[b]	0.29	< 0.001
Pro flux from Gln	-	22.2[a]	3.4[b]	2.0[b]	5.0[b]	0.27	< 0.001

[1]Glu = glutamate, Gln = glutamine, Ala = alanine, Pro = proline
[2]Pro flux from Glu and Gln are not reported
[3]Means with different superscripts are significantly different from each other
[4]NS = non-significant

Discussion

In late term chicken embryos, over 80% of glucose entry is derived from new glucose synthesis as demonstrated in **Chapter 3** from either *in ovo* amino acids or glycerol portion of triglycerides. The objective of this study was to determine whether Glu and Gln by virtue of their high concentrations in the egg contents and also their pivotal location in the central metabolic pathways, are major carbon skeleton donors to glucose synthesis, NEAA synthesis and energy metabolism.

Isotopomer analysis of blood glucose after *in ovo* injection of [U-^{13}C]Glu and [U-^{13}C]Gln in this study showed only minimal labelling of glucose isotopomers. Thus, in this study Glu and Gln were not significant contributors to glucose synthesis in day 19 chicken embryos. This is in spite of the fact that Glu and Gln form ~ 15% of the amino acid content of the egg protein and also with a 2-fold higher availability of these amino acids in the egg than required for net tissue growth of the embryo by day 18 (Ohta *et al*., 1999). This result suggests that other substrates, especially glycerol from triglyceride breakdown may be the major carbon donor for gluconeogenesis. Even though various substrates including lactate, pyruvate, dihydroxyacetone, glycerol and amino acids can be potential precursors for glucose synthesis as previously demonstrated in isolated chicken hepatocytes (Brady *et al.*, 1979), their relative contributions vary with the tissue of interest, stage of development and the localization of rate limiting enzymes (e.g. phosphoenol pyruvate carboxykinase). Thus the extent of contribution of various substrates to gluconeogenesis during different stages of embryonic development needs further investigation.

Another interesting observation in this study was that following *in ovo* administration of [U-^{13}C]Glu and [U-^{13}C]Gln, the enrichments of lower isotopomers of both Glu and Gln (M+1, M+2, M+3) were low in the blood. Only less than 10% of the tracer carbon was recycled as M+1, M+2, M+3 isotopomers in blood Glu and Gln. Splanchnic bed metabolism of Glu and Gln in most mammals is characterised by extensive oxidation of these amino acids by the intestinal mucosa with only less than half of glutamine and less than 20% of Glu appearing in the portal blood from the luminal side (Windemueller & Spaeth, 1980; Matthews et al. 1993; Reeds et al. 1996). This would thus result in extensive recycling of Glu and Gln carbon leading to appearance of lower isotopomers (M+1, M+2, M+3) in NEAA associated with Krebs cycle in blood and post hepatic tissues. High recycling of Glu and Gln carbon and resulting appearance of M+1, M+2, M+3 isotopomers would also indicate in vivo synthesis of these amino acids from other carbon substrates and also increased Krebs cycle activity. However in this study, Glu and Gln M+1, M+2, M+3 isotopomer enrichments and also the corresponding enrichments in alanine, aspartate and the Krebs cycle intermediates were low. This suggests minimal in vivo synthesis of these amino acids through the Krebs cycle. Thus the metabolism of Glu and Gln in developing embryos may be different from those animals consuming oral diets. Further as the embryo mobilizes Glu and Gln directly from the egg contents, and maintains high concentrations of these two amino acids in the blood, especially for Gln, only minimal synthesis of these amino acids may be required by the embryonic tissues. Previous literature which indicated that the developing embryo only uses less than half of these amino acids compared to what is available in the egg

(Ohta *et al*., 1999) also supports this hypothesis and further suggests that *in ovo* availability of these amino acids may not be a limitation for growth and development.

The differences observed in the uptake of [M+5] Glu and Gln between tissues is an indication of the metabolic requirement of the tissues for these amino acids during the particular stage of development. [U-^{13}C]Glu and [U-^{13}C]Gln injections, both resulted in, muscle followed by intestines accumulating the highest [M+5] label which is a reflection of tissue uptake of Glu and Gln. Thus the uptake of Glu and Gln was highest in the muscle followed by intestine, kidney and liver. Further, with the exception of the liver, the uptake of Gln was higher in all the tissues than Glu. Even though the metabolic roles of these amino acids in chicken embryonic tissues needs to be further defined, the high tissue uptake of Gln, especially in muscle is consistent with the literature in other species correlating Gln uptake with high rates of protein synthesis (Wu and Thompson, 1990; Watford and Wu, 2005) as is the case in developing tissues. The rate of inter-conversion of these amino acids between each other is also high in all the tissues as indicated by the [M+5] Glu enrichment with [U-^{13}C]Gln tracer and [M+5] Gln enrichment with [U-^{13}C]Glu tracer which in turn suggests high activities of phosphate dependent glutaminase and glutamine synthetase.

Even though [M+5] Glu and Gln isotopomer enrichments were lowest in the liver, the contribution of Glu and Gln to proline was highest in the liver (24%). Glu and Gln flux to proline in all other tissues were more than 60% lower when compared to the liver. Previous research by Wu et al., (1995) identified the lack of pyrroline-5-carboxylase synthase enzyme in chicken enterocytes which is essential for the conversion of Glu and Gln in to proline. In fact proline flux from Glu and Gln was minimal in intestine in this

study. However the expression patterns and activity of pyrroline-5-carboxylase synthase in liver is not well defined. Further, any appearance of an [M+5] proline after administration of *in ovo* [U-^{13}C]Glu or Gln can only occur through the conversion of Glu and Gln to proline thus suggesting that all the pathway enzymes necessary for this conversion are active in the liver.

In summary, embryos from the small and large eggs, despite a significant difference in body weight on day 19 of embryonic development, maintained similar rates of Glu and Gln metabolism with virtually no contribution of these amino acids towards gluconeogenesis, and thus glycogen synthesis. The results also indicate that metabolism of Glu and Gln to proline probably accounts for the shortage of proline in the developing chick embryo (Ohta *et al*., 1999).

CHAPTER 5: EXPERIMENT 4

CONTRIBUTION OF GLYCEROL TO GLUCOSE, GLYCOGEN AND NON-ESSENTIAL AMINO ACID SYNTHESIS IN EMBRYOS FROM SMALL AND LARGE CHICKEN EGGS

Abstract

The objective of this study was to determine the contribution of glycerol to glucose and non-essential amino acid (NEAA) synthesis in 14 and 19 day old chicken embryos from small and large eggs. [U-^{13}C]Glycerol (14 mg in 75 μL water) was injected into the chorio-allantoic fluid of small (mean ± standard deviation, 56.6 ± 0.88g; n = 7 per age group) and large (71.7 ± 1.09g; n =7 per age group) eggs for four consecutive days prior to tissue and blood collection on day 14 and 19 of embryonic development. Blood and tissues were analyzed by GC-MS for ^{13}C-mass isotopomer distribution in glycerol, glucose, glycogen and NEAA. Injection of [U-^{13}C]glycerol (M+3) resulted in significant enrichments of [M+1], [M+2] and [M+3] isotopomers in blood glucose and liver and muscle glycogen. These enrichments were higher ($P < 0.05$) in small egg embryos on day 14 of embryonic development in both blood glucose and glycogen. All the blood glucose isotopomers were more highly ($P < 0.05$) enriched on day 19 of embryonic development compared to day 14. Liver and muscle glycogen isotopomer enrichments were lower ($P < 0.05$) than blood glucose enrichments on day 19 suggesting the role of other three carbon units (e.g. amino acids) in glycogen synthesis. Injection of [U-^{13}C]glycerol (M+3) resulted in significant enrichments of [M+1], [M+2] and [M+3] isotopomers in alanine in all the tissues, with aspartate and glutamate isotopomers more highly enriched ($P < 0.05$) in liver than blood and other tissues. In summary glycerol is a major precursor for glucose synthesis in chicken embryos and the indirect pathway is responsible for most of the liver and muscle glycogen; a major substrate being glycerol.

Key words: Chicken, Embryo, Glycerol, Glucose, Amino acid, Metabolism

Introduction

The avian egg at incubation has < 3% available carbohydrates (Romanoff and Romanoff, 1967) and in consequence the developing chicken embryo has to maintain high rates of gluconeogenesis from early stages of incubation. In fact, plasma glucose, detected as early as day 4 of incubation, increases steadily from 6 – 8 mM early during embryonic development to 10 – 12 mM at two to three weeks post hatch (Hazelwood, 1971) parallel to an increase in gluconeogenic enzymes (Pearce, 1971; 1977). Glycogen content in the liver and muscle which serves as a vital source of energy for hatching also starts accumulating early (day 6) during embryonic development (by the uronic acid pathway), peaks by day 12, declines to 50% by day 13 and further increases to over 400% by day 20 (Hazelwood, 1971).

The synthesis of glucose and glycogen by the embryo is dependent on the substrates available *in ovo* with amino acids and the glycerol portion of the triglycerides being the major substrates. However, a wide variety of substrates including lactate, pyruvate, dihydroxyacetone, glyceraldehydes, amino acids and fructose have the potential to contribute to glucose synthesis in chicken (Langslow, 1978; Brady *et al.*, 1979). Further, substrate preference can vary with availability, stage of embryonic development and localization of pathway enzymes (e.g. isoforms of phosphoenolpyruvate carboxykinase) in different tissues. Thus, while lactate may be a major contributor to gluconeogenesis in isolated chicken hepatocytes (Brady *et al.*, 1979), and also during anaerobic respiration (Moran, 2007); amino acids and glycerol were found to be the major metabolic substrates in kidney for gluconeogenesis in post hatch chicks (Watford *et al*., 1981, Magnuson *et al*., 2003).

The major substrates for glucose synthesis during chicken embryonic development are not well defined. In a previous study from our lab (**Chapter 4**) glutamate and glutamine were found to be non-significant contributors to blood glucose synthesis in day 19 embryos. Further as embryos develop in a lipid rich environment, with extensive lipid breakdown in the yolk sac and the liver during the latter half of incubation for energy production (Deeming and Ferguson, 1991; Sato *et al*., 2006), the glycerol portion of triglycerides is a good and abundant three carbon source for glucose synthesis.

The objective of this study was to determine the contribution of glycerol to gluconeogenesis, glycogen synthesis and NEAA synthesis during the latter half of embryonic development. We hypothesize that the contribution of glycerol to gluconeogenesis, glycogen synthesis and NEAA synthesis will be lower in small egg embryos compared to large egg embryos, due to their initial lower yolk content (Lourens *et al*., 2006). An *in ovo* [U-^{13}C] glycerol stable isotope injection approach was developed and utilized to characterize glycerol metabolism.

Materials and Methods

Egg Incubation and Experimental Protocol

The experimental protocol was approved by the Animal Care and Use Committee of the University of Maryland.

Fertilized, small (mean ± standard deviation, 56.6 ± 0.88g; n = 14) and large (71.7 ± 1.09g; n = 14) eggs were obtained from Perdue Farms, Inc., Salisbury, MD from a broiler flock of the same age (40 wks old). All eggs were incubated at a standard temperature and relative humidity of 37°C and 65% respectively. On day 9 of incubation

all eggs were candled for viable embryos. Two groups each from small and large eggs were incubated for collection on days 14 and 19 of incubation.

***In ovo* [U-^{13}C]Glycerol Tracer Validation**

A pilot study was conducted to determine the time-course for enrichments of glucose and NEAA to reach isotopic (e.g. M+2, M+3) and isotopomer (e.g. M+2:M+3) steady-states following *in ovo* injection of [U-^{13}C]glycerol. [U-^{13}C]glycerol (14 mg in 75 μL of sterile water) was injected into the chorio-allantoic fluid of 4 eggs of similar weight starting on day 14 of incubation. The air space end of the egg was sterilized with 70% ethanol before piercing the egg shell. A 25 gauge needle was used to inject the [U-^{13}C]glycerol solution, which was deposited 2-3 mm beneath the egg shell membrane into the chorio-allantoic fluid. The tracer was assumed to mix uniformly with the fluid compartment into which it was injected and absorbed by the developing embryo through the extensive chorio-allantoic capillary network. After 1, 2, 3 and 4 days of administering the [U-^{13}C] glycerol, one egg was removed and sampled for blood glucose and liver NEAA isotopomer enrichments.

***In ovo* [U-^{13}C]Glycerol Injection**

One group each from small and large eggs was randomly selected on day 11 or 16 for injection of [U-^{13}C]glycerol (99 atom percent ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, MA). Based on the pilot study, it was determined that isotopic and isotopomer steady state is achieved after four consecutive days of administering [U-^{13}C]glycerol. Thus, in the main experiment, each egg was administered into the chorio-allantoic fluid [U-^{13}C]glycerol (14 mg in 75 μL of sterile water) for four consecutive days before sample collection on the fifth day.

Sample Collection and Analysis

Following [U-^{13}C]glycerol injection for four days, each group of eggs was dissected on the fifth day to collect blood and tissue samples. Thus, a group each of small and large eggs was dissected on days 14 and 19 of incubation. After removing the egg shell surrounding the air cell, the egg shell membrane was carefully peeled off to expose the extra embryonic membranes. Whole egg contents were then carefully transferred to a petri-dish taking care that the vitelline vessels (artery and vein) were on the top and clearly visible. Embryos were blood sampled by making a small nick on the vitelline vessels and blood was withdrawn into a glass pasture pipette with a rubber bulb. Blood was then transferred into a 2 ml tube and frozen immediately at -20ºC for later analysis. Liver, intestine, muscle and kidney tissues were dissected, rinsed with ice-cold normal saline to remove excess blood and other debris, and transferred into 2 ml plastic tubes for storage at -80ºC.

Glycerol Enrichments

Glycerol was extracted from blood (~300µL) with 1.5mL of methanol, thoroughly vortexed and centrifuged for 10 min at 4,000 rpm. The supernatant was transferred to a V-vial, and blown down completely under N_2 gas. The dried sample was processed (Gilker *et al*., 1992) by adding 50µL of heptafluoro-butyryl isobutyl anhydride plus 50µL of ethyl acetate and heating at 70ºC for 15min. The derivative was blown down under N_2 and the sample reconstituted in ethyl acetate for gas chromatography-mass spectrometry (GC-MS) The sample was separated using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1µm Hewlett-Packard) with helium as carrier gas prior to GC-MS (5973N Mass Selective Detector coupled to a 6890 Series GC System, Agilent, Palo Alto, CA)

under methane negative chemical ionization conditions. The glycerol isotopomers monitored were ions (mass-to-charge) 680 to 683.

Glucose and Glycogen Enrichments

For determination of blood glucose enrichments, samples (100 μL) were acidified with ice-cold 15% sulpho-salicylic acid (w/v) and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The elute containing free glucose was collected after passing the supernatant over 0.5g of cation exchange resin. The solution was concentrated by freeze drying and analysed by GC-MS for glucose enrichment after forming the di-O-isopropylidene acetate derivative of glucose. After separation using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1μm Hewlett-Packard) with helium as carrier gas, selective ion monitoring of ions with m/z 287 – 292 was performed with MS under electrical ionization mode (Hannestad and Lundblad, 1997).

For determination of glycogen enrichments, liver (100mg) or muscle (200mg) were homogenized in 0.5 to 1.0mL of ice-cold 30% sulpho-salicylic acid (w/v) and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The glycogen pellet was extracted by adding ice cold ethanol (95%) to the supernatant (2:1) and centrifuging for 20 min at 13,000 rpm. The isolated glycogen pellet was washed twice with 0.5mL of ice cold ethanol (95%) to get rid of any residual glucose and spun down for 10 min at 13,000 rpm. The glycogen pellet was dried for 2 to 3 hrs at room temperature and free glucose liberated by incubating the pellet with 0.5mg amlyloglucosidase enzyme (31.2 units / mg solid; Sigma-aldrich, St. Louis, MO) in 250μL of 0.3M acetic acid and 2M acetate buffer (1:1; pH = 4.5) at 55ºC for 1hr. The

incubation mixture was freeze dried and processed for GC-MS by forming the di-O-isopropylidene acetate derivative as described for glucose enrichments.

Amino acid Enrichments

For all blood (100 μL) and tissue (50 mg) samples, NEAA were isolated by cation-exchange (AG 50W-X8 resin, 100-200 mesh; Bio-Rad Laboratories, Hercules, CA) and amino acids eluted from the resin with 2 volumes of 2 *M* NH_4OH followed by one volume of water. The eluate was freeze dried, reconstituted in 250 μL of double distilled water, dried under a stream of N_2 gas, and amino acids converted to their heptafluoro-butyryl isobutyl derivatives (MacKenzie and Tenaschuk, 1979ab) prior to separation by GC (Heliflex® AT™-Amino acid, 25 m × 0.53 mm × 1.20 μm, Alltech). Selected ion monitoring was done by gas chromatography-mass spectrometry (GC-MS; 5973N Mass Selective Detector coupled to a 6890 Series GC System, Agilent, Palo Alto, CA) under methane negative chemical ionization conditions. The following ions of mass-to-charge (m/z) were monitored: Alanine 321 – 324, Aspartate 421 – 425, Glycine 307 – 309, Serine 533 – 536, Proline 347 – 352, Glutamine 361 – 366, Glutamate 435 – 440 and Arginine 778 – 784.

The normalized crude ion abundances for glucose and amino acids were corrected for the natural abundance of stable isotopes present in the original molecule and that contributed by the derivative ^{13}C using the matrix approach (Fernandez *et al*., 1996). Natural isotopomer distributions in unlabelled glucose and amino acids were quantified from blood samples taken from embryos that had not received the isotopic tracer. Corrected enrichments are reported as moles of tracer (M+*n*) per 100 moles of tracee (M+0) for the calculations described below.

Statistical Analysis

After verifying for assumptions of normality and homogeneity of variance, results were analyzed by ANOVA using the mixed procedure of SAS (version 8.0, SAS Institute, Inc. Cary, NC) with small and large eggs as treatment groups and days of incubation as blocks. Treatment means were compared by Tukey-Kramer multiple comparison test. Data are presented as least square means ± SEM and the differences are considered significant at $P < 0.05$ while $P < 0.1$ is considered a trend.

Results

Embryo weights

Embryonic weights were significantly higher ($P < 0.05$) for the embryos from large eggs on both day 14 (11.4 ± 0.53g vs. 13.3 ± 0.47g) and 19 (31.0 ± 0.58g vs. 36.7 ± 0.34g) of incubation.

Figure 5.1 shows the time-course of enrichments of [M+2] and [M+3] glucose isotopomers and the ratio of [M+2] / [M+3] in embryonic blood after 1, 2, 3 or 4 days of daily injection of [U-^{13}C] glycerol into incubating eggs. By day 4, glucose isotopic and isotopomer steady state had been attained. Thus, in the main study, glycerol tracer was administered for 4 consecutive days prior to blood and tissue sampling.

Even though blood glycerol enrichments were measured (**Table 5.1**), the data was not used to calculate the contributions to glucose and glycogen synthesis due to the high variation between embryos.

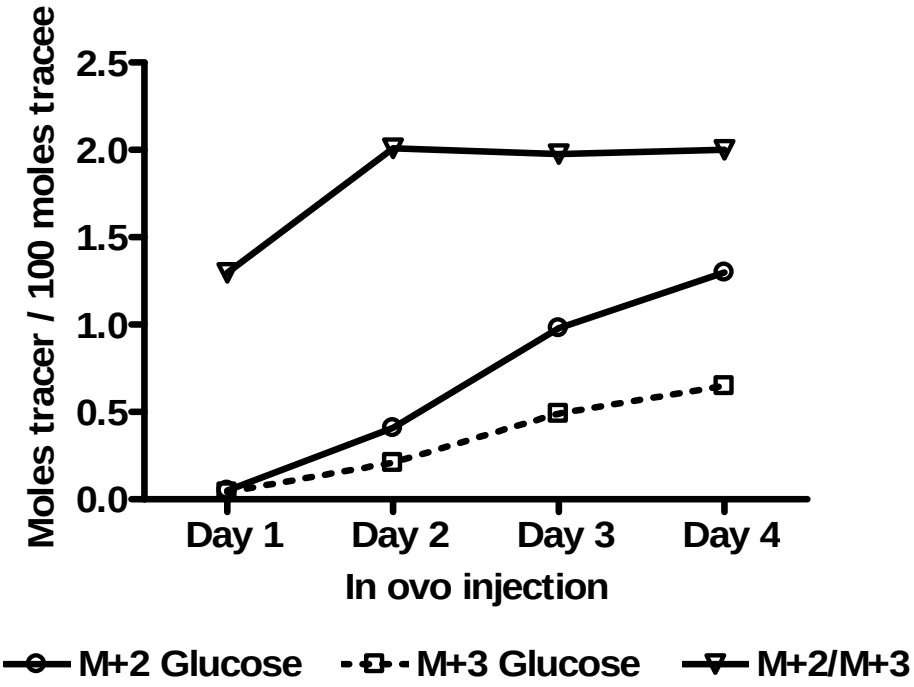

Figure 5.1: Enrichments of M+2 and M+3 isotopomers and the ratio of M+2 / M+3 during 4 days of [U-^{13}C] glycerol injection for validating the tracer approach. Glucose isotopomers were in metabolic steady state by four days of tracer injection.

Glucose and Glycogen Enrichments

Blood glucose and tissue glycogen enrichments are presented in **Table 5.2**. *In ovo* injection of [U-^{13}C]glycerol (M+3) resulted in significant enrichments of [M+1], [M+2], [M+3] in blood glucose, liver glycogen and muscle glycogen. On day 14 of embryonic development, all the blood glucose isotopomers were higher ($P < 0.05$) in small egg embryos compared to the large egg embryos. However on day 19, the variation between individual embryos was greater. Thus, even though the mean blood glucose isotopomer enrichments were higher in small egg embryos, the enrichment differences between small and large egg embryos did not achieve statistical significance. Blood glucose enrichments on day 19 of embryonic development were higher ($P < 0.05$) than on day 14.

On day 14 of embryonic development, [M+1], [M+2] and [M+3] in muscle glycogen from small egg embryos tended to be higher relative to the large egg embryos ($P < 0.1$); however no differences were observed on day 19. Enrichments of these isotopomers in liver glycogen were similar between small and large embryos on both day 14 and 19. Glycogen enrichments did not change from day 14 to 19 in liver. However, day 14 muscle glycogen enrichments of [M+1] ($P = 0.11$), [M+2] ($P = 0.042$) and [M+3] ($P = 0.026$) isotopomers were higher on day 14 of embryonic development in both small and large groups.

Table 5.1: Isotopomer enrichments of blood glycerol in 14 and 19 day old chicken embryos injected *in ovo* with [U-^{13}C]glycerol[1]

Day of Incubation	Egg type[2]	[M + 1]	[M + 2]	[M + 3]
D14	**S**	2.7 ± 1.27	2.7 ± 1.07	264.6 ± 203.28
D14	**L**	2.2 ± 0.87	2.2 ± 0.78	136.0 ± 56.56
D19	**S**	2.3 ± 1.12	6.1 ± 2.11	572.7 ± 257.43
D19	**L**	1.0 ± 0.37	5.9 ± 2.77	233.8 ± 131.27

[1]Enrichments expressed as moles tracer per 100 moles of tracee;
Values are means of 5 to 7 embryos
[2]**S** = small, **L** = large

Table 5.2: Isotopomer enrichments in blood glucose, liver glycogen and muscle glycogen in 14 and 19 day old chicken embryos injected *in ovo* with [U-^{13}C]glycerol[1]

Day of Incubation	Egg type[2]	M+1		M+2		M+3		M+4		M+5		M+6	
		Blood Glucose Isotopomer Enrichments											
D14	**S**	3.6	±0.43	3.7	±0.42	2.6	±0.32	0.13	±0.028	0.08	±0.018	0.12	±0.029
	L	2.1	±0.36	2.2	±0.37	1.4	±0.30	0.04	±0.010	0.03	±0.008	0.02	±0.007
D19	**S**	8.8	±1.19	9.8	±1.25	8.0	±1.23	0.79	±0.166	0.52	±0.114	0.67	±0.181
	L	5.9	±1.90	6.1	±2.12	4.9	±1.89	0.45	±0.234	0.31	±0.158	0.44	±0.218
		Liver Glycogen Isotopomer Enrichments											
D14	**S**	2.8	±0.63	2.6	±0.61	1.7	±0.41	0.15	±0.046	0.07	±0.019	0.27	±0.112
	L	2.0	±0.41	1.8	±0.30	1.0	±0.20	0.08	±0.030	0.05	±0.031	0.44	±0.074
D19	**S**	1.7	±0.35	2.2	±0.50	1.3	±0.41	0.14	±0.074	0.11	±0.059	0.07	±0.038
	L	1.6	±0.33	2.0	±0.44	1.3	±0.40	0.08	±0.037	0.06	±0.031	0.03	±0.020
		Muscle Glycogen Isotopomer Enrichments											
D14	**S**	2.6	±0.49	2.5	±0.41	2.6	±0.49	0.14	±0.053	0.09	±0.033	0.21	±0.097
	L	1.3	±0.40	1.5	±0.28	1.3	±0.39	0.04	±0.011	0.02	±0.011	0.08	±0.036
D19	**S**	1.2	±0.23	1.6	±0.18	1.3	±0.15	0.09	±0.023	0.07	±0.022	0.25	±0.083
	L	0.76	±0.169	1.1	±0.22	1.0	±0.22	0.13	±0.033	0.06	±0.024	0.57	±0.203

[1]Enrichments expressed as moles tracer per 100 moles of tracee;
Values are means of 5 to 7 embryos
[2]**S** = small, **L** = large

Contribution of Glycerol to NEAA synthesis

The isotopomer distributions in alanine, aspartate and glutamate are presented in **Appendix 3A, B and C** respectively.

For all the amino acids, isotopomer enrichments were different ($P < 0.05$) between tissues and also between blood and tissues. Alanine isotopomers were generally higher enriched in blood and tissues of small embryos, however this was a trend ($P < 0.1$) only on day 14 of embryonic development with no differences on day 19. A similar trend was observed with both aspartate and glutamate isotopomers with only day 14 liver and muscle aspartate isotopomers achieving statistical significance ($P < 0.01$).

Discussion

Egg yolk contains 60-70% triglycerides which are extensively oxidized during the latter half of embryo development, providing more than 90% of the energy requirements of the embryos (Sato *et al*., 2006). Oxidation of triglyceride will release the glycerol 3 carbon units which can enter the glycolytic pathway at the triose phosphate level. Glycerol can contribute its carbon through reverse glycolysis towards the synthesis of glucose and also through forward glycolysis towards the synthesis of pyruvate and NEAA, associated with Krebs cycle activity. The objective of this study was to determine the contribution of glycerol to glucose and glycogen synthesis and also to NEAA synthesis using a [U-^{13}C]glycerol tracer.

In ovo injection of glycerol (M+3) resulted in significant enrichments of [M+1], [M+2] and [M+3] isotopomers in blood glucose with the enrichments higher on day 19. In fact, the [M+3] glucose isotopomer was enriched 3-fold higher than [M+3] glucose on day 14. This suggests a greater contribution of glycerol carbon to glucose synthesis

during day 19. Glycerol concentrations on day 14 and day 18 of embryonic development were found to be similar in broiler and layer embryos (Sato *et al*., 2006). Assuming that the glycerol concentrations during the latter half of incubation are maintained, a probable reason for the dilution in glucose enrichments from glycerol tracer, observed on day 14 in this study may be the contribution from other unlabeled carbon sources. Gluconeogenic amino acids being the most probable candidates, the contribution of these amino acids to glucose synthesis may be significant during early stages of embryo development. There was a significant increase in [M+1] and [M+2] isotopomers in blood glucose from day 14 to 19 of embryonic development suggesting high recycling of glycerol to glucose. Thus, with increasing rates of gluconeogenesis with embryonic development and with a fixed nutrient pool, the high recycling rates would aid in conserving glycerol carbon for glucose synthesis.

The higher enrichments of [M+1], [M+2] and [M+3] isotopomers in blood glucose of small embryos could suggest a larger proportion of glycerol carbon being transferred to glucose in these embryos. However, this needs further validation as the actual proportional contribution of glycerol to glucose could not be calculated in this study due to high variability in blood glycerol enrichments. Further, differences in yolk solids have been detected between small and large eggs from the same flock (Lourens et al., 2006) with the small eggs having lower yolk content. Thus the higher isotopomer enrichments observed in blood glucose from [U-^{13}C]glycerol may reflect the relatively smaller glycerol precursor pool in small egg embryos.

The carbon precursors for glycogen synthesis during embryonic development are not well defined. Significant enrichments in [M+1], [M+2] and [M+3] isotopomers in

liver and muscle glycogen suggests that glycerol is a major precursor for glycogen synthesis. Glycogen synthesis can occur via direct phosphorylation of glucose and incorporation of glucose-6-phosphate into glycogen or indirectly through the incorporation of glycolytic intermediates. In humans and rats, evidence exists that only a small proportion of intact glucose is incorporated in to glycogen and the majority of glycogen synthesis occurs via the indirect pathway (Katz and McGarry, 1984; Huang and Veech, 1988). This certainly seems to be the case in developing chicken embryos with high incorporation of glycerol carbon into glycogen as reported in this study. Further, another study from our lab found indicated minimal synthesis of glycogen from blood glucose on embryonic day 15. Enrichment of isotopomers in glucose units released from glycogen following *in ovo* [U-^{13}C]glucose tracer injection was insignificant (data not reported). The differences between isotopomer enrichments in blood glucose and the enrichments in liver and muscle glycogen following *in ovo* [U-^{13}C]glycerol injection were higher on day 19 embryonic compared to the differences on day 14. Thus on day 19 the contribution of unlabeled three carbon units to glycogen synthesis may be greater compared to initial stage of incubation.

In ovo [U-^{13}C]glycerol injection resulted in significant enrichment of [M+3] alanine which was similar between blood and the tissue samples suggesting that glycerol is a major source for alanine synthesis. Isotopomer enrichments of [M+1], [M+2] and [M+3] aspartate and glutamate were higher in liver compared to all other tissues which is consistent with the known function of liver as the primary organ of lipid metabolism. Mass isotopomer profiles in different tissues vs. blood were different and thus blood may

not always reflect whole body tissue metabolism and specific tissue nutrient requirements.

In summary, significant enrichments of blood glucose and liver and muscle glycogen after administration of [U-^{13}C]glycerol, suggest that glycerol is a major carbon source for glucose and glycogen synthesis in developing chicken embryos. Further, differences in enrichments between small and large embryos suggest that initial yolk content may be the limiting factor determining the partitioning of glycerol carbon for glucose synthesis in small vs. large embryos.

Summary and Conclusions

The hypothesis that small eggs perform more poorly compared to chicks from larger eggs because of their limited supplies of specific macronutrients during embryonic development was tested in this thesis. The research hypothesis was formulated with the objective of deciphering the regulation of metabolic pathway fluxes during chicken embryonic development in response to nutrient availability. Universally labeled stable isotopes ([U-^{13}C]glucose, glutamate, glutamine and glycerol) were used for the experiments as the redistribution of their carbon skeletons through central metabolic pathways allowed simultaneous quantification of glucose and amino acid metabolism.

The results form the four studies demonstrate the ability of the developing embryo to alter nutrient fluxes through central metabolic pathways depending on the stage of development and nutrient requirements of the tissues. For example, with embryonic age, the availability of glucose *in ovo* was increased by increasing gluconeogenesis. Glutamate and glutamine were not major precursors to glucose synthesis in day 19 embryos. However, following [U-^{13}C]glycerol injection significant labeling was detected both in blood glucose and liver and muscle glycogen. Thus, glycerol was a major carbon precursor for glucose and glycogen synthesis. Further, with increasing glucose production, glucose carbon had a greater role in embryonic non-essential amino acid synthesis and energy metabolism via the Krebs cycle.

Mass isotopomer distribution patterns in various tissues demonstrated differences in nutrient fluxes between tissues indicating the flexibility of these tissues in adapting their metabolism based on nutrient availability and tissue needs. For example, the flux of proline from glutamate and glutamine was 3 to 4 fold higher in liver when compared to

other tissues. A further example in blood demonstrated reduced glycolytic flux with *in ovo* supplementation with glucose and/or amino acids.

Even though the literature on macronutrient composition of the egg is extensive, our knowledge regarding macronutrient utilization and nutrient partitioning in developing chicken embryos is limited mostly to early information from indirect calorimetry and *in vitro* studies . Knowledge of nutrient use by the embryo is particularly important due to the metabolic transition during late term embryonic development from a lipid environment *in ovo* to exogenous carbohydrates post hatch. This period is critical in the development of the post hatch chick with high mortality rates and lower growth rates associated with inefficient metabolic transition. Thus knowledge of the nutrient fluxes and partitioning through central pathways will provide us a picture of specific tissue requirements which will in turn allow us to tailor macronutrient compositions for optimal growth and development of the chick.

Appendix 1A

Tracer: Tracee ratios of blood glucose isotopomers (M+1, 2…..) after *in ovo* [U-^{13}C]glucose injection; Study 1, Chapter 2[1].

Day of Incubation	Egg type[2]	M+1	M+2	M+3	M+4	M+5	M+6
D12	**S**	1.31 ± 0.189	1.21 ± 0.154	0.38 ± 0.044	0.00 ± 0.00	0.00 ± 0.00	6.95 ± 0.739
	L	1.18 ± 0.096	1.02 ± 0.104	0.38 ± 0.049	0.00 ± 0.00	0.00 ± 0.00	18.67 ± 1.535
D14	**S**	2.74 ± 0.320	1.10 ± 0.201	0.28 ± 0.058	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.165
	L	2.66 ± 0.245	1.08 ± 0.100	0.30 ± 0.041	0.00 ± 0.00	0.00 ± 0.00	1.85 ± 0.237
D16	**S**	2.60 ± 0.398	0.72 ± 0.119	0.17 ± 0.034	0.00 ± 0.00	0.00 ± 0.00	2.91 ± 0.417
	L	1.27 ± 0.157	0.61 ± 0.133	0.16 ± 0.038	0.00 ± 0.00	0.00 ± 0.00	2.15 ± 0.361
D18	**S**	3.48 ± 0.700	2.06 ± 0.704	0.51 ± 0.181	0.00 ± 0.00	0.00 ± 0.00	3.84 ± 1.957
	L	3.64 ± 0.598	1.54 ± 0.301	0.37 ± 0.073	0.00 ± 0.00	0.00 ± 0.00	2.83 ± 0.829

[1]Values are means ± standard error of five embryos expressed as moles tracer / 100 moles tracee
[2]**S** = small, **L** = Large

Appendix 1B

Tracer: Tracee ratios of alanine isotopomers in blood, liver (**Table 1**), intestine and muscle (**Table 2**) after *in ovo* [U-^{13}C]glucose injection; Study 1, Chapter 2[1].

Table 1

Day of incubation	Egg type[2]	Blood			Liver		
		M+1	M+2	M+3	M+1	M+2	M+3
D12	**S**	0.46 ± 0.052	0.21 ± 0.043	0.39 ± 0.039	0.65 ± 0.099	0.33 ± 0.067	0.17 ± 0.033
	L	0.64 ± 0.062	0.33 ± 0.046	0.78 ± 0.316	0.68 ± 0.047	0.42 ± 0.059	0.24 ± 0.062
D14	**S**	0.78 ± 0.050	0.24 ± 0.059	0.41 ± 0.047	0.99 ± 0.094	0.42 ± 0.086	0.19 ± 0.041
	L	0.85 ± 0.101	0.23 ± 0.034	0.43 ± 0.043	1.07 ± 0.107	0.44 ± 0.033	0.20 ± 0.028
D16	**S**	0.65 ± 0.060	0.08 ± 0.022	0.24 ± 0.051	0.63 ± 0.030	0.19 ± 0.025	0.10 ± 0.008
	L	0.40 ± 0.081	0.10 ± 0.037	0.16 ± 0.036	0.48 ± 0.089	0.24 ± 0.073	0.13 ± 0.013
D18	**S**	1.18 ± 0.334	0.62 ± 0.200	1.17 ± 0.479	1.32 ± 0.321	0.71 ± 0.150	0.39 ± 0.081
	L	0.67 ± 0.102	0.26 ± 0.066	0.53 ± 0.097	0.70 ± 0.059	0.28 ± 0.042	0.20 ± 0.035

Table 2

Day of incubation	Egg type[2]	Intestine			Muscle		
		M+1	M+2	M+3	M+1	M+2	M+3
D12	**S**	0.54 ± 0.104	0.37 ± 0.063	0.51 ± 0.061	0.52 ± 0.085	0.33 ± 0.058	0.39 ± 0.088
	L	0.50 ± 0.036	0.30 ± 0.036	0.50 ± 0.114	0.58 ± 0.052	0.38 ± 0.024	0.53 ± 0.124
D14	**S**	0.86 ± 0.129	0.37 ± 0.081	0.30 ± 0.044	0.85 ± 0.096	0.35 ± 0.070	0.37 ± 0.082
	L	0.89 ± 0.115	0.38 ± 0.049	0.42 ± 0.126	0.89 ± 0.064	0.38 ± 0.034	0.38 ± 0.049
D16	**S**	0.64 ± 0.080	0.26 ± 0.060	0.20 ± 0.089	0.65 ± 0.041	0.19 ± 0.016	0.17 ± 0.022
	L	0.38 ± 0.070	0.26 ± 0.059	0.21 ± 0.049	0.44 ± 0.058	0.24 ± 0.061	0.19 ± 0.046
D18	**S**	1.15 ± 0.191	0.57 ± 0.092	0.50 ± 0.092	1.35 ± 0.162	0.85 ± 0.093	0.65 ± 0.104
	L	0.66 ± 0.094	0.32 ± 0.058	0.25 ± 0.035	0.75 ± 0.108	0.40 ± 0.054	0.31 ± 0.034

[1]Values are means ± standard error of five embryos expressed as moles tracer / 100 moles tracee

[2] **S** = small, **L** = Large

Appendix 1C

Tracer: Tracee ratios of aspartate isotopomers in blood, liver (**Table 1**), intestine and muscle (**Table 2**) after *in ovo* [U-^{13}C]glucose injection; Study 1, Chapter 2[1].

Table 1

Day of incubation	Egg type[2]	Blood						Liver					
		M+1		M+2		M+3		M+1		M+2		M+3	
D12	**S**	0.59 ±	0.121	.	.	0.02 ±	0.001	0.77 ±	0.157	0.33 ±	0.064	0.15 ±	0.026
	L	0.93 ±	0.152	.	.	0.02 ±	0.035	0.90 ±	0.241	0.38 ±	0.127	0.16 ±	0.189
D14	**S**	1.21 ±	0.076	0.11 ±	0.036	0.03 ±	0.006	1.53 ±	0.129	0.40 ±	0.110	0.18 ±	0.040
	L	1.32 ±	0.054	0.09 ±	0.029	0.04 ±	0.021	1.53 ±	0.211	0.45 ±	0.052	0.23 ±	0.030
D16	**S**	0.75 ±	0.062	0.22 ±	0.039	0.09 ±	0.024	1.01 ±	0.040	0.22 ±	0.035	0.09 ±	0.013
	L	0.58 ±	0.059	0.04 ±	0.014	0.02 ±	0.003	1.41 ±	0.237	0.68 ±	0.194	-	-
D18	**S**	0.54 ±	0.135	0.33 ±	0.091	0.15 ±	0.064	2.08 ±	0.444	0.88 ±	0.180	0.24 ±	0.051
	L	0.47 ±	0.070	0.33 ±	0.107	0.13 ±	0.052	1.19 ±	0.134	0.37 ±	0.071	0.11 ±	0.017

Table 2

Day of incubation	Egg type[2]	Intestine						Muscle					
		M+1		M+2		M+3		M+1		M+2		M+3	
D12	**S**	0.69 ±	0.071	0.17 ±	0.025	0.03 ±	0.004	0.80 ±	0.068	0.24 ±	0.023	0.01 ±	0.003
	L	0.83 ±	0.167	0.22 ±	0.079	0.03 ±	0.030	0.83 ±	0.131	0.44 ±	0.055	0.10 ±	0.036
D14	**S**	1.40 ±	0.274	0.48 ±	0.154	0.11 ±	0.065	1.00 ±	0.059	0.40 ±	0.057	0.16 ±	0.078
	L	1.09 ±	0.079	0.40 ±	0.083	0.07 ±	0.032	0.98 ±	0.074	0.35 ±	0.039	0.11 ±	0.050
D16	**S**	1.22 ±	0.096	0.38 ±	0.087	0.09 ±	0.046	0.89 ±	0.064	0.32 ±	0.043	0.04 ±	0.022
	L	0.74 ±	0.083	0.28 ±	0.046	0.09 ±	0.027	0.66 ±	0.030	0.32 ±	0.042	0.07 ±	0.028
D18	**S**	1.51 ±	0.203	0.44 ±	0.063	0.10 ±	0.010	1.48 ±	0.202	0.51 ±	0.071	0.07 ±	0.008
	L	1.11 ±	0.126	0.39 ±	0.077	0.10 ±	0.025	1.09 ±	0.112	0.56 ±	0.046	0.12 ±	0.027

[1]Values are means ± standard error of five embryos expressed as moles tracer / 100 moles tracee

[2] **S** = small, **L** = Large

Appendix 1D

Tracer: Tracee ratios of glutamate isotopomers in blood, liver (**Table 1**), intestine and muscle (**Table 2**) after *in ovo* [U-^{13}C]glucose injection; Study 1, Chapter 2[1].

Table 1

Day of incubation	Egg type[2]	Blood			Liver		
		M+1	M+2	M+3	M+1	M+2	M+3
D12	**S**	0.46 ± 0.085	0.22 ± 0.036	0.04 ± 0.004	0.87 ± 0.072	0.41 ± 0.065	0.16 ± 0.012
	L	0.60 ± 0.066	0.25 ± 0.040	0.06 ± 0.009	0.96 ± 0.047	0.47 ± 0.037	0.18 ± 0.019
D14	**S**	1.70 ± 0.304	1.04 ± 0.327	0.10 ± 0.029	1.51 ± 0.090	0.78 ± 0.110	0.16 ± 0.024
	L	1.82 ± 0.304	1.00 ± 0.312	0.10 ± 0.027	1.60 ± 0.192	0.84 ± 0.117	0.16 ± 0.017
D16	**S**	2.25 ± 0.418	2.21 ± 0.532	0.22 ± 0.054	1.47 ± 0.071	0.78 ± 0.029	0.1 ± 0.028
	L	0.94 ± 0.182	0.45 ± 0.177	0.04 ± 0.008	1.21 ± 0.146	0.65 ± 0.108	0.26 ± 0.116
D18	**S**	3.69 ± 0.995	3.55 ± 1.414	0.36 ± 0.180	2.18 ± 0.339	1.66 ± 0.337	0.20 ± 0.037
	L	3.33 ± 0.535	3.35 ± 0.874	0.41 ± 0.139	1.56 ± 0.127	1.01 ± 0.097	0.18 ± 0.028

Table 2

Day of incubation	Egg type[2]	Intestine			Muscle		
		M+1	M+2	M+3	M+1	M+2	M+3
D12	**S**	0.82 ± 0.061	0.29 ± 0.043	0.07 ± 0.007	1.07 ± 0.089	0.30 ± 0.039	0.05 ± 0.008
	L	0.95 ± 0.055	0.35 ± 0.025	0.09 ± 0.012	1.09 ± 0.065	0.39 ± 0.026	0.05 ± 0.003
D14	**S**	1.58 ± 0.158	0.76 ± 0.220	0.06 ± 0.023	1.29 ± 0.056	0.46 ± 0.051	0.05 ± 0.013
	L	1.26 ± 0.155	0.48 ± 0.097	0.09 ± 0.016	1.33 ± 0.092	0.45 ± 0.065	0.04 ± 0.004
D16	**S**	1.28 ± 0.055	0.67 ± 0.072	0.16 ± 0.005	1.28 ± 0.059	0.51 ± 0.048	0.04 ± 0.004
	L	0.80 ± 0.058	0.27 ± 0.038	0.10 ± 0.014	0.88 ± 0.037	0.25 ± 0.020	0.02 ± 0.003
D18	**S**	1.73 ± 0.260	0.97 ± 0.191	0.17 ± 0.018	1.88 ± 0.266	1.03 ± 0.219	0.15 ± 0.019
	L	1.38 ± 0.153	0.71 ± 0.124	0.13 ± 0.010	1.39 ± 0.264	0.74 ± 0.194	0.13 ± 0.018

[1]Values are means ± standard error of five embryos expressed as moles tracer / 100 moles tracee

[2] **S** = small, **L** = Large

Appendix 1E

Tracer: Tracee ratios of Krebs cycle intermediates isotopomers in liver after *in ovo* [U-^{13}C]glucose injection; Study 1, Chapter 2[1].

	Day of incubation	Egg type[2]	(moles tracer / 100 moles tracee)		
			M+1	**M+2**	**M+3**
Lactate	**D 16**	**S**	1.15 ± 0.045	0.46 ± 0.062	0.11 ± 0.011
		L	0.74 ± 0.061	0.41 ± 0.069	0.10 ± 0.019
	D 18	**S**	2.69 ± 0.537	1.45 ± 0.314	0.30 ± 0.072
		L	1.73 ± 0.302	0.79 ± 0.232	0.10 ± 0.021
Pyruvate	**D 16**	**S**	0.82 ± 0.046	0.24 ± 0.022	0.10 ± 0.012
		L	0.34 ± 0.099	0.28 ± 0.077	0.13 ± 0.017
	D 18	**S**	2.21 ± 0.416	1.15 ± 0.238	0.31 ± 0.057
		L	1.31 ± 0.224	0.51 ± 0.128	0.13 ± 0.019
Succinate	**D 16**	**S**	0.71 ± 0.095	0.49 ± 0.019	0.04 ± 0.008
		L	0.48 ± 0.065	0.43 ± 0.042	0.04 ± 0.010
	D 18	**S**	2.18 ± 0.479	1.20 ± 0.219	0.16 ± 0.038
		L	1.39 ± 0.202	0.61 ± 0.096	0.08 ± 0.009
Malate	**D 16**	**S**	1.60 ± 0.145	0.53 ± 0.056	0.08 ± 0.004
		L	1.15 ± 0.082	0.50 ± 0.096	0.08 ± 0.018
	D 18	**S**[*]	2.37 ± 0.534	1.14 ± 0.296	0.07 ± 0.045
		L[*]	1.88 ± 0.046	0.56 ± 0.044	0.06 ± 0.014
Oxaloacetate	**D 16**	**S**	2.38 ± 0.114	0.64 ± 0.040	0.06 ± 0.035
		L	2.24 ± 0.111	0.60 ± 0.085	0.09 ± 0.016
	D 18	**S**	. .	. .	. .
		L	. .	. .	. .
α-Ketoglutarate	**D 16**	**S**	1.18 ± 0.069	0.87 ± 0.052	0.05 ± 0.011
		L	0.39 ± 0.081	0.50 ± 0.049	0.13 ± 0.042
	D 18	**S**[*]	2.15 ± 0.521	1.13 ± 0.356	0.12 ± 0.072
		L	1.61 ± 0.198	1.08 ± 0.161	0.07 ± 0.047

[1]Values are means ± standard error of five embryos

[*]Values are means ± standard error of three embryos

[2]**S** = small, **L** = Large

Appendix 2A

Tracer: Tracee ratios of blood glucose isotopomers (M+1, 2…..) in day 12 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Nutrient Treatments[2]	Egg Type[3]	M+1		M+2		M+3		M+4		M+5		M+6	
C	**S**	0.70	±0.341	0.78	±0.243	0.28	±0.073	0.00	±0.00	0.30	±0.081	4.67	±1.271
	L	0.97	±0.259	0.98	±0.267	0.34	±0.083	0.00	±0.00	0.27	±0.059	4.22	±0.984
G	**S**	1.47	±0.214	1.51	±0.174	0.51	±0.055	0.00	±0.00	0.28	±0.024	4.43	±0.448
	L	0.73	±0.276	0.99	±0.254	0.35	±0.087	0.00	±0.00	0.19	±0.042	2.92	±0.682
AA	**S**	3.05	±0.319	2.77	±0.206	0.97	±0.079	0.00	±0.00	1.23	±0.265	18.21	±3.969
	L	2.78	±0.372	2.60	±0.397	0.90	±0.138	0.00	±0.00	0.45	±0.113	6.45	±1.590
G+AA	**S**	1.80	±0.270	1.60	±0.260	0.52	±0.089	0.00	±0.00	0.51	±0.069	7.90	±1.130
	L	1.19	±0.135	1.09	±0.074	0.37	±0.028	0.00	±0.00	0.40	±0.124	6.02	±1.894

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.
[3]**S** = small, **l** = Large.

Appendix 2B

Tracer: Tracee ratios of blood glucose isotopomers (M+1, 2…..) in day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Nutrient Treatments[2]	Egg Type[3]	M+1		M+2		M+3		M+4		M+5		M+6	
C	**S**	6.35	±2.109	6.32	±2.544	1.85	±0.964	0.34	±0.241	0.40	±0.195	4.11	±1.832
	L	7.43	±0.978	6.36	±0.721	1.56	±0.187	0.20	±0.047	0.19	±0.021	1.90	±0.217
G	**S**	6.87	±1.047	6.51	±1.250	1.90	±0.457	0.28	±0.085	0.33	±0.091	3.51	±0.954
	L	9.20	±0.650	8.59	±0.716	2.59	±0.313	0.39	±0.054	0.38	±0.090	3.56	±0.926
AA	**S**	8.10	±1.899	7.14	±1.890	2.04	±0.691	0.32	±0.155	0.31	±0.108	2.91	±0.818
	L	6.35	±0.815	6.19	±0.886	1.69	±0.325	0.22	±0.062	0.23	±0.047	2.24	±0.443
G+AA	**S**	7.37	±1.070	6.55	±0.808	1.74	±0.297	0.25	±0.056	0.31	±0.059	3.46	±0.688
	L	6.58	±0.531	6.30	±0.612	1.77	±0.199	0.21	±0.038	0.33	±0.044	3.63	±0.509

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.
[3]**S** = small, **L** = large.

Appendix 2C

Tracer: Tracee ratios of blood alanine isotopomers (M+1, 2 and 3) in day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Nutrient Treatments[2]	Egg Type[3]	[M + 1]	[M + 2]	[M + 3]
C	S	1.57 ±0.454	1.76 ±0.460	1.36 ±0.513
	L	1.92 ±0.182	1.61 ±0.149	1.18 ±0.143
G	S	1.78 ±0.234	1.59 ±0.260	1.58 ±0.379
	L	2.12 ±0.211	2.39 ±0.618	1.67 ±0.247
AA	S	1.55 ±0.468	1.32 ±0.358	1.05 ±0.356
	L	0.94 ±0.139	0.86 ±0.105	0.73 ±0.093
G+AA	S	0.90 ±0.070	0.84 ±0.070	0.70 ±0.075
	L	0.56 ±0.057	0.63 ±0.064	0.58 ±0.076

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.
[3]**S** = small, **L** = large.

Appendix 2C contd

Tracer: Tracee ratios of alanine isotopomers (M+1, 2 and 3) in liver, intestine (Table 1), muscle and kidney (Table 2) of day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Table 1

Nutrient Treatments[2]	Egg Type[3]	Liver						Intestine					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	**S**	0.64	±0.052	0.72	±0.057	0.50	±0.064	0.99	±0.088	1.00	±0.081	1.06	±0.154
	L	0.75	±0.125	0.79	±0.102	0.47	±0.030	0.93	±0.117	0.91	±0.087	0.93	±0.080
G	**S**	0.74	±0.064	0.78	±0.046	0.51	±0.029	1.03	±0.086	1.05	±0.073	1.15	±0.130
	L	0.60	±0.092	0.69	±0.063	0.42	±0.044	1.05	±0.065	1.05	±0.054	1.33	±0.141
AA	**S**	0.86	±0.089	0.87	±0.080	0.65	±0.043	1.14	±0.137	1.17	±0.108	1.18	±0.162
	L	0.80	±0.060	0.84	±0.058	0.62	±0.050	0.93	±0.074	1.01	±0.072	0.96	±0.083
G+AA	**S**	0.84	±0.059	0.84	±0.048	0.64	±0.033	1.05	±0.039	1.06	±0.038	1.19	±0.066
	L	0.94	±0.042	0.93	±0.033	0.60	±0.029	0.91	±0.046	0.98	±0.033	1.03	±0.029

Table 2

Nutrient Treatments[2]	Egg Type[3]	Muscle						Kidney					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	**S**	1.40	±0.050	1.35	±0.091	1.29	±0.165	1.09	±0.061	1.11	±0.078	0.83	±0.063
	L	1.30	±0.096	1.22	±0.095	1.10	±0.086	1.27	±0.201	1.02	±0.122	0.70	±0.069
G	**S**	1.31	±0.058	1.29	±0.049	1.38	±0.072	2.08	±0.215	1.71	±0.216	2.14	±0.495
	L	1.37	±0.141	1.30	±0.092	1.25	±0.081	2.02	±0.179	1.67	±0.205	2.16	±0.563
AA	**S**	1.30	±0.048	1.35	±0.069	1.18	±0.098	1.62	±0.226	1.41	±0.178	1.35	±0.171
	L	1.08	±0.078	1.22	±0.070	1.00	±0.084	1.69	±0.182	1.42	±0.138	1.23	±0.132
G+AA	**S**	1.17	±0.048	1.29	±0.048	1.42	±0.115	1.30	±0.137	1.31	±0.083	1.18	±0.137
	L	1.10	±0.076	1.20	±0.075	1.16	±0.094	1.25	±0.092	1.25	±0.081	1.12	±0.081

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.

[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.

[3]**S** = small, **L** = large.

Appendix 2D

Tracer: Tracee ratios of blood aspartate isotopomers (M+1, 2 and 3) in day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Nutrient Treatments[2]	Egg Type[3]	[M + 1]	[M + 2]	[M + 3]
C	S	1.88 ±0.310	0.08 ±0.052	0.06 ±0.018
	L	3.23 ±0.512	0.19 ±0.083	0.05 ±0.033
G	S	1.87 ±0.318	0.22 ±0.080	0.20 ±0.179
	L	0.91 ±0.058	0.35 ±0.064	0.10 ±0.019
AA	S	3.37 ±0.636	0.30 ±0.078	0.06 ±0.027
	L	1.06 ±0.166	0.17 ±0.034	0.03 ±0.013
G+AA	S	2.05 ±0.380	0.25 ±0.030	0.06 ±0.029
	L	1.73 ±0.128	0.24 ±0.027	0.05 ±0.011

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.
[3]**S** = small, **L** = large.

Appendix 2D contd

Tracer: Tracee ratios of aspartate isotopomers (M+1, 2 and 3) in liver, intestine (Table 1), muscle and kidney (Table 2) of day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Table 1

Nutrient Treatments[2]	Egg Type[3]	Liver						Intestine					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	**S**	1.92	±0.096	1.03	±0.083	0.28	±0.036	0.94	±0.053	0.43	±0.036	0.13	±0.012
	L	2.32	±0.246	1.14	±0.115	0.25	±0.016	0.91	±0.052	0.37	±0.021	0.12	±0.005
G	**S**	2.08	±0.153	1.13	±0.078	0.31	±0.017	1.10	±0.059	0.48	±0.045	0.15	±0.017
	L	2.00	±0.147	1.05	±0.078	0.31	±0.024	1.09	±0.046	0.60	±0.036	0.17	±0.014
AA	**S**	1.01	±0.023	0.84	±0.072	0.38	±0.075	0.78	±0.058	0.72	±0.066	0.09	±0.027
	L	1.47	±0.101	1.07	±0.063	0.35	±0.027	0.71	±0.034	0.67	±0.165	0.14	±0.021
G+AA	**S**	1.10	±0.058	1.03	±0.054	0.49	±0.112	0.86	±0.068	0.73	±0.136	0.41	±0.097
	L	1.86	±0.114	1.33	±0.064	0.44	±0.038	0.77	±0.052	1.18	±0.193	0.21	±0.038

Table 2

Nutrient Treatments[2]	Egg Type[3]	Muscle						Kidney					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	**S**	1.21	±0.130	0.45	±0.074	0.10	±0.012	0.80	±0.134	0.25	±0.096	0.19	±0.073
	L	1.28	±0.082	0.50	±0.037	0.13	±0.030	0.81	±0.172	0.70	±0.089	0.27	±0.054
G	**S**	1.16	±0.067	0.43	±0.022	0.09	±0.009	1.16	±0.074	0.41	±0.036	0.32	±0.051
	L	1.54	±0.066	0.54	±0.047	0.08	±0.002	1.32	±0.128	0.48	±0.087	0.26	±0.070
AA	**S**	1.22	±0.058	0.63	±0.074	0.10	±0.016	2.72	±0.188	0.76	±0.045	0.29	±0.019
	L	1.01	±0.071	0.54	±0.049	0.13	±0.018	2.56	±0.239	0.73	±0.062	0.23	±0.031
G+AA	**S**	1.18	±0.031	0.50	±0.064	0.13	±0.016	1.60	±0.212	0.99	±0.306	0.12	±0.047
	L	1.12	±0.040	0.62	±0.029	0.15	±0.009	0.88	±0.087	0.80	±0.173	0.12	±0.043

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.

[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.

[3]**S** = small, **L** = large.

Appendix 2E

Tracer: Tracee ratios of blood glutamate isotopomers (M+1, 2 and 3) in day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Nutrient Treatments[2]	Egg Type[3]	[M + 1]	[M + 2]	[M + 3]
C	S	1.78 ±0.290	1.14 ±0.256	0.10 ±0.048
	L	2.50 ±0.413	1.86 ±0.492	0.13 ±0.062
G	S	1.85 ±0.211	1.23 ±0.259	0.21 ±0.057
	L	2.88 ±0.321	2.51 ±0.347	0.22 ±0.059
AA	S	1.92 ±0.296	1.33 ±0.381	0.18 ±0.049
	L	1.56 ±0.143	0.79 ±0.089	0.14 ±0.010
G+AA	S	1.93 ±0.076	1.11 ±0.076	0.09 ±0.026
	L	1.71 ±0.131	1.04 ±0.073	0.07 ±0.026

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.
[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.
[3]**S** = small, **L** = large.

Appendix 2E contd

Tracer: Tracee ratios of glutamate isotopomers (M+1, 2 and 3) in liver, intestine (Table 1), muscle and kidney (Table 2) of day 20 chicken embryos after *in ovo* [U-^{13}C]glucose injection; Study 2, Chapter 3[1].

Table 1

Nutrient Treatments[2]	Egg Type[3]	Liver						Intestine					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	S	1.28	±0.034	1.32	±0.108	0.42	±0.167	1.47	±0.102	0.80	±0.101	0.10	±0.007
	L	1.58	±0.123	1.50	±0.114	0.26	±0.069	1.34	±0.093	0.78	±0.069	0.09	±0.009
G	S	1.80	±0.074	1.56	±0.077	1.02	±0.125	1.65	±0.082	0.89	±0.074	0.11	±0.009
	L	1.49	±0.104	1.40	±0.128	0.79	±0.087	1.68	±0.139	1.10	±0.080	0.14	±0.007
AA	S	2.00	±0.139	1.65	±0.166	0.44	±0.118	1.61	±0.161	1.00	±0.161	0.10	±0.022
	L	1.87	±0.086	1.47	±0.094	0.13	±0.053	1.19	±0.093	0.77	±0.046	0.10	±0.012
G+AA	S	2.04	±0.079	1.62	±0.062	0.24	±0.045	1.61	±0.115	0.99	±0.116	0.09	±0.019
	L	1.80	±0.042	1.48	±0.070	0.18	±0.012	1.44	±0.060	0.76	±0.036	0.08	±0.006

Table 2

Nutrient Treatments[2]	Egg Type[3]	Muscle						Kidney					
		[M + 1]		[M + 2]		[M + 3]		[M + 1]		[M + 2]		[M + 3]	
C	S	1.79	±0.239	1.18	±0.236	0.14	±0.033	1.19	±0.257	0.81	±0.166	0.17	±0.043
	L	2.03	±0.171	1.44	±0.147	0.16	±0.014	1.20	±0.036	1.20	±0.094	0.14	±0.039
G	S	1.74	±0.074	1.07	±0.100	0.13	±0.016	1.79	±0.097	1.08	±0.108	0.13	±0.015
	L	2.23	±0.150	1.67	±0.108	0.17	±0.011	2.07	±0.103	1.41	±0.110	0.13	±0.030
AA	S	2.11	±0.267	1.42	±0.225	0.17	±0.033	1.53	±0.080	0.88	±0.151	0.09	±0.030
	L	1.79	±0.098	0.98	±0.074	0.13	±0.010	1.60	±0.125	0.87	±0.111	0.09	±0.021
G+AA	S	2.08	±0.134	1.22	±0.119	0.16	±0.017	0.77	±0.111	0.71	±0.116	0.12	±0.065
	L	1.81	±0.082	1.03	±0.060	0.14	±0.010	0.66	±0.088	0.70	±0.097	0.02	±0.022

[1]Values are means ± standard error of five to nine embryos expressed as moles of tracer per 100 moles of tracee.

[2]**C** = control, **G** = glucose, **AA** = amino acids, **G+AA** = glucose plus amino acids.

[3]**S** = small, **L** = large.

Appendix 3A

Tracer: Tracee ratios of alanine isotopomers (M+1, 2 & 3) in blood and tissues of 14 and 19 day old chicken embryos after *in ovo* [U-^{13}C]glycerol injection; Study 4, Chapter 5[1].

Day of Incubation	Egg Type[2]	[M + 1]	[M + 2]	[M + 3]
		Blood		
D14	**S**	0.64 ±0.127	0.73 ±0.104	1.16 ±0.136
	L	0.31 ±0.101	0.46 ±0.099	0.80 ±0.157
D19	**S**	1.03 ±0.129	1.16 ±0.134	1.24 ±0.120
	L	0.68 ±0.306	0.79 ±0.298	0.91 ±0.294
		Liver		
D14	**S**	1.37 ±0.222	1.31 ±0.157	0.97 ±0.106
	L	0.66 ±0.136	0.75 ±0.108	0.62 ±0.119
D19	**S**	1.53 ±0.266	1.57 ±0.242	1.02 ±0.168
	L	0.97 ±0.333	1.04 ±0.287	0.55 ±0.135
		Intestine		
D14	**S**	0.89 ±0.178	0.86 ±0.128	1.53 ±0.302
	L	0.35 ±0.111	0.45 ±0.083	0.81 ±0.134
D19	**S**	1.20 ±0.179	1.18 ±0.190	2.35 ±0.472
	L	0.66 ±0.174	0.77 ±0.185	1.62 ±0.460
		Muscle		
D14	**S**	0.80 ±0.138	0.84 ±0.116	1.14 ±0.145
	L	0.44 ±0.099	0.49 ±0.090	0.77 ±0.144
D19	**S**	1.40 ±0.177	1.44 ±0.185	1.34 ±0.173
	L	0.92 ±0.193	0.91 ±0.192	0.91 ±0.214
		Kidney		
D14	**S**	1.42 ±0.213	1.16 ±0.138	1.50 ±0.147
	L	0.76 ±0.183	0.73 ±0.135	0.93 ±0.189
D19	**S**	1.80 ±0.220	1.63 ±0.196	1.93 ±0.172
	L	1.30 ±0.388	1.17 ±0.336	1.27 ±0.367

[1]Values are means ± standard error of 6 to 7 embryos expressed as moles of tracer per 100 moles of tracee.
[2]**S** = small, **L** = Large.

Appendix 3B

Tracer: Tracee ratios of aspartate isotopomers (M+1, 2 & 3) in blood and tissues of 14 and 19 day old chicken embryos after *in ovo* [U-^{13}C]glycerol injection; Study 4, Chapter 5[1].

Day of Incubation	Egg Type[2]	[M + 1]	[M + 2]	[M + 3]
		Blood		
D14	**S**	0.50 ±0.067	0.21 ±0.034	0.06 ±0.018
	L	0.34 ±0.046	0.15 ±0.035	0.05 ±0.013
D19	**S**	0.48 ±0.066	0.41 ±0.072	0.07 ±0.017
	L	0.40 ±0.097	0.19 ±0.050	0.06 ±0.013
		Liver		
D14	**S**	2.43 ±0.291	1.56 ±0.180	0.26 ±0.033
	L	1.47 ±0.226	0.93 ±0.148	0.15 ±0.024
D19	**S**	5.23 ±0.723	3.16 ±0.454	0.46 ±0.079
	L	3.30 ±0.933	1.90 ±0.563	0.36 ±0.127
		Intestine		
D14	**S**	0.91 ±0.107	0.37 ±0.037	0.09 ±0.024
	L	0.54 ±0.087	0.19 ±0.037	0.07 ±0.011
D19	**S**	1.57 ±0.205	0.63 ±0.088	0.35 ±0.066
	L	1.05 ±0.214	0.51 ±0.112	0.26 ±0.051
		Muscle		
D14	**S**	1.01 ±0.109	0.36 ±0.039	0.08 ±0.008
	L	0.70 ±0.089	0.24 ±0.037	0.05 ±0.008
D19	**S**	1.76 ±0.251	0.52 ±0.044	0.06 ±0.007
	L	1.10 ±0.277	0.41 ±0.097	0.04 ±0.012
		Kidney		
D14	**S**	0.98 ±0.079	0.41 ±0.043	0.09 ±0.019
	L	0.76 ±0.117	0.29 ±0.033	0.08 ±0.015
D19	**S**	1.76 ±0.188	0.62 ±0.078	0.17 ±0.022
	L	1.23 ±0.281	0.47 ±0.109	0.13 ±0.040

[1]Values are means ± standard error of 6 to 7 embryos expressed as moles of tracer per 100 moles of tracee.
[2]**S** = small, **L** = Large.

Appendix 3C

Tracer: Tracee ratios of glutamate isotopomers (M+1, 2 & 3) in blood and tissues of 14 and 19 day old chicken embryos after *in ovo* [U-^{13}C]glycerol injection; Study 4, Chapter 5[1].

Day of Incubation	Egg Type[2]	[M + 1]	[M + 2]	[M + 3]
		Blood		
D14	**S**	0.81 ±0.077	0.58 ±0.061	0.09 ±0.010
	L	0.61 ±0.069	0.43 ±0.075	0.07 ±0.015
D19	**S**	1.48 ±0.202	1.10 ±0.171	0.12 ±0.023
	L	1.13 ±0.286	0.82 ±0.269	0.10 ±0.035
		Liver		
D14	**S**	1.54 ±0.173	1.49 ±0.184	0.19 ±0.029
	L	1.05 ±0.124	0.94 ±0.173	0.13 ±0.026
D19	**S**	3.03 ±0.427	3.24 ±0.469	0.34 ±0.063
	L	1.86 ±0.412	1.90 ±0.544	0.25 ±0.088
		Intestine		
D14	**S**	0.91 ±0.054	0.53 ±0.081	0.07 ±0.007
	L	0.68 ±0.076	0.32 ±0.051	0.05 ±0.010
D19	**S**	1.54 ±0.189	1.04 ±0.145	0.18 ±0.027
	L	1.34 ±0.319	0.91 ±0.257	0.18 ±0.063
		Muscle		
D14	**S**	1.04 ±0.121	0.57 ±0.060	0.08 ±0.012
	L	0.88 ±0.082	0.37 ±0.061	0.06 ±0.012
D19	**S**	1.90 ±0.222	1.22 ±0.172	0.10 ±0.024
	L	1.46 ±0.335	0.97 ±0.306	0.09 ±0.042
		Kidney		
D14	**S**	1.09 ±0.130	0.67 ±0.078	0.09 ±0.012
	L	0.74 ±0.102	0.49 ±0.078	0.06 ±0.017
D19	**S**	1.88 ±0.207	1.22 ±0.144	0.12 ±0.017
	L	1.48 ±0.374	0.99 ±0.284	0.10 ±0.042

[1]Values are means ± standard error of 6 to 7 embryos expressed as moles of tracer per 100 moles of tracee.
[2]**S** = small, **L** = Large.

BIBLIOGRAPHY

Applegate TJ, Dibner JJ, Kitchell ML, Uni Z, Lilburn MS. Effect of turkey (Meleagridis gallopavo) breeder hen age and egg size on poult development. 2. Intestinal villus growth, enterocyte migration and proliferation of the turkey poult. Comp Biochem Physiol B Biochem Mol Biol. 1999;124(4):381-9.

Applegate TJ, Lilburn MS. Effect of turkey (Meleagridis gallopavo) breeder hen age and egg size on poult development. 1. Intestinal growth and glucose tolerance of the turkey poult. Comp Biochem Physiol B Biochem Mol Biol. 1999;124(4):371-80.

Applegate TJ, Lilburn MS. Independent effects of hen age and egg size on incubation and poult characteristics in commercial turkeys. Poult Sci. 1996;75:1210–6.

Belo PS, Romsos DR, Leville GA. Blood metabolites and glucose metabolism in the fed and fasted chicken. J Nutr. 1976;106(8):1135-43.

Bequette, B.J., N.E. Sunny, S.W. El-Kadi, and S.L. Owens. Application of stable isotopes and mass isotopomer distribution analysis to the study of intermediary metabolism of nutrients. J. Anim. Sci. 2006;84(E. Suppl.):E50-E59.

Brady LJ, Romsos DR, Leveille GA. Gluconeogenesis in isolated chicken liver cells. Comp Biochem Physiol B. 1979;63:193.

Brunengraber H, Kelleher JK, Des Rosiers C. Applications of mass isotopomer analysis to nutrition research. Annu Rev Nutr. 1997;17:559-96.

Burelle Y, Fillipi C, Péronnet F, Leverve X. Mechanisms of increased gluconeogenesis from alanine in rat isolated hepatocytes after endurance training. Am J Physiol Endocrinol Metab. 2000;278(1):E35-42.

Burley RW, Vadehra DV. The avian egg: Chemistry and Biology. John Wiley & Sons. 1989.

Calder AG, Garden KE, Anderson SE, Lobley GE. Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U-(13)C amino acids as internal standards. Rapid Commun Mass Spectrom. 1999;13(21):2080-3.

Corssmit EP, Romijn JA, Sauerwein HP. Regulation of glucose production with special attention to nonclassical regulatory mechanisms: a review. Metabolism. 2001;50(7):742-55.

Chotinsky D, Toncheva E, Profirov Y. Development of disaccharidase activity in the small intestine of broiler chickens. Br Poult Sci. 2001;42:389–93.

Christensen VL, Donaldson WE, Nestor KE, McMurtry JP. Effects of genetics and maternal dietary iodide supplementation on glycogen content of organs within embryonic turkeys. Poult Sci. 1999;78:890–8.

Christensen, VL, Wineland MJ, Fasenko GM, Donaldson WE. Egg storage effects on plasma glucose and supply and demand tissue glycogen concentrations of broiler embryos. Poult Sci. 2001;80:1729–35.

Coggan AR. Use of stable isotopes to study carbohydrate and fat metabolism at the whole-body level. Proc Nutr Soc. 1999;58(4):953-61.

Coles BA, Croom WJ, Brake J, Daniel LR, Christensen VL, Phelps CP, Gore A, Taylor IL. In ovo peptide YY administration improves growth and feed conversion ratios in week-old broiler chicks. Poult Sci. 1999;78:1320–22.

Coles BA, Croom J, Daniel LR, Christensen VL, Taylor IL. In ovo peptide YY administration and jejunal glucose transport in hatchling turkey poults: Effects of dosage and genotype. Int J Poult Sci. 2003;2(1):1-6.

Deeming DC, Ferguson MW. Egg incubation: Its effects on embryonic development in birds and reptiles. Cambridge University Press. 1991.17-28.

Des Rosiers C, Fernandez CA, David F, Brunengraber H. Reversibility of the mitochondrial isocitrate dehydrogenase reaction in the perfused rat liver. J Biol Chem. 1994;269(44):27179-82.

El-Kadi SW, Baldwin RL 6th, Sunny NE, Owens SL, Bequette BJ. Intestinal protein supply alters amino acid, but not glucose, metabolism by the sheep gastrointestinal tract. J Nutr. 2006;136(5):1261-9.

Fanguy RC, Misra LK, Vo KV, Blohowiak CC, Krueger WF. Effect of delayed placement on mortality and growth performance of commercial broilers. Poult Sci. 1980;59:1215-20.

Fell DA. Increasing the flux in metabolic pathways: A metabolic control analysis perspective. Biotechnol Bioeng. 1998;58:121-4.

Fernandez CA, Des Rosiers C, Previs SF, David F, Brunengraber H.. Correction of ^{13}C mass isotopomer distributions for natural stable isotope abundance. J. Mass. Spectrom. 1996;31: 255-262.

Floyd JC Jr, Fajans SS, Conn JW, Knopf RF, Rull J. Stimulation of insulin secretion by amino acids. J Clin Invest. 1966;45(9):1487-502.

Freeman BM. The mobilization of hepatic glycogen in Gallus domesticus at the end of incubation. Comp Biochem Physiol. 1969;28(3):1169-76.

Gilker CD, Pesola GR, Matthews DE. A mass spectrometric method for measuring glycerol levels and enrichments in plasma using 13C and 2H stable isotopic tracers. Anal Biochem. 1992;205(1):172-8.

Goodridge AG. Conversion of [U-14C]Glucose into carbon dioxide, glycogen, cholesterol and fatty acids in liver slices from embryonic and growing chicks. Biochem. J. 1968;108(4):655-61.

Hager JE, Beane WL. Post hatch incubation time and early growth of broiler chickens. Poult Sci. 1983;62:247-54.

Hannestad U, Lundblad A. Accurate and precise isotope dilution mass spectrophotometry method for determining glucose in whole blood. Clin Chem. 1997;43:794-800.

Harms RH, Hussein SM. Variations in yolk: albumen ratio in hen eggs from commercial flocks. J. Appl. Poultry. Res. 1993; 2:166-70.

Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. Annu Rev Nutr. 1984;4:409-54.

Hazelwood RL. Endocrine control of avian carbohydrate metabolism. Poult Sci. 1971;50(1):9-18.

Heitmann RN, Bergman EN. Glutamine metabolism, interorgan transport and glucogenicity in the sheep. Am J Physiol. 1978;234(2):E197-203.

Heitmann RN, Bergman EN. Glutamate interconversions and glucogenicity in the sheep. Am J Physiol. 1981;241(6):E465-72.

Heitmann RN, Hoover WH, Sniffen CJ. Gluconeogenesis from amino acids in mature wether sheep. J Nutr. 1973;103(11):1587-93.

Hohlweg A, Hare T, Milakofsky L, Nibbio B, Tran Q, Epple A. Hormonal effects on amino acids and related compounds in plasma, amniotic fluid, and allantoic fluid of the chicken embryo. Gen Comp Endocrinol. 1999;114(3):378-86.

Huang MT, Veech RL. Role of the direct and indirect pathways for glycogen synthesis in rat liver in the postprandial state. J Clin Invest. 1988;81(3):872-8.

Jenkins SA, Porter TE. Ontogeny of the hypothalamo-pituitary-adrenocortical axis in the chicken embryo: a review. Domest Anim Endocrinol. 2004;26(4):267-75.

John TM, George JC, Moran Jr ET. Metabolic changes in pectoral muscle and liver of turkey embryos in relation to hatching: influence of glucose and antibiotic treatment of eggs. Poult Sci. 1988;67:463–9.

Kalhan SC. Stable isotopic tracers for studies of glucose metabolism. J. Nutr. 1996;126:362S-68S.

Kaloyianni M, Freedland RA. Contribution of several amino acids and lactate to gluconeogenesis in hepatocytes isolated from rats fed various diets. J Nutr. 1990;120(1):116-22.

Katz J, McGarry JD. The glucose paradox. Is glucose a substrate for liver metabolism? J Clin Invest. 1984;74(6):1901-9.

Katz J, Paul Lee WN, Pat A. Wals, Bergner EA. Studies of glycogen synthesis and the Krebs cycle by mass isotopomer analysis with [U-13C] glucose in rats. J Biol Chem. 1989;264(22):12994-3001.

Katz J, Tayek JA. Gluconeogenesis and the Cori cycle in 12-, 20-, and 40-h-fasted humans. Am J Physiol. 1998;275(*Endocrinol. Metab. 38*):E537-42.

Landau BR. Quantifying the contribution of gluconeogenesis to glucose production in fasted human subjects using stable isotopes. Proc Nutr Soc. 1999;58(4):963-72.

Langslow DR. Gluconeogenesis in birds. Biochem Soc Trans. 1978;6(6):1148-52.

Lobley GE. Control of the metabolic fate of amino acids in ruminants: a review. J Anim Sci. 1992;70(10):3264-75.

Lourens A, Molenaar R, van den Brand H, Heetkamp MJW, Meijerhof R, Kemp B. Effect of egg size on heat production and the transition of energy from egg to hatchling. Poult Sci. 2006;85:770–6.

Lu JW, McMurtry JP, Coon CN. Developmental changes of plasma insulin, glucagon, insulin-like growth factors, thyroid hormones, and glucose concentrations in chick embryos and hatched chicks. Poult Sci. 2007;86(4):673-83.

MacKenzie KL, Tenaschuk D. Quantitative formation of N(O,S)-heptafluorobutyryl isobutyl amino acids for gas chromatographic analysis I. Esterification. J Chromatography A. 1979;171:195–208.

MacKenzie KL, Tenaschuk D. Quantitative formation of N(O,S)-heptafluorobutyryl isobutyl amino acids for gas chromatographic analysis" II. Acylation. J Chromatography A. 1979;173:53–63.

Magnuson MA, She P, Shiota M. Gene-altered mice and metabolic flux control. J Biol Chem. 2003;278(35):32485-8.

Mallette LE, Exton JH, Park CR. Control of gluconeogenesis from amino acids in the perfused rat liver. J. Biol. Chem. 1969;244(20): 5713-23.

Matthews DE, Marano MA, Campbell RG. Splanchnic bed utilization of glutamine and glutamic acid in humans. Am J Physiol. 1993;264:E848–54.

Miller RG, Keshen TH, Jahoor F, Shew SB, Jaksic T. Compartmentation of endogenously synthesized amino acids in neonates. J Surg Res. 1996;63(1):199-203.

Moran Jr. ET. Nutrition of the developing embryo and hatchling. Poult Sci. 2007;86:1043-49.

Murray HA. Physiological ontogeny. A. Chicken embryos. II. Catabolism. Chemical changes in fertile eggs during incubation. Selection of standard conditions. J. Gen. Physiol. 1925;5:1-37.

Nitsan Z, Ben-Avraham G, Zoref Z, Nir I. Growth and development of the digestive organs and some enzymes in broiler chicks after hatching. Br Poult Sci. 1991;32(3):515-23.

Noy Y, Sklan D. Energy utilization in newly hatched chicks. Poult Sci. 1999;78(12):1750-6.

Noy Y, Sklan D. Posthatch development in poultry. J Appl Poult Res. 1997;6:344–54.

Noy Y, Sklan D. The effect of different types of early feeding on performance in chicks and poults. J Appl Poult Res. 1999a;8:16–24.

Noy Y, Sklan D. Yolk and exogenous feed utilization in the posthatch chick. Poult Sci. 2001;80:1490–95.

Noy Y, Sklan D. Yolk utilisation in the newly hatched poult. Br Poult Sci. 1998;39:446–51.

Noy Y, Uni Z, Sklan D. Routes of yolk utilisation in the newly hatched chick. Br Poult Sci. 1996;37:987–96.

Obst BS, Diamond J. Ontogenesis of intestinal nutrient transport in domestic chickens (Gallus gallus) and its relation to growth. The Auk. 1992;109(3):45-64.

Ohta Y, Tsushima N, Koide K, Kidd MT, Ishibashi T. Effect of amino acid injection in broiler breeder eggs on embryonic growth and hatchability of chicks. Poult Sci. 1999;78:1493–98.

Pascual M, Jahoor F, Reeds PJ. Dietary glucose is extensively recycled in the splanchnic bed of fed adult mice. J Nutr. 1997;127(8):1480-8.

Pascual M, Jahoor F, Reeds PJ. In vivo glucose contribution to glutamate synthesis is maintained while its contribution to acetyl coA is lowered in adult mice fed a diet with a high fat:carbohydrate ratio. J Nutr. 1998;128:733–9.

Pearce J. Carbohydrate metabolism in the domestic fowl. Proc Nutr Soc. 1971;30:254-9.

Pearce J. Some differences between avian and mammalian biochemistry. Int J Biochem. 1977;8:269-75.

Peebles ED, Li L, Miller S, Pansky T, Whitmarsh S, Latour MA, Gerard PD. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poult Sci. 1999;78(10):1435-42.

Peebles ED, Zumwalt CD, Doyle SM, Gerard PD, Latour MA, Boyle CR, Smith TW. Effects of breeder age and dietary fat source and level on broiler hatching egg characteristics. Poult Sci. 2000;79(5):698-704.

Reeds PJ, Berthold HK, Boza JJ, Burrin DG, Jahoor F, Jaksic T, Klein PD, Keshen T, Miller R, Stoll B, Wykes LJ. Integration of amino acid and carbon intermediary metabolism: studies with uniformly labeled tracers and mass isotopomer analysis. Eur J Pediatr. 1997;156:S50-58.

Reeds PJ, Burrin DG. Glutamine and the bowel. J Nutr. 2001;131(9 Suppl):2505S-8S.

Reeds PJ, Burrin DG, Jahoor F, Wykes L, Henry J, Frazer EM. Enteral glutamate is almost completely metabolized in first pass by the gastrointestinal tract of infant pigs. Am J Physiol. 1996;270:E413–8.

Rennie MJ. An introduction to the use of tracers in nutrition and metabolism. Proc Nutr Soc. 1999;58(4):935-44.

Ritz CW, Hulet RM, Self BB, Denbow DM. Growth and intestinal morphology of male turkeys as influenced by dietary supplementation of amylase and xylanase. Poult Sci. 1995;74(8):1329-34.

Romanoff AL, Romanoff AJ. The biochemistry of the avian embryo: a quantitative analysis of prenatal development. John Wiley & Sons.1967.

SAS, 2006. Statistical Analysis Software (Release 8.02). Cary, NC, SAS Inst. Inc.

Sato M, Tachibana T, Furuse M. Heat production and lipid metabolism in broiler and layer chickens during embryonic development. Comp Biochem Physiol A Mol Integr Physiol. 2006 Mar;143(3):382-8.

Savon S, Hakimi P, Hanson RW. Expression of the genes for the mitochondrial and cytosolic forms of phosphoenolpyruvate carboxykinase in avian liver during development. Biol Neonate. 1993;64(1):62-8.

Sklan D, Geyra A, Tako E, Gal-Gerber O, Uni Z. Ontogeny of brush border carbohydrate digestion and uptake in the chick. Br J Nutr. 2003;89:747-53.

Sklan D, Noy Y. Hydrolysis and absorption in the small intestines of posthatch chicks. Poult Sci. 2000;79:1306–10.

Speake BK, Murray AM, Noble RC. Transport and transformations of yolk lipids during development of the avian embryo. Prog Lipid Res. 1998;37(1):1-32.

Stoll B, Burrin DG, Henry J, Yu H, Jahoor F, Reeds PJ. Substrate oxidation by the portal drained viscera of fed piglets. Am J Physiol. 1999;277(1 Pt 1):E168-75.

Sulistiyanto B, Akiba Y, Sato K. Energy utilisation of carbohydrate, fat and protein sources in newly hatched broiler chicks. Br Poult Sci. 1999;40(5):653-9.

Sunny NE, El-Kadi SW, Oba M, Owens SL, Angel R, Bequette BJ. Gluconeogenesis and Krebs cycle metabolism in 2-day-old fed and fasted chicks. J Anim Sci. 2004;87(Suppl. 1):313.

Tako E, Ferket PR, Uni Z. Effects of in ovo feeding of carbohydrates and β-hydroxy-β-methylbutyrate on the development of chicken intestine. Poult Sci. 2004;83:2023-28.

Tserng KY, Kalhan SC. Estimation of glucose carbon recycling and glucose turnover with [U-13C] glucose. Am J Physiol. 1983;245(Endocrinol. Metab. 8):E476-82.

Turro I, Dunnington EA, Nitsan Z, Picard M, Siegel PB. Effect of yolk sac removal at hatch on growth and feeding behavior in lines of chickens differing in body weight. Growth Dev Aging. 1994;58(2):105-12.

Uni Z, Ferket PR, Tako E, Kedar O. In ovo feeding improves energy status of late-term chicken embryos. Poult Sci. 2005;84(5):764-70.

Uni Z, Tako E, Gal-Garber O, Sklan D. Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. Poult Sci. 2003;82(11):1747-54.

Veech RL, Fell DA. Distribution control of metabolic flux. Cell Biochem Funct. 1996;14(4):229-36.

Vieira SL, Moran, Jr ET. Broiler chicks hatched from egg weight extremes and diverse breeder strains. J. Appl. Poultry Res. 1998;7:392-402.

Watford M, Hod Y, Chiao YB, Utter MF, Hanson RW. The unique role of the kidney in gluconeogenesis in the chicken. The significance of a cytosolic form of phosphoenolpyruvate carboxykinase. J Biol Chem. 1981;256(19):10023-7.

Watford M, Wu G. Glutamine metabolism in uricotelic species: variation in skeletal muscle glutamine synthetase, glutaminase, glutamine levels and rates of protein synthesis. Comp Biochem Physiol B Biochem Mol Biol. 2005;140(4):607-14.

Windmueller HG, and Spaeth AE. Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate. J Biol Chem. 1980;255:107–12.

Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. J Biol Chem. 1974;249(16):5070-9.

Wu G, Flynn NE, Yan W, Barstow DG Jr. Glutamine metabolism in chick enterocytes: absence of pyrroline-5-carboxylase synthase and citrulline synthesis. Biochem J. 1995;306:717-21.

Wu G. Intestinal mucosal amino acid catabolism. J Nutr. 1998;128:1249-52.

Wu GY, Thompson JR. The effect of glutamine on protein turnover in chick skeletal muscle in vitro. Biochem J. 1990;265(2):593-8.

Wykes L, Jahoor F, Reeds PJ. Gluconeogenesis measured with [U-13C]glucose and mass isotopomer analysis with apo-B-100 amino acids in pigs. Am J Physiol (Endocrinol Metab, 37). 1998;274:E365–76.

Yeung D, Oliver IT. Gluconeogenesis from amino acids in neonatal rat liver. Biochem J. 1967;103(3):744-8.