

ABSTRACT

DE OLIVEIRA, JEAN EDUARDO. Effects of *In ovo* Feeding on Turkey Embryos Development, Energy Status, Intestinal Maturation, Gene Expression and Post-hatch Development. (Under the direction of Peter R. Ferket.)

Glycogen stored in liver and muscles are the main energy source during hatching of poultry. Turkey embryos are very sensitive to perturbations in energy metabolism because they have a wider hatching window than chicken embryos. Mortality of turkey embryos during late-term incubation is high relative to chickens, and many surviving hatchlings have compromised vitality. *In ovo* feeding (IOF) improves the energy status and gut development of perinatal poultry by supplementing the amnion with nutrients, which is then orally consumed by the embryo prior to hatch. A survey of amnion and embryonic development was done to determine the best time, maximum volume and solution characteristics for IOF. Several IOF formulations containing combinations of carbohydrates, proteins and enteric modulators were tested in turkey eggs, with emphasis on comparing hydrolyzed soy protein to egg white. IOF was found to improve energy status at hatch, but subsequent growth performance results were inconsistent. Therefore, further studies were designed to better understand late-term turkey embryo metabolism, using a customized focused oligonucleotide microarray. A gene expression survey using this array was performed on liver, pectoral muscle, hatching muscle, duodenum, and ileum of turkey embryos from 20 days of incubation until hatch. The embryos were found to switch from yolk fat to carbohydrate-based metabolism around 22 days of incubation, and potential substrates were identified to be tested *in ovo*. A nutritive solution containing metabolic co-factors was then formulated and tested in a subsequent IOF experiment. At hatch, poult treated with this latest IOF formulation exhibited better quality scores and

more symmetric legs than controls. Gene expression patterns of liver, pectoral muscle, hatching muscle, duodenum and ileum confirmed IOF favored skeletal development of poult. This dissertation research clearly demonstrated that *in ovo* feeding technology can enhance energy status and advance the development of hatchlings, resulting in superior poult quality.

Effects of *In ovo* Feeding on Turkey Embryos Development, Energy Status, Intestinal Maturation, Gene Expression and Post-hatch Development

by
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Biography

Native of Sao Paulo, Brazil, I graduated in Animal Science from Universidade Estadual Paulista (UNESP)-Jaboticabal, Brazil in December 1998. While in college I always had an interest in poultry and research, keeping an internship at the poultry nutrition lab from sophomore year until graduation. I was also selected in my sophomore year to participate in a government sponsored program called Special Training Program (PET) for students showing higher academic potential, where I was stimulated to develop academic, leadership, and community skills. In 2002, I completed my Master of Science Degree in Animal Science with emphasis in poultry nutrition at Universidade Federal de Vicosa, Brazil. In the fall of 2003, I came to United States to start my doctoral studies in Nutrition under the direction of Dr. Peter Ferket. I was stimulated to get a minor in biotechnology to work towards integrating molecular biology to nutrition. During my time as a student at NC State I served as Poultry Science Graduate Student Representative at the University Graduate Students Association, Poultry Science Graduate Student Association President, and Poultry Science Departmental Ambassador for International Students, and president of the NC State Brazilian Students Association.

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Chapter 1

Introduction and Literature Review

Poultry Production Overview

Historic advances in poultry industry

Poultry meat and eggs are high-quality protein, and during the last 50 years they have become among the most affordable food staple for people throughout the world. As technological development advanced during the twentieth century, the world's population became more urbanized (Lobo, 2006) and dependent upon concentrated large-scale food production that can be easily and efficiently transported to metropolitan areas. The poultry industry led this transformation from subsistence agriculture to integrated production to feed this growing urbanized society, elevating the consumption of poultry products ahead of other animal products as the least expensive and most popular animal protein source (Hammerstedt, 1999).

Modern chickens were domesticated from the Red Jungle Fowl (*Gallus gallus*) over the last four or five thousand years for eggs and meat, for game and for exhibition (Winter and Funk, 1960; Roberts, 1998; Klasing, 2005). Chickens were imported to the new world from Europe and Asia during the 1800's, where they were raised for eggs and meat in the backyards of most homes. Indeed, the most profitable part of the poultry business until the early 1920's was for competitive exhibitions rather than for food (Winter and Funk, 1960; Hanke et al., 1972). But then the profit on poultry production shifted from the exhibitions fancy feather breeds to the egg and meat production poultry

(Winter and Funk, 1960; Hanke et al., 1972). Emphasis on poultry meat and egg production accelerated after Herbert Hoover's 1928 presidential campaign promise of "A chicken in every pot...and a car in every garage." The outcome was so successful that since 1992 poultry meat has been the most-consumed meat in the US, surpassing beef and pork, based in the retail weight series (Smith, 2006b).

Modern turkeys are native to North America and were domesticated from *Meleagris gallopavo* by the Aztec Indians in what is now Mexico as far back as AD1000 (Roberts, 2002, Klasing, 2005). Early European explorers brought turkeys back to their noble underwriters who further selected them for show and feasts. Commercial rearing became important in early 19th century, after domesticated turkeys were brought back from Europe and backcrossed with wild turkeys (Hanke et al., 1972; Roberts, 2002; Klasing, 2005).

The growth of the poultry industry during the last 7 decades has been phenomenal. In 1939, 660.5 million chickens and 27.9 million turkeys were raised in the United States (Winter and Funk, 1960). In 2006, 10.9 billion broiler chickens (20 billion kg) and 261.9 million turkeys (3.3 billion kg) were produced (USDA National Agriculture Statistics Service, 2007). The average consumption of chicken in the US today is 36.3 kg per capita (Lefens, 2007), and per capita consumption of turkey is growing faster than chicken during the last five years (Smith, 2007a). Worldwide 67.6 billion kg of chicken meat is produced every year (van der Sluis, 2005), and the biggest poultry meat exporter is Brazil with 2.85 million metric tons in 2006, followed by the US with 2.34 million metric tons (Shane, 2007). Considering such high poultry production in

the USA and throughout the world, any small percentage change in poultry production has an economic impact worth millions of dollars to the poultry and associated industries.

There are several reasons for the success of the poultry industry and the phenomenal growth in consumer demand for its products, including rapid application of scientific discoveries, specialization into integrated production systems, and free acceptance in world market.

Since its' beginning, the poultry industry has being strongly dependent on research (Smith, 2006b). Large scale production was only possible after the discovery of vitamins, improvement of artificial incubation, introduction of disease test kits, vaccination, and breeder genetic selection application (Winter and Funk, 1960; Hanke et al., 1972; Morris, 1980). This continuous industry progress is based on the application of scientifically-proven technologies that help poultry growers improve production yield, bird health and nutrition, housing, genetics and reproduction, food quality, safety, and efficiency (Smith, 2006b). The best example of dramatic advances can be seen in animal performance, especially improvements in body weights at market. A typical 1957 chicken strain reached 539 g of body weight at 42 days of age, while a 2001 commercial broiler chicken strain reached 2,672 g at the same 42 days of age, which constitutes remarkable progress (Havenstein et al., 2003). The production characteristics of turkeys have also improved significantly during the last 4 decades. Modern 2003 strains were observed to weigh approximately twice the weight of the 1966 turkey strain from 112 to 196 days of age (Havenstein et al., 2007). This improvement in growth performance and meat yield was driven by costumer preference for white breast meat products, and the industry's focus on greater meat yields per unit of input costs. The average market weight of

broilers slaughtered in the US reached a record high of 2.54 kg in 2006 (Smith, 2006a). Today, most of the broiler and turkey carcasses are deboned and further processed and the amount of poultry meat produced are sustained by heavier birds, even when the number of birds decreases (Smith, 2006a). Such accomplishment could only be reached by constant research and innovation to overcome challenges, and there is still more room for improvement.

Another reason why the poultry industry has grown so much is attributed to having a refined and specialized segmentation. A few breeding companies provide elite genetic stock, while production companies grow out hybrid crosses to produce the final product (Hammerstedt, 1999). The grower receives a technology package and mainly concentrates on managing the birds, while genetic companies concentrate in producing a more efficient bird every generation.

Finally, the phenomenal growth of the poultry industry would not have occurred if not for few social and trade barriers. There are no barriers against poultry meat, like religious or cultural impediments that exist to other meats. This allied to attractive prices, led exports to boom world wide, providing to the industry an even greater incentive for growth. In conclusion, poultry is the perfect animal product to feed a globalized world.

Poultry Industry challenges

The challenges that the poultry industry faces are the opportunities for research and development. According to Smith, (2006b), the poultry industry must commit and coordinate with research to remain as the leading food animal sector. A global commitment to solving the poultry industry challenges is necessary because multinational poultry production companies now produce a significant share of the worldwide trade

poultry products. The main issues forecasted by specialists are disease pandemic, feed stuff contamination, environmental impact, shortage of carbohydrate sources, metabolic diseases, and increasing production costs (Shelton, 2006; Smith, 2006b).

To reduce production costs, it is important to improve early growth performance characteristics in poultry. As demands for decreasing market weight for age continue, it will become more important to improve growth and development during the brooding phase (Hulet, 2007). This is especially important to the turkey industry, where fertile eggs and day old poults cost more than they do in the chicken industry (Schaal and Cherian, 2007). Genetic selection has created a more efficient bird, driving costs down (Hammerstedt, 1999), but that would be negated if poults do not survive the first week of life. Therefore, early survival of is a major concern for high-yield poultry strains, particularly to the turkey industry.

Starting turkey poults

Turkey industry's main challenges are to improve poult yield and quality from hatcheries, reduce mortality and stunted growth during brooding, and have a more uniform bird at market age independent of the season. Management-related problems during brooding and growing account for over 6% of flock mortality, and for 10 to 30% depression in growth relative to the genetic potential. Weak poults suffer from metabolic disorders related to the transition from egg to the precocial poult (Noble et al., 1999; Christensen et al., 2003c; Christensen et al., 2007).

Hammerstedt, (1999) pointed out that production effectiveness starts with reproduction, including hatchability and post hatch survival. Because market weight for age has decreased during the last 4 decades without change in the duration of incubation,

the incubation period has increased to represent 30 to 40% of the productive life of meat poultry (Hulet, 2007). Therefore, the incubation and brooding periods has gained more relative importance to a successful rearing of meat poultry than ever before, and this trend is expected to continue as increases in post-hatch growth rate are achieved.

Early mortality and stunted growth is not only costly to the turkey industry, but it negatively impacts animal welfare concerns. Two common conditions have been reported as responsible for these losses, starve-outs and flip-over poult. Poults that do not develop and eventually die during the first 5 days post-hatch are commonly called “starve-outs” (Christensen et al., 2003c). “Starve-out” poult typically have exhausted their carbohydrate reserves, which adversely affects their ability to thermoregulate and use their yolk reserves (Donaldson, 1995). This inability to utilize yolk reserves to support tissue growth and development consequently increases the incidence of unabsorbed yolk sacs observed at carcass processing of surviving birds (Buhr et al., 2006). Thus, high mortality is usually seen 5-7 days post hatch, and the ones that survive never recover (Horrox, 2003), causing increased size variation at slaughter that adversely affects the uniformity of meat cuts (Horrox, 2006b).

Poor poult quality at hatch can also be exhibited by an increased incidence of poult that flip on their back, paddle their feet, and cannot get back on their feet without assistance. If these “flip-over” poult are put back on their feet, they cannot regain balance, they are weak, and they vocalize loudly, indicating loss of neurological control, distress, and lethargy (Noble et al., 1999). The same authors reported high mortality rates among flip-overs (37.8% of the flock) during the 2 days post-hatch. Christensen et al., (2003c) observed that these flip-over poult have smaller hearts and lower plasma

concentration of thyroid hormones than normal poult. Moreover, the incidence of flip-overs poult is higher among poult that have delayed hatch from small eggs (Noble et al., 1999).

Christensen et al., (2007) proposed a solution to avoid starve-outs, flip-overs and improve early development and uniformity in turkey poult by feeding them readily available carbohydrates as soon as possible after hatching. Early access to feed, as it will be discussed later, improves post-hatch survival and growth (Uni and Ferket, 2004) by improving gut development, digestion and absorption capacity, thereby overcoming some of the challenges faced by the neonatal poult during the first week post hatch. For the neonatal poult to initiate feeding and early growth, an appetite and a functional digestive system is necessary (Christensen et al., 2007).

Hatchery Holding Period

It is common practice in the poultry industry to hold poult without feed and water for many hours after hatch. Poult may remain for up to 36 hours after hatching before they are pulled from the hatching cabinet, and then it may take an additional 72 hours before they are be serviced and transported to brooder farms where they finally have access to feed and water. Poult servicing includes sexing, toe trimming, snood removal, beak trimming, and injection of antibiotics (Donaldson and Christensen, 1991, Donaldson et al., 1991). After enduring this stress of servicing poult are often held in transportation boxes stacked in a dim room for up to 24 hours so they can recover and endure the stress of transport and placement. Donaldson et al., (1991) confirmed that poult recuperate liver glycogen concentration to levels prior to servicing when held for 24 hours, however this glycogen status recover occurs at the expense of catabolizing their

own protein reserves (Donaldson, 1995; Keirs et al., 2002) since they have no access to feed or water during this time.

Several studies were performed to evaluate the impact of this early fasting period on poult development, comparing hatchlings that were held for 24 hours with those given *ad libitum* access to feed and water immediately after they were removed from the hatcher. Careghi et al., (2005) observed that broiler chicks fed immediately after hatch showed higher weight gain later in life as compared to the held chicks, and that late hatchers benefit more from early access to feed. Uni et al., (1998) demonstrated that early fasting clearly delays gut maturation, affecting the development of mucosal morphology and intestinal enzyme activity. Fed poult and chicks also have more goblet cells per villus, and more proliferating enterocytes, in contrast to more apoptotic cells in fasted birds (Uni et al., 1998; Potturi et al., 2005; Smirnov et al., 2006). According to (Potturi et al., 2005), poult fed immediately after hatch had 5.0 μ m longer and 6.8 μ m wider intestinal villi, and 5.6 μ m deeper villi crypts than fasted-held poult. These fasted poult also had more aerobic bacteria in their small intestine (Potturi et al., 2005). Feed restriction early in life also programmed birds for obesity later in life (Zhan et al., 2007) by permanently altering energy related enzyme production and function. Velleman and Mozdziak, (2005) found reduced muscle growth among chicks that experienced a 72 hour of fasting after hatch. Because of the literature cited above, there is great interest in ways to aid poult nutrition before placement, as Careghi et al., (2005) suggested to provide an energy source in the hatch basket and during transport.

There are several other management practices and conditions that can accentuate the adverse effects of a long post-hatch holding period, including egg storage period, egg size, and hatch window.

Egg storage time

The adverse effects of post-hatch holding time on poult vigor can be accentuated by long pre-incubation egg storing time. Careghi et al., (2005) reported that extended egg storage time (over 7 days) and hatchery holding time negatively affect poult early growth. Eggs stored for over a week causes increased number of embryonic abnormalities, mortality, slower embryonic metabolism, and delays or prolongs the internal pipping stage (Decuypere and Bruggeman, 2007).

By the time of oviposition, the chicken embryo has already developed inside the hen for 18 to 21 hours, having about 40,000 to 60,000 cells (Fasenko, 2007). Turkey embryos are at less mature stages of development than chickens at time of lay (Gupta and Bakst, 1993, Fasenko, 2007). Fertile eggs will continue development if adequate incubation conditions are provided. Edwards determined that chicken embryos do not develop but still survive if stored at 21°C (Edwards, 1902). The temperature below which embryonic development does not occur is termed physiological zero, but the preferred term is embryonic diapause (Fasenko, 2007). Bakst and Gupta, (1997) found that the turkey embryo physiological zero is lower than chickens, at 15°C. Even though the embryo is not growing during storage, the young embryo, who depends on glucose to survive, is still consuming the limited amount of available glucose. Embryos from eggs stored for 14 days are not behind in development, but their metabolism is slower throughout incubation (Fasenko, 2007). This includes lower concentration of plasma

hormones, and reduced efficiency in maintaining glycogen reserves in muscle and heart (Christensen et al., 2001b; Fassenko, 2007). As a consequence, the energy stores of such embryos are smaller, contributing to even lower energy status at hatch. Mather and Laughlin, (1977) observed that embryos from eggs stored for more than seven days may take up to 12 hours to resume development when set at the incubator. This observation was later confirmed with turkeys by Fassenko, (2007).

Egg size/breeder age

Breeder hen age and egg size are two other factors that can aggravate holding time. These two factors are often associated since older hens lay bigger eggs (Decuypere and Bruggeman, 2007). The first egg laid of each clutch is also bigger than subsequent eggs (Zakaria et al., 2005). Embryos from hens at the end of the lay cycle hatch earlier, and thus, these hatchlings stay longer in the incubator, depleting their energy stores (Joseph and Moran, 2005b). Eggs from older hens also have higher egg conductance due to larger eggshell pores, losing more weight during incubation, and stimulating embryos to hatch earlier (Christensen et al., 1996; Funderbunk et al., 2005). Eggs that lose weight at an accelerated rate during incubation yield embryos and hatchlings that are more dependent on gluconeogenesis and are more adversely affected by nutrient deprivation and poor brooding conditions (Peebles et al., 2005).

As the embryo grows tissues demanding oxygen are formed, but oxygen supply is fixed. At a certain point a plateau in oxygen consumption is reached. This plateau stage is essential for maturation of several processes in embryo development. Christensen et al., (1996) showed that because eggs from older hens have higher shell conductance their embryos reach the plateau stage later. Whether cause or consequence, these embryos

grow muscle more efficiently. Because muscle requires more oxygen, embryos from older hens have less liver glycogen available for hatching and post-hatch survival. Breeder hen age also affect circulating thyroid hormone levels of embryos, T4, which stimulates growth, was higher in embryos from older hen, whereas T3, which promotes glycogen metabolism, was higher in embryos from younger hens. This hen age effect on thyroid function in embryos may be a direct consequence of the nutrients available in the egg. The hen deposits more yolk at expense of egg white as she ages. Because yolk lipids supply energy for growth and carbohydrate plays a major role at hatching, a hatching egg with more yolk and less albumen yields heavier poults that do not always survive the rigors of hatching until feed intake initiates (Christensen et al., 1996). Consequently, hatchability declines as hens get older (Nestor et al., 1972). Research is needed to alleviate the adverse effect of age and egg size by helping these troubled embryos to accumulate and utilize glycogen to fuel hatch.

Hatch window

Hatch window is defined as the time it takes from the first embryo hatched to the time the hatch is pulled. A broad hatch window may exceed a duration of 36 to 48 hours, which further delays the access to feed in addition to the 24 hours or more required for poult servicing, holding, and transport to the brooder farm (Careghi et al., 2005). The optimum time to pull the poults from the hatcher is often difficult to determine among hatches with a wide hatch window because embryos do not hatch all at the same time. If the poults are pulled from the hatcher too soon, many late-hatched poults are unnecessarily disposed; but if the poults are pulled too late, many early-hatched poults will be dehydrated and depleted of their energy reserves, thus compromising performance

and final weight (Bamelis et al., 2005; Careghi et al., 2005). In contrast, determining the optimum hatch pull time is much easier for hatching with a narrow hatch window, and there are fewer poult quality problems (Careghi et al., 2005; Joseph and Moran, 2005b). Depending on incubation conditions the hatch window of time can be 24 to 36 hours, or more.

Hatch window duration may be affected by several factors, including breeder age, incubation temperature, egg storage time, and location in the incubator Wyatt, et al., (1985) reported that broiler chicks from older hens began hatching 6 hours sooner than chicks from younger flocks. In a similar experiment, Joseph and Moran, (2005b) reported that early hatches stayed in the hatcher up to 32 hours post-emergence, while late hatches were held up to 6 hours. Eggs incubated in higher temperatures hatch earlier (Christensen et al., 2001a; Christensen et al., 2003a; Christensen et al., 2003b; Hulet et al., 2007). Position in the incubator affects egg temperature because of differences in air flow. Egg shell temperature may be higher than incubator temperature, especially after mid incubation when the embryo is producing heat, thus subjecting the embryos to heat stress (Hulet et al., 2007). We have already discussed the effects of egg storage time on length of incubation, an extended hatch window is expected if eggs are stored for different amounts of time and then are set together.

In summary, there are many factors that may delay the initiation of feeding, and it is a challenge to manage all these factors to reduce their impact on hatchability, viability and performance. New technologies are welcome and necessary to address these problems, but their implementation will not be easy. Some hatchery management experts are suggesting a revolutionary way to hatch and handle chicks and poults to minimize the

delay to feed intake initiation. The eggs are transferred to special units where they hatch with feed and water available, and where they stay for a couple of days before being shipped out. This results in chicks and poults equivalently one day ahead in development compared to standard industry practice (Horroxx, 2006a), but this new hatchery technology would require heavy investments in new equipment by hatcheries.

Consequences of Selection

Great advances in poultry breeding and genetic selection for valuable production traits have been achieved during the last 50 years. Many have predicted that the genetic limit for growth was reached and slower progress will be seen from then on (Shelton, 2006), but we still see advances every year. The formula for success depends on two main branches of research, genetics and nutrition. The first creates a bird with higher potential, and the second has to find a way to help that bird to achieve as much of that potential as possible.

Constant selection for performance, especially heavier weights, has its consequences. One big setback is that selection for higher post-hatch growth is negatively correlated with embryo survival (Nestor and Noble, 1995; Christensen et al., 2000). Collin et al., (2007) pointed out that selection for breast meat yield leads to poor visceral system. This phenomenon was explained by Foye (2005), who said that selection for growth is pushing precocial poultry to become more altricial by directing resources to growth instead of viscera maturation.

One of the consequences of the reduction of visceral capacity relative to body size among modern broilers selected for rapid growth is exemplified by the increasing incidence of ascites. Ascites have been linked to limited cardiopulmonary capacity of the

animal to attend body demands. One of the strategies to reduce the incidence of ascites is to slow down early growth by feed restriction; but if it is too severe, the restriction may not be compensated later in life, resulting in lower weights and yields at processing (Ozkan et al., 2006). Selection for weight gain also increases risk of obesity, lower fertility, and lower hatchability (Joseph and Moran, 2005a).

The value of poultry industry has increased tremendously in the last twenty years. The broiler industry has grown from \$5.68 billion in 1985 to \$20.9 billion in 2005. Turkey production has grown 77% during the same period. Associated with this increase in meat poultry production was annual increase in broiler and turkey eggs set in hatcheries of 98% and 33%, respectively. Although considerable progress was been made in the performance characteristics of breeders and commercial poultry, hatchability rate has improvement little during the last twenty years. Indeed, 20% of chicken and turkey hatching eggs do not yield hatchlings, and the combined monetary losses of low hatchability represented \$500 million to the industry in 2005. Each 1% increase in hatchability would result in \$25 million in return, so finding ways to improve hatchability and early survival have a significant economic impact (Schaal and Cherian, 2007). According to Keirs et al., (2007), only a small portion of non-hatched embryos have anatomical abnormalities, so the majority of them should have hatched and survived. Most of the knowledge on embryonic development is based on research conducted over 60 years ago. It is now apparent that more emphasis on optimizing the growth and maturity of the developing embryo is needed to maximize post-hatch growth and development (Hulet, 2007).

Poultry Embryo Development

Embryonic use of egg nutrients

Egg composition

Embryo development apart from the hen allows poultry producers to handle large number of animals, but on the down side all nutrients must be present in the egg to sustain embryonic development by the time the egg is laid (Fasenko, 2007). Composition of a typical chicken egg is presented on Table 1.1. There is much less information available on the composition of turkey egg, mostly because they are only produced for the purpose of turkey reproduction rather than as a human food source. An 80g turkey egg comprises of about 30% yolk and 55-60% albumen (Applegate and Lilburn, 1998). Reidy et al., (1994) determined egg composition from two of the three major turkey breeds, and the average composition values among breeds over different hen ages are presented in Table 1.2. Although the relative composition of eggs from chickens and turkeys are similar, turkey eggs have higher total nutrients because of their larger size. These egg nutrients will be all that is available to nurture the developing embryo. However, it is noteworthy that there is very little energy reserves as carbohydrates in the egg (Romanoff, 1967), being most of lipids in the yolk fraction. Ninety eight percent of the free carbohydrates are present as 0.5% glucose in the albumen (Davis and Reeves, 2002).

Table 1.1. Approximate composition of a 60g chicken egg

	Egg weight %	Water %	% on dry matter basis			
			Protein	Lipids	Carbohydrate	Minerals
Egg yolk	31	51	30	60	1.2	2.2
Egg white	58	88	75	0.08	4.2	1.7
Egg shell	11	0.3	0.7	-	-	98
Total	100					

Adapted from: Davis and Reeves (2002)

Table 1.2. Approximate composition of a 90g turkey egg

	Egg weight %	Water %	% on dry matter basis	
			Protein	Lipids
Egg yolk	30.1	46.3	28.6	59.5
Albumen	59.2	88.7	78.3	-
Egg shell	10.7	-	-	-
Total	100	-	-	-

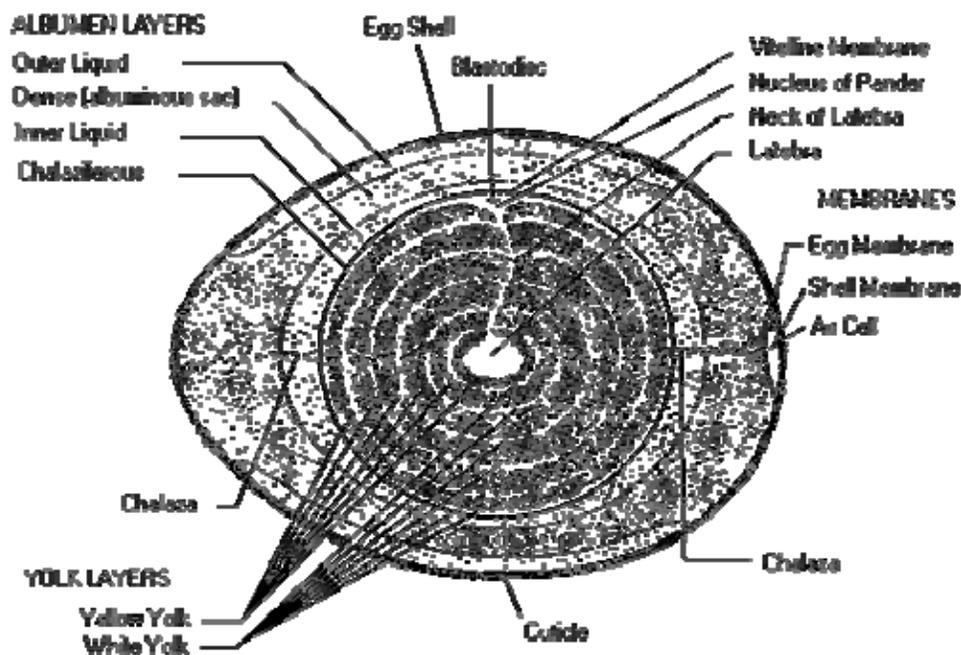
Adapted from: Reidy et al. (1994).

Egg structure

A schematic structure of the avian egg before incubation is illustrated by Figure 1.1. These structures simply keep the little embryo alive and protected until incubation starts.

Beginning with the outside of the egg moving inward, the egg shell has a porous calcium carbonate structure that allows gas exchange and two membranes. The outer membrane is attached to the shell, while the inner membrane retracts when egg cools down forming the air cell space on the large end of the egg. The next component is the egg white, composed of three layers of protein generally called albumen. The albumen layers are named based on their density as outer thin, dense or thick and inner thin. The central structure is the yolk, composed of multiple layers of lipids enveloped by a

membrane. Resting on top of the yolk is the blastodisc where the embryo resides after the egg is fertilized. Concentric yolk layers can be differentiated by the amount of pigment deposited depending whether they were formed during daylight or night darkness. Linking the yolk to each end of the egg are the chalazas that act like bungee cords to keep the yolk horizontally centered in the egg. These rudimentary structures are present at the time of lay but they rapidly change once incubation proceeds.



**Figure 1.1. STRUCTURE OF THE HEN'S EGG
SHOWN BY A SECTION THROUGH THE LONG AXIS**

From <http://www.msstate.edu/dept/poultry/avianemb.htm#stages> (Smith, 2007b)

Embryo development during Incubation

Moran, (2007) presented a comprehensive review on the development of the avian embryo. Moran divided embryonic development in three distinct periods: 1) the

establishment of the germ; 2) completion of embryonic formation; and 3) preparation for emergence.

During the establishment of the germ, the embryo and its sustaining structures resume cell proliferation from the 40,000 to 60,000 cells already present at oviposition (Fasenko, 2007). The energy expended at this time arises by glycolysis of glucose present at the outer thin layer of the albumen. The embryo metabolism is mainly anaerobic, accumulating lactate because of low O₂ diffusion of the primitive hemoglobin. The vascular system is rapidly established and germ invaginations lead to formation of the chorionic sac and the allantoic cavity. The different egg compartments during incubation are illustrated by Figure 1.2. Egg turning is crucial during this period to allow proper formation of egg compartments and to give embryonic access to glucose present in the outer thin. The inner cell layer of the amniotic membrane secretes amniotic fluid in which the embryo floats, keeping the embryo from drying and protecting it from shock. The chorion surrounds all embryonic structures and serves as a protective membrane. The allantois grows larger as the embryo grows, until it fuses with the chorion forming the chorio-allantoic membrane, responsible for exchanging oxygen and carbon dioxide with the environment, and for storing nitrogenous waste (Smith, 2007b). The yolk sac membrane selects the nutrients being up taken from yolk sac reserves, which includes lipids, protein, minerals and vitamins. The yolk sac membrane can also modify these nutrients and serve as their short-term storage. Egg storage and storage conditions prior to incubation can impair formation of a fully functional choriallantois, affecting recovery of nutrients.

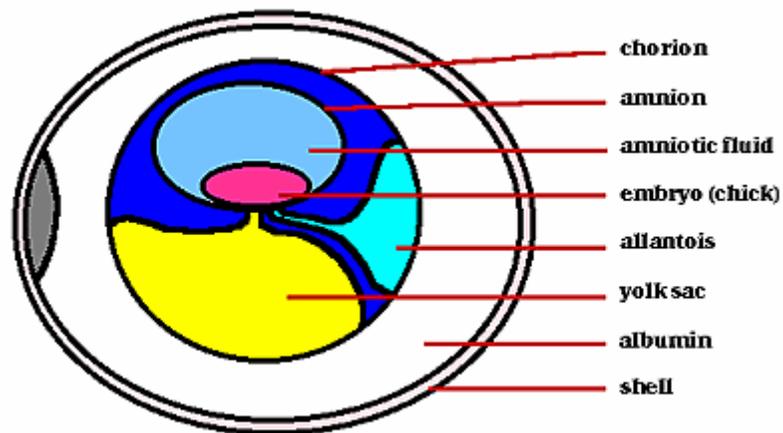


Figure 1.2. Schematic representation egg compartmentalization during incubation.

From: <http://www.biology.eku.edu/RITCHISO/avianreproduction.html> (Ritchison, 2007)

The second third of incubation is marked by a fully developed vascular system, with the choriallantois able to assure O_2 - CO_2 exchange. The embryo then undergoes the first drastic switch of its metabolism, from carbohydrate to fully dependent on lipid oxidation as the energy source. The embryo grows very fast in size during this phase. Essential fatty acids are preserved for cell membrane synthesis while saturated fatty acids are consumed to sustain the increasing caloric needs of formed tissues. Turkey embryos are structurally complete by 24 days of incubation, when choriallantoic respiration capacity is reached in the previously mentioned plateau stage (Christensen et al., 1996). The embryo then undergoes another critical period: the transition for emergence.

In preparation for emergence, embryonic size and movements cause rupture of the membrane that separated the albumen and amniotic fluid, causing them to mix. The embryo then orally consumes the amniotic fluid, which passes through the gastrointestinal system. At this stage of intestinal development, enterocytes of the

duodenum and jejunum are able to absorb macromolecules of protein, in a process similar to mammalian absorption of colostrums. Such consumption continues until the albumen-amniotic fluid disappears and internal pipping begins. Since embryonic skeletal tissue development is complete at this point, the nutrients absorbed are used for visceral organs maturation and most of it is stored as glycogen. The main protein present in the fluid is called ovomucoid, which has extensive amounts of carbohydrate. Gluconeogenesis from albumen proteins is channeled to form glycogen, while the amino acids are spared for protein synthesis. Glycogen is deposited in liver and muscle. Some residual albumen-allantoic fluid ingested enters the yolk and continues to express digestive enzymes obtained while passing through gastrointestinal systems. Digestion of nutrients then can occur in the yolk and be absorbed by yolk sac cell villi, especially VLDL, which accumulates triglycerides in sub-dermal locations of the embryo. Cholesterol accumulates in the liver, causing it to grow in size and become yellow in appearance. At the same time HDL aggregates in granules and are covered with calcium from circulation. These granules remain in the yolk sac until pipping. Dissolution of mammary knobs adjacent to the choriallantois-shell membrane interface mobilizes great amounts of calcium, which rises in the circulation favoring calcification of the skeleton, which was mainly cartilage until then. Now the embryo is ready to start pipping the weakened shell and membranes (Moran, 2007).

Emergence starts when the embryo breaks the choriallantois and the inner shell membrane near the air cell, in what is called internal pipping. At this point the embryo must initiate pulmonary respiration, since the outer shell membrane is losing contact with the shell. This is a critical period because limited supply of oxygen suppresses

continuing use of lipids as energy, so metabolism switches again to the anaerobic catabolism of glucose from glycogen reserves producing lactate. The remaining yolk sac is retracted into the abdominal cavity, and peripheral blood is recovered into the embryo. A relatively great amount of energy is used to sustain embryonic pipping movements to break the shell, and body rotation. Shell piercing is achieved by the coordination of pipping muscle movements and the egg tooth of the beak. The pipping or hatching muscle is a specialized muscle located in the back of the head. Hatching muscle fibers are exclusively anaerobic and rely on glycogen stored there previously. It also has a special nervous system to coordinate its movements. External air access now provides enough oxygen for oxidation of fatty acids and lactate recovery in the liver. At this point the choriallantois can no longer extract calcium from the shell, so calcium mobilization begins from HDL granules. The embryo continues breaking the shell, rotating and using the feet to push until it is free from the shell (Moran, 2007). The blood vessels linking the navel to the shell membrane are detached, and the hatching process is over.

Although yolk sac lipoproteins continue to be an important nutrient source at this time, fatty acid recovery from preformed body depots appears to dominate. Extensive hepatic cholesterol in place at hatch rapidly dissipates along with the depots. Cholesterol, together with depot essential fatty acids, enables continued membrane formation and growth. A high concurrent demand for glucose appears to reside with its need to support growth of glycolytic muscle. In the intestine, cell proliferation is stimulated by feed intake to replace embryonic enterocytes, able to absorb macromolecules, by mature ones, able to produce digestive enzymes and to absorb external feed nutrients. The complete

transition may take up to two weeks post-hatch, and is delayed if feeding is delayed (Moran, 2007).

Turkey poult peculiarities

Genetic advances are forcing commercial poultry towards altricial development, as was pointed out by Foye, (2005). Early nutrition and incubation have become key factors for successful poultry production and it offers the greatest opportunity for improvement (Hulet, 2007). The development of body functions starts early during the embryonic phase in precocial birds, especially poultry. This early and very sensitive developmental phase is of high significance for the adaptability of the organism later in life (Tzschentke, 2007).

The turkey is more prone to perinatal problems than chickens because of it has a thicker egg shell (lower conductance), longer incubation period (28 versus 21 days), and longer hatching window (exacerbated by longer storage since less eggs are available). These disadvantages often increases the incidence of weak poults by the time of placement, resulting in 2-5% mortality during brooding phase (Carver et al., 2000; Christensen et al., 2003c; Uni and Ferket, 2004). Bakst et al., (1997) described that we can not use the same staging procedure for chickens and turkeys because there are several differences between their developments. Chicken morphogenetic development is more advanced by the time of oviposition (Gupta and Bakst, 1993; Bakst et al., 1997), but more research is necessary to confirm if this is related to higher hatchery losses among turkeys compared to chickens.

It has been well documented that weaker poults are associated with depleted glycogen energy reserves by the time of hatching (Donaldson and Christensen, 1991,

Donaldson et al., 1991; Donaldson, 1995; Christensen et al.; 2001b, Christensen et al., 2003c), and recovering glycogen reserves is done at the expense of muscle degradation by gluconeogenesis (Keirs et al., 2002). These poult may be too weak to eat and drink thus initial losses may never be compensated.

Late-term embryo tissue specificities

To study turkey embryo and poult nutrition and metabolism, it is important to identify which are the most metabolic active tissues: liver, pectoral muscle, hatching muscle, and intestines. The following is a brief explanation why these tissues are metabolically important for the perinatal turkey.

As in all animals, the liver is the most metabolically active tissue in the body of turkey embryos. It is the only organ in which all metabolic pathways and metabolic enzymes are active; some of them are present exclusively in the liver. Blood coming from vitelline circulation, digestive tract or peripheral tissues pass through the liver. In the liver nutrients absorbed can be stored, modified or exported to other parts of the body, while waste components from other tissues can be metabolized for excretion, or recycled back into useful metabolites. Major energy metabolic pathways like glycolysis, gluconeogenesis, tricarboxylic acid cycle (TCA), pentose phosphate, glycogenesis and glycogenolysis are most active in the liver, due to its role in controlling energy homeostasis. Extensive lipid metabolism also occurs in the liver, like exporting stored and *de novo* synthesized cholesterol, triacylglycerol and phospholipids packaged or repackaged as low-density lipoproteins (LDL), very low density lipoproteins (VLDL) and high-density lipoproteins (HDL). To keep energy homeostasis, hepatic cells are under fine control of circulating hormones, including insulin, glucagon, corticosteroids and

thyroid hormones. In poultry embryos, the liver is especially important to produce glucose through gluconeogenesis. The major gluconeogenic organ in animals is the liver, with small contributions from the kidney cortex (Matthews and Holde, 1990a). This is crucial because from when internal pipping begins until the poult is fed, gluconeogenesis is the only source of glucose production for the embryo and poult (Donaldson, 1995), and only the liver has glucose-6-phosphatase, the enzyme needed to export glucose (Matthews and Holde, 1990a). This is in contrast to what happens in mammals, which have a constant supply of maternal glucose (Pearce and Brown, 1971). For example, gluconeogenesis and glycogenesis rapidly develop in rats only after birth (Pearce and Brown, 1971). Some cells of the brain, blood, heart, and muscle depend exclusively on glucose as their energy substrate under anaerobic conditions (like hatching) (Krebs, 1972). Gluconeogenesis from amino acids also require deamination and the liver plays a key role in metabolizing nitrogen to be transported in a less toxic form to the kidney where it can be excreted (Matthews and Holde, 1990b), or to the allantois in the case of the embryo. Because of its increased role in late-term embryo metabolism, the liver shows extensive growth during the last phase of incubation, growing proportionally faster than the rest of the embryo (Romanoff, 1967). The avian's liver has an even greater importance in carbohydrate metabolism because their hearts lack the enzymes of the Cori cycle, so lactate produced during increased glycolysis can only be recycled by hepatic tissue (Pearce and Brown, 1971; Christensen et al., 2003c).

The breast muscle (*Pectoralis major*) of the avian embryo is metabolically important mainly because of its relatively large size. Even though the pectoral muscle contains less glycogen per unit mass, it accounts for the greatest quantity of total

glycogen stored in the body (Foye et al., 2006). Therefore, it is important to study the pectoral muscle when tracking the destiny of circulating glucose during late incubation. The pectoral muscle is also the tissue of highest economic value (Smith, 2006a). Genetic selection of turkeys for increased breast meat yields may have contributed to some of the metabolic challenges the poult is experiencing nowadays. Larger breasts retaining constant concentration of glycogen may reduce glucose available for liver storage. Since genetic selection keeps increasing pectoral muscle size, this trend is not going to stop. New technologies opened the door to even greater emphasis in breast meat. Geneticists may include some selected key genes into selection strategy to improve pectoral muscle (Velleman, 2007). The pectoral muscle is also the possible source of protein mobilized to supply amino acids for gluconeogenesis if energy reserves are depleted after hatch (Donaldson, 1995; Keirs et al., 2002; Warner et al., 2006). This could mean less pectoral muscle at market age, and consequently economic losses. Thus, there is a need to study the role the pectoral muscle plays in embryonic metabolism during embryo development, hatching and brooding.

The importance of the hatching muscle to the developing embryo was already discussed above, but it is worth emphasizing some more key points. The *Complexus* muscle or hatching muscle is a large paired neck muscle that overlies the *spinalis* and *biventer cervicus* muscles (Gross, 1985), extending from the dorsal surface of the transverse process of some of the anterior cervical vertebrae to the occipital crest of the skull (George and Berger, 1966). Each half is divided into three segments with neuromuscular junctions in between them. It acts by extending the head dorsally and laterally. Another characteristic of this muscle is its increase in size towards the time of

pipping, and then shrinking during 3 to 8 days post-hatch (John et al., 1987). The function of this muscle is to help the embryo pierce the membranes and the shell (Moran, 2007). Enlargement of the hatching muscle forces the egg-tooth to push against the shell, thereby causing the shell to crack (Smail, 1964). There are not many studies on structural and metabolic aspects of this muscle in relation to its function, but what is known is that it has two types of muscle fibers. The majority of the fibers are glycolytic only (able to use glucose exclusively), and a few are oxidative-glycolytic (able to use glucose and fatty acids). The muscle swelling happens with accumulation of high amounts of water and glycogen granules, which is reduced by half after hatch (John, et al., 1987). Glycolytic fibers produce lactate and are more active during pipping and hatching, while oxidative-glycolytic fibers are active after hatch, coinciding with rising amounts of free fatty acids in this tissue (John et al., 1987). The *complexus* muscle has its independent nerve control (Gross, 1985; Moran, 2007) to coordinate its movements. Because it is one of the most active muscles during emergence, it has higher energy demands than any other tissue of the body. So the hatching muscle is an important tissue to consider when studying late-term embryo energy metabolism and hatchability.

During egg incubation bird embryos do not invest much into gut development, but at the end of the incubation period rapid visceral growth and maturation occurs. The hatched poult must have a functional digestive system, because their nutrient reserves are limited (Christensen et al., 2007), and it must start eating. The sooner the gastrointestinal tract achieves its functional capacity, the sooner the young bird can utilize dietary nutrients and efficiently grows to its genetic potential (Uni et al., 2006). It is understandable that the embryo would not spend a lot of resources growing intestinal

tissue during embryonic development since their nutrients are being absorbed by the yolk sac membrane directly into the vitelline circulation (Richards, 1997). Also intestinal cells require high energy for maintenance and have very high turnover. The major event to trigger change in this system is amnion consumption, which constitutes the first substrate to pass through intestinal tract for absorption (Uni et al., 2006). At this time there is an abrupt change in nutrient source from yolk lipids to protein and some carbohydrates arriving in the gut (Uni et al., 1998). Intestinal mucosa cells called enterocytes are produced in the crypt and migrate to the tip of the villus to replace embryonic mucosa. At the same time they mature from secretory to digestive/absorbing cells. This pattern of maturation is programmed to starting a couple days before hatch, and will persist throughout the bird's life. Even though maturation is programmed to happen, substrate presence seems to accelerate development. During migration, enterocytes acquire differential functions for digestion, including expression of enzymes such as disaccharidase, peptidase, and alkaline phosphatase (Uni et al., 1998). Uni et al., (1998) showed that villus volume increased rapidly in all segments of the intestine starting at 2 days post-hatch. Duodenum completed development by 7 days post-hatch, whereas jejunum and ileum continued until day 14. Crypt depth increases two to three fold as age advances, being greatest in the duodenum and least in the ileum. Production of major enzymes, like sucrase, maltase and alkaline phosphatase, also increases with age.

Birds have a greater capacity to digest disaccharides than mammals immediately post-hatch. Even before hatch there are reports of pancreatic and brush-border enzymatic activity in the chicken embryo intestine (Uni and Ferket, 2004), but not much is known about other enzymes and transporters. It was also previously discussed that not having

immediate access to feed causes loss of intestinal structure and delays maturation (Uni et al., 1998; Uni and Ferket, 2004; Christensen et al., 2007). To emphasize the importance of a mature gut, it is worth mentioning the study done by Applegate et al., (2005) who compared turkey poults and pekin ducklings. Both species have the same egg size and incubation length, but ducklings grow faster with the difference being their higher intestinal growth and maturation compared to poults of the same age. Hypothetically, any improvement in early gut maturation and digestive capacity would be valuable to help give poults a good start post-hatch.

Ways to Improve Early Development

Because turkey poults are more difficult to hatch and brood than chickens, early development has been a focused area of research. (Uni and Ferket, 2004) described it as the “hatchling quality” phenomena, where hatchlings do not survive the critical post-hatch adjustment period and many survivors exhibit stunted growth, inefficient feed utilization, reduced disease resistance, or poor meat yield; this all costs the poultry industry over \$200 million a year. As discussed below, many options to alleviate these losses have being suggested, and many have been tested.

While changes in the way hatcheries handle poults have not been implemented during the last 3 decades, a lot of studies have been focusing on early feeding. One hypothesis is that having the first feed formulated to improve gut development could compensate for the previous long period of fasting. For example, feeding yeast extract to turkey poults has been shown to accelerate gut maturation in comparison to poults fed regular diets (Solis de los Santos et al., 2007). Even just the physical presence of solids with no nutritive value in the intestine is enough to stimulate maturation and growth, but

with no lasting effects (Noy and Sklan, 1998b). Early feeding stimulates gastrointestinal motility and use of yolk sac nutrients necessary for growth (Noy and Sklan, 1998b; Noy and Sklan, 1998a; Noy and Sklan, 1999b; Noy and Sklan, 2001).

Offering glucose in drinking water was the earliest initiative to improve chick energy status, but the practice was later found to be detrimental because glucose suppresses gluconeogenesis, which is the main source of energy to the early hatchling (Donaldson, 1995). One other option is to offer feed in the transport boxes, which led to the design of products like Oasis¹, which is made of protein, carbohydrate, fat and fiber in a green granular form. The product attracts chicks and poults to eat during transport or when dressed on top of feed trays in the brooder house. Although gavage of nutrients is not practical under commercial conditions, it was found to be effective as Oasis in increasing body weight and breast meat yield (Noy and Sklan, 1999a). Even at market age early fed birds were 8-10% heavier and had 7-9% bigger breasts than birds held without feed (Noy and Sklan, 1998b; Noy and Sklan, 1999a).

Another option tested was subcutaneous injection of nutrients at servicing. Gluconeogenic substrates like amino acids and vitamins were injected subcutaneously in just hatched broiler chicks and resulted in 10% gain in body weight in 24 hours and nearly double their weights in 96 hours due to reduced tissue catabolism (Keirs et al., 2002; Peebles et al., 2006). Another study testing the injection of glucose and alanine concluded that under proper brooding conditions and timely feed provision, growth was not improved (Donaldson, 1995; Keirs et al., 2002; Peebles et al., 2006). John, et al., (1987) tested the effect of immersing eggs in a glucose-antibiotic solution, and saw no

¹ Novus International, Inc., MO

differences in glycogen reserves, but an increase on lactate, indicating increased glycolysis.

From all options tested, early feeding seems to be the most advantageous, while gavage, injections and egg dipping showed to be unpractical or have inconsistent results. These inconsistent results emphasize that there is not enough information about avian embryo physiology and nutrition to predict responses. Although early feeding enhances development of hatched birds, it is too late for embryos to overcome struggles during pipping and hatching. It is also difficult to measure improvements on poult quality other than body weight and growth.

Assessing Poult Quality

A good-quality 1-day-old chick is a crucial link between the hatchery and the broiler farmer. The usual criterion to assess chick quality is personal observation and judgment of personnel, with no clear standards. The relationship of body weight at hatch with slaughter weight is unclear, disqualifying chick weight as a good indicator of chick quality must be considered.

Two scoring systems have been proposed to provide a better assessment of poult quality (Decuypere and Bruggeman, 2007). The first one is called the Pasgar Score, in which the chick starts with a score of 10 and loses points when abnormalities are observed (Boerjan, 2006; Decuypere and Bruggeman, 2007). The other scoring system was proposed by Tona et al., (2003), where parameters receive points depending on their importance, with totals ranging from 0 to 100 (100 being the best chick). These scoring systems have shown good correlation with body weight at 6 weeks of age, where birds showing high scores at hatch were the heaviest birds at slaughter. The most important

chick quality traits of the two evaluation systems is associated with retracted yolk and naval (Decuypere and Bruggeman, 2007).

An increasing number of turkeys develop some sort of leg problem. The most common skeleton problems are dyschondroplasia, twisted leg, rickets, kinky back, brittle bone disease, and general lameness (Zhou et al., 2007a). Birds with leg problems have trouble moving and thus do not eat and drink normally, ending as small birds at processing and more condemnations when associated with infection. Zhou et al. emphasized that skeleton integrity is important for meat processing and cause significant economic losses (Zhou et al., 2007a). The source of the problem seems to trace back to initial bone formation when calcium is mobilized from eggshell for skeleton calcification. Incubation conditions or limited egg nutrients can prevent normal bone development, which can be seen in long bones like leg shanks of the hatched poult or chick. At 18 days of incubation, the turkey embryo skeleton is still all cartilage, with solid bone mass appearing between E18 and 3 days post hatch. Ossification is mainly completed by 7 days pf age (Simsa and Ornan, 2007). Shank length can be used as an indicator of poult quality. Leg relative asymmetry was a parameter created to determine hatchling quality; it uses the ratio between right and left shank length to assess proper use of nutrients for bone development (Moller et al., 1995; Moller and Cuervo, 2002; Moller and Manning, 2003; Yalcin et al., 2003; Yalcin et al., 2004; Yalcin et al., 2005).

Among the factors that affect quality, Decuypere and Bruggeman, (2007) highlighted the following: egg storage time (the longer, the worse), age of breeder (eggs from older hens result in poor quality chicks), incubation conditions (humidity, temperature, turning and gaseous environment), and embryo physiology (heat production,

hormones, gas exchange, energy stores). Studying egg size and heat production, Lourens et al., (2006) concluded that temperature has a big influence on poult quality, and large eggs had more remaining yolk energy. Egg turning regulates accumulation of protein in amniotic fluid, affecting embryo growth, hatchability and consequently poult quality. Turning also influences thyroid hormone levels and corticosteroid production, affecting embryonic response to stress (Tona et al., 2005). The same authors even suggested that ceasing to turn eggs negatively affects poult quality through genome imprinting. Decuypere and Bruggeman, (2007) hypothesized that conditions prior to hatch can modify embryo physiology in ways that can not be evaluated, such as changes in the expression of genes involved in growth.

In summary, there is a problem in turkey production with poor hatchability, poult quality, early survival, and stunted growth. A combination of factors contribute to the picture, including breeder hen age, egg size, egg conductance, egg storage, incubation conditions, hatch window, poult servicing, holding time at the hatchery, transport to brooder farm, and brooding conditions. The worse case scenario seems to be with large eggs, from older hens, stored for over a week, incubated in less than ideal conditions, submitted to long holding and transportation periods, finally placed in lower brooding temperatures. Unfortunately some sort of combination of these factors can not always be avoided in practical conditions, and that is why we need to consider new ways to help these poults must be considered.

Aiding Embryo Nutrition *in Ovo*

***In ovo* feeding concept**

The greatest opportunity to facilitate improvements in hatchability and hatchling viability was, for a long time, to change incubation conditions. In the early days of poultry industry, Smith (1937) advised farmers to invest in turkey hen nutrition because after the egg was laid, it was impossible to increase any of the food essentials for development and growth of the embryo until it hatches. In the beginning of the 80's, *in ovo* vaccination against Marek's disease was proven to be effective against early exposure to the virus (Sharma and Burmester, 1982). These same authors developed a successful method of *in ovo* injection. Experimental injection of small amounts of drugs, vaccines and nutrients in the egg during incubation was tested along the years. A summary of research papers mentioning *in ovo* administration is presented on Table 1.3.

Table 1.3. Summary of research papers mentioning *in ovo* administration of substances.

Article	<i>In ovo</i> injected substance	Target
Balaban and Hill, 1971	L-thyroxine, thiourea	embryo
Al-Murrani, 1982	Amino acids	yolk
Decuyper et al., 1982	Iopanoic acid	allantoic circulation
Sharma and Burmester, 1982; 1984	vaccines	amnion/embryo
Sharma et al., 1984	vaccines	amnion/embryo
Sharma, 1985	vaccines	amnion/embryo
Wakenell and Sharma, 1986	vaccines	amnion/embryo
Iqbal et al., 1987	methimazole	allantoic circulation
Hargis et al., 1989	growth hormone	albumen
Ahmad and Sharma, 1992, 1993	vaccines	amnion/embryo
Whitfill et al., 1992a	vaccines	amnion/embryo
Whitfill et al., 1992b	vaccines	amnion/embryo
Sarma et al., 1995	vaccines	amnion/embryo
Johnston et al., 1997.	vaccines	amnion/embryo
Edens, et al., 1997.	lactobacillus	air cell, amnion, embryo
Kocamis et al., 1999.	growth hormone	albumen (pre-incubation)
Henry and Burke, 1999.	testosterone, antiandrogen	albumen (pre-incubation)
Coles et al., 1999.	peptide YY	air cell
McReynolds, et al., 2000.	antibiotics	amnion
Williams and Brake, 2000.	fungicides, mold inhibitors	air cell
Williams et al., 2000.	contaminated air	air cell
Wu et al., 2000.	antibodies against adipocyte membrane	allantoic circulation
Kocamis et al., 2000.	insulin-like growth factor-I	albumen
Ohta and Kidd, 2001.	amino acids	yolk
Ohta et al., 2001.	amino acids	yolk
Jochemsen and Jeurissen, 2002.	detectable particles and vaccines	amnion
Weber et al., 2004.	Eimeria	air cell
Tako et al., 2004.	carbohydrates, HMB	amnion
Uni et al., 2005.	carbohydrates, HMB	amnion
Tako et al., 2005.	zinc-methionine egg white, HMB,	amnion
Foye, 2005	carbohydrates, arginine	amnion
Moore, 2005	egg white, HMB	amnion
Zhai et al., 2005, 2006	L-carnitine	amnion
Smirnov et al., 2006.	carbohydrates	amnion
Foye et al., 2006.	egg white, HMB, carbohydrates	amnion
Kim et al., 2006.	antibodies against myostatin	albumen or yolk
Matsushita et al., 2006.	pesticides	albumen (pre-incubation)
Pedroso et al., 2006.	glucose	amnion
Kim et al., 2007.	antibodies against myostatin	yolk

The *in ovo* studies presented on Table 1.3 show a variety of substances being injected in different compartments of the egg. The first studies of Balaban and Hill (1971), Al-Murrani (1982) and Sharma and Burmester (1982), Sharma and Burmester (1984) determined that injecting solutions in the air cell or on the chorioallantoic membrane depressed hatchability. Balaban and Hill (1971) proved that hatchability is dependent on thyroid hormones, which was later confirmed by Decuypere et al. (1982). Al-Murrani (1982) was the first to attempt improving embryo body weight by adding amino acids to the yolk sac of chicken embryos at 7 days of incubation. He concluded that embryos used the extra protein to grow heavier only when they reached late embryonic growth, and that supplemented chicks were heavier all the way to market age. His idea was not to make *in ovo* supplementation commercially viable, but to prove that laying hens needed additional protein in their diets. Most of the studies done during the following two decades were focused on *in ovo* vaccination (Johnston et al., 1997). Research was done to test the effect of growth hormone (Hargis et al., 1989; Kocamis et al., 1999), testosterone (Henry and Burke, 1999), peptide YY (Coles et al., 1999), antibiotics (McReynolds et al., 2000), insulin-like growth factor-I (Kocamis, et al., 2000), pathogens (Williams et al., 2000; Weber, et al., 2004), and fungicides and toxic compounds (Williams et al., 2000; Matsushita et al., 2006). The objective of this research was not to improve poultry production, but to demonstrate embryo response within the egg's closed environment. Ohta resumed Al-Murrani's work, now with the goal to improve hatchability and chick weight (Ohta and Kidd, 2001; Ohta et al., 2001). As Al-Murrani, (1982), they injected a mixed amino acid solution similar to the amino acid profile of egg white into different embryonic compartments on day 7 of incubation. They

concluded that the yolk sac or the extra-embryonic cavity was the best target for amino acid injection at 7 days of incubation to positively affect hatchability. However, injecting this amino acid solution into the amnion at 7 days of incubation resulted in embryonic death within 24 hours. Yolk and extra-embryonic coelom supplemented chicks hatched heavier than the controls (Ohta and Kidd, 2001), and had higher amino acids concentration in their tissues (Ohta et al., 2001).

In ovo vaccination has become a widely adopted practice by the poultry industry (Johnston et al., 1997), and after the technology was patented (Sharma and Burmester, 1984). In fact, *in ovo* techniques are mentioned by Smith (2006b) as one of the biggest contributions of poultry research, along with feather sexing, nutritional strategies to avoid leg problems, blood screening to eliminate pathogen carriers, and genome sequencing.

Uni and Ferket, (2003) invented and patented the concept of administrating a nutritive solution into the amniotic fluid so as to “feed” supplemental nutrients to the embryo which consumes the amniotic fluid prior to hatch. According to Uni and Ferket, (2004), if early access to feed is critical for early development post-hatch, then feeding the embryo before hatch by *in ovo* administration would be expected to enhance hatchability, and development of the digestive tract, and increase body weight and nutritional status of the hatchling. The advantage of *in ovo* feeding over early feeding is the possibility of helping the struggling embryos to hatch. *In ovo* feeding may not replace the benefits of early feeding, but it should potentialize its effects if both practices were combined, and at least minimize the adverse effects of post-hatch holding if the poults do not have access to early feeding.

The main difference between *in ovo* feeding (IOF) and other *in ovo* applications done in the past is that IOF specifically targets the amnion to deliver nutrients that will be presented to the intestinal mucosa when orally consumed by the embryo just prior to hatch (Uni and Ferket, 2003; Uni and Ferket, 2004; Uni et al., 2006). As for *in ovo* vaccination, a special needle is introduced through the shell and membranes until the tip reaches the target (the amnion in the case of IOF), delivering nutrients without harming the embryo (Figure 1.3).



Figure 1.3. Schematic representation on *in ovo* injection

This technology may revolutionize early nutrition and even incubation practices. The challenge is to overcome the IOF formulation and delivery constraints so it can be commercially applied to achieve a better return on investment.

In ovo constraints

Enting et al., (2007) tested breeder diets with different nutrient densities and concluded that available practical diets produced eggs with reduced egg white compared to a control diet. The authors concluded that the amount of egg white in these eggs at the time of lay was insufficient to sustain proper embryonic development. This is one situation where IOF can be beneficial to the embryos. However, several technical questions need to be addressed for IOF to succeed under commercial conditions: 1) what is the best time to inject (embryo age or stage of development)? 2) how much volume can or need to be injected? 3) what is the best osmolality of the solution so it will not harm amnion/embryo osmotic balance? 4) what nutrients and how much can be *in ovo* fed without increasing metabolic load or causing toxicity to the embryo? Therefore, questions related to the physical constraints of the egg and metabolic constraints of the embryo must be answered.

The idea of supplementing carbohydrates to chicks and poults into the amnion before hatch may seem simple, but it is technologically difficult. As already mentioned, just giving glucose in the drinking water to chicks at placement ended up being detrimental because it suppressed gluconeogenic enzymatic activity (Donaldson, 1995). So the kind of nutrient that can be *in ovo* fed and how it will affect embryonic metabolism must be carefully studied.

A popular trade magazine asked the questions: “Can nutritionists find ways to put more vitality into day-olds?”, and “Will it be via breeder feed or by direct application into the egg?” (Horrox, 2006b). These seem to be fair questions to ask as we attempt to solve

the problems of low chick quality. But since it is difficult to influence egg composition via hen nutrition, *in ovo* feeding offers an intriguing solution.

Effects of *in ovo* feeding

Preliminary in ovo feeding research

As disclosed in the patent by Uni and Ferket, (2003) *in ovo* feeding may include the amnion supplementation of nutrients like protein, amino acids, carbohydrates, vitamins, and enteric modulators (like beta-hydroxy-beta-methylbutyrate (HMB), organic minerals, lectins, fatty acids and antioxidants in IOF formulations. HMB was chosen to be the first enteric modulator to be tested. HMB is a metabolite of the essential amino acid leucine that occurs naturally at about 5% of leucine metabolism through a minor pathway (Foye, 2005).

Ferket et al. (2005) furthered knowledge on IOF constraints in a study of amnion volume and pH of turkey eggs, and the effects of solution osmolality on hatchability. The osmolality study tested saline solutions ranging from 150 to 1500 mOsm (milliosmoles), and concluded that chick hatchability was optimum when solution osmolality was between 400 to 600 mOsm, with unacceptable rates with solutions exceeding 800 mOsm. Amnion volume of turkey eggs were on average 3.5 mL, pH=6.5 and osmolality 322 mOsm at 21 days of incubation, 2.0 mL, pH=6.5 and 322 mOsm on 22 days of incubation, 1.0 mL, pH=7.3 and 318 mOsm at 23 days of incubation. Negligible amounts of amnion were found from 24 days of incubation onto hatch. Based on these findings the IOF solution volume limits for turkeys was determined to be between 1.0 and 2.0 mL, injected at 23 days of incubation.

The original research disclosed in the patent (Uni and Ferket, 2003) included 8 trials using either chicken eggs, turkey eggs or both. The first trial tested the effect of injecting increasing amounts of HMB (0 to 100 mcg) at 24 days of incubation on turkey embryo hatchability, liver glycogen concentration, and plasma glucose concentration. Supplementation of HMB has being shown to increase carcass quality of steers, reduce early broiler mortality, and increase broiler chicken bodyweight, breast yield, and carcass yield (Fuller and Nissen, 1994; Nissen et al., 1994; Van Koevering et al., 1994). The conclusion of this research was that HMB concentrations between 0.1 and 1.0 mcg increased hatchability, all doses increased liver glycogen, and 0.1 mcg resulted in highest plasma glucose concentration. The second trial tested a more complex carbohydrate solution composed of 10% sucrose, 10% maltose, 5% dextrin dissolved in 0.9% NaCl solution. One milliliter of this formula was injected into the amnion of chicken embryos on day 18 of incubation. Compared to the non-injected control eggs, IOF eggs showed no effect on hatchability, but IOF yielded heavier chicks, with bigger and more mature intestines. IOF birds maintained heavier weights until the end of the trial at 35 days of age. The third trial used a formula containing 5% sucrose, 5% maltose and 15% dextrin dissolved in 0.9% NaCl, also using same volume and embryonic age. This IOF treatment resulted in chicks 3 to 5% heavier than the controls through 25 days of age. The forth trial used the following formulation: 2.5% sucrose, 2.5% maltose, 12% dextrin, 8.25% egg white protein, in 0.9% saline (NaCl), injected 1 mL in broiler chicken eggs on day 18 of incubation. This formulation, simulating the nutrient composition of a typical chick diet, resulted in 3 to 5% increase in body weight up to 14 days of age, as compared to control chicks.

Turkey formulas were tested in the fifth trial with 24% egg white protein in 9% saline, primarily because poultts have a higher protein requirement than chicks. Two milliliters of this formula were injected at 23 days of incubation. This IOF formula increased body weights by 4 to 8% from 1 to 12 days post-hatch in comparison to control poultts. The next turkey formula (sixth trial) added some carbohydrates and HMB, with composition including 5% maltose, 12% dextrin, 8.5% egg white protein and 0.1% HMB, all dissolved in 0.9% saline solution. The volume injected was between 1 to 3 mL per egg. Here the body weights IOF poultts were 3 to 8% greater than controls. A simpler formula containing 24% egg white protein and 0.1% HMB in 0.9% saline solution was used in the seventh trial, and it resulted in poultts 4 to 6% heavier than the controls up until 24 days post-hatch. Finally, the last IOF formula tested had 5% maltose, 12% dextrin, 8.5% egg white protein and 0.1% HMB in 0.9% saline injected in chicken eggs, and 6% dextrin, 15% egg white protein, 0.1% HMB in 0.9% saline injected in turkey eggs (1 and 2 mL/egg, respectively). These formulas increased chick hatchability by 15.6% and turkey hatchability by 17.5%, proving that IOF effectively helps hatchlings in their struggle to emerge from the shell.

Based on these promising results, the patent was approved, and a Binational Agriculture Research and Development Fund (<http://www.bard-isus.com>) grant was shared between Dr. Uni² and Dr. Ferket³ to further develop the technology. Uni's research concentrated on IOF of chickens, while Ferket's group concentrated on IOF of

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³ North Carolina State University, Raleigh, NC

turkeys. Subsequently, Pfizer-Embrex, Inc.⁴ joined the project to develop an automated system capable to commercialize the IOF technology for the poultry industry.

Chicken in ovo feeding research

Since the early work by Uni and Ferket, (2003), considerable progress has been made in identifying why or how IOF increases hatchability rate and body weights, as described by a series of research publications. The first IOF paper was published by Tako et al., (2004). In this study three IOF formulations were tested in chicken eggs: 1) CHO solution (25 g of maltose/L, 25 g of sucrose/L, 200 g of dextrin/L, and 5 g of NaCl/L); 2) HMB solution (1 g of HMB/L in 5 g of NaCl/L); and 3) CHO+HMB solution (25 g of maltose/L, 25 g of sucrose/L, 200 g of dextrin/L, 1 g of HMB/L in 5 g of NaCl/L). The control was a non-injected egg group. Each egg was injected with 1 mL of the respective IOF solution at 17.5 days of incubation. The intestinal morphology results showed that IOF birds had enhanced intestinal development by increasing villi size and disaccharidases enzyme capacity. In comparison to controls, body weight of IOF chicks increased 3.2% at hatch and 5.3% at 3 days post-hatch. The best results were found when birds received HMB alone or with carbohydrates.

Uni et al. (2005) injected 1 mL of a solution containing 25g/L maltose, 25g/L sucrose, 200g/L dextrin, 1g/L HMB and 5g/L NaCl, in 17.5 days of incubation chicken eggs, in two separated trials. In comparison to the control, this IOF treatment increased hatchability 5 to 6%, increased liver glycogen 2 to 5 fold, and increased pectoral muscle size 6 to 8% among birds up to 25 days post-hatch. These authors concluded that IOF

⁴ Triangle Research Park, Durham, NC

HMB and carbohydrates at 17.5 days of incubation improved late-term embryo energy status and post-hatch growth.

Tako et al. (2005) tested IOF zinc-methionine (ZnMet) as a metabolic effector for chicken embryos. At 17 days of incubation, they injected 1 mL IOF solution containing 0.5g/L ZnMet and 5g/L NaCl, and compared it to a 5g/L NaCl saline control solution. The ZnMet solution increased mRNA expression of the intestinal zinc transporter (ZnT1), brush border enzymes activity, and increase in intestinal villus surface area 15 to 47%. The authors concluded that IOF ZnMet triggered the expression of genes involved in digestion and absorption of amino acids and glucose, indicating greater intestinal maturity.

In ovo feeding also affected chicken intestinal surface mucus production (Smirnov et al., 2006). The mucus layer over the gastrointestinal tract mucosa serves as a diffusive barrier, which protects the absorptive cells from pathogens, provides an adequate environment for brush border enzymes to function, and facilitates nutrient absorption. Mucus is made of mucin protein secreted by specialized cells called goblet cells. Smirnov et al., (2006) *in ovo* fed chicken embryos 1mL/egg a test solution containing either carbohydrates (15 g of maltose/L, 15 g of sucrose/L, 150 g of dextrin/L, and 5 g NaCl/L) or a saline control solution (75 g of NaCl/L) at 17.5 days of incubation. These researchers observed the carbohydrate-treated group had greater villus surface area from 19 days of incubation until 3 days post-hatch, higher goblet cell density of jejunum villi, and higher mucin gene expression than the saline-control birds. They concluded that the presence of carbohydrate in the intestinal lumen of the chick embryo might be a trigger for the number of goblet cells and their production of acidic mucin.

The later research confirm the earlier patent claims by Uni and Ferket, (2003) that IOF enhances chick energy status and gut maturation, but the search for a commercially feasible formulation with consistent results is yet to be done. Evidently, IOF formulations containing carbohydrates and HMB do affect embryonic metabolism, which may not always result in better hatchability or heavier body weights.

Turkey in ovo feeding research

The effects of HMB, egg white protein (EWP) and carbohydrates IOF to turkey embryos were addressed by Foye et al., (2006). They injected eggs with 1.5 mL of the respective IOF solutions on day 23 of incubation. The treatments were: 1) 18% EWP, 2) 18% EWP + 0.1% HMB, 3) 0.1% HMB and 4) 20% dextrin + 0.3% maltose, all dissolved in 0.9% NaCl solution. The control group was made of non-injected eggs. At hatch, the IOF treatment groups were 6.0, 3.2 and 3.3% heavier than the controls for treatments EWP, EWP+HMB, and HMB, respectively. This difference was not sustained by 3 and 7 days post-hatch, with the exception of birds IOF HMB alone. The pectoral muscle was larger for EWP and HMB treatments than controls only at day of hatch. Feeding embryos protein increased liver glycogen reserves, while HMB and carbohydrates increased muscle glycogen. The authors concluded that EWP stimulated gluconeogenesis and glycogen storage in the liver, while HMB and carbohydrates caused insulin release, shutting down gluconeogenesis yet stimulating muscle glucose uptake and local glycogen storage. The effects on body weight were variable between the treatments at different ages, with EWP giving the best weights at hatch, but lowest weights at 7 days. Foye et al., (2006) concluded that future studies must be conducted to determine the effects of IOF amino acids and their metabolites on carbohydrate

metabolism during avian development. Based on these findings, it is hard to predict whether protein or carbohydrates would benefit turkey embryos the most. The effects of a combination of protein and carbohydrates fed *in ovo* have also not yet been addressed.

In her dissertation Foye (2005) also tested the IOF effects of the amino acid arginine in turkeys. Arginine was investigated as an IOF solution ingredient because previous studies indicated that it can stimulate insulin secretion and thus could add value to HMB and carbohydrates effects on insulin. Turkey eggs received 1.5 mL of IOF solutions on day 23 of incubation. The treatments tested in a series of experiments included saline solutions containing 0.7% arginine, 18% EWP with or without 0.1% HMB, and 20% dextrin and 3% maltose. Contrary to the authors expectations, HMB did not enhanced pectoral muscle glycogen or size as compared to the controls; arginine and EWP with or without HMB increased liver glycogen at hatch, but not thereafter, and body weight was increased during the first two weeks post-hatch. HMB, arginine and EWP confirmed their role as gluconeogenic substrates by increasing glucose-6-phosphatase activity. IOF carbohydrates enhanced activity of the brush border enzymes sucrase-isomaltase (IS) and leucine aminopeptidase (LAP) at 7 days of age. HMB, arginine, and EWP enhanced activity of the same brush border enzymes at hatch, in addition to glucose transporter SGLT-1, peptide transporter Pept-1 and intestinal enzymes maltase and aminopeptidase (AP). This response likely occurred because arginine and HMB increased insulin-like growth factors (IGF-I and II) in plasma, stimulating gut cells proliferation and maturation (Foye, 2005).

Moore (2005) tested the effect of IOF turkey embryos on pectoral muscle satellite cell mitotic activity. IOF solutions injected contained just 0.4% NaCl (control) or 18%

EWP + 0.1% HMB in 0.4% NaCl solution. Each egg received 1.5 mL of the respective IOF solution at 22 days of incubation. *In ovo* feeding HMB and EWP was expected to increase satellite cell proliferation, but this effect was not among poult observed until 7 days post-hatch, nor was body weight, pectoral muscle size, or weight gain. The author's discussion emphasized that he may not have observed treatment differences because these experimental poult had immediate access to feed and water after hatch, as opposed to commercial industry poult are typically held for 24 to 48 hours without food or water. Moore (2005) also stressed that he may have missed the chance to observe an IOF treatment effect because the most important period of muscle cell proliferation occurs between 25 days of incubation and 1 day post-hatch.

As observed among the in chicken IOF research, the effects of different IOF solution ingredients do not always include increased body weights or better growth performance.

Important Metabolic Pathways associated with Perinatal Nutrition

The effects of different IOF solution ingredients on chicks and turkey embryos are not always predictable and simple to understand. The choice of IOF ingredients used was based on the assumption that the nutrients should be similar to what the hatchling will be fed after hatch in order to promote intestinal development and metabolic adaptation. The embryo relies on gluconeogenesis as its energetic supply from hatch until feed intake initiation (Donaldson, 1995). Foye (2005) demonstrated that IOF components (carbohydrates and HMB) that induce insulin release impaired gluconeogenesis, while protein and amino acids stimulated gluconeogenic enzymes. The embryo undergoes radical changes as the end of the incubation approaches (Uni and Ferket, 2004). Just

before hatch, the hatchling's full body size is reached, and its vital organ systems (e.g. respiratory and digestive systems) must become functional (Moran, 2007). At that same time, the embryo is engaging in the process of hatching. Because nutrients available to the embryo are very limited just before hatch, an amazingly orchestrated sequence of metabolic changes within a very short time. These metabolic changes determine which pathway will be active, depending on substrate and oxygen availability. Little is known about how these metabolic events occur and how they can be modified by management and nutritional interventions. Thus, it is necessary to understand intermediary metabolism of the late-term embryo before one can understand the impact *in ovo* feeding certain nutrients or compounds.

The following section will introduce the major metabolic pathways related to energy metabolism and briefly comment on the possible flux energetic substrates can follow. The metabolic pathways of interest include glycolysis, gluconeogenesis, TCA cycle, pentose phosphate pathway, glycogenesis, glycogenolysis, fatty acid synthesis and fatty acid oxidation.

Glycolysis and gluconeogenesis

Glucose present in eukaryotic circulation is rapidly taken up by cells. Once inside the cell, glucose can be used to generate energy or be stored as glycogen or fat. Glycolysis and gluconeogenesis pathways with their respective enzymes are illustrated by Figure 1.4. Glycolysis is the main pathway where glucose can be phosphorylated, so its' destiny can be decided. The flux through a pathway is determined by the amount of enzymes and its affinity for the substrate. Through glycolysis, glucose can be reduced to 2 pyruvate units plus 6 moles of ATP. Pyruvate can enter the TCA cycle for further

oxidation, producing more ATP. Because glycolysis does not require oxygen, it is the main pathway to obtain ATP when oxygen supply is limited. Pyruvate is converted to lactate during oxygen shortage, and lactate can be recycled back later by hepatic cells through the Cori Cycle (Figure 1.5).

Gluconeogenesis is the major way to produce glucose from other carbon-rich components like amino acids, glycerol and other carbohydrates (Figure 1.4). Glucose is produced by gluconeogenesis only in the liver and kidney, so it can be exported to other tissues or polymerized and stored as glycogen by liver cells for later use. Other tissues can oxidize glucose for energy or make up their own glycogen or lipid stores.

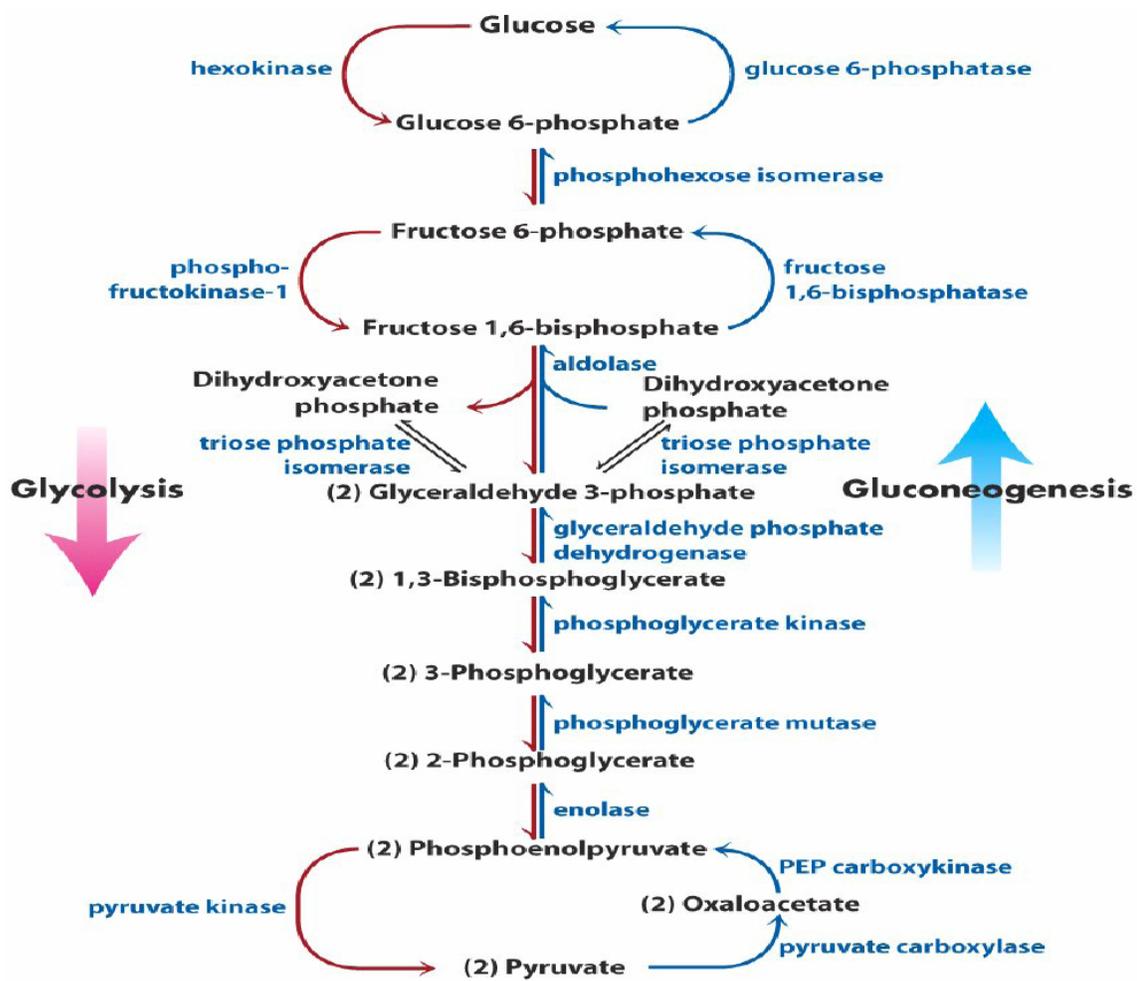


Figure 1.4. Glycolysis (red) and gluconeogenesis (blue) pathways and enzymes.

From: mcb.berkeley.edu/courses/mcb102/handouts/Buchanan/Lecture%204%20rev.pdf -

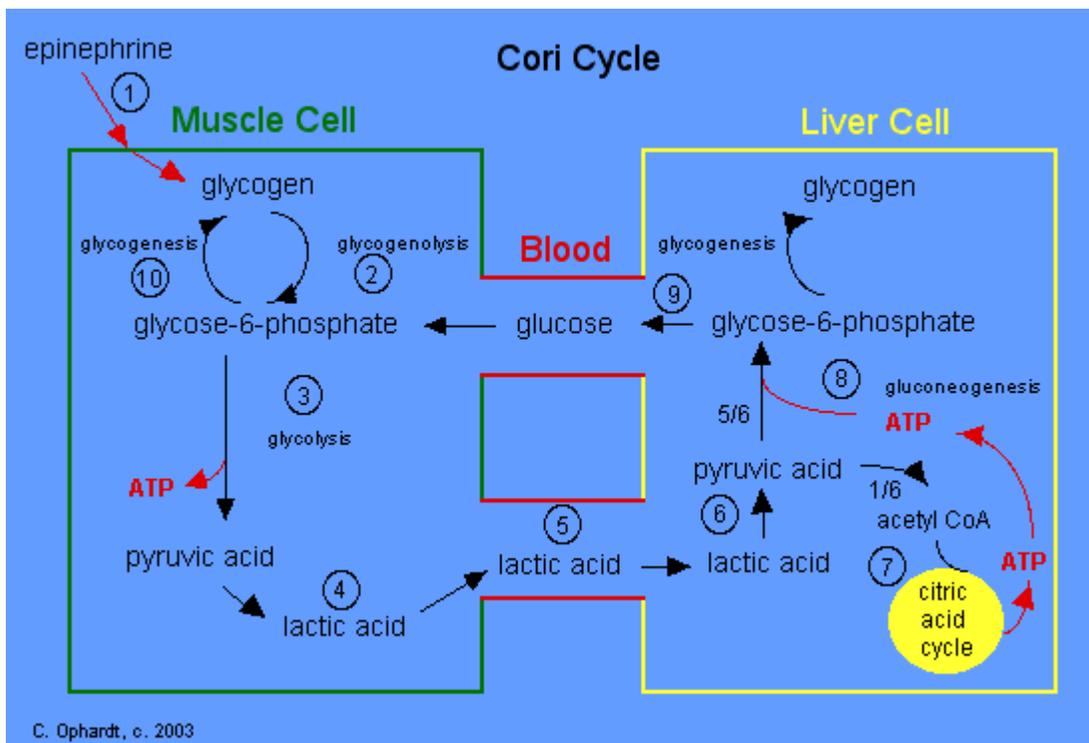


Figure 1.5. The Cori cycle

From: <http://www.elmhurst.edu/~chm/vchembook/604glycogenesis.html>

The Cori cycle will be active during two periods of incubation, when glycolysis production of pyruvate exceeds TCA cycle capacity. The first period occurs during the first week of incubation when the embryo is using mainly glucose and choriallantoic respiration is being established. The second period occurs during internal pipping, when choriallantoic membrane is detaching from the shell and the embryo breaks the membranes during emergence. These are the periods when large amounts of lactate will be produced and the liver will process it back to pyruvate.

TCA Cycle

The tricarboxylic acid cycle (TCA) or Krebs cycle is the major pathway to produce ATP in a living cell or organism. The intermediates and enzymes of TCA cycle

are illustrated by Figure 1.6. Pyruvate is converted to acetyl-CoA which enters the cycle forming citrate. As citrate is converted to several intermediates, energy-rich molecules of NADH, GTP and FADH₂ are produced. NADH and FADH₂ are reducing equivalents that will later result in 24 ATP produced for each glucose unit through the electron transport chain. Other than producing energy, the TCA cycle may receive or donate intermediaries to other pathways. Carbon backbones from many origins enter the TCA cycle in different points as one of the intermediates. Because oxidative production of ATP requires oxygen, the TCA cycle flux is limited by oxygen availability. When lipids are mobilized from fats, fatty acids produce acetyl-CoA which enters the TCA to produce energy. Because two carbon units are lost as CO₂ through TCA cycle, and acetyl-CoA has 2 carbons, fatty acids can not be converted back to glucose through gluconeogenesis.

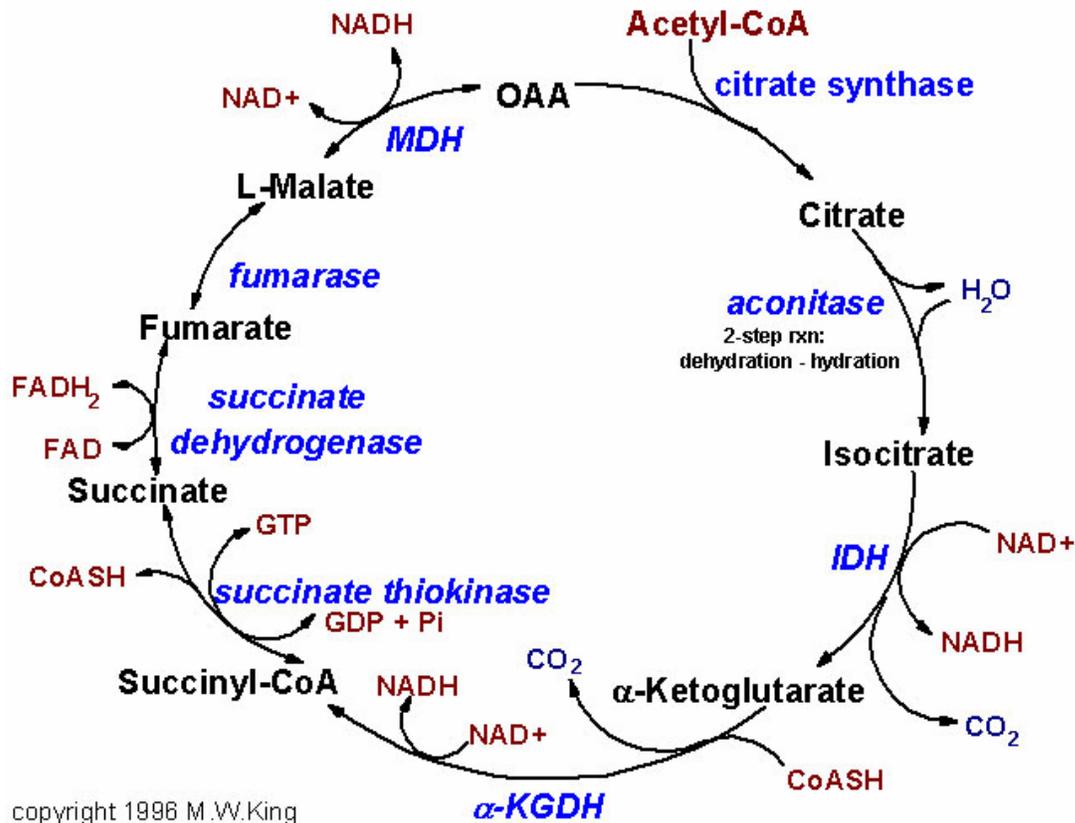


Figure 1.6. The TCA cycle showing enzymes, substrates and products. The abbreviated enzymes are: IDH = isocitrate dehydrogenase and α -KGDH = α -ketoglutarate dehydrogenase.

From: <http://web.indstate.edu/theme/mwking/tca-cycle.html>

During embryonic development this pathway is the major source of energy, generating ATP. Because it depends on oxygen for oxidation, TCA cycle must be shut down during internal pipping.

Glycogenesis and glycogenolysis

If glucose is not needed for energy, limited amounts are stored as long branched chains of glucose called glycogen. The glycogen synthesis pathway is illustrated by Figure 1.7. Glycogen can be converted back to glucose for short-term emergency supply of glucose. Glycogen is stored in liver and muscle cells. Glucose-6-Phosphate is

produced by the first step of glycolysis, and then, glucose is diverted to produce glycogen through glycogenesis (Figure 1.7). When glycogen is mobilized through glycogenolysis (Figures 1.8 and 1.9), it yields back glucose-6-P. Glucose-6-Phosphate depends on the gluconeogenic enzyme glucose-6-Phosphatase to leave the cell, otherwise it can only continue through the glycolytic pathway. Only liver and kidney cells produce glucose-6-phosphatase and thus, can export glucose to circulation (Figure 1.8). Under normal conditions, fasting triggers the use of stored glycogen to provide energy until the next meal. If fasting is prolonged, glycogen reserves are depleted and lipids are mobilized for oxidation. If oxygen limitation prohibits the use of fat, glucose must be produced through gluconeogenesis from other compounds to supply energy needs. The enzymes of glycogenesis and glycogenolysis are under very strict control by hormones and other enzymes to ensure a constant supply of glucose to tissues.

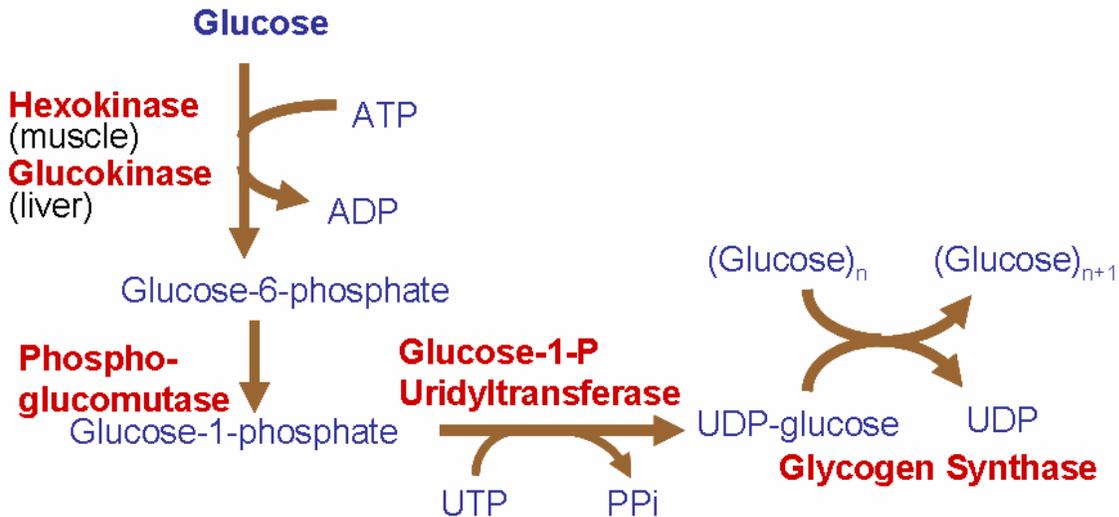


Figure 1.7. Pathway of glycogen synthesis (glycogenesis)

From: www.biochem.arizona.edu/classes/bioc460/fall/html/power/lec31.ppt

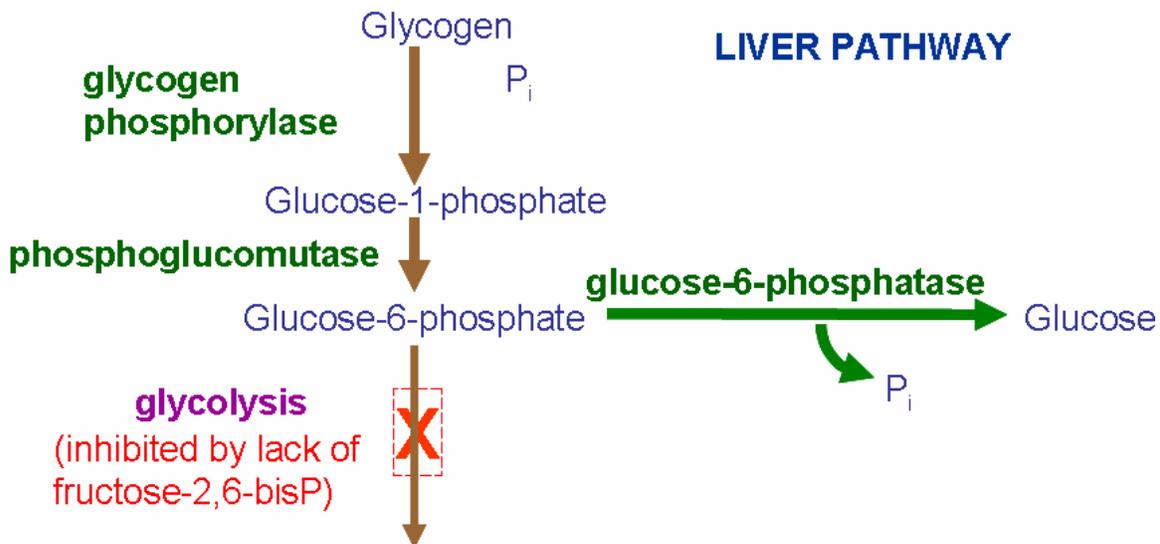


Figure 1.8. Glycogenolysis and the fate of glycogen in the liver.

From: www.biochem.arizona.edu/classes/bioc460/fall/html/power/lec31.ppt

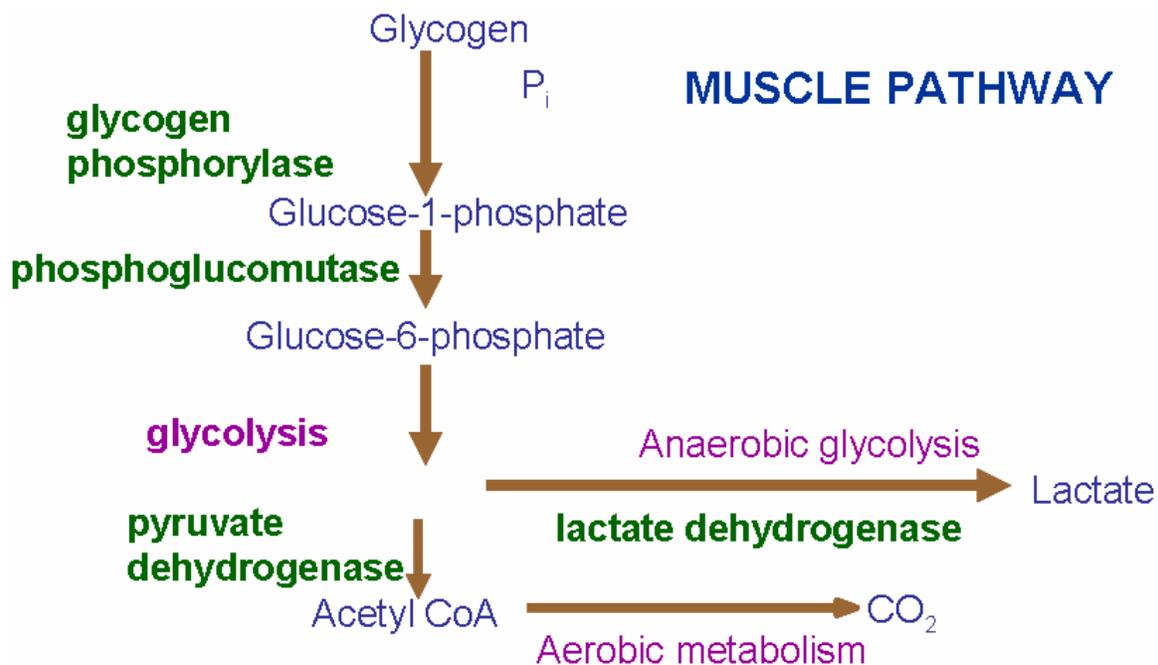


Figure 1.9. Glycogenolysis and the fate of glycogen in muscle
 From: www.biochem.arizona.edu/classes/bioc460/fall/html/power/lec31.ppt

The importance of these pathways to the poultry embryo has been discussed above, but it can be summarized as follows: After internal pipping, reduced oxygen availability limits lipid oxidation; so the embryo prepares for that constraint by orally consuming the amniotic fluid and using its nutrients to store energy as glycogen. The embryo depends on stored glycogen for muscle activity, heat production, and body maintenance during and after hatch, until feed is consumed. If the embryo depletes its glycogen reserves, hatchling quality and survival are compromised.

Pentose phosphate pathway

The pentose phosphate pathway (Figure 1.10) (also called Phosphogluconate Pathway, or Hexose Monophosphate Shunt) is a process that serves to generate NADPH

and the synthesis of pentose (5-carbon) sugars. There are two distinct phases in the pathway. The first is the oxidative phase, in which NADPH is generated, and the second is the non-oxidative synthesis of 5 carbon sugars (Lefens, 2007). The complete pathway is illustrated by Figure 1.10. This is an important pathway in embryonic tissues because pentose sugars are necessary for DNA and RNA synthesis. Since there are minimal amounts of nucleic acids in the freshly laid egg, they must be synthesized by the embryo during incubation (Reddy et al., 1952).



Figure 1.10. Pentose phosphate pathway intermediaries and enzymes

From: http://www.uic.edu/classes/phar/phar332/Clinical_Cases/vitamin%20cases/thiamin%20cases/thiamin/ppp.gif

Fatty acid synthesis and beta oxidation

When acetyl-CoA is produced in excess, it is a sign of abundant energy in the cell, so it is used to synthesize fatty acids. Fatty acids are packed on a glycerol backbone forming the fat units known as triacylglycerol. When glucose availability is low and oxygen supply is not limited, fatty acids are mobilized from fat, liberating acetyl-CoA so that it can be oxidized to produce ATP. This pathway is called beta oxidation.

The first step of fatty acid synthesis is the conversion of acetyl-CoA to Malonyl-CoA by adding carbon from bicarbonate, as illustrated by Figure 1.11. Malonyl-CoA then has its CoA unit replaced by the acyl carrier protein (ACP) and receives an acetyl-ACP unit in the first reaction to produce a fatty acid in a process called condensation (Figure 1.12). Then, malonyl-CoA units are added to the growing fatty acid chain (Figure 1.13).

Fatty acid oxidation is very similar to the inversion of fatty acid elongation (Figure 1.14). Acetyl-CoA units can then enter TCA cycle to produce ATP.

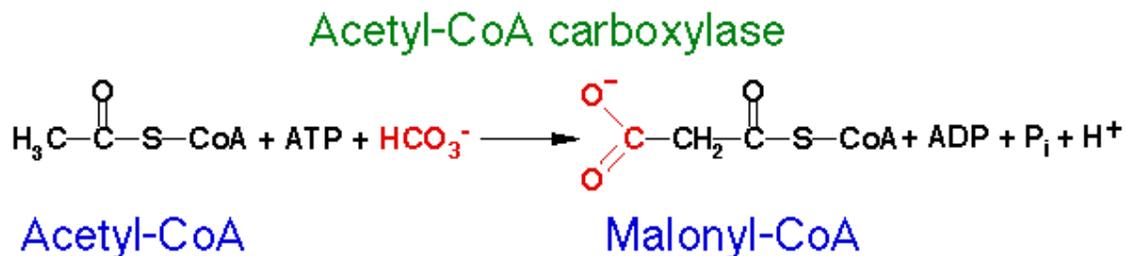


Figure 1.11. Malonyl-CoA synthesis from acetyl-CoA and bicarbonate.
From: <http://138.192.68.68/bio/Courses/biochem2/FattyAcid/FASynthesis.html>

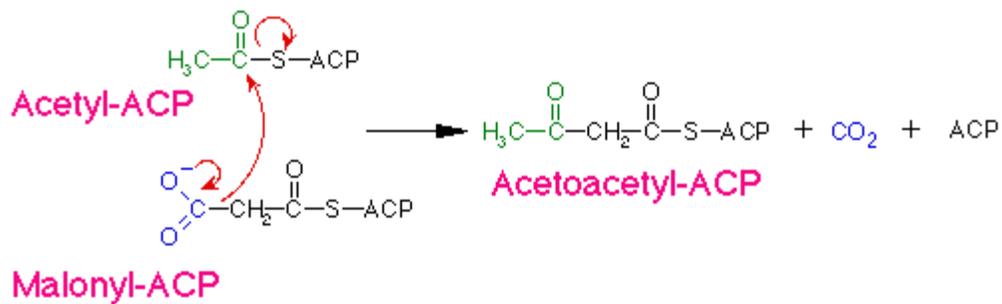


Figure 1.12. Formation of the first bond on fatty acid synthesis

From: <http://138.192.68.68/bio/Courses/biochem2/FattyAcid/FASynthesis.html>

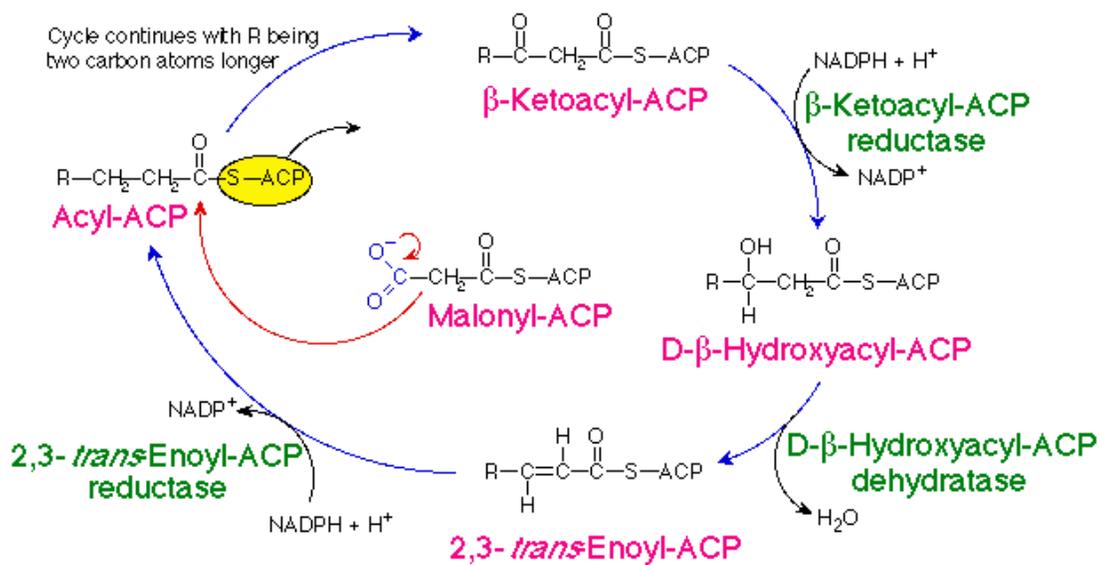


Figure 1.13. Fatty acid elongation pathway.

From: <http://138.192.68.68/bio/Courses/biochem2/FattyAcid/FASynthesis.html>

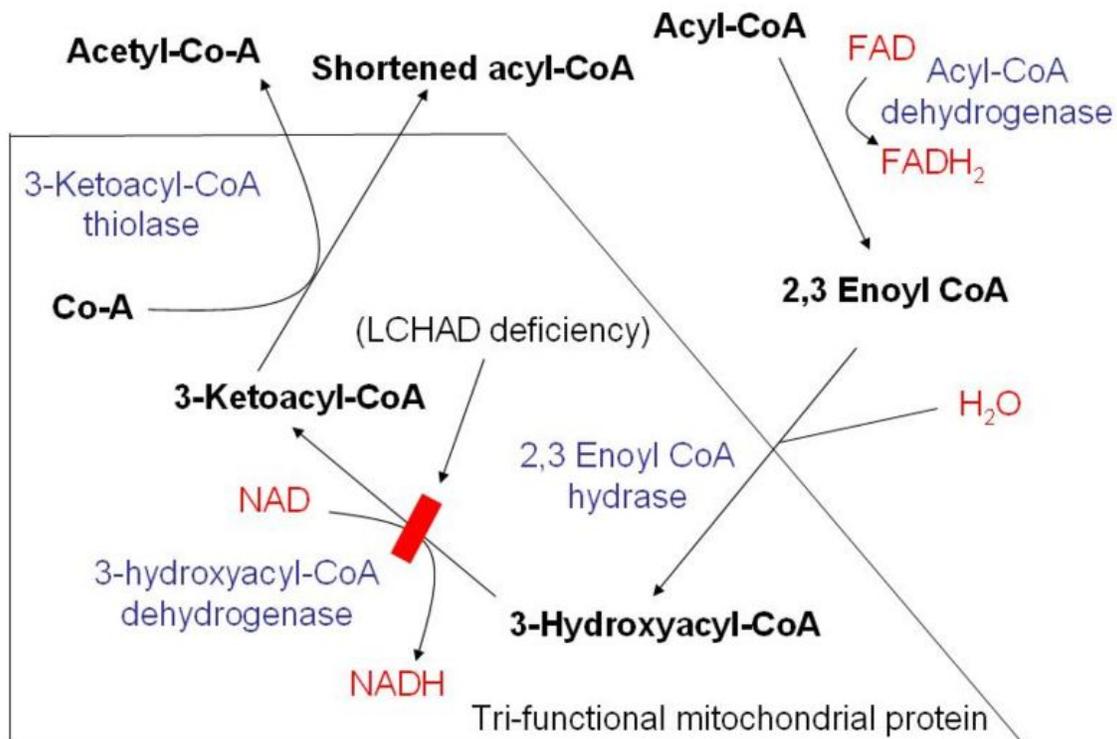


Figure 1.14. Fatty acid beta oxidation.

From: http://en.wikipedia.org/wiki/Beta_oxidation

Fatty acids are already stored in egg yolk as triacylglycerol and phospholipids to be used for energy and membrane synthesis. Beta oxidation is a very important means to produce energy during incubation, but it is inhibited prior to hatch because of the limited oxygen supply and little triacylglycerol left in the yolk. Beta oxidation connects with TCA by acetyl-CoA reacting with oxaloacetate to form citrate, what is catalyzed by the enzyme citrate synthase.

Metabolic pathways interaction

The pathways presented previously do not act as separated units but rather are all connected in the living organism. A general representation of how these pathways connect to each other is illustrated by Figure 1.15. All absorbed nutrients will enter one or more of these pathways, so they can be used, stored, modified, combined or excreted

from the cell or organism. The complexity of this system requires careful control to guarantee survival first, and then growth and reproduction. The amount and kind of substrates that enter the system trigger the production of hormones that spread messages telling the cells in the organism what to do. The environment also has a big impact on metabolism, influencing hormone production and release. In the cell, hormones and other metabolites activate or inhibit genes that express enzymes in these pathways. In the case of poultry and embryo energy metabolism, the main hormones involved in controlling these pathways are insulin, glucagon, catecholamines, and thyroid hormones.

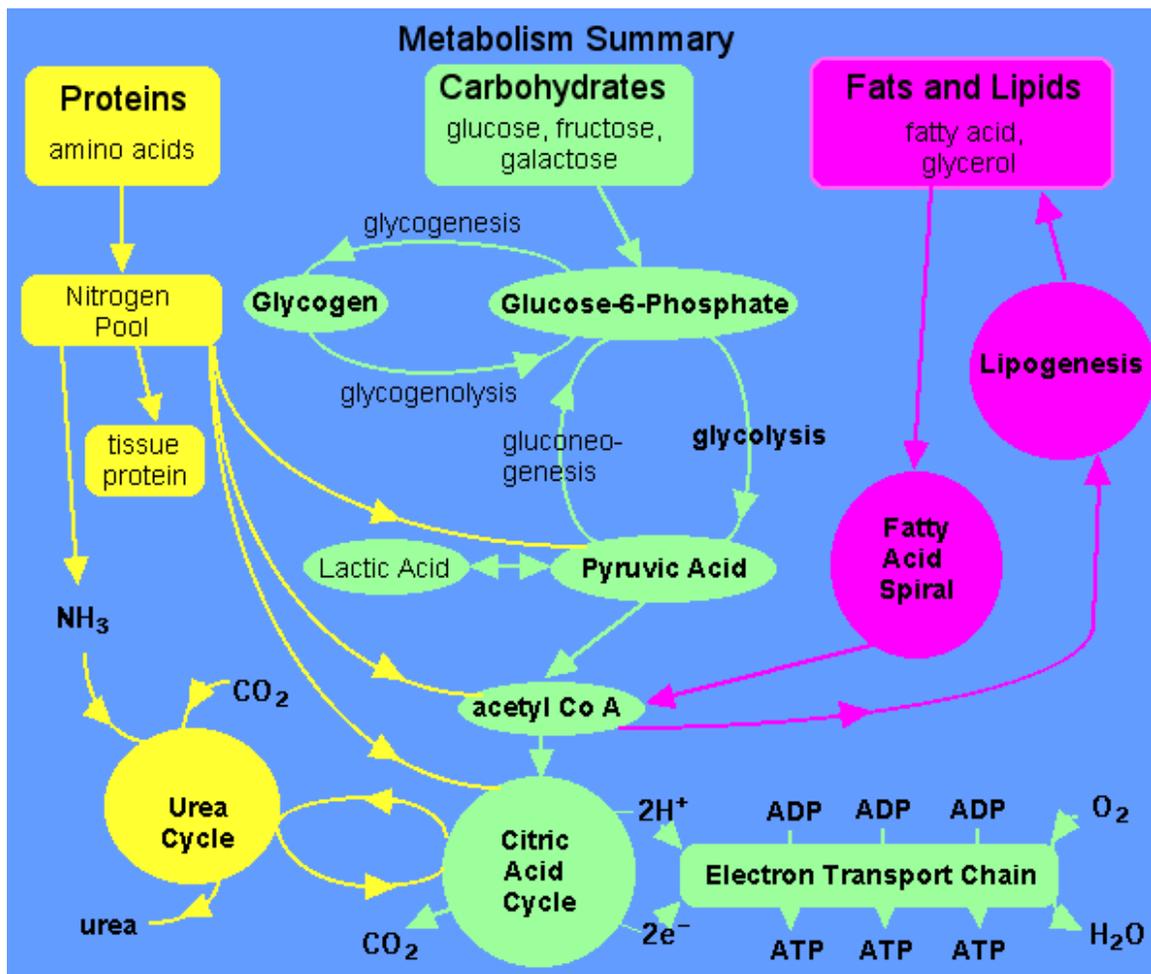


Figure 1.15. Graphic representation of metabolic major pathways and how they relate to each other.

From: <http://www.elmhurst.edu/~chm/vchembook/604glycogenesis.html>

Hormonal control of poultry embryo metabolism

Genome wide analysis identified insulin, glucagon, T3, T4, IGF-I, IGFII genes as being most involved in metabolism, affecting phenotypic traits in chickens (Zhou et al., 2007b).

Insulin and glucagon are closely related to energy in all tissues of the bird (Lu et al., 2007). These hormones are produced by the pancreas and their release into blood circulation depends on the animal's energy status. Insulin is the hormone released after a meal, signaling abundance of circulating glucose. It permits cells throughout the body to uptake glucose and cause the synthetic processes to occur. Insulin stimulates glycolysis, TCA cycle, glycogenesis and fatty acid synthesis. Glucagon is the hormone of fasting, indicating low glucose availability, so only cells that rely of glucose as their only energetic substrate will take it. Glucagon also triggers glycogen and fat mobilization throughout the body. In the liver, gluconeogenesis is stimulated by glucagon to produce glucose from other substrates so it can be made available to other tissues through circulation.

Lu et al. (2007) summarized what is known about these hormones in poultry embryos. In chicken embryos, insulin regulates the concentration of amino acids and related compounds in plasma, amniotic fluid, and allantoic fluid. It also accelerates embryonic morphological development. Other than insulin, the same gene also expresses insulin like growth factors I and II (IGF-I and IGF-II). In poultry as in mammal species, these IGFs stimulate growth, development and intermediary metabolism. Specifically important, IGF-I and II stimulate hepatic glycogen, RNA and protein synthesis. IGFs were also found in amniotic fluid, where they may play a role in regulating amino acid

compounds. Their research found that insulin levels increased significantly as the embryo ages, reaching a plateau during pipping and hatching, and rising again 24 hours after the chick starts feeding. Parallel to that pattern, glucagon levels are low throughout incubation, and then start rising three days before hatch, peaking during pipping and hatching. Glucagon levels drop by 40% 24 hours after the chick starts feeding. Plasma glucose levels follow the insulin pattern, increasing throughout incubation, and plateau during pipping and at hatching. A significant reduction in plasma glucose levels happens 4 days prior to hatch. Concurrently, there is a peak on insulin levels along with amnion consumption by the embryo and glycogen accumulation in liver and muscle tissues. IGF-I levels increase during incubation, but are reduced during hatch. After hatch, IGF-I levels rapidly increase and keep increasing all the way until 21 days post-hatch. IGF-I levels have been identified as important factors in stimulating intestinal mucosa growth and cell maturation (Foye, 2005). IGF-II levels also increase during incubation and remain much higher than IGF-I all the way until hatch, when it quickly drops to very low levels. This indicates that IGF-II may play a bigger role in growth and development during incubation, while IGF-I becomes the major player after hatch.

Because embryo growth hormone levels are low during incubation, Lu et al. (2007) hypothesized insulin's role as a possible growth factor for the embryo. They found indications of insulin acting as an important promoter of protein deposition during the period of rapid embryonic growth. Moreover, insulin levels are much more sensitive to amino acids than to carbohydrate levels in chick embryos. This makes sense since there is very little glucose in the egg as compared to high levels of amino acids in the amnion and albumen.

Glucagon appears to be the dominant pancreatic hormone in birds, maintaining them in constant hyperglycemia. Glucagon levels in avian plasma are 2 to 3 times greater than in mammals. Glucagon rises when the embryo need of glucose is increased, precisely during the first 10 days of incubation and during the last week before hatch (Lu et al., 2007). Lu et al. (2007) confirmed previous research by Picardo and Dickson (1982) claiming that hepatocytes' mobilization of glycogen is not affected by insulin levels 3 days before hatch, which could grant liver cells capacity to synthesize and utilize glycogen simultaneously. Glucagon also plays a role during the embryonic transition from lipid to carbohydrate metabolism, as Langslow et al. (1979) proposed adipocytes became sensitive to glucagon levels only after hatch. This is important so that subcutaneous adipose stores are not used until the first day post-hatch. In contrast to other species or among chickens post-hatch, glucagon levels may be low or high during rapid growth stages of incubation, so it seems to function as an embryonic growth-stimulating factor in a complex way or by interacting with other hormones (Lu et al., 2007). In simple terms, Langslow et al. (1979) concluded that chick embryos could be in a fasting status, while hatched chicks may be in a feasting status hormone wise.

Last, but not least in importance, are the thyroid hormones triiodothyronine (T3) and thyroxine (T4), which are involved in numerous physiological processes, including regulation of heat production, mobilization of glycogen reserves, embryo muscle growth (Christensen et al., 1996), and stimulus for hatching (Christensen and Biellier, 1982; Christensen et al., 1982; Christensen et al., 2003b; Christensen et al., 2003c). Lu et al. (2007) observed constant levels of T3 during mid incubation, but it peaked the day before hatch. T4 levels reached high levels and stayed high during amnion consumption

(between days 17 and 20 of incubation), decreasing after hatch. Elevated thyroxine levels are considered important for stimulating a variety of developmental and metabolic processes necessary for hatching. The sharp rise in T3 was associated to embryonic switching to lung respiration. Both T3 and T4 are positively correlated with embryonic body weight during late incubation (Lu, et al., 2007).

Lu et al. (2007) concluded that the most important hormones contributing to growth are insulin and IGF-II during embryogenesis, and IGF-I and T4 from hatch to 3 weeks post-hatch. Glucagon modifies growth and function, while thyroid hormones are critical for hatching. The relationship between energy status and the endocrine system during early development is not completely understood, and it requires further study (Lu et al., 2007).

Studying Nutrition Through Gene Expression

Era post chicken genome

The chicken genome was completed and published in 2004, granting the chicken with the title of first farm animal to have its genome sequenced (Lamont, 2006; Siegel et al., 2006). A new era in poultry research started with the completion of the chicken genome, opening the door to innumerable applications (Lamont, 2006).

Even before Mendelian theory of genetic selection was suggested, chickens already had a proud history in practical genetics, qualifying them to be the first to be sequenced. The available genome sequence place the chicken as member of the “elite club” of species that can use full power modern genomic technology. Now it is possible

to map and assay the genotype with great precision, and we are just starting to accumulate data on expression patterns of all genes (Siegel et al., 2006).

Methods of measuring gene expression

The best way to study intermediary metabolism would be by measuring the present amount of proteins in a cell or organism (Spielbauer and Stahl, 2005), including enzymes, hormones and structural components. Proteins are produced by gene expression. The science of producing a quantitative representation of the complete protein expression pattern of a cell, a tissue, an organ or an organism under exactly defined conditions is called proteomics (Spielbauer and Stahl, 2005). Genes in the DNA sequence are transcribed into messenger RNA (mRNA), and then mRNA is translated into proteins. Parameters like mRNA stability, protein degradation, post-translational modifications and others prevent making a statement over the current amount of measured protein, because the classical molecular biology still can not make a strict connection between the amount of mRNA and protein (Spielbauer and Stahl, 2005). The discovery of a method to reverse transcribe RNA into stable DNA opened the door to the new world of the science called transcriptomics. Now RNA expression patterns can be produced, and functional genomics is trying to correlate transcriptomics to protein amounts and function (Spielbauer and Stahl, 2005).

During the early 1990s, a protocol was perfected to synthesize small segments of DNA in laboratory in what was called polymerase chain reaction (PCR). In time, PCR was expanded to include amplifying RNA molecules as their complementary DNA (cDNA), in a procedure termed “reverse transcriptase” or RT-PCR. This reaction mimics

the process used by viruses to synthesize DNA from an RNA template. The enzyme able to perform this task is reverse transcriptase. Through reverse transcription unstable mRNA turns into more stable cDNA, creating a powerful tool for examining the entire population of mRNAs expressed in a cell at a particular time (Harris, 2000).

The first step to study mRNA populations is to isolate the RNA from all other cell components. RNAs are very sensitive to enzymatic degradation. To protect RNA from degradation, tissues can be treated in two ways: snap-freezing in liquid nitrogen, or immersion in a powerful RNase inhibitor, such as TRIzol or RNA-Later buffers (Garosi et al., 2005). The purified RNA extract can be studied in many ways, like RT-PCR, real time PCR, Northern Blot and microarrays (Harris, 2000; Yadetie et al., 2004; Spielbauer and Stahl, 2005).

One of the most powerful tools in molecular biology is the polymerase chain reaction (PCR). With time, several adaptations of the original protocol that used DNA as a template appeared in the literature, including the possibility to use mRNA as template (RT-PCR), rapid amplification of cDNA ends (RACE), competitive PCR, real-time PCR, Taqman⁵, SYBR green and hybridization probes (Ruiz and Bok, 1993; Wiseman, 2002). All of those are variations of the same principle can be used to make many copies of DNA or RNA using unspecific (random) primers, or copies of one specific gene using specific primers. With every cycle, the PCR products exponentially increase in number (Wiseman, 2002) until they can be detected. Real time PCR is one way to quantify the amount of specific genes by comparing the increasing number of copies of a desired sequence *versus* the ones of a constantly expressed house-keeping gene (Wiseman, 2002). It can also be used to compare expression of total RNAs from different origins to

⁵ Applied Biosystems, Foster City, CA

identify which genes are differently expressed (Harris, 2000). This technique is still one of the most used ways to study gene expression, but it has some limitations. One of the biggest concerns when doing PCRs is contamination. Because it works by making many copies of small amounts of RNA or DNA, even the smallest contamination with foreign sequences can completely compromise the results, giving false positives or negatives results. It can also amplify RNAs that were not meant to be translated, like products of degradation (Harris, 2000). Another problem with PCR is that if the ideal conditions for that specific reaction are not matched, like number of cycles, correct dye, amount of sample, amount of primers and amount of reagents, no product is obtained. For this reason, RT-PCR requires extensive optimization and validation before it can be successfully used (Yadatie et al., 2004). Test sensitivity is another issue, with big variation when results from different laboratories are compared (Wiseman, 2002). Others reported PCR-related problems include staining, densitometry, imaging, heteroduplex formation between target and standard, and the use of complicated nonsymmetrical detection algorithms (Wiseman, 2002). For all these reasons, plus cost and time, researchers must limit the number of samples and genes that can be studied by PCR (Wiseman, 2002).

Northern Blot was another method created to identify and quantify RNAs (Zhan et al., 1997). In Northern blotting, agarose gel electrophoresis of RNA is followed by transfer of the RNA onto porous solid supports, typically nylon or nitrocellulose membranes, so they can be scanned for identification. Quantification can also be done by dot/slot blot hybridization with probes performed after transfer to membrane without size separation (Yadatie et al., 2004). It works by blotting the gel onto a filter and hybridized

with a target to detect a particular species of mRNA as a distinct band or spot (van Zyl, 2005). The advantage of this technique over PCR is that it does not require much optimization and validation (Yadatie et al., 2004). The limitations of this technique are that it is very laborious and time consuming (Sato et al., 1993; Yadatie et al., 2004), and sensitive non-radioactive assays are still being perfected (Sato et al., 1993; Zhan et al., 1997; Yadatie et al., 2004), so only a small number of genes can be studied at a time (Yadatie et al., 2004).

After the year 2000, a new technology became available, called microarrays (Page et al., 2003). Microarray experiments rely on the same principle of hybridization (base pairing) used in Northern blotting, so it is sometimes referred to as “reverse Northern” (van Zyl, 2005). Oligonucleotides or cDNAs are spotted (probes) onto nylon filters or glass slides and hybridized with a target made from an mRNA population of interest. Usually, targets are made by reverse-transcribing mRNA into single-stranded cDNA in the presence of labeled nucleotides (van Zyl, 2005). The biggest advantage of microarrays over traditional methods for gene expression analysis, such as RT-PCR, Northern blotting or real time PCR, is that a large number of genes can be measured in a single assay (Spielbauer and Stahl, 2005). Printing a large number sequence spots in a very small area in a specific pattern required automation and precision that could only be achieved by robotics, so the principles of microarray technology are miniaturization, parallelization and automation (Spielbauer and Stahl, 2005). Thanks to that, researchers are now able to study thousands of genes in one experiment, creating a “still picture” of the mRNAs present in a cell culture, plan or animal tissue, or even a whole organism at a determined time point (Harris, 2000).

The ability to access information about a great number of genes is especially advantageous to study complex metabolic patterns. Entire pathways can have their enzymes mapped to determine the possible flux of metabolites. The possible applications of microarray technology in nutritional studies will be discussed next.

Microarray technology and its application in nutritional studies

Microarrays are used to survey thousand of genes in single experiment. The output of a microarray experiment is called a “gene expression profile” (Gibson, 2003). The core principle relies on the fact that the amount of transcript can be estimated by the amount hybridized to a complementary probe. Each probe represents the complement of at least a part of a transcript that might be expressed in a tissue (Gibson, 2003).

Dr. Harris explained the microarray technique as fixing cDNAs or oligos containing a particular sequence referred as “expressed sequence tag” (EST) to a glass slide, while mRNA samples from a tissue is converted to corresponding cDNA and labeled with either Cy3 or Cy5 fluorescent dye. When fluorescent cDNAs from 2 samples labeled with different dyes are hybridized together on the slide, the specific product that corresponds to the ESTs in the DNA array pairs with it and can be measured by scanning the slide. The slide is scanned with 2 different wave lengths, so Cy3 fluoresces green and Cy5 fluoresces red. Various degrees of red and green can be measured by the scanner, so a probe favoring either color is a sign of differential expression of that population of mRNAs (Harris, 2000). In hybridization arrays, the DNA with a defined identity tethered to a solid medium is referred to as ‘probe’ and the labeled DNA as ‘target’ (Spielbauer and Stahl, 2005).

The steps involved in a microarray experiment include RNA extraction, labeling, microarray construction, hybridization, scanning and statistical analysis (Harris, 2000, Gibson, 2003; Bauer et al., 2004; Garosi et al., 2005; Spielbauer and Stahl, 2005).

RNA extraction and quality control

RNA quantity and quality are vitally important to assure reliability and reproducibility of the results (Harris, 2000). Already discussed was how careful RNA must be manipulated to avoid RNase degradation. Even with all care, different tissues yield different quantities of RNA. The final extract must be free of RNases, proteins and genomic DNA. The quality control may include spectrophotometry and gel electrophoresis, to assess RNA concentration and integrity. If the recovered RNA amount is too small, a PCR amplification step of the RNA may be included, but it is recommended to be kept to a minimum (Harris, 2000). Another option is to pool RNA samples from different sources within the same treatment group (Garosi et al., 2005). Degradation is the greatest risk, so all instruments, tubes and reagents must be RNase-free and the samples must be refrigerated while in use, or frozen at minus eighty degrees Celsius when stored.

Labeling

there are three common methods of labeling adopted by most scientists: direct labeling, indirect labeling, and dendrimer labeling (Harris, 2000). Direct labeling incorporates deoxyribonucleotide triphosphate fluorescently labeled during reverse transcription. Indirect labeling incorporates nucleotides containing a reactive aminoallyl

residue during transcription and then on a subsequent reaction succinyl esters of Cy3 or Cy5 are covalently coupled to the amino-allyl-labeled cDNAs (Harris, 2000; Spielbauer and Stahl, 2005). Both methods depend on the frequency of incorporation of the modified nucleotides, but indirect labeling is preferred because the fluorescent nucleotides are bulkier, which decreases incorporation. The dendrimer labeling method uses a reverse transcription reaction primed with an oligonucleotide containing a specific capture sequence. The cDNAs with the capture sequence is hybridized to fluorescently labeled dendrimers and then to the array. This method requires less starting material, but it costs more. Even though indirect labeling is laborious and time-consuming, it is still the most popular because it results in higher yields, with better dye incorporation, and longer cDNAs, which increases specific hybridization (Harris, 2000).

Array construction

The most popular methods of microarray construction include the use of platforms, custom cDNA arrays, and custom oligo arrays (Harris, 2000). Platforms like Affymetrix GeneChip relies on *in situ* synthesis of 25-mer oligonucleotides using photomasks, which means that each probe is individually synthesized on the chip surface (Spielbauer and Stahl, 2005). Customizable pre-synthesized cDNA or commercialized oligos 50 to 70 base pairs long can be printed on the slide surface. Even though *in situ* probe synthesis is considered to be more reliable and reproducible (Garosi et al., 2005), they require sophisticated and expensive equipment, while DNA printed arrays are affordable for academic research laboratories (Spielbauer and Stahl, 2005). Pre-made oligos also give the advantage of fabricating them in high quality and defined quantities (Spielbauer and Stahl, 2005). Automated spotting and rigorous quality control can

minimize a great part of variation introduced in customized arrays (Garosi et al., 2005). In these arrays, the oligonucleotides are covalently attached to the slide surface, which is coated with functional groups (Harris, 2000; Spielbauer and Stahl, 2005), each spot corresponding to one gene (Harris, 2000). After DNA fixation, the slides are chemically reduced, and salt and impurities are removed, so they can be stored for several months (Spielbauer and Stahl, 2005). For these reasons oligonucleotide arrays have become increasingly popular (Garosi et al., 2005; Spielbauer and Stahl, 2005).

All available technologies used in automated microarray fabrication, photolithography, inkjet printing and contact printing (Spielbauer and Stahl, 2005), were usually made of full genome chips, but printed oligo spots (contact printing) can create gene focused arrays. Scientists are moving away from full or nearly full genome coverage to focused arrays. The main advantage of this approach is the ability to retain the power of microarray technology for monitoring a set of selected genes involved in a pathway of interest, with more flexibility but with only a fraction of the cost of the regular arrays. They are even sensitive enough to detect single copy number changes (Carvalho et al., 2004). The disadvantages are that these arrays are printed with sequences from gene oligos available from deduced genome assemblies, and the identity of the genes can change as more accurate sequences are reported. They also need the inclusion of a reliable baseline signal, which can be achieved by increasing the number of technical replications of the genes of interest on the array, and addition of internal and external control genes (Garosi et al., 2005).

Hybridization

The microarrays are hybridized and washed according to the slide manufacturer's recommendations (Spielbauer and Stahl, 2005). In this step equal amounts of labeled cDNAs are hybridized on the microarray slide under specific temperature and time, protected from light. It usually includes a pre-hybridization, the hybridization itself and a post-hybridization wash with different buffer solutions and nuclease-free water⁶. After they are dried, the slides are ready for laser scanning.

Scanning

Scanning is an important step of a microarray experiment because once scanned, all data, whether high or poor quality, can not be changed. The major scanner settings are laser power and voltage. Lower laser power minimizes photo-bleaching or saturation, but lower signals can be missed. High power results in the inverse situation, catching lower signals, but causing bleaching if high intensity signals. Between the two, there is more concern about avoiding signal saturation (Spielbauer and Stahl, 2005). (Lyng et al., 2004) determined that for most available scanners, the best relationship between intensity and tube voltage is within the intensity range 200-50,000 (mean spot intensity). The same slide can be scanned more than once in different laser powers so signals on the extreme range can be correctly measured (Garosi et al., 2005).

⁶ Corning Inc. Pronto! Universal Microarray System Protocol

Data processing and statistical analysis

Image processing

The first step in data analysis is the quantification of signal and background intensities. The raw scanner generated data is processed using a software package. This step is called image analysis and consists of isolating the region of the image corresponding to each individual spot from the background around it, so it can be converted into an intensity value. Often it is necessary to perform visual inspections, and if necessary, manual correction of grid placement on each microarray image (Garosi et al., 2005). Subtracting the background from foreground intensity in each channel gives spot intensity for calculating the expression ratio between the two channels.

There are four methods to deal with background: local background subtraction, morphological opening, constant background subtraction, and not correcting for background at all. (Yang et al., 2001) suggested that when the software package does not have a morphological opening option, it is preferred to calculate the log-ratios without background subtraction. The product is a data intensity file that will be used for statistical analysis.

Data normalization

Dye effects (Cy3 signal is usually higher than Cy5) and array-to-array variations can be corrected by normalization procedures. Especially in high density arrays, the use of multiple testing procedures in the statistical analysis is essential to avoid producing just a list of arbitrary genes, rather than meaningful true results (Spielbauer and Stahl,

2005). When using whole-genome arrays, there is a risk of having solutions with no problems (Harris, 2000), and getting more data than one can digest (Page et al., 2003).

Because microarrays follow many and long protocols with multiple steps, there are many possible sources of variation, so precautions must be taken to reduce variability (Garosi et al., 2005). For example, printer head, chip lot, reagent lot, day of extraction, scanners, and technicians can introduce variation (Page et al., 2003). To help isolate and account for variation, microarray experimental designs include both technical and biological replicates. Biological replicates are independent biological samples (e.g. same tissue from different individuals of the same treatment group) and they take care of target population variability. Technical replicates are repetition of a microarray experiment using the same RNA samples, and they account for variability between and within slides (Garosi et al., 2005). To reduce technical variability, samples that are from the same group or those that will be hybridized together should be extracted and labeled at the same time (Page et al., 2003). Although best practices are followed, there is still need to perform data normalization. The normalization process aims to balance many of the systematic variations present in an array experiment (Garosi et al., 2005). There are several normalization techniques available, but they all follow the principle of identifying genes that are not affected by experimental conditions and show a ratio with the reference sample equal to one. Another method called global normalization uses the mean or median values instead of a reference. Alternative methods include linear regression analysis, log centering, rank invariant, and Chen's ratio statistics. Lowess normalization uses locally weighted linear regression to smooth data by comparing one spot with its neighboring spots. During the process, housekeeping genes that are constantly expressed,

genomic DNA which is double stranded, and external control genes that should not hybridize, and negative controls can be used as internal controls, helping to identify the 'noise' of a microarray experiment (Garosi et al., 2005).

Testing approaches

The statistical approaches used to analyze microarray data can vary depending on the situation. When comparing just two conditions, Student's t test can be used to find genes differently expressed, but when performing thousands of t tests a large number of false negatives can be expected. This problem can be solved by calculating the false discovery rate, which is the proportion of false positive genes in the total set of differentially expressed genes by methods like Benjamini, Bonferroni, and Bonferroni step-down. These methods estimate false discovery rate and attempt to correct it. When performing a test, one must choose a test and set a p-value cutoff (e.g. a 0.05 false positive rate) (Wolfinger et al., 2001). The rigor of the test will depend on how it calculates the false discovery rate; some like Bonferroni are very rigorous, while others not so much like FDR. Another method is the Bayesian approach, which improves the confidence in array data with low number of replicates by assuming that genes with similar expression have similar measurement errors. The ANOVA approach is a generalization of the t test when comparing more than two conditions. In ANOVA, a model is built to estimate and account for known sources of variation so the treatment effect can be isolated. It has the advantage of possible inclusion of interesting variations like gene regulation, day of labeling and different dyes (Garosi et al., 2005). Mixed ANOVA models are preferred with experiments including biological replicates

(Spielbauer and Stahl, 2005). Some platform arrays have their own software, and now there are many being created to analyze all sort of arrays (Garosi et al., 2005).

Generally, a two-fold or greater difference in expression over normalization is considered distinct expression (Harris, 2000). Even though fold difference is the most common way to quantify differences, it is not the only method. There are even better quantitative ways, such as standardized mean difference (Page et al., 2003). Because microarrays statistical analysis is not simple, collaboration with a biostatistician is strongly recommended (Page et al., 2003). Validation of the results can be done by repeating the experiment by a different technique, like PCR or Northern blot, for the genes that were differently expressed by microarrays. In one report, microarray results gave fold change gene expression values closely correlated with the ones calculated by Northern blots (Casey et al., 2005).

Page et al. (2003) encouraged nutritionists to forge ahead into microarray research, considering clear definition of objectives, appropriately designing of experiments, choosing adequate sample size, applying statistical rigor to data analysis, and paying attention to data handling and management issues. For example, hybridization schemes depend on experimental design, which will also define number of samples, array printing and statistical analysis. Even though arrays can be used to generate hypothesis, better results can be obtained when a clear goal is defined from the beginning to meet those objectives. These will help define important starting points, like the number of biological and technical replicates. Page et al. (2003) suggested that it is more appropriate to choose sample size based on control of false discovery rate (FDR) and the expected discovery rate (EDR), which are mathematical estimations. In any case they advise not to

use fewer than five observations per treatment or it would limit the statistical methods that could be used, with 40 observations or more being ideal.

Finding one gene or a group of genes that are differently expressed must be followed by successful interpretation on the data in a biological context (Garosi et al., 2005). It is necessary to regularly update information on the genes present on the arrays, like gene names and annotations. This information is available through online databases. Tools like principal component analysis can help assess similarity or dissimilarity of expression profiles, and it facilitates selection of relevant genes from the data. Visualization of gene expression changes in pathways can also help (Garosi et al., 2005).

Experimental design

There are two basic types of array experimental designs: the reference design and the loop design (Garosi et al., 2005). In the loop design, each sample is compared directly with other samples in a circular or multiple-pairwise fashion (interwoven loop). This approach has greater statistical power than the reference design, so the ability to detect differences is maximized. The reference design compares each sample with a common RNA reference sample, serving as a common denominator between different array hybridizations (Garosi et al., 2005). This method is preferred when a large number of samples are analyzed. Graphical examples of both designs are illustrated by Figure 1.16. It is necessary to be sure that each sample is labeled at least once with each dye, and that the comparisons we want to make are possible with the design chosen.

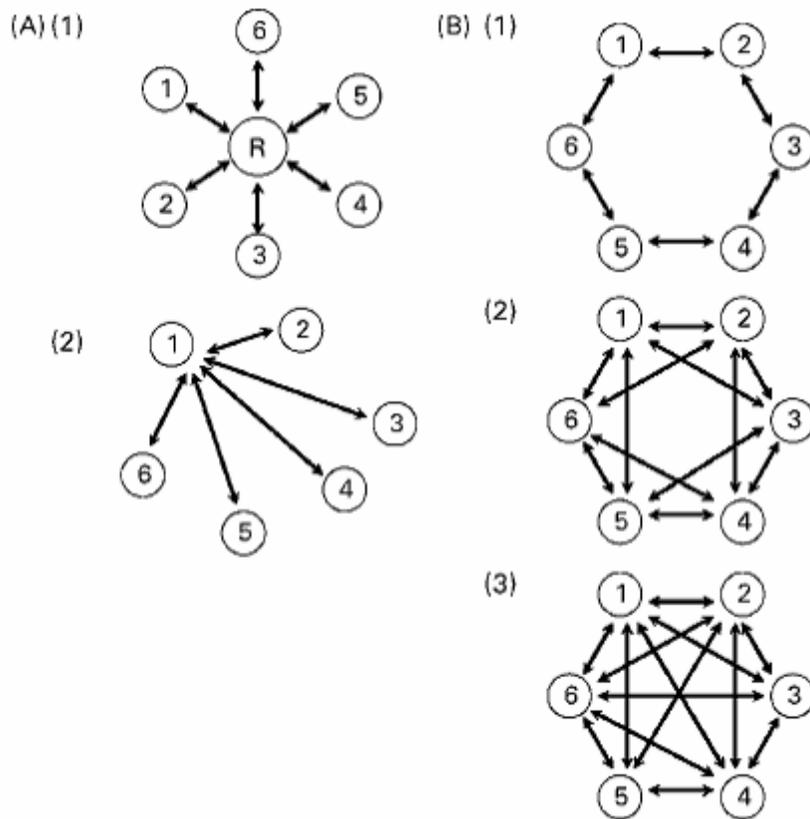


Figure 1.16. Common array experimental designs. (A) Reference design: (1) each sample is tested against a single, common standard or (2) to one of the tested samples. (B) Loop design: (1) simple loop, and (2, 3) interwoven loop design. Each sample is compared head-to-head with the other sample in a circular manner; each comparison is performed with a dye swap.

From: (Garosi et al., 2005)

Application in nutritional studies

The diet is the most important environmental factor, with permanent effects on the genome. The effect of nutrition on gene expression is defined as nutrigenomics (Spielbauer and Stahl, 2005). The actual way in which macro or micronutrients produce desirable changes in metabolism is largely unknown. DNA chip technology will open up new ways to study nutrition in more depth. The study of nutrigenomics could help to identify these effects and facilitate the prevention of common diet-related problems

(Spielbauer and Stahl, 2005). In fact DNA microarrays will make major contributions to nutritional research (Page et al., 2003).

Identification of cellular response to a specific nutritional signal may provide ways to decipher the mechanism by which a nutritional signal is transduced into a given response. Today, little is known about the molecular mechanisms by which the genome perceives nutritional signals and mobilizes the organism to respond (Spielbauer and Stahl, 2005). As we learn about the essentiality of nutrients, it is clear that they interact with genetic material in the cell nuclei, either directly or indirectly, causing changes in gene expression, so the population of mRNAs is affected, and not necessarily on that specific nutrient's metabolism (Harris, 2000). Because DNA chip technology allows simultaneous screening of a large number of genes, it can give a comprehensive, detailed picture of changes in gene expression, shedding light on complex regulatory interactions (Spielbauer and Stahl, 2005). In nutritional sciences, whole-genome gene expression profiles could provide new insights into nutrient-gene interactions and diet-related mechanisms underlining alterations in gene expression (Spielbauer and Stahl, 2005), giving a qualitative and quantitative diagnostic (Harris, 2000). Currently DNA microarrays are applied to analyze RNA expression levels, but they can be used for gene discovery, sequence identification, mapping, polymorphism detection, and hypothesis generation (Gibson, 2003; Spielbauer and Stahl, 2005). Also, RNA and protein arrays are being created using the same technology (Yadatie et al., 2004; Spielbauer and Stahl, 2005). The interaction of all these technologies to give them meaning must be a combined effort of scientists from many disciplines.

Other nutritional applications could be in the detection of genetically modified organisms, presence of animal products in the feed, and bioequivalence of food and drugs (Page et al., 2003).

Chicken arrays

Since the chicken genome became available, microarray techniques have also improved and they are being used more often. Several microarray experiments using chickens can be found in the literature. They cover a variety of applications. Here is a brief summary of the more relevant ones.

Min et al. (2003) used microarrays to create a profile of changes in gene expression of chickens infected with two species of *Eimeria*. They sampled the duodenum and jejunum regions of the intestine. The glass array was spotted with 400 cDNA clones selected from the chicken EST database, including 12 chicken cytokines. After primary infection 99 genes were affected by *E. acervulina* and 51 by *E. maxima*. The secondary infection cause changes in 46 and 25 transcripts, respectively for *E. acervulina* and *E. maxima*. The authors concluded that the genes and their pattern affected by both *Eimeria* species were similar, but more research is needed to understand host protective responses.

Also in 2003, an international research consortium project launched a system-wide chicken DNA array based on cDNA libraries from chicken liver, fat, breast, leg muscle, pineal gland and reproductive tract. They printed 2 arrays, the first being chicken metabolic/somatic systems array with 11,000 gene sequences and the second a neuroendocrine/reproductive system array with 8,000 sequences. In a test done to compare liver samples of hypothyroid *versus* hyperthyroid chickens, they found 14

differently expressed genes that were verified by PCR. They also compared liver gene expression of 16 days of incubation embryos with those of hatchlings and found 8 genes that are up regulated in embryos compared to chicks and 9 genes that were higher expressed in chicks when compared with embryos. The authors concluded that they successfully developed and tested these system-wide arrays and that they are now available to be used by several research groups (Cogburn et al., 2003). Since then, the consortium have been working in profiling several tissues in different situations like before and after hatch, fast and re-fed chickens, fast *versus* slow growing chickens and so on (Cogburn et al., 2004). Chicken arrays used for cancer research were developed by The Fred Hutchinson Cancer Research Center in Seattle and tested by Black et al. (2004), who identified 27 genes whose expression is increased 3 fold or more in v-Jun-transformed cells. In a different study, Afrakhte and Schultheiss (2004) created an array with 11,000 clones identified from the chick precardiac mesoendoderm and were able to select multiple cardiac-specific genes, including several that have not been characterized before. In 2005, the same cancer research group published their chicken array, now known as the 13k chicken array, with sequences isolated from 24 different tissues, and with 11,447 non-redundant ETSs and 160 control spots. They now advertise this chicken array for research groups interested in genome wide screening (Burnside et al., 2005).

In China, a group is using the chicken as a model to study excessive lipid accumulation. The Beijing Genomics Institute created a 9,024 chicken cDNA array that was used in that study. They compared a commercial line of broilers with a local chicken breed, and found 42 genes differently expressed, when blasted with the chicken genome available at the GeneBank database. These genes were mainly related to lipid and energy

metabolism, some were transcription and splicing factors, and some related to protein synthesis and degradation. Four genes were verified by Northern blot and PCR with similar results across the different techniques (Wang et al., 2006).

As needed, researchers are creating their own focused arrays to target some specific tissue or research interest. Smith et al. (2006) developed a 5K microarray focused on immune function. This array is now another one available for purchase to scientists interested in studying chicken immunology. Another 5k chicken microarray was created to study the neuroendocrine system. It was tested by Ellestad et al. (2006) with chicken embryo's pituitary glands, and they found 352 cDNAs that were differently expressed as the embryos aged from 10 to 17 days of incubation. From this group of genes they could identify 141 genes related to thyrotrophic differentiation, 69 indicating growth hormone mRNA production and 61 related to prolactin mRNA synthesis. They concluded that the array was useful not only to identify when these genes start being expressed, but also other numerous transcription factors and signaling molecules not previously associated in pituitary development. Affymetrix developed and commercialize a GeneChip Chicken Genome Array, designed with 28,000 chicken transcripts plus 17 avian virus transcripts for disease detection (Affymetrix, 2007).

From these reports we can conclude that several groups are producing their own chicken arrays and making them available to others, mostly using cDNA libraries and looking at large number of genes not all of them yet identified with annotation. There are still no reports of chicken arrays used specifically for practical nutrition studies. There are also no reports of arrays being tested with turkey RNA samples, what was expected since the turkey genome is still being sequenced.

Dissertation Research Outline

Based on results from previous *in ovo* feeding (IOF) research, which were already discussed, this dissertation is a continuation of the *in ovo* feeding project at North Carolina State University. The main objectives of these studies were to move from the proved concept to a more practical focus, going toward future industry application. To make IOF feasible, there was a need to study the physiological effects of changing injection site from the side to the top of the egg, since the injecting machine prototype was going to target the amnion from that position. The next couple trials focused on finding a protein source with characteristics that were compatible with sound industry manufacturing practices and animal response. Results from these trials revealed our lack of information about embryo physiology during the perinatal period, so the last half of this research introduced the use molecular biology tools to better understand the physiology of development of late-term turkey embryos.

Preliminary research

Site of Injection

Since the IOF concept was created, the way of injecting the solutions was to locate the amnion by candling the egg, marking an area on the shell where the risk of hitting the embryo or blood vessels was minimal, punching a hole in the shell, and then manually injecting the nutritive solution. The selected area was usually somewhere on the side of the egg. The industry partner on this project⁷ developed a means to automate *in ovo* feeding. *In ovo* vaccination machines that are commercially available already hit the amnion 89% of the time (Sharma and Burmester, 1982), injecting from the top of the

⁷ Pfizer-Embrex Inc., Research Triangle Park, Durham, NC

large end of the egg. Thus, Pfizer-Embrex Inc., purposed to improve the accuracy of amnion targeting via injection through the air cell end of the egg without the need of candling. To confirm the benefits of IOF *versus* other possible ways to supplement poults at hatch, we also tested gavaging the birds with the same nutritive solution at hatch or injecting the solution subcutaneously. A research trial was carried out to compare IOF from the side, IOF from the top, gavage and subcutaneous injection to non-supplemented embryos or poults.

Hypotheses: 1) IOF can be successfully be done by injecting from the top of the egg. 2) Feeding a nutritive solution *in ovo* promotes greater benefits to poults than gavage or neck injection.

Conclusions: Injection from the top hit the amnion in 90% of the time, which was considered acceptable, thus the first hypothesis was accepted. The IOF-treated poults had equal or superior performance for the parameters tested, thus we accepted the hypothesis that IOF-treated poults benefit more from nutrients fed *in ovo* then by other methods.

Results of this trial are presented on Appendix 1 of this dissertation.

IOF Solution Osmotic limits

Another issue addressed was to define the osmotic limits for IOF solutions turkeys. The original experiment that defined these limits was performed by Uni and Ferket (2003), who tested IOF saline (NaCl) solutions ranging from 400 to 1200 mOsm using, in chicken eggs. A similar osmotic constraint study in turkey embryos using carbohydrates was necessary because it had never been determined before and a different osmotic response to energetic substrates was expected to be different than for a mineral salt.

Hypotheses: Osmotic limits for turkey embryos are the same as those for chicken embryos.

Conclusion: Based on poult hatchability and body weight, IOF solution osmolalities between 550 and 850 mOsm were considered safe, with estimated best value at 708 mOsm. The range determined for chickens were between 400 and 600 mOsm. Since turkey osmotic limits seems to be higher the limit for chickens, we reject our hypothesis. Results of this trial are presented at Appendix 2 of this dissertation.

Phase 1: Evaluation of hydrolyzed soy as an IOF Solution Ingredient

As reviewed in the chicken and turkey IOF research sections, several formulas with different ingredients and concentrations were tested. The first phase of this research was to identify and test ingredients used in IOF formulas with a focus on the protein source. Previous IOF research used egg white protein (albumen) as the only protein source because it most closely matched the amino acid balance requirements of the embryo, but there are some technical drawbacks with its use in a commercial IOF solution. Commercially available as a chemical-grade in powder, egg albumin is difficult to dissolve in saline solution and it will coagulate into an insoluble mass after heat sterilization necessary to minimize microbial contamination after IOF solution injection into the egg. In order to use EWP in IOF solutions, the saline solution had to be prepared by dissolving NaCl at the concentration of 0.4% in distilled water, and then autoclaved to secure sterility. The 0.4% concentration was preferred over the 0.9% used in the beginning of the IOF research to reduce the osmotic load but still get the benefits the sodium offering enhanced enterocyte nutrient absorption. The powder substrates, sugars or albumen were then dissolved in that saline solution. EWP and dextrin required at least

1 hr of gentle agitation to prepare a homogenous solution, but the EWP could not be completely dissolved, creating a risk of needle clogging during *in ovo* injection. Moreover, high incidence of eggs injected with IOF solutions containing EWP were suffered microbial contamination, which could be traced to the EWP source. Therefore, another protein source that had better functional properties for IOF technology had to be identified and tested. Since soybean meal is the main protein source for poult after hatch, soluble soy products were tested for use in IOF formulas.

Hypotheses: 1) Soluble soybean protein could be used in IOF formulas without risk of contamination; and 2) Use of hydrolyzed soy is as effective as EWP as an IOF solution constituent.

To test these hypotheses, 5 research trials were conducted to evaluate the level of hydrolyzed soy could be included in the IOF solution with or without carbohydrates without adversely affecting the embryo or post-hatch poult. The results of trials 1 and 2 are presented on Chapter II of this dissertation. Trials 3 and 4 are in Chapter III, and trial 5 results are in Chapter IV.

Conclusion: The first hypothesis that hydrolyzed soy protein is highly soluble and does not offer risk of contamination was accepted. The second hypothesis that hydrolyzed soy would be a good alternative protein to EWP for use in IOF formulations was rejected because it contributed to excessive osmolality and did not yield consistent improvement in hatchability or poult performance. Evidently, a protein source that was less osmotically active than hydrolyzed soy is needed for IOF applications. This series of experiments also revealed that more research to define the IOF volume and osmolality constraints was necessary.

Phase 2: determining IOF volume and osmolality constraints

As concluded in phase 1, the IOF volume and osmolality constraints had to be determined before proceeding with IOF solution ingredient evaluation. In order to complete these objectives, several questions had to be answered: How much amnion is present in turkey eggs throughout late development? What is the fluid osmolality and viscosity of the amnion and embryo that constrain IOF osmolality limits? What is the pattern of amniotic fluid consumption by turkey embryos of different breeds?

Hypothesis: Characterization of amnion and the turkey embryo can help determine IOF constraints.

A series of experiments were conducted to measure embryo growth, amniotic fluid volume, osmolality and viscosity from 20 to 28 days of incubation, using eggs from 3 different breeds.

Conclusions: Information on amnion volume, osmolality and viscosity of different turkey breeds, along with embryonic growth and IOF solution osmolality is useful to determine the optimum volume that can be injected into the amnion, the best time of injection, and the osmotic limits of the amnion-embryo system.

Phase 3: late-term turkey embryo gene expression survey

After determining the constraints associated with IOF, more information about the changes in metabolism of the late-term embryo was needed to identify appropriate nutritional substrates that could serve as IOF ingredients. Comprehensive literature search yielded insufficient information about late-term turkey embryo metabolism to help identify potential nutritional substrates as IOF ingredients. Therefore, microarray

technology was used to profile metabolism of the late-term embryo, particularly those pathways associated with energy metabolism.

Hypotheses: 1) A focused oligo microarray based on the chicken genome sequences can be used to screen turkey embryo tissues and map changes in energy metabolism during the last week of incubation. 2) Evidence of gene expression of key metabolic enzymes will identify potential IOF substrates that could enhance energy flux to benefit embryo vitality and physic-morphological development.

A microarray was constructed using all known unique oligonucleotide sequences corresponding to enzymes of metabolic pathways, hormones, intestinal digestive enzymes and transporters. Two experiments were carried out, the first using a prototype 96 genes array and the second using an updated 300 gene array. In both experiments, turkey embryos were sampled from around 20 days of incubation until hatch. The RNA samples used in the microarrays experiments were extracted from liver, pectoral muscle, hatching muscle and intestine tissues.

Conclusion: The hypothesis that a microarray based on chicken sequences can be used to evaluate turkey RNA samples was accepted, implying that the sequences in both species are similar enough for successful target and probe hybridization. Also accept was the hypothesis that the array information can be used to map embryo development of all metabolic pathways represented in the array. Evidently, the embryo has the enzymatic capacity to metabolize identified substrates at the IOF target stage of development, but this must be confirmed by a subsequent IOF treatment study investigation.

Phase 4: Effect of *in ovo* feeding on turkey embryo gene expression – energy metabolism and intestinal development

This last phase of research in this dissertation was done to confirm that gene microarray technology is useful to map the changes in metabolism after *in ovo* feeding of nutrients identified as candidate substrates in phase 3 in comparison to non-injected control turkey embryos.

Hypotheses: 1) Differences in metabolism between IOF and control embryos can be measured by microarray technology. 2) These differences can be correlated with other parameters like poult quality and tissue glycogen concentration. 3) Consequences of feeding substrates identified by previous array experiment could be predicted and proven.

To test these hypotheses an experiment was design to compare regular non-injected turkey embryos with embryos IOF 0.4 mL of a solution containing metabolic co-factors. In contrast to previous IOF studies with turkeys, proteins and sugars were excluded from the IOF formulation because of two major technical constraints. First, IOF target studies using a modified Inovoject® machine (Pfizer-Embrex Inc., Durham, NC) identified the highest IOF delivery accuracy into the amnion at 24 days of incubation. At this stage of embryonic development, an injection volume of .4 ml saline was found to have negligible effect on hatchability. Second, the microarray survey of late-term embryos done in phase 3 identified that metabolic enzymes associated with glycogen accumulation from caloric substrates (proteins and carbohydrates) is down regulated at 24 days of incubation, whereas glycogenolysis and lactate utilization is up-regulated as the hatching process begins. Therefore, only substrates and co-factors that complement the unique metabolic state of the E24 turkey embryo was included in the IOF solution.

Embryonic tissues sampled on days 22, 24, 25, 26 of incubation and at hatch included the liver, pectoral muscle, hatching muscle, duodenum and jejunum. The extracted RNA was used in microarrays experiments using the 300 genes array created to screen energy related pathways and intestinal development.

Conclusions: The first hypothesis that differences in metabolism between IOF and non-fed can be detected by microarray was accepted, since a total of 21 genes were differently expressed. The expression profiled could be correlated to poult quality scores and a tissue glycogen concentration, thus the second hypothesis was accepted. However, the third hypothesis could not be adequately confirmed because the changes in gene expression caused by IOF were not as straight forward as predicted. More research is needed to improve the ability to predict responses to a certain nutrient IOF to turkey embryos.

Conclusions

In ovo feeding of Carbohydrates and protein can enhance poult quality, livability and performance as demonstrated by previous research. Nonetheless, there still not one formula composition able to satisfy all the constraints associated with the commercial application of IOF technology to yield consistent improvement in bird performance. Investigating constraints in volume and osmolality of amniotic fluid helped define IOF formulation specifications to be delivered at different stages of late-term incubation (22 to 23 days of incubation). New technologies like microarrays can greatly add to our current knowledge in understanding turkey embryo metabolism and development, which could result in improvement in turkey poult hatchability, quality, survival and early performance.

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Chapter 2

“Effect of carbohydrates, hydrolyzed soybean protein and methionine fed *in ovo* on turkey poult hatchability and energy status at hatch”

Abstract

About twenty percent of turkey eggs set do not hatch, and as many as thirty percent suffer from growth depression during brooding. Many of these weak poults do not survive the first week post-hatch. *In ovo* feeding (IOF) has been demonstrated to be a method to enhance turkey embryo energy status and gut maturation, with the goal of increasing hatchability and post-hatch development. Previous studies included egg white protein (EWP) in IOF solutions to supply turkey embryos more protein, found to be more effective than in chickens. However, EWP does not dissolve easily in a saline IOF solution, and it cannot be heat-sterilized. A more soluble and heat-stable alternative to EWP, hydrolyzed soy protein (HSP), was evaluated for IOF application. Two experiments tested IOF solutions containing different levels of HSP combined with carbohydrate (CHO) and β -hydroxyl- β -methylbutyrate (HMB), with and without methionine, and compared it to non-injected controls (NC) and embryos *in ovo*-fed CHO alone. IOF solutions containing CHO or 4% HSP increased hatchability in the first trial. In contrast, 8% HSP with or without methionine reduced hatchability in both trials, although hatched poults of these treatments were heavier at hatch as compared to NC ($P < 0.06$). IOF of protein increased liver glycogen concentration measured in the first trial. All IOF-treated poults consumed more feed than NC poults during the first 6 hours

post-hatch in the second trial. IOF of HSP may improve poult viability and vigor at hatch, but more research is needed to learn how to avoid its adverse effect on hatchability.

Introduction

Turkey poults face many challenges during late-term incubation, including limited oxygen supply, amnion consumption, switching from lipid to carbohydrate metabolism, glycogen accumulation, internal and external pipping, until they finally hatch (Donaldson and Christensen, 1991; Christensen et al., 2003; Uni and Ferket, 2004; Moran, 2007). Many embryos struggle during these events and may enter one or more phases of the General Adaptation Syndrome (GAS) (Donaldson et al., 1991). Many problems identified with these embryos are associated to a low energy status (Christensen et al., 1996; Uni et al., 2005; Uni et al., 2006). Depleted energy reserves can result in poor quality poults, stunted growth, or death during hatch or the first days post-hatch (Uni and Ferket, 2004). Feeding embryos *in ovo* was suggested as a way to help embryos overcome these challenges by adding nutrients to the amniotic fluid that will be orally consumed prior to internal pipping (Uni and Ferket, 2003). Previous studies tested solutions containing carbohydrates (Tako et al., 2004), egg white protein (Foye, 2005; Foye et al., 2006), and intestinal modulators like zinc methionine and β -hydroxyl- β -methylbutyrate (HMB) (Tako et al., 2004; Tako et al., 2005; Foye et al., 2006). Turkey poults are believed to have higher protein requirements than chicks early in life, most of which is supplied by dietary inclusion of soybean meal (Donaldson and Christensen, 1991; Donaldson, 1995). The amino acid methionine is supplemented in poultry diets because it is the first limiting amino acid in corn-soy based diets (National Research Council, 1994). Egg white was the logical protein source to be fed *in ovo* because it is the predominant protein source

available to the embryos (Foye, 2005), but its use in commercial IOF applications is not feasible because of functional and microbial quality constraints. Egg white protein (EWP) does not completely dissolve in the saline IOF solutions, and it foams upon agitation. Moreover, EWP is susceptible to microbial contamination and it cannot be heat-sterilized without coagulation. In contrast, hydrolyzed soy protein (HSP), used predominantly in microbial fermentation applications, is soluble in saline solutions and it can be heat-sterilized with minimal change to its physical properties (Kerry Bio-Sciences, 2007). The objective of this study was to evaluate the efficacy of HSP as a protein ingredient of IOF solutions for turkey embryos. This objective was addressed by two experiments that evaluated the effects of levels of HSP in saline IOF solutions containing carbohydrates or HMB on hatchability and indicators of energy status, and compared this treatment to negative and positive controls. Because HSP has lower methionine content in comparison to EWP, the efficacy of methionine supplementation to IOF solutions containing HSP was also evaluated.

Material and Methods

Experiment 1

Four-hundred Nicholas turkey eggs from 12 weeks of lay hens were obtained from a commercial source¹ and incubated² at the North Carolina State University Turkey Unit³. At 20 days of incubation, eggs were candled and divided into 4 groups with similar weight distribution, averaging 90g (± 15). Eggs were injected with IOF solution according to assigned treatments at 24 days of incubation (E). The treatments tested were: 1) non-

¹ Prestage Farms, Clinton, NC

² Jamesway incubator setter and hatcher model 252

³ Lake Wheeler Field Laboratory, Raleigh, NC

injected controls (NC); 2) IOF carbohydrates (13% dextrin + 5% glucose + 0.1% HMB) (CHO); 3) IOF carbohydrates plus 8% HSP⁴ (6% dextrin + 8% HSP + 0.1% HMB) (CP1); and 4) IOF carbohydrates plus 4% HSP (10% dextrin + 6% maltose + 4% HSP + 0.1% HMB) (CP2). All IOF solutions were prepared in a 0.4% NaCl sterile solution, and formulated to an osmolality of 650 mOsm, which was determined by Ferket et al. (2005) to be the upper limit to prevent hatchability problems. A standard curve of solution osmolality X HSP concentration in 0.4% saline was determined in order to identify the maximum inclusion level of HSP within a 650 mOsm IOF solution (Figure 1). Osmolality was measured using a Micro Osmometer⁵.

At the day of injection eggs were removed from the incubator, candled and marked on the side of the egg to locate the amnion. After the eggs were sprayed with a chlorine-based disinfecting solution using hand plant mister, a hole was punched at the marked spot on the shell over the amnion using a 22 gauge needle secured on a rubber stopper, and 1.5 ml of the respective IOF solution was injected into the amniotic cavity. All eggs remained outside the incubator for the same amount of time to complete the IOF treatment, including the NC treatment group. After injection, the injection hole was sealed with cellophane tape and the eggs were placed back in the hatching incubator.

Experiment 2

Four-hundred Hybrid turkey eggs from 12 weeks of lay hens were obtained from the same source¹ and incubated² as in experiment 1. At 20 days of incubation the eggs were removed from the incubator, candled to remove infertile eggs, and divided into 4 groups with similar weight distribution, averaging 80g (± 15). Eggs were injected on 24E

⁴ HY SOY, Kerry Bio-Science, Chicago, IL

⁵ Model 3300, Advanced Instruments, Inc., Norwood, MA

with 1.5 mL of IOF solution according to assigned treatments. The treatments tested were: 1) non-injected controls (NC); 2) IOF carbohydrates (13% dextrin + 6% glucose + 0.1% HMB) (CHO); 3) IOF carbohydrates plus HSP (6% dextrin + 8% HSP + 0.1% HMB) (CHP); and 4) IOF carbohydrates, HSP, and methionine⁶ (6% dextrin + 8% HSP + 0.31% methionine + 0.1% HMB) (CPM). As in experiment 1, the IOF formulations were made in 0.4% NaCl sterile saline to be 650 mOsm. Methionine supplementation level in treatment 4 IOF solution containing HSP was chosen to achieve an amino acid profile similar to egg white amino acid profile. The IOF procedure was done in the same manner as for experiment 1, with eggs returning to the hatching incubator afterwards.

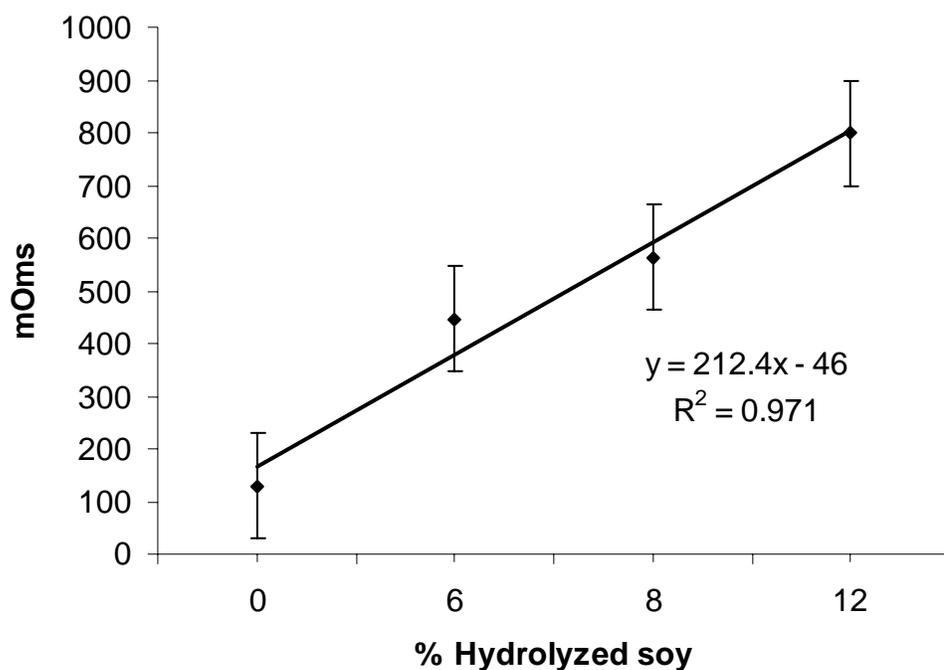


Figure 2.1. Influence of % hydrolyzed soy protein dissolved in 0.4% saline solution on osmolality (mOsm).

⁶ DL-Methionine, Sigma-Aldrich, Inc., St. Louis, MO

Tissue Sampling and Glycogen Determination

In both experiments, the hatch was pulled at 672 hours of incubation (28E) and counted by treatment to determine percent hatchability. The poults were then individually weighted and 15 from each treatment group were randomly selected for sampling. In the first experiment, sampled poults were euthanized by cervical dislocation and livers were removed, and placed immediately on ice for future glycogen analysis. In both experiments, remaining yolk sac was removed from 15 poults and weighted to calculate yolk-free body weight (YFBW). Liver samples were frozen at -20C until they were processed for glycogen analysis. Glycogen concentration was determined by colorimetric method of iodine binding (Dreiling et al., 1987). Remaining poults were euthanized using CO₂. The experimental protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Feeding Behavior Test

Poults hatched in the second experiment were submitted to a feeding behavior test to observe eagerness to feed intake initiation by determining the amount of feed consumed over a 6 hr period. Four hr post-hatch, 60 poults of the same IOF treatment were placed in transport boxes and allotted 30g of turkey starter feed each in 100 cm² trays. The boxes were open and placed under brooding heat lamps. The amount of feed remaining on each tray was recorded after one, three and six hours after placement.

Statistical Analysis

Both experimental designs were completely randomized, with four treatments and 100 replicates (poults) per treatment, with exception of % yolk, YFBW and glycogen

analysis which had 15 replicates per treatment. The data was analyzed using general linear models procedure of SAS (SAS Institute. 2004) for analysis of variance. Comparisons between treatments were made using least-square-means test (lsmeans), and differences were considered significant at $P < 0.05$, when not otherwise indicated.

Results and Discussion

Experiment 1

Hatchability results are shown on Table 2.1. CHO and CP2 treated poultts had hatchability 6-7% higher than NC (Table 2.1), evidently a consequence of fewer live pipped embryos at hatch (Table 2.1). A decreased rate of live pipped embryos among the NC embryos demonstrated their struggle to hatch, which was minimized by IOF. Struggling embryos can eventually deplete their energy sources and die. Embryo mortality and those pipping reduced hatchability of the CP1 group by 6% below NC, indicating distress embryos. About 3% of embryos were unintentionally injected into the right pectoral muscle with the CHO and CP2 IOF solutions (Table 2.1), which is typical of *in ovo* injection applications (Sharma and Burmester, 1982).

Body weights at hatch are presented in Table 2.2, and YFBW and percent remaining yolk are presented in Table 2.3. Poultts *in ovo*-fed 8% HSP (CP1) were heavier than poultts of all other treatments (Table 2.2). This difference in BW was likely attributed to increased residual yolk reserves, since there was no difference in YFBW among treatments (Table 2.3). Apparently, IOF solutions containing HSP delayed yolk utilization, which can also be influenced by accelerated hatching time. Metabolic stress, caused by embryo's need to metabolize amino acids could have accelerated hatching and

forced earlier yolk sac internalization. Higher mortality of CP1 embryos before hatch (Table 2.1), accompanied by heavier weights of surviving pouls (Table 2.2), may be a sign that this treatment favored the survival of big embryos to hatch over the small ones. The larger embryos may have survived and benefited from CHP because they had a higher capacity to metabolize extra amino acids delivered *in ovo*.

Liver weights and liver glycogen concentrations are presented in Table 2.4. There were no differences in liver weights, but poult *in ovo*-fed HSP (CP1 and CP2) had higher liver glycogen concentration (Table 2.4) than the NC and CHO poult, indicating higher energy status. Liver glycogen concentration was lowest among the CHO poult, which disagreed with previous authors who reported higher liver glycogen concentrations after IOF of carbohydrates (Foye, 2005; Uni et al., 2005; Uni et al., 2006).

Table 2.1 – Effect of IOF treatment on hatchability and mortality rates of turkey embryos (Experiment 1).¹

Treatment	Hatched (%)	Live pipped (%)	Dead embryos		
			Pipped (%)	Embryo hit (%)	Non-pipped (%)
Non-injected controls (NC)	80.30	12.12	1.52	0.00	6.06
IOF carbohydrates (CHO)	89.23	7.69	0.00	3.08	0.00
IOF 8% soy prot.+CHO (CP1)	78.87	9.86	4.23	0.00	7.04
IOF 4% soy prot.+CHO (CP2)	85.51	2.90	4.35	2.90	4.35

¹Values are expressed as a percentage of about 100 total viable eggs placed in the hatching incubator at 24 days of incubation.

Table 2.2 – Effect of IOF treatment on body weights poult after hatch (Experiment 1)¹

Treatment	Number of Hatched	
	Poult (n)	Body Weight (g)
Non-injected controls (NC)	235	65.81 ^b
IOF carbohydrates (CHO)	255	67.67 ^b
IOF 8% soy prot.+CHO (CP1)	230	69.07 ^a
IOF 4% soy prot.+CHO (CP2)	250	67.12 ^b
SEM	-	0.77

¹Values represent means n hatched poult.

^{a,b}Means within columns with different letters differ significantly (P<0.05).

Table 2.3 – Effect of IOF treatment on yolk-free body mass and yolk sac size relative to total body weight of poult at hatch (Experiment 1)¹

Treatment	Yolk-free BW (g)	Yolk sac (% of BW)
Non-injected controls (NC)	59.39 ^a	8.83 ^b
IOF carbohydrates (CHO)	60.87 ^a	9.91 ^{ab}
IOF 8% soy prot.+CHO (CP1)	61.17 ^a	11.20 ^a
IOF 4% soy prot.+CHO (CP2)	59.90 ^a	10.32 ^{ab}
SEM	1.14	0.862

¹Values are means of 15 replicate poult per treatment.

^{a,b}Means within columns with different letters differ significantly (P<0.06).

Table 2.4 – Effect of IOF treatment on liver weights and liver glycogen concentration of poult at hatch (Experiment 1)¹

Treatment	Glycogen (mg/g)	Liver weight (g)
Non-injected controls (NC)	86.06 ^B	1.61 ^A
IOF carbohydrates (CHO)	63.56 ^C	1.66 ^A
IOF 8% soy prot.+CHO (CP1)	109.58 ^A	1.73 ^A
IOF 4% soy prot.+CHO (CP2)	107.86 ^A	1.68 ^A
SEM	10.44	0.051

¹Values are means of 15 replicate poult per treatment.

^{A,B,C}Means within columns with different letters differ significantly (P<0.01)

Experiment 2

The treatment effects on hatchability observed in experiment 2 are presented in Table 2.5. The IOF treatment groups receiving the HSP resulted in 3 to 6% lower hatchability rate than the NC and CHO treatment groups. This response agreed with the response observed in the first experiment, where the IOF solution containing 8% HSP (CP1) reduced hatchability by 3% as compared to NC and CHO (Table 2.1). This reduction in hatchability was attributed to increased rate of live pips (Table 2.5). As observed in experiment 1, about 3% of the dead embryos were injected into the right breast muscle during IOF procedure (Tables 2.1 and 2.5).

The treatment effects on BW and YFBW at hatch are presented in Tables 2.6 and 2.7, respectively. Again the CHP treatment, which is the same as the CP1 treatment of experiment 1, resulted in heavier poult at hatch than the NC and CPM treatments, with

poults fed CHO not differing from the other treatments (Table 2.6). Although heavier BW was associated with increased retained yolk mass in the first experiment, no such association was observed in the second experiment. There was no difference in percent retained yolk or YFBW among treatments (Table 2.7). This difference in yolk utilization between experiments may be due to differences between breeds (Nicholas vs. Hybrids), or differences in egg size (first trial eggs were 10g heavier than eggs of second trial).

The treatment effects on feeding behavior are presented in Table 2.8. All IOF treatment groups consumed more feed during the 6 hr observation period and at a greater rate of feed intake initiation than the NC treatment group (Table 2.8). Apparently, the IOF treated poults had more energy available for active feeding behavior and greater appetite than the non-IOF poults. So, even though including HSP in the IOF formulation may have overloaded embryonic metabolism with excess non-essential amino acids, poults that survived the CHP, CPM, and CHO treatments were more active and eager to eat within 8 hours after hatch than NC poults. Higher disposition to feeding is a sign of better energy status and more mature digestive capacity of IOF poults (Uni et al., 2006). Methionine supplementation of the IOF solutions had no effect on any of the parameters studied, so correcting amino acid balance did not resolve the negative effects of HSP.

Table 2.5 – Effect of IOF treatment on hatchability and mortality rates of turkey embryos (Experiment 2) .¹

<i>Treatment</i>	Hatchability (%)	Live pip (%)	Dead pip (%)	Embryo hit (%)
Non-injected controls (NC)	95.29	4.71	0.00	0.00
IOF carbohydrates (CHO)	92.94	4.71	0.00	2.35
IOF soy prot.+CHO (CHP)	88.24	7.06	0.00	3.53
IOF soy prot.+CHO+Met (CPM)	89.41	9.41	1.18	0.00

¹Values are expressed as a percentage of about 100 total viable eggs placed in the hatching incubator at 24 days of incubation.

Table 2.6 – Effect of IOF treatment on body weights poult after hatch (Experiment 1)¹

Treatment	Number of Hatched	
	Poults (n)	Body Weight (g)
Non-injected controls (NC)	81	56.48 ^b
IOF carbohydrates (CHO)	79	56.95 ^{ab}
IOF soy prot.+CHO (CHP)	75	58.03 ^a
IOF soy prot.+CHO+Met (CPM)	76	57.06 ^{ab}
SEM	-	0.57

¹Values represent means n hatched poults.

^{a,b}Means within columns with different letters differ significantly (P<0.05).

Table 2.7 – Effect of IOF treatment on yolk-free body mass and yolk sac size relative to total body weight of poults at hatch (Experiment 1)¹

Treatment	Yolk-free BW (g)	Yolk sac (%)
Non-injected controls (NC)	51.41	7.69
IOF carbohydrates (CHO)	52.13	8.65
IOF soy prot.+CHO (CHP)	51.90	7.14
IOF soy prot.+CHO+Met (CPM)	54.19	6.69
SEM	1.23	0.715

¹Values are means of 15 replicate poults per treatment.

No means were significantly different (P<0.05)

Table 2.8 – Behavioral activity expressed as time spent to consume pre-starter feed by day old turkey poults in-ovo fed with different nutritive solutions¹

Treatment	Feed remaining (g)			
	Time 0	1 hour	3 hours	6 hours
Non-injected controls (NC)	30.00	23.95	13.91	5.65
IOF carbohydrates (CHO)	30.00	22.05	8.77	0.40
IOF soy prot.+CHO (CHP)	30.00	21.13	5.08	0.15
IOF soy prot.+CHO+Met (CPM)	30.00	23.92	8.69	0.00

¹Values represent a mean of 60 poults

Conclusions

The results of these two experiments demonstrated that turkey poults benefit from protein fed *in ovo*, by increasing body weights at hatch, increasing liver glycogen concentration, and enhancing feed intake initiation behavior. However, some negative effect of including HSP in IOF solutions on hatchability suggest that more research is necessary to determine the right amount of protein and the best formulation to be fed *in ovo*.

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Chapter 3

“Effect of *in ovo* feeding saline solutions with or without carbohydrate and protein on turkey poult hatchability, energy status and growth up to 14 days of age”

Abstract

Many physical or chemical characteristics of the egg or conditions during incubation can cause metabolic distress of turkey embryos and thus adversely affect the hatchability of poults. Low energy status often results in poor vitality or even death from the time of internal pipping until the first couple days post-hatch. Two experiments were conducted to investigate the effects of *in ovo* feeding (IOF) a saline solution or saline containing other nutrients (beta-hydroxy-beta-methylbutyrate, hydrolyzed soy protein and dextrin) on energy status and growth performance of turkey poults from 1 to 14 d post-hatch. In comparison to non-injected controls, IOF of saline or other nutrients did not affect hatchability, body weight, or organ size at hatch, but it increased glycogen concentration in the liver and in the hatching muscle. Toms gained more weights than hens throughout the studies. Pectoral muscle size was the same for all treatments at 14 days of age. Although higher energy status did not affect body weight or pectoral muscle at 14d, some differences were observed during development, so other quality or immune parameters not included in this study may have also been affect. Future research will address this possibility and improvements to *in ovo* feeding formulation.

Introduction

Turkey poults are considered difficult to start because of high incidence of weak poults (Christensen et al., 2003), “flip-overs” (Noble et al., 1999), and “starve-outs” (Christensen, et al., 2003). Mortality rate during the first week after hatch may be as high as 6% of the flock placement, and 10 to 30% of the surviving poults may exhibit depressed growth (Christensen et al., 2003). These viability problems among so many poults are predominately a consequence of their low energy status, which were depleted during hatching, servicing, holding, and transport (Wyatt et al., 1985; Donaldson and Christensen, 1991; Donaldson et al., 1991; Uni and Ferket, 2004; Careghi et al., 2005). Compounding this effect is the poor ability to replenish their energy reserves once they gain access to feed because of enteric immaturity and low digestive capacity (Uni et al., 1998; Uni et al., 1999). *In ovo* feeding (IOF) embryos nutrients to the amnion prior to hatch was proposed by Uni and Ferket (2003) to help increase poult quality and survival. Previous studies demonstrated that IOF can enhance energy status (Foye et al., 2006) and gut maturation of developing embryos (Tako et al., 2004; Smirnov et al., 2006). Turkey poults are believed to require more protein in their diets than broiler chicks, so Foye (2005) investigated the addition of a protein source in IOF solutions. Egg white was the logical protein source to be fed *in ovo* because it is the predominant protein source available to the embryos (Foye, 2005), but its use in commercial IOF applications is not feasible because of functional and microbial quality constraints. Egg white protein (EWP) does not completely dissolve in the saline IOF solutions, and it foams upon agitation. Moreover, EWP is susceptible to microbial contamination and it cannot be heat-sterilized

without coagulation. In contrast, hydrolyzed soy protein (HSP), used predominantly in microbial fermentation applications, is soluble in saline solutions and it can be heat-sterilized with minimal change to its physical properties (Kerry Bio-Science, 2007). The objective of this study was to evaluate the effects of IOF turkey embryos with HSP combined with carbohydrates on hatchability, poult energy status and post-hatch development.

Material and Methods

Two experiments were carried out each utilizing 840 Nicholas turkey eggs from 12 week of lay hens obtained from a commercial hatchery¹. These eggs were incubated using standard industry procedure at Pfizer-Embrex². At 20 days of incubation (E), the eggs were candled to remove infertile eggs, and then they were sorted into 3 groups with a similar distribution of weights. *In ovo* injection was performed manually on 24E, injecting from the blunt side of the egg, and caring that all eggs stayed outside the incubator for the same amount of time. The treatments tested were: 1) non-injected control (NC), 2) IOF 0.4% NaCl solution (SC), and 3) IOF carbohydrate-protein solution (6% dextrin + 8% HSP³ + 0.1% HMB⁴ + 0.4% NaCl) (CHP). All procedures were performed the same in both experiments, with the only difference being the volume of the IOF solution administered into the amnion, which were 1.5 mL and 1.0 mL for experiments 1 and 2, respectively. At about 672 hrs of incubation (28E), all hatching trays were pulled out, and the number of hatched and unhatched eggs was counted. Fifteen poults per treatment were randomly selected for sampling, while the remaining

¹ Prestage Farms, Clinton, NC

² Research Triangle Park, Durham, NC

³ Kerry Bio-Science, Chicago, IL

poults were transported to NCSU Turkey Research Unit⁴, where they were sexed, neck tagged, weighed, and divided by sex and treatment among 12 floor pens containing about 55 poults per pen. At 7 and 14 days post-hatch, the poults in each pen were individually weighed, and the feed consumption per pen was measured to determine feed conversion for each age period.

Tissue Sampling and Glycogen Analysis

At day of hatch, sample birds (15 replicates per treatment) were euthanized by cervical dislocation and liver, pectoral muscle and hatching muscle were harvested and immediately placed on ice for about 2 hours, and then stored at -20C until processing for glycogen content analysis. Glycogen concentration [GLY] was measured by the iodine binding method (Dreiling et al., 1987). The remaining yolk sac was removed and weighed to calculate yolk-free body weight (YFBW). Fifteen birds per treatment were also sampled at 7 and 14 days post-hatch to determine pectoral muscle weight.

Housing and Animal care

Each of the 12, 10 m² floor pens was furnished with 4 cm of soft pine shavings as litter, and equipped with a 125 watt bulb heat lamp, an automatic water drinker⁵ and a tube feeder⁶. Each pen was populated with 55 male or female poults and assigned to one of the three IOF treatments. These poults were housed in a curtained-sided, temperature-controlled building equipped with ceiling-mounted mixing fans to prevent air stratification and incandescent lights scattered throughout the house for uniform

⁴ Lake Wheeler Field Laboratory, Raleigh, NC

⁵ Plasson[®]

⁶ Shenandoah[®]

illumination. Temperature inside the house was kept between 29 and 31C during the first week, and then it was gradually reduced by 3C every week. The birds received 23 hr light: 1 hr dark each day, and water and feed was offered *at libitum*. The same feed formulated to meet or exceed NRC requirements (National Research Council, 1994) was offered to all treatment pens.

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

Statistical Analysis

The experiment was designed as a 3 X 2 completely randomized factorial, consisting of 3 IOF treatments and 2 genders. Each gender X IOF treatment combination was replicated in two pens, each containing 55 poult. Samples collected at day of hatch were analyzed with 15 replicates per treatment. The data was analyzed using the general linear models GLM procedure of SAS (SAS Institute. 2004) and differences between means were tested by least-square-means (lsmeans) with significance at $P < 0.05$.

Results

The IOF solution osmolality used in both experiments was determined⁷ to be 120 mOsm for the SC solution and 620 mOsm for CHP. Accuracy of manual IOF injection technique was validated to exceed 90% amnion target by opening 40 eggs injected with 0.3% coumasie blue dye in ethanol.

In the first experiment, hatchability was 87.96% for NC, 88.51% for SC and 83.02% for CHP. Hatchability of the second experiment was 86.50% for NC, 89.4% for

⁷ Micro Osmometer Model 3300, Advanced Instruments, Inc., Norwood, MA

SC and 90.47% for CHP. Apparently, the hatchability rate of the CHP treatment group was 5% lower than the other 2 treatments in the first experiment, but this disadvantage was eliminated by reducing the volume of IOF solution injected in the second trial.

There were no interaction effects between IOF treatment and gender for any of the studied parameters, except for pectoral muscle weight at 7d of age in the first experiment. BW of poultts according to treatment and gender in both experiments are presented on Table 3.1. There was no IOF treatment or gender effects on BW at hatch in either experiment (Table 3.1). Yolk weight averaged 6.07g (± 0.57) and 3.35g (± 0.27) in experiments 1 and 2, respectively. YFBW averaged 55.14g (± 1.12) and 49.07g (± 0.94) in experiments 1 and 2. However, there were no significant treatment effects on yolk weight or YFBW. The treatment effects on weight and [GLY] of the tissues sampled at hatch are presented in Table 3.2. Although IOF treatment did not affect tissue size at hatch, [GLY] was significantly affected in all tissues with exception of pectoral muscle in experiment 1 (Table 3.2). IOF of saline or CHP increased [GLY] in the hatching muscle and liver, as compared to NC in experiment 1 (Table 3.2). In experiment 2, [GLY] in hatching muscle was highest in the CHP treatment group, followed by SC and least for NC. The SC treatment group had higher [GLY] in pectoral muscle than the other two treatment groups. In contrast, liver [GLY] was higher in the CHP treatment group than in the SC group, with the NC group intermediately (Table 3.2).

No significant interaction effects between IOF treatments and gender were found on BW at 7 and 14d. BW of poultts from both experiments on day 7 and 14 is presented on Tables 3.3 and 3.4, respectively. The WG data during all age phases in both experiments is presented in Table 3.5. There was no difference between IOF treatments

for BW on day 7 in experiment 1, but 7 d BW of the CHP-treated poult was greater than the SC poult, with NC not differing from either group (Table 3.3). As expected, males were heavier than females at 7 d in both experiments. There were no differences between IOF treatments for BW at 14 d in either experiment, but males were consistently heavier than females (Table 3.4). The only significant IOF treatment effect on WG was observed during the 1-7 d period of experiment 2, where SC poult had lower WG than NC poult, but not significantly different from CHP poult (Table 3.5). Males gained more weight than females in all periods and in both trials (Table 3.5).

The IOF and gender effects on pectoral muscle weight in experiments 1 and 2 are presented in Table 3.6. With the exception of the NC treatment group, pectoral muscle weights were heavier for males than females at 7d of age in experiment 1.

Table 3.1 – Effects of *in ovo* feeding (IOF) and gender on body weight of turkey poult at hatch (experiments 1 and 2)¹

Treatment	Experiment 1		Experiment 2	
	Body Weight (g)	N	Body Weight (g)	N
<i>In ovo</i> feeding				
Non-injected control (NC)	60.47	212	52.61	223
IOF ² Saline (SC)	61.12	212	52.46	229
IOF carbohydrate-protein (CHP)	60.70	209	52.88	226
SEM ³	0.36	-	0.41	-
Gender				
Males	61.24	315	52.77	330
Females	60.28	318	52.53	348
SEM	0.29	-	0.33	-

None of the means on the same column were statistically different at P=0.05

¹Values are main effect means for IOF treatments and gender. There were no significant (P>0.05) IOF treatment X gender effects observed.

²IOF = *in ovo* feeding

³SEM = standard error of the mean

Table 3.2 – Effect of *in ovo* feeding (IOF) treatment on weights and glycogen concentration of hatching muscle, pectoral muscle, and liver in poult at hatch (Experiments 1 and 2)¹.

Parameter	IOF Treatment ²			
		NC	SC	CHP
Experiment 1				
Hatching muscle	Weight (g)	0.395 ^{ns}	0.390 ^{ns}	0.455 ^{ns}
	Glycogen (mg/g)	36.43 ^B	55.67 ^A	60.21 ^A
Pectoral muscle	Weight (g)	1.48 ^{ns}	1.38 ^{ns}	1.51 ^{ns}
	Glycogen (mg/g)	39.23 ^{ns}	46.85 ^{ns}	39.69 ^{ns}
Liver	Weight (g)	1.52 ^{ns}	1.44 ^{ns}	1.47 ^{ns}
	Glycogen (mg/g)	133.65 ^b	156.05 ^a	162.40 ^a
Experiment 2				
Hatching muscle	Weight (g)	0.417 ^{ns}	0.411 ^{ns}	0.438 ^{ns}
	Glycogen (mg/g)	21.01 ^C	36.21 ^B	50.92 ^A
Pectoral muscle	Weight (g)	1.46 ^{ns}	1.62 ^{ns}	1.37 ^{ns}
	Glycogen (mg/g)	40.30 ^B	55.27 ^A	44.07 ^B
Liver	Weight (g)	1.28 ^{ns}	1.25 ^{ns}	1.34 ^{ns}
	Glycogen (mg/g)	83.23 ^{ab}	74.67 ^b	94.74 ^a

^{a,b}Means with different lower case letter superscripts within a row are significantly different (P<0.05)

^{A,B,C}Means with different upper case letter superscripts within a row are significantly different (P<0.01)

^{ns}Means not significant (P<0.05)

¹Values are means of 15 replicate birds per treatment.

²IOF= *in ovo* feeding, NC= non-injected control, SC= *in ovo* fed saline solution, CHP= *in ovo* fed carbohydrate and protein

Table 3.3 – *In ovo* feeding treatment and gender main effects on body weight of turkey poult at 7d of age (Experiment 1 and 2).¹

IOF ² Treatment Effect	Experiment 1		Experiment 2	
	Body Weight (g)	N	Body Weight (g)	N
Non-injected control (NC)	162.94 ^{ns}	209	126.07 ^{ab}	220
IOF ² Saline (SC)	167.42 ^{ns}	206	121.68 ^b	227
IOF Carbohydrate-protein (CHP)	165.59 ^{ns}	206	127.59 ^a	220
SEM ³	3.08	-	1.29	-
Gender				
Males	170.91 ^a	313	131.19 ^A	324
Females	159.72 ^b	308	119.04 ^B	343
SEM	2.51	-	1.05	-

^{a,b}Means with different lower case letters within a column are significantly different (P<0.05)

^{A,B}Means with different upper case letters within a column are significantly different (P<0.01)

^{ns}Means not significant (P<0.05)

¹Values are main effect means for IOF treatments and gender. There were no significant (P>0.05) IOF treatment X gender effects observed

²IOF = *in ovo* feeding

³SEM = standard error of the mean

Table 3.4 – *In ovo* feeding treatment and gender main effects on body weight of turkey poult at 14d of age (Experiment 1 and 2)¹

IOF ² Treatment	Experiment 1		Experiment 2	
	Body Weight (g)	N	Body Weight (g)	N
<i>In ovo</i> feeding				
Non-injected control (NC)	380.36 ^{ns}	190	299.23 ^{ns}	203
IOF Saline (SC)	386.06 ^{ns}	186	290.16 ^{ns}	209
IOF Carbohydrate-protein (CHP)	392.16 ^{ns}	183	299.37 ^{ns}	199
SEM ³	7.07	-	2.58	-
Gender				
Males	404.00 ^A	276	313.14 ^A	298
Females	368.39 ^B	283	279.36 ^B	313
SEM	5.77	-	2.10	-

^{A,B}Means with different upper case letter superscripts within a column are significantly different (P<0.01)

^{ns}Means not significant (P<0.05)

¹Values are main effect means for IOF treatments and gender. There were no significant (P>0.05) IOF treatment X gender effects observed.

²IOF = *in ovo* feeding

³SEM = standard error of the mean

Table 3.5 – Main effects of *in ovo* feeding treatment and gender on weight gain of turkey poult during 1-7d, 7-14d and 1-14d of age (Experiments 1 and 2)¹

IOF ² Treatment	Experiment 1			Experiment 2		
	-----Days-----					
	1-7	7-14	1-14	1-7	7-14	1-14
Non-injected control (NC)	102.36 ^{ns}	217.48 ^{ns}	319.95 ^{ns}	73.47a	171.41 ^{ns}	245.27 ^{ns}
Saline (SC)	106.22 ^{ns}	218.78 ^{ns}	324.76 ^{ns}	69.41b	169.41 ^{ns}	239.06 ^{ns}
Carbohydrate-protein (CHP)	104.87 ^{ns}	226.23 ^{ns}	331.00 ^{ns}	74.47ab	168.86 ^{ns}	245.80 ^{ns}
SEM ³	2.86	4.26	6.80	1.57	2.63	2.41
Gender						
Males	109.59a	233.52A	342.74A	78.26A	180.02A	261.08A
Females	99.38b	208.13B	307.73B	66.52B	159.77B	225.68B
SEM ³	2.34	3.48	5.56	0.94	2.15	1.97

^{a,b}Means with different lower case letter superscripts are significantly different (P<0.05).

^{A,B}Means with different upper case letter superscripts are significantly different (P<0.01).

^{ns}Means not significant (P<0.05)

¹Values are main effect means for IOF treatments and gender. There were no significant (P>0.05) IOF treatment X gender effects observed.

²IOF = *in ovo* feeding

³SEM = standard error of the mean

Table 3.6 – Pectoral muscle weight at 7 days of age of male and female *in ovo* fed (IOF) turkeys of treatment 1¹

IOF Treatment	Gender	Pectoral muscle weight (g)
Non-injected control (NC)	Male	13.334 B
	Female	13.623 AB
IOF ² Saline (SC)	Male	12.343 B
	Female	15.224 A
IOF carbohydrate-protein (CHP)	Male	15.069 A
	Female	13.037 B

^{A,B}Means with different upper case letter superscripts are significantly different (P<0.01)

¹Values are mean of 15 poults. Standard error of the mean = 0.80

²IOF = *in ovo* feeding

Table 3.7 – Effects of *in ovo* feeding and sex on pectoral muscle weight of turkey poults at 14d of age on experiment 1¹, and at 7d and 14d of age in experiment 2¹.

Treatment	Experiment 1		Experiment 2	
	14d	Pectoral muscle		14d
		(g)		
<i>In ovo</i> feeding				
Non-injected control (NC)	50.39 ^{ns}	9.83 ^{ns}		37.59 ^{ns}
IOF ² Saline (SC)	50.90 ^{ns}	9.94 ^{ns}		35.50 ^{ns}
IOF carbohydrate-protein (CHP)	50.80 ^{ns}	9.61 ^{ns}		36.22 ^{ns}
SEM ³	1.37	0.58		1.15
Sex				
Males	52.80A	10.05 ^{ns}		38.42A
Females	48.59B	9.54 ^{ns}		34.45B
SEM	1.12	0.47		0.94

^{A,B}Means with different upper case letter superscripts are significantly different (P<0.01)

^{ns}Means not significant (P<0.05)

¹Values are main effect means for IOF treatments (20 replicates) and gender (60 replicates). There were no significant (P>0.05) IOF treatment X gender effects observed

²IOF = *in ovo* feeding

³SEM = standard error of the mean

Discussion

Poults, that use most of their energy reserves to hatch and sustain themselves until feed intake initiation, are at risk energy starvation, resulting in impaired growth or death (Christensen et al., 2003). Flip-overs and starve-outs poults are the consequences of these conditions as commonly observed in the field (Noble et al., 1999; Christensen et al., 2003). Liver glycogen is the most important energy reserve to sustain poults during the

perinatal period, and it has been used to indicate poult energy status (Donaldson et al., 1991; Donaldson, 1995; Christensen et al., 1996; Christensen et al., 2003). Poults are able to restore their liver and muscle glycogen reserves after hatch by pectoral muscle catabolism and gluconeogenesis, especially if they do not have immediate access to feed and water (Donaldson et al., 1991; Keirs et al., 2002). *In ovo* feeding has been shown to improve energy status of perinatal chicks and poults by increasing glycogen reserves, and stimulating gut maturation that help poults utilize feed nutrients (Uni and Ferket, 2003; Uni and Ferket, 2004; Ferket et al., 2005). In this study, a slight reduction in hatchability was observed when embryos were *in ovo*-fed the 1.5 ml solution containing CHP in experiment 1, but not when the IOF volume was reduced to 1.0 mL in experiment 2. In contrast, *in ovo* feed either 1.5 ml or 1.0 ml saline had no adverse effect on hatchability. Considering the difference in osmolality between the CHP and SC IOF solutions (620 mOsm *versus* 120 mOsm), some of the CHP treated embryos may have suffered greater osmotic stress or metabolic overload than the SC treated embryos, resulting in reduced hatchability.

Even though all poults started with similar BW, residual yolk and YFBW at hatch (Table 3.1), differences in other parameters demonstrated that the IOF poults were metabolically different than the NC poults. For example, poults IOF CHP had more remaining glycogen reserves in the livers and hatching muscles at hatch than NC poults and, to a lesser degree, the SC poults (Table 3.2). Similar improvements in glycogen reserves were observed by Uni et al. (2005) who *in ovo* fed chickens saline solutions containing carbohydrates, and by Foye et al. (2006) who *in ovo* fed turkeys saline solutions containing egg white protein. These differences in energy status at hatch were

not enough to result in higher weight gain later in life, so IOF poults weighed the same at the end of both studies (Tables 3.4 and 3.5). The fact that there were no BW differences between SC and CHP solutions fed *in ovo* demonstrates the value of NaCl as an energy modulator in poultry embryos. Although sodium chloride has no energetic value, it changed the use of innate energy available in the egg and embryo, resulting in more glycogen accumulated in pectoral muscle (Table 3.2) at expense of liver, which may have been the reason why SC poults gained less weight during the first week post-hatch than NC poults (Table 3.5, experiment 2).

As expected, male poults had greater post-hatch weight gain than females, with both genders being equally affected by the IOF treatments, as there was generally no IOF treatment X gender interaction effects observed. The only exception was pectoral muscle mass at 7d (Table 3.6), where NC females did not differ from NC males, while SC benefited females and CHP benefited males. This effect was probably a consequence of differences in pectoral muscle deposition between sexes, where female precocity leads to slightly higher muscle than males (Table 3.6, Experiment 1, NC). The difference in muscle mass between genders was accentuated by IOF saline. We hypothesize that IOF saline increased muscle breakdown for gluconeogenesis as this treatment had numerically smaller pectoral muscle at hatch (Table 3.2), and males were still recovering from it at 7d. Interestingly, IOF CHP did not affect females, which had pectoral muscle mass similar to controls, but it could be used by males to produce more pectoral muscle. Compensatory gain after 7d of age dissipated the treatment effects, so only differences between genders persisted at 14d (Table 3.7).

Liver weights of poult at hatch were similar to values reported by Donaldson et al. (1991) and Christensen et al. (1996), but hepatic [GLY] concentrations were in general about 4-fold higher than those reported by these authors or by Foye et al. (2006). Breast [GLY] reported here were very similar to concentrations measured by Foye et al. (2006). The higher hepatic glycogen reserves among the CHP treated poult in this study may indicate that these poult may have an advantage immediately after hatch, but this benefit may not last beyond 14 days.

Although preliminary research estimated highest poult body weight with IOF solution osmolality of 708 mOsm (Appendix 2, page 268), this osmolality value as well as the one used herein (620 mOsm) may have been too high, leading the embryos to osmotic stress and hindering them from full benefits of IOF.

Conclusions

High osmolality of *in ovo* feeding solution associated to hydrolyzed soy protein anti-nutritional factors reduced benefits of *in ovo* feeding turkey embryos, but it still resulted on increased poult energy status and eagerness to feed.

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Chapter 4

“Effect of incubation conditions on energy status and performance of *in ovo* fed turkey poults from 25 days of incubation to 14 days of age”

Abstract

Turkey poults are very sensitive to poor incubation conditions, causing them to struggle during hatch until feed intake initiation because of low energy status. Nutrient supplementation of the amnion by *in ovo* feeding (IOF) has been demonstrated to improve embryonic energy status and to promote gut maturation. This study was designed to investigate the effects of two different incubation profiles (P1 and P2), similar to single stage or multistage temperature profiles, respectively, on IOF turkey poults. Six hundred turkey eggs were divided between the two hatcheries that followed distinct incubation profiles. Each hatchery was assigned an equal number of eggs divided among three treatments: 1) non-IOF controls (NC), 2) IOF saline (SC), and 3) IOF carbohydrates, egg white protein, sodium chloride and beta-hydroxy-beta-methylbutyrate (CHP). IOF eggs were injected with 1.5 ml of the respective solution into the amnion at 23E. Eggs from each treatment were sampled at 25E to collect liver, pectoral muscle and hatching muscle for glycogen analysis. At hatch, the number of hatched poults and unhatched eggs was counted and the same tissues were sampled for glycogen analysis. Poults incubated with both profiles and IOF treatments were raised until 14d to compare growth performance between profiles, IOF treatments, and gender. Although incubator temperatures in P2 registered higher than in P1 during hatching (37.9 vs. 37.0C, respectively), hatchability or poult weight at hatch were not adversely affected. However,

the P1 poult accumulated more liver glycogen and performed better during rearing than poult from P2. In comparison to the NC group, hatchability was reduced by about 15% in the CHP IOF group due to a microbial contaminated ingredient source, but their post-hatch performance was better. The best growth performance was observed among males in the CHP treatment group. These results demonstrate that heat distressed conditions during the last stages of incubation can adversely affect post-hatch performance and appetite of poult, but this could be ameliorated by IOF.

Introduction

The eggs of oviparous species must be laid containing all necessary nutrients for embryonic development. The hen specifically synthesizes and deposits the egg nutrients, so egg composition is relatively constant (Whitehead, 1991). According to Boerjan (2006) poultry embryos will develop if given adequate incubation conditions, like temperature, humidity and turning. When these conditions are not ideal, embryonic development may be compromised. Studies showed that if poultry eggs are not turned or they are turned incorrectly, embryonic compartments are not properly formed, and the incidence of embryonic malformations, mal positioning and albumen misuse are increased (Tona et al., 2005). Temperature and egg storage can affect incubation period. For example, high temperatures shorten the incubation period (Hulet et al., 2007), and long egg storage prolongs time to hatch (Tona et al., 2003). Even though egg composition is kept relatively constant, breeder nutrition and age can cause changes depending on body pool of nutrients and synthetic capacity (Whitehead, 1991). For example, the amount of yolk increases at expense of albumen as the hen ages, altering the amount of substrate available to the embryo (Joseph and Moran, 2005). A combination of these

conditions, plus changes in genetics due to selection for higher yields, cause turkey poults to struggle to hatch and have low energy status by the time they hatch. If energy stores are depleted, survival and performance can be compromised (Donaldson et al., 1991).

One possible way to aid embryo energy needs is to provide extra nutrients *in ovo*. *In ovo* feeding (IOF) consists in injecting a nutritive solution into the amnion prior to embryo amniotic fluid consumption (Uni and Ferket, 2003). IOF nutrients will supply the embryo with extra substrate for glycogen storage and tissue growth, improving poult quality, performance and survivability (Uni and Ferket, 2004). Foye et al. (2006) tested feeding turkey embryos *in ovo* with nutritive solutions containing different amounts of egg white protein, sodium chloride, carbohydrates (dextrin and maltose) and β -hydroxyl- β -methylbutyrate (HMB), and compared their response to non-injected control poults. They concluded that IOF enhanced body weight (BW) and glycogen status of poults during the neonatal period.

According to French (1997), at the beginning of incubation embryo temperature is usually slightly lower than incubator temperature, but from mid-incubation onwards metabolic heat production from the embryo may cause embryo temperature to rise above incubator temperature. Single-stage incubator settings can be adjusted to match embryonic temperature needs, but multi-stage incubators have constant cycles of similar temperatures throughout incubation. High embryonic temperatures occur more frequently in multi-stage systems, especially if air flow is not uniform. Heat-stressed embryos may respond differently to hatching challenges and to IOF. Practical application of IOF implies that embryos incubated in a variety of conditions must show positive responses.

Therefore, the objective of this study was to evaluate the same IOF formulation on eggs from the same flock, but incubated under different conditions.

Material and Methods

Incubation and IOF

Turkey eggs were obtained from first-cycle Hybrid breeder hens in the 6th week of lay and divided into two groups of 600 eggs each assigned to different hatcheries. One group of eggs was incubated at Pfizer-Embrex Research Laboratory¹, and the other group was incubated at the North Carolina State University Turkey Research Unit². Both hatcheries were equipped with Jamesway 252 incubator setters and hatchers, but different incubation profiles were followed (Table 4.1). The first hatchery used an incubation profile (P1) with decreasing set points to help with removal of metabolic heat as the embryos growth, similar to what happens in single stage incubation. The second hatchery profile kept incubator set temperature constant, letting the temperature increase due to less removal of metabolic heat from the eggs in later stages of incubation (P2), as usually happens in multistage incubation. All other procedures were performed in the same manner in both hatcheries. At 20 days of incubation (E), all eggs were candled to remove infertile eggs and then divided into 3 groups with similar average egg weights (73.3 ± 10 g) and weight distribution. Each group of eggs were randomly assigned to one of 3 IOF treatments: 1) non-injected control (NC), 2) IOF saline (0.4% NaCl) (SC) or 3) IOF nutritive solution (20% egg white+5% dextrin+0.1% HMB+0.4% NaCl) (CHP). At 23E eggs from each treatment were removed from the incubator, one treatment at the time, to perform IOF. First, the eggs were disinfected and then manually injected from the top of

¹ Research Triangle Park, Durham, NC

² Lake Wheeler Field Laboratory, Raleigh, NC

the blunt end of the egg with a specially designed needle³. Then 1.5 mL of pre-warmed (*ca.* 37C) IOF solution was injected into the amnion according to the assigned treatments. The injection needles were dipped in a chlorinated disinfectant between egg injections to minimize microbial cross-contamination by the injection procedure. Eggs of NC groups were removed from incubator for the same duration of time as IOF-treatment group eggs (*ca.* 20 minutes). Once the IOF procedure was completed, the eggs were transferred to hatching baskets and placed in the hatchers. The number of external pipped eggs and hatched poult was counted each day at P2, starting on 25E until day of hatch. On the morning of 28E, the hatches were pulled and the number of hatched poult, pipped eggs and dead embryos was recorded to evaluate hatchability.

Animal Husbandry

At day of hatch, poult from both hatcheries were sexed, tagged and individually weighed. The poult were then transported to a temperature controlled curtained-sided facility containing 12 10m² pens. Floor pens were topped with 4 cm of soft pine shavings, and equipped with one heating lamp⁴, one drinker⁵ and one feeder⁶. Twenty five poult from each hatchery were placed in the same pen, being of the same sex and IOF treatment. Each of the 6 treatment combinations were replicated twice inside the house. Feed and water were provided *ad libitum* throughout the study and all pens received the same diet formulated to match or exceed NRC requirements (National Research Council, 1994). Feed remaining in the feeders was weighed at 7 and 14 days to calculate feed intake (FI) and feed conversion (FC) for each pen. All experimental protocols were

³ Proprietary of Pfizer-Embrex, Research Triangle Park, Durham, NC

⁴ Model 54411 Heave Gauge Aluminum Base, Hog Slats, Inc., Newton Gove, NC

⁵ Plasson[®] automatic drinker

⁶ Shenandoah[®] tube feeder

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

Tissue Sampling and Glycogen Analysis

At 25E 5 eggs from each treatment combination were sampled to collect the hatching muscle (HM) and liver for glycogen analysis. The eggs were opened and the embryos euthanized by cervical dislocation, followed by tissue harvesting. The same tissues, along with the *pectoralis* muscle (breast), were collected from 8 poult per treatment at day of hatch for the same purpose. All tissues were immediately place on ice and then frozen at -20C until processing. Glycogen concentration was determined using the iodine binding assay as described by Foye et al. (2006). At 7 and 14 days of age, 8 birds per treatment were sampled to determine relative pectoral muscle weight.

Feeding Behavior Test

Previous studies revealed that IOF chicks and poults had more mature digestive and absorptive systems than their non-IOF counterparts (Foye 2005; Foye et al., 2006; Uni et al., 2006). More mature guts associated with better energy status could result in poults that are more active and eager to feed. A simple feeding test was conducted to look for indications of such behavior. As soon as birds were place in their respective pens (Time 0), each pen received a 10 cm² feeding tray containing 5g/poult of feed (Oasis⁷). Two hours later (0-2 hours) the trays were carefully removed and replaced by a new tray containing the same amount of feed (5g/poult). At the end of another two hour period (2-4 hours) all trays were removed. The feed remaining in both trays were weighted to

⁷ Novus International Inc., St. Louis, MO

determine average FI per bird during the first 2 hours (0-2 hours) after placement, during the 2 to 4 hour period (2-4 hours), and during the total 4 hours (2-4 hours) after placement.

Statistical Analysis

Glycogen data from 25E and day of hatch was analyzed by mixed ANOVA according to the model,

$$Y = \mu + HAT + IOF + HAT*IOF + e,$$

with hatchery (*HAT*), *in ovo* feeding (*IOF*) and their interaction (*HAT*IOF*) as fixed effects. Day of hatch BW was analyzed by mixed ANOVA according to the model,

$$Y = \mu + HAT + IOF + GDR + HAT*IOF + HAT*GDR + IOF*GDR + HAT*IOF*GDR + e,$$

with hatchery (*HAT*), *in ovo* feeding (*IOF*), gender (*GDR*), and their interactions (*HAT*IOF*, *HAT*GDR*, *IOF*GDR*, *HAT*IOF*GD*) as main effects. The 7d and 14d performance data was also analyzed by mixed ANOVA according to the model,

$$Y = \mu + HAT + IOF + GDR + HAT*IOF + HAT*GDR + IOF*GDR + HAT*IOF*GDR + PEN + e,$$

with hatchery (*HAT*), *in ovo* feeding (*IOF*), gender (*GDR*) and their interactions (*HAT*IOF*, *HAT*GDR*, *IOF*GDR*, *HAT*IOF*GD*) as main effects, and pen as a random effect. These statistical analysis were conducted by the Fit Model procedure in JMP (SAS Institute. 2005). Means were separated by *t*-student or Tukey test and considered significant when $P < 0.05$.

Results

The IOF solutions were tested to determine their osmolality⁸. Osmolality of the SC and CHP solutions were 133.0 mOsm and 492.3 mOsm, respectively. The number of pipping embryos and hatched poult starting at 25E until 28E at P2 are presented in Table 4.3. Based on these data, IOF embryos started external pipping earlier than NC, and at 26E more than 4% of IOF poult had already hatched (Table 4.3). NC poult had a narrower hatching window than the IOF treatments, with all poult hatching within a 48 h period (Table 4.3). Table 4.2 shows the hatchability results at P1. As observed at P2, P1, the CHP treatment group had lower hatchability than the SC and NC treatment groups (Table 4.2). This reduction on hatchability seems to be a result of increased number of dead embryos and late pipping embryos. These embryos apparently died from some sort of contamination as determined by visual examination. Microbiological testing of the IOF ingredients identified the egg white protein as the source of the contamination.

The results of the feeding behavior test are illustrated by Figure 4.1. Apparently, the CHP-treated poult consumed more feed during the first 2 hours after placement than the SC or NC poult (Figure 4.1).

There were no treatment interaction effects on HM weight, HM glycogen concentration, or liver glycogen concentration at 25E. The results for HM weight and liver glycogen concentration are presented in Table 4.4. There were no differences between IOF treatments for HM weight, which averaged 0.558g, but P2 embryos had bigger HM than P1 embryos (Table 4.4). There were no differences between any of the treatments for HM glycogen concentration (average 8.05 mg/g).

⁸ Micro Osmometer Model 3300, Advanced Instruments, Inc., Norwood, MA

Table 4.5 presents the results of the interaction between IOF and hatchery for liver weight at 25E. Poult IOF CHP and incubated at P2 had heavier livers than P1 CHP and P2 SC, with all other treatments showing intermediary liver sizes (Table 4.5). There was also no interaction between IOF and hatchery for HM weight, HM glycogen concentration or for yolk as percent of BW at hatch (Table 4.6). Moreover, there were no differences among the IOF treatments. HM of poult incubated at P1 were bigger than HM of P2 (Table 4.6), but poult from P1 had less glycogen in HM and less percent remaining yolk than poult from P2 (Table 4.6).

Pectoral muscle weight and glycogen concentration at hatch are shown in Table 4.7. Interaction between factors was significant for pectoral muscle at hatch. The pectoral muscle of the CHP treated poult incubated at P1 were heavier than those from NC and CHP poult incubated at P2, with NC and SC from P1 and SC from P2 showing intermediary muscle weights (Table 4.7). There were no differences among treatments for liver weight or for its glycogen concentration at hatch, which averaged 1.352 g and 12.68 mg/g, respectively.

The mean of the individual poult's body weights (BW) and weight gains (WG) from hatch to 14d of age are presented in Table 4.8. Means of treatment pens for feed intake, weight gain and feed conversion are presented in Table 4.9. No significant treatment interaction effects were observed. At hatch (day 0), there were no differences in BW among IOF treatments or incubation profiles, but male poult were heavier than female poult (Table 4.8). At 7d, poult from P1 were heavier than poult from P2, and this difference was maintained throughout the rest of the study (Table 4.8). At 14d, CHP poult were heavier than SC and NC poult, and males were heavier than females. WG

was higher for CHP and SC than for NC poult, beginning at 7d post-hatch (Table 4.8). The WG of CHP poult was also higher than NC poult throughout the 0-14d period (Table 4.8). The poult subjected to IOF tended to have higher feed intake than the NC poult, and the best feed conversions were found for males of NC and IOF treatments (Table 4.9).

Table 4.1. Incubator temperatures measured in hatcheries 1 and 2.

Period	Incubator Temperature (C)	
	P1	P2
1-14E	37.6	37.4
15-23E	37.3	37.5
24-28E	37.0	37.9

Table 4.2. Number of eggs external pipped, hatched poult, and dead embryos at hatch from turkey poult *in ovo* fed (IOF) saline or a carbohydrate-protein solution (CHP) and non-injected poult incubated under P1.

Treatment	P1 Hatchability		
	Pipped (%)	Hatched (%)	Dead (%)
Non-injected	5.23	92.12	2.00
IOF ¹ saline	6.50	92.19	0.66
IOF ¹ CHP ²	10.00	79.99	8.67

¹IOF=*in ovo* feeding

²CHP=carbohydrate and protein solution

Table 4.3. Number of eggs external pipped, hatched poult, and dead embryos from day 25 of incubation (E) to day of hatch, from turkey poult *in ovo* fed (IOF) saline or a carbohydrate-protein solution (CHP) and non-injected poult incubated under P2.

Treatment	25E		26E		27E		Day of Hatch		Dead (%)
	Pipped	Hatched	Pipped	Hatched	Pipped	Hatched	Pipped	Hatched	
----- (% of total Eggs) -----									
Non-injected	0.0	0.0	40.85	1.22	31.71	56.71	7.32	90.85	1.83
IOF ¹ saline	0.61	0.0	42.10	4.88	25.61	68.90	6.71	91.46	1.83
IOF ¹ CHP ²	4.26	0.0	27.44	4.26	20.73	60.98	10.98	75.00	14.02

¹IOF=*in ovo* feeding

²CHP=carbohydrate and protein solution

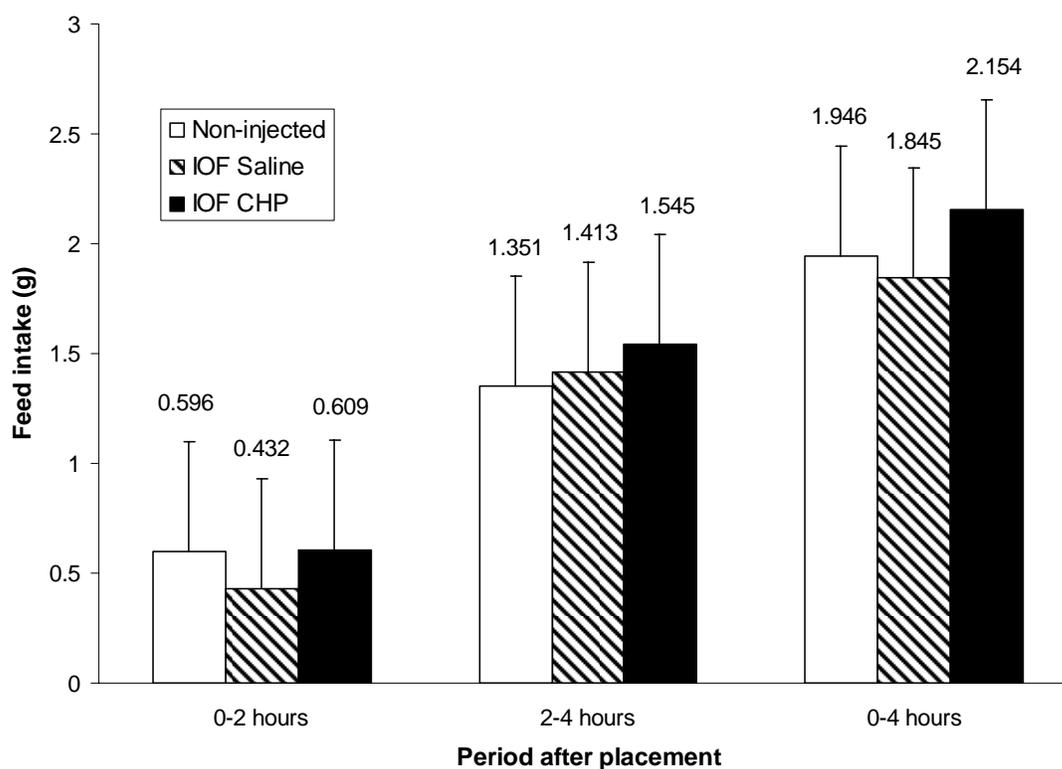


Figure 4.1. Feed intake of control and *in ovo* fed (IOF) turkey poults [saline or carbohydrate-protein solution (CHP)] during the first four hours after placement.

Table 4.4. Main effects of IOF treatment and incubation profile on hatching muscle size and liver glycogen concentration at 25E.¹

IOF Treatment	Hatching Muscle (g)	Liver Glycogen (mg/g)
Non-injected	0.585 ^a	52.625 ^{ab}
IOF ² saline	0.558 ^a	80.586 ^a
IOF ² CHP ³	0.531 ^a	35.153 ^b
Incubation Profile		
P1	0.490 ^b	75.858 ^a
P2	0.632 ^a	36.385 ^b

^{a,b}Means with different superscripts within a column differ significantly ($P < 0.05$)

¹Main effect means of IOF treatment are averages of 10 replicates poults. Main effects means of hatchery are averages of 15 replicate poults.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Table 4.5. Effect of *in ovo* feeding (IOF) treatment on liver weights of 25E embryos incubated in two different temperatures profiles.¹

Treatment		
Incubation Profile	IOF	Liver (g)
P1	Non-injected	0.711 ^{ab}
	IOF ² saline	0.779 ^{ab}
	IOF ² CHP ³	0.702 ^b
P2	Non-injected	0.789 ^{ab}
	IOF ² saline	0.686 ^b
	IOF ² CHP ³	0.906 ^a

^{a,b}Means with different superscripts within a column differ significantly (P<0.05)

¹Mean values are averages of 5 replicate poult per treatment.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Table 4.6. Main effects of IOF treatment and hatchery on hatching muscle weight and glycogen concentration, and yolk as percent of body weight at hatch.¹

IOF Treatment	Hatching Muscle (g)	Hatching muscle glycogen (mg/g)	Yolk (%)
Non-injected	0.341 ^a	1.404 ^a	5.282 ^a
IOF ² saline	0.336 ^a	2.489 ^a	6.434 ^a
IOF ² CHP ³	0.298 ^a	3.015 ^a	5.913 ^a
Incubation Profile			
P1	0.403 ^a	1.173 ^b	4.972 ^b
P2	0.247 ^b	3.432 ^a	6.844 ^a

^{a,b}Means with different superscripts within a column differ significantly (P<0.05)

¹ Main effect means of IOF treatment are averages of 16 replicates poult. Main effect means of hatchery are averages of 24 replicate poult.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Table 4.7. Effect of IOF treatment on pectoral muscle weight and glycogen concentration of poult incubated different temperature profiles at day of hatch.¹

Treatment			
Incubation Profile	IOF	Pectoral muscle (g)	Pectoral muscle glycogen (mg/g)
P1	Non-injected	1.289 ^{ab}	1.996 ^c
	IOF ² saline	1.258 ^{ab}	0.013 ^d
	IOF ² CHP ¹	1.532 ^a	5.687 ^a
P2	Non-injected	1.157 ^b	4.846 ^a
	IOF ² saline	1.445 ^{ab}	2.968 ^b
	IOF ² CHP ³	1.181 ^b	2.581 ^{bc}

Means with different superscripts within a column differ significantly (P<0.05)

¹Mean values are averages of 8 replicate poult per treatment.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Table 4.8. Main effects of IOF treatment, incubation profile, and gender on body weight (BW) and weight gain (WG) from hatch (0d) to 14 days of age (14d).¹

IOF Treatment	Body Weight			Weight Gain		
	0d	7d	14d	0-7d	7-14d	0-14d
Non-injected	52.72 ^a	131.21 ^a	290.80 ^b	78.43 ^a	159.54 ^b	237.67 ^b
IOF ² saline	53.51 ^a	133.74 ^a	293.35 ^b	80.10 ^a	159.81 ^b	239.76 ^{ab}
IOF ² CHP ³	53.21 ^a	137.00 ^a	309.55 ^a	83.77 ^a	171.15 ^a	253.22 ^a
Incubation Profile						
P1	52.96 ^a	137.25 ^a	304.87 ^a	84.18 ^a	166.76 ^a	250.08 ^a
P2	53.34 ^a	130.71 ^b	290.93 ^b	77.35 ^b	160.25 ^b	237.02 ^b
Gender						
Male	53.49 ^a	136.28 ^a	308.28 ^a	82.64 ^a	171.18 ^a	253.26 ^a
Female	52.81 ^b	131.69 ^a	287.52 ^b	78.93 ^a	155.82 ^b	233.84 ^b

^{a,b}Means with different superscripts within a column differ significantly ($P < 0.05$)

¹ Main effect means of IOF treatment are averages of 256 replicate poult. Main effect means of hatchery are averages of 384 replicate poult. Main effect means of gender are averages of 384 replicate poult.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Table 4.9. Effect of IOF treatment on the growth performance of male and female turkey poult from hatch to 14 days of age.¹

Treatment	Gender	Feed Intake (g)	Weight Gain (g)	Feed Conversion
Non-injected	Male	175.82	166.24	1.06
	Female	194.34	152.76	1.27
IOF ² saline	Male	186.68	166.99	1.12
	Female	207.81	152.96	1.36
IOF ² CHP ³	Male	223.09	212.16	1.05
	Female	200.49	155.56	1.29

¹Values are means of 2 replicate pens containing *ca.* 128 poult.

²IOF=*in ovo* feeding

³CHP=carbohydrate and protein solution

Discussion

Incubation temperature profile was the main difference observed in this study. According to Table 4.1, incubation temperature in P1 was gradually decreased from 37.6 to 37.0C, while incubation temperature in P2 gradually increased from 37.4 to 37.9C (Table 4.1). Temperatures measured at P1 represent the profile where temperatures can be adjusted to match embryonic needs at each stage of development, which can be

obtained in single stage incubators. Temperatures observed in P2 are similar to what is usually seen in multistage incubators, where temperatures can not be adjusted to match needs of both early and late term embryos, so temperatures are higher than late-term embryo needs. Even though this difference in incubation profile did not affect hatchability or poult BW at hatch, it clearly affected the performance of these poult post-hatch, with P1 poult showing superior performance (Table 4.8). Moreover, the P1 poult had the bigger pectoral muscles with higher glycogen concentration at hatch than those hatched in P2 (Table 4.7). In contrast, P2 poult amassed bigger HM at the expense of liver [GLY] than P1 poult at 25E (Table 4.4), regressing by hatch (Table 4.6) probably due to protein catabolism. Similar differences between poult from hatcheries using single stage or multistage incubators were found by Warner et al. (2006).

We believe that lower temperatures early in incubation at P2 may have played a role in the poult inferior performance, what agrees with the findings of Funderburk et al., (2006), where poult from egg incubated in lower temperatures showed lower body weights at the end of 7d.

IOF treatment effects were independent of gender or hatchery. The reason IOF embryos started hatching earlier (Table 4.3) is not clear, but it could be because of accelerated development or because of incubation distress. The higher incubation temperatures in P2 may have caused heat distress, resulting in inferior performance as compared to the poult of P1. Despite the negative effects of microbial contamination on the hatchability of the CHP-treated poult (Tables 4.2 and 4.3), these poult exhibited superior WG and BW starting at 7d of age through until the end of this study (14d). The enhanced growth performance among the CHP-treated poult may be a consequence of

greater digestive capacity as observed by Foye et al. (2006) who used a similar IOF formulation. Evidently, the CHP-treated poult had greater appetite at placement than poult of the other treatment groups (Figure 4.1). Although the male poult of both NC and CHP treatment groups had the same feed conversion, only the CHP poult was heavier because of higher feed intake (Table 4.9). Some studies demonstrated a positive correlation between BW at 7 d of age and age of slaughter as influenced by early feeding (Noy and Sklan, 1998, Noy and Sklan, 1999), but a similar relationship is yet to be demonstrated in turkeys after *in ovo* feeding. Poults subjected to the SC IOF treatment responded intermediately between those subjected to the NC and CHP treatments, indicating that they received only partial benefit of *in ovo* feeding.

Conclusion

In ovo feeding, gender and incubation profile affected embryo development, energy use and post-hatch performance. The best performance was found among male poults that were *in ovo* fed a saline solution containing protein and carbohydrate and incubated using single stage temperature profile.

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Chapter 5

“Effect of *in ovo* feeding on glycogen status of perinatal turkeys”

Abstract

In ovo feeding (IOF) improves turkey poult quality and viability, but the formulation must be optimized relative to appropriate control treatments. The objective of this study was to compare different controls and IOF formulations. At 23 days of incubation (E), 840 Hybrid turkey eggs were sorted into 5 groups of similar weight distribution and assigned to the following treatments: 1) non-injected control (NC); 2) shell-hole punch control (HC); 3) 1.5 ml IOF saline control solution (SC); 4) 1.5 ml carbohydrate IOF solution (CHO); and 5) 1.5 ml carbohydrate-protein solution (CHP). Hatchability rate (HR) and body weights (BW) were recorded at hatch, and then the poults were randomly distributed by treatment and sex among 12 replicate pens. Glycogen concentration [GLY] of liver (L), pectoral muscle (BM), and pipping muscle (PM) were determined at 25E (8 eggs/treatment) and hatch (10 poults/treatment). BW and weight gain (WG) were determined at 7 and 14 d. IOF treatments increased HR in comparison to controls (93% and 93% for CHO and CHP versus 90%, 91%, and 91% for NC, HC, and SC, $p < .05$). CHO and CHP had 2% higher BW over controls (NC, HC, and SC) at hatch ($p < .05$). BW of toms was greater than hens, but there was no difference among treatments after 7d. HC had higher WG at 7d when compared with CHO ($p < .05$). There were no treatment effects on [GLY] in PM or L at 25E. At hatch, PM [GLY] was greatest for CHO, intermediate for CHP, and lowest for NC and SC (16.8, vs. 8.6, vs. 2.8 and 4.1 mg/g, respectively, $p < .05$). BM [GLY] was greatest for NC, followed by CHP than CHO and SC (2.2, 1.3, .7,

and .4 mg/g, respectively). L [GLY] was higher for CHO than CHP, NC, and SC (11.9 vs. 4.8, 6.6, and 5.7 mg/g, respectively, $p < .05$). IOF solutions containing CHO or CHP increased HR and BW at hatch, probably due improved energy status. Since HC and SC affected [GLY] in tissues and WG when compared with NC, they can not be considered controls for IOF studies

Introduction

Genetic selection of commercial meat poultry has succeeded to yield great growth potential (Hammerstedt, 1999). As the number of days for modern broilers and turkeys to achieve market weight decreases with each generation of selection, the neonatal growth period becomes of greater relative importance to their total productive life (Uni and Ferket, 2004; Ferket et al., 2005). Turkey poults are particularly at risk because they must endure the stress of hatching, some waiting in the hatching cabinet for over 24 hours after hatch, then they are subjected to the stress of handling and transport for another 24 hours before given access to feed and water (Donaldson and Christensen, 1991; Donaldson et al., 1991). Consequently, many poults succumb to impaired growth and as many as 4-6% do not survive the first week of life (Carver et al., 2000; Christensen et al., 2003; Uni and Ferket, 2004).

The major energy source available for poults to hatch and sustain them until feed intake begins comes from glycogen reserves stored in the liver prior to hatch (Donaldson 1995; Christensen et al., 1999). In order to improve the glycogen status of neonatal poultry, Uni and Ferket (2003) proposed *in ovo* feeding (IOF): injecting a nutrient solution into the amnion of the embryo prior to internal pipping. Several studies have demonstrated that IOF accelerates enteric development and maturation before and after

hatch (Uni and Ferket, 2004; Tako et al., 2005; Smirnov et al., 2006; Uni et al., 2006), improves hatchability (Ferket et al., 2005), increases body weight and relative pectoral muscle size at hatch (Ferket et al., 2005; Uni et al., 2005), and improves energy status (Uni et al., 2005). Previous research in our laboratory have demonstrated that IOF of several nutrients have beneficial effects, including carbohydrates (Tako et al., 2004), sodium chloride (Tako et al., 2005; Smirnov et al., 2006), arginine (Foye, 2005), egg white protein (Foye, 2005; Foye et al., 2006), zinc-methionine (Tako et al., 2005) and β -hydroxy- β -methylbutyrate (HMB) (Tako et al., 2004; Foye et al., 2006).

Since soybean meal is the primary dietary protein source for turkey poult (Donaldson, 1995), it was hypothesized that hydrolyzed soy protein (HSP) would be a good candidate ingredient for IOF solution formulation. Previous studies showed that poult IOF HSP had enhanced energy status (Chapters 2 and 3), superior feed intake (Chapter 2), and suffered less negative effects of heat stress during incubation (Chapter 4). The objective of the study reported herein was to evaluate the efficacy of an IOF solution containing hydrolyzed soy in comparison to control treatments, including IOF solutions containing carbohydrates, saline, and sham injections. IOF Treatment efficacy was evaluated by hatching performance indicators, hatchling body weight, and glycogen reserves in liver, hatching muscle, and pectoral muscle.

Material and Methods

Eight-hundred-forty fertile Hybrid turkey eggs were obtained from a commercial hatchery¹, sorted by weight, and divided into 5 treatment groups (175 eggs per treatment) with similar weight distribution (87.5 g, \pm 12.5), and incubated at Pfizer-Embrex Inc.²

¹ Prestage Farms, Clinton, NC

² Research Triangle Park, Durham, NC

according to typical commercial practices. At 23 days of incubation (23E), each IOF treatment group was injected through the air cell with an IOF solution (1.5 mL/egg) into the amniotic fluid. The 5 experimental treatments were: 1) non-injected control (NC); 2) shell hole punch control (HC); 3) 1.5 mL IOF saline control solution (.4% NaCl, 136 mOsm) (SC); 4) 1.5 mL carbohydrate IOF solution (.4% NaCl + .2% HMB³ + 20% dextrin⁴ + 1.5% maltose⁴ + 3% sucrose⁴, 496 mOsm) (CHO); and 5) 1.5 mL carbohydrate-protein solution (.4% NaCl + .2% HMB + 5% HSP⁵ + 10% dextrin, 496 mOsm) (CHP). Technical accuracy of injection into the amnion was validated to be over 90% by injecting 40 eggs with 0.3% coumasie blue dye in ethanol and observing the location of the dye upon immediate embryo breakout. All eggs, including NC, were outside the incubator for about 20 minutes to allow for time to complete the IOF injection procedure. After injection, the eggs of each treatment group were placed in hatching baskets (175 Eggs per basket per treatment). Hatched poults were removed from the incubator at 672 hours of incubation (28E), and the percentage hatch, pips, and unhatched eggs was determined. The unhatched eggs were opened to determine the incidence of live and dead embryos.

Tissue Sampling and Glycogen Determination

Liver and hatching muscle were collected at 25E from 8 embryos from treatment groups 1, 3, 4 and 5. Treatment 2 (HC) embryos were not sampled at this time to reduce the number of samples, since glycogen storage in HC group was not expected to differ from NC. These tissue samples were immediately placed on ice for about 4 hours and

³ Metabolic Technologies Inc., Ames, IA

⁴ Sigma-Aldrich, Inc., St. Louis, MO

⁵ Kerry Bio-Science, Chicago, IL

then stored at -20°C until they were processed for glycogen analysis. Within 2 hours after the hatchlings were removed from the hatcher, they were sexed, neck tagged, and weighed. Liver, pectoral muscle, and hatching muscle samples were collected from ten poult per group from treatments 1, 3, 4, and 5, placed immediately on ice for about 4 hours, and then stored at -20°C until they were processed for glycogen content analysis. All treatments were sampled in the same manner at 7 days post-hatch to collect liver and pectoral muscle for glycogen analysis. A colorimetric method of iodine reduction (Uni et al., 2005) was used to determine glycogen concentration [GLY] (mg glycogen/g fresh tissue) in liver, breast, and hatching muscle.

Housing and Animal Care

Poults were housed in a temperature-controlled curtain-sided facility containing 12 10 m² pens. Each pen was top-dressed with 4 cm of soft pine shavings at the start of the experiment. Ventilation was provided by natural air movement through appropriately adjusted curtain sides and air mixing fans located on the ceiling throughout the house. The house temperature was kept between 29-31 C during the first week, and then gradually decreased 3 C each week. The house was illuminated with incandescent lights for 23 hours per day during the experimental period. Heat lamps⁶ with 125 watt bulbs provided supplemental heat for each pen. Each pen was populated with 10 birds from each treatment, either males or females, totaling 50 poults per pen. Feed and water were provided *ad libitum* throughout the duration of the study. All pens received the same diet, which met or exceeded NRC (1994) recommendations. Visual health inspection of all birds was performed daily, and weights of dead or culled birds were recorded. The poults

⁶ Model 54411 Heave Gauge Aluminum Base, Hog Slats, Inc., Newton Gove, NC

were individually weighted at 7 and 14 days of age. The experimental protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee.

Statistical Analysis

Data were analyzed using the general linear models (GLM) procedure of SAS (SAS Institute, 2004) for analysis of variance (ANOVA). Hatchability and performance data were statistically analyzed as a completely randomized 5 X 2 factorial arrangement with 5 embryo treatments and 2 genders (identified at time of sampling or post-hatch vent observation). Glycogen data was analyzed as completely randomized with 4 treatments (NC, SC, CHO and CHP). Differences among treatments were compared using least-square-means test (lsmeans) with significance at $P < 0.05$.

Results

Significant treatment effects were observed at the time of internal pipping (25E). The proportion of embryos that had internally pipping at 25E is presented in Table 5.1. The NC and CHP embryos started pipping earlier than SC and CHO embryos, as indicated by a higher percentage of internal pipping. Figure 5.1 illustrates these same 2 groups having bigger hatching muscles, as compared to the CHO-treated embryos, and the SC group with hatching muscle of intermediary size. Figure 5.2 illustrates hatching muscle [GLY] at 25E. Both IOF treatments that supplied caloric nutrients (CHO and CHP) had lower hatching muscle glycogen concentration than the SC and NC embryos. In contrast, the same CHO and CHP treatment groups had greater liver [GLY] at 25E than NC and SC control groups (Figure 5.3).

There were significant treatment effects observed on hatchability and body weights on the day of hatch. All the IOF treatment groups (SC, CHO, and CHP) had 2% higher hatchability rates because of fewer dead embryos than the NC and HC treatment groups (Table 5.2). Poults fed CHO or CHP were significantly heavier at hatch than NC and HC control poults, with SC poults not differing from either group (Figure 5.4). However, these differences in hatchling BW were no longer observed at 7 or 14 days post-hatch (Table 5.3). As expected, the male poults gained more weight and were heavier throughout the whole study than the females.

The IOF treatments had different effects on body glycogen reserves at hatch, depending on the tissue of origin. The CHO treated poults had highest [GLY] in hatching muscle, significantly higher than the CHP as well as the SC and NC poults. Although the combination of carbohydrates and protein in the IOF solution resulted in lower hatching muscle [GLY] than carbohydrates alone (CHO), the CHP treatment still resulted in higher [GLY] than the SC and NC treatments (Figure 5.5). In contrast to hatching muscle, liver [GLY] at hatch was significantly increased only among the CHO treated poults (Figure 5.6). In contrast to what was observed for hatching muscle and liver, pectoral muscle [GLY] was highest among the NC treatment group (Figure 5.7). Pectoral muscle [GLY] was lowest among the SC treatment group, but it was incrementally increased as the caloric content of IOF treatment increased as in CHO and CHP, respectively.

Table 5.1. Effect of *in ovo* feeding (IOF) treatment on percentage of internal pipping of eggs sampled at 25 days of incubation.¹

Treatment	Pipped (% of Sampled)
Non-injected control (NC)	50.0%
IOF Saline (SC)	37.0%
IOF carbohydrates (CHO)	37.0%
IOF CHO+Protein	62.5%

¹Values are expressed as a percentage of 8 sampled eggs per treatment.

Table 5.2. Effect of *in ovo* feeding (IOF) treatment on hatchability of fertile eggs .

Treatment	Hatched		Unhatched	
	n ¹	% ²	N ¹	% ²
Non-injected control (NC)	152	91.6	14	8.4
Shell hole control (HC)	152	91.0	15	9.0
IOF Saline (SC)	162	93.1	12	6.9
IOF Carbohydrates (CHO)	151	93.8	10	6.2
IOF CHO+Protein (CHP)	154	92.8	12	7.2

¹n = number hatched or unhatched

²Percentage of total viable embryos observed at time of *in ovo* feeding (23E).

Table 5.3. Effect of *in ovo* feeding (IOF) treatment on body weight and weight gain of poults at 7 and 14 days post-hatch.¹

Treatment	Body Weight (g)		Weight gain (g)		
	7d	14 d	1-7d	7-14d	1-14d
Non-injected control(NC)	127.02	308.58	72.91 ^{ab}	181.11	254.63
Shell hole control (HC)	129.62	316.21	75.80 ^a	186.16	262.53
IOF Saline (SC)	127.88	306.41	73.33 ^{ab}	180.06	253.64
IOF carbohydrates (CHO)	126.42	312.67	71.44 ^b	185.45	257.63
IOF CHO+Protein (CHP)	128.59	315.81	73.22 ^{ab}	187.02	255.97
Statistical Analysis		----- (P-Value) -----			
Treatment	0.3042	0.2043	0.0838	0.1611	0.3937
SEM ²	1.164	3.562	1.109	2.449	3.413

¹Values are means of about 128 poults per treatment.

²SEM = standard error of the mean

^{a,b}Means with different superscripts within a column differ significantly (P<0.05).

Table 5.4. Effect of gender on body weight and weight gain of turkey poults at 7 and 14 dayspost-hatch.¹

Gender	Body Weight (g)		Weight gain (g)		
	7d	14 d	1-7d	7-14d	1-14d
Male	134.85	329.54	79.99	195.15	273.84
Female	120.98	294.24	66.69	172.77	239.91
P-Value	0.0001	0.0001	0.0001	0.0001	0.0001
SEM ²	0.731	2.257	0.696	1.552	2.210

¹Values are mean of 320 replicate poults per treatment.

²SEM = standard error of the mean

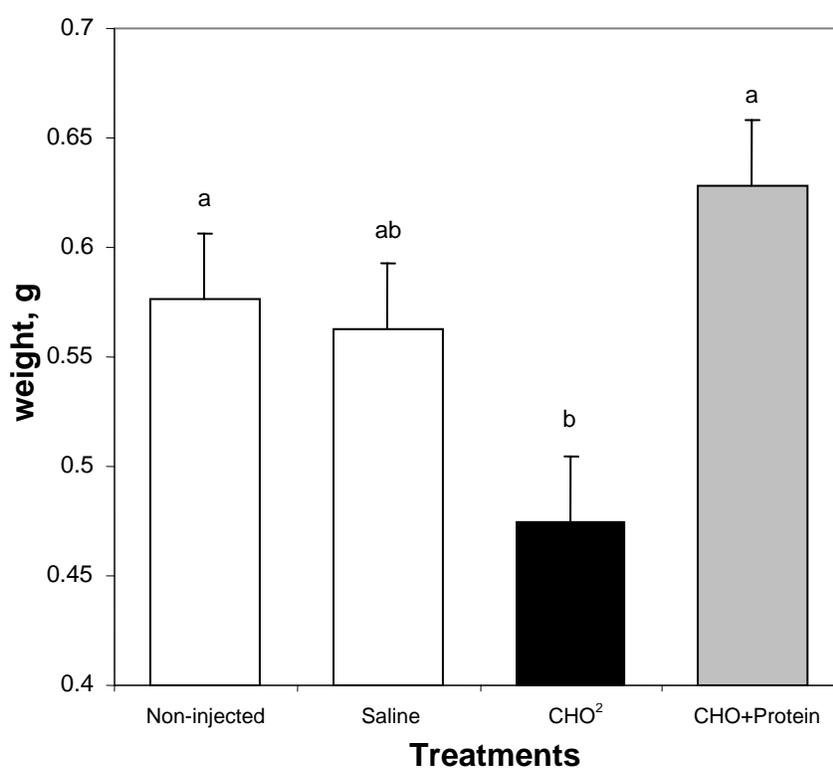


Figure 5.1. Effect of *in ovo* feeding treatment at 23 days of incubation on hatching muscle weight of turkey embryos at 25 days of incubation.¹

¹Values are means of *ca.* 8 sampled embryos per treatment.

²CHO= carbohydrates.

^{a,b}Bars with different letters differ significantly (P<0.05)

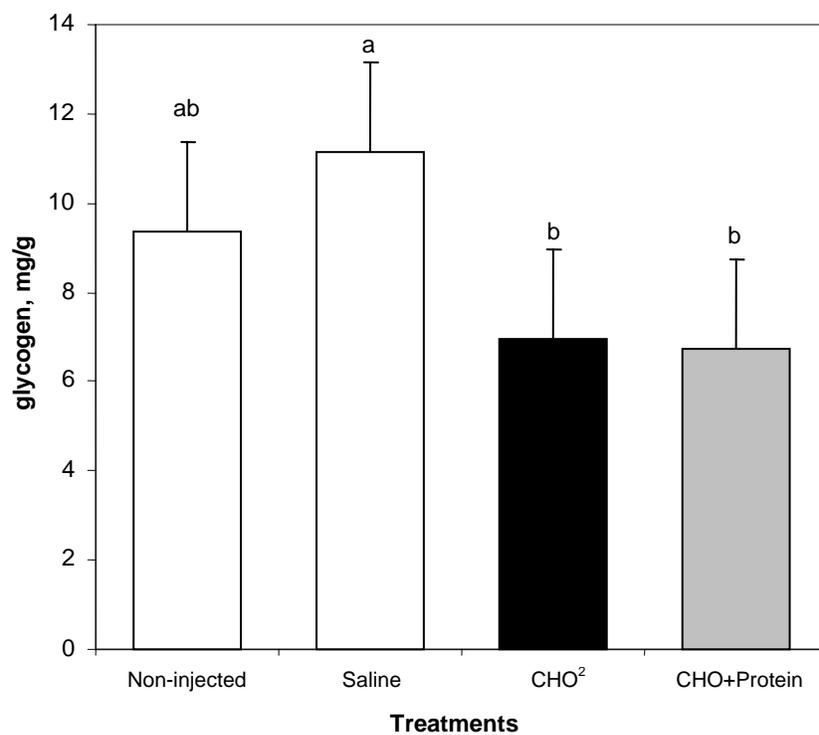


Figure 5.2. Effect of *in ovo* feeding treatment at 23 days of incubation on hatching muscle glycogen concentration of turkey embryos at 25 days of incubation,¹

¹Values are means of *ca.* 8 sampled embryos per treatment.

²CHO= carbohydrates.

^{a,b}Bars with different letters differ significantly ($P < 0.05$)

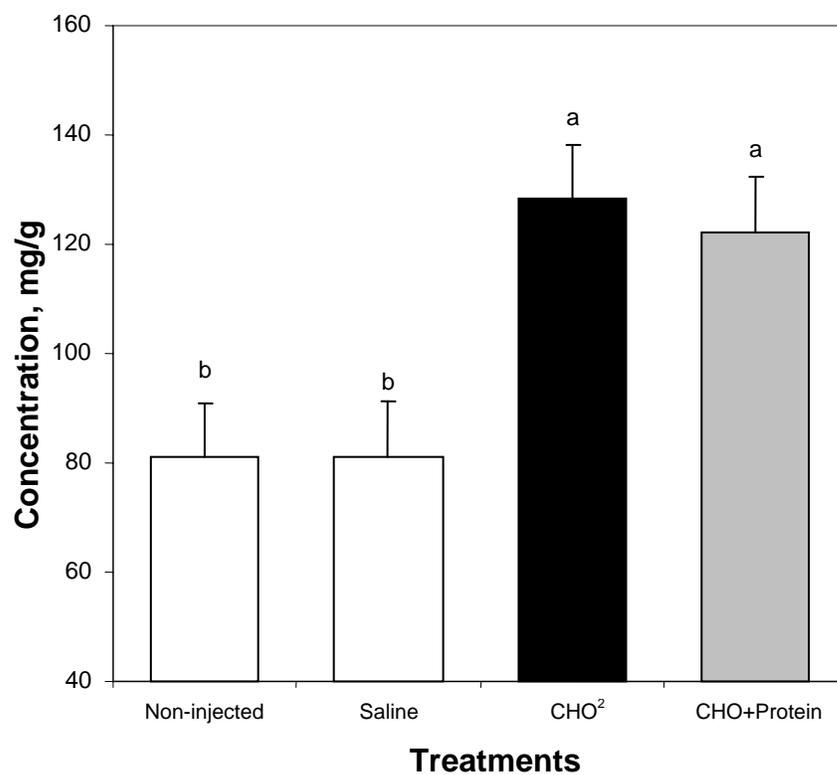


Figure 5.3. Effect of *in ovo* feeding treatment at 23 days of incubation on liver glycogen concentration of turkey embryos at 25 days of incubation.¹

¹Values are means of *ca.* 8 sampled embryos per treatment.

²CHO= carbohydrates.

^{a,b}Bars with different letters differ significantly ($P < 0.05$)

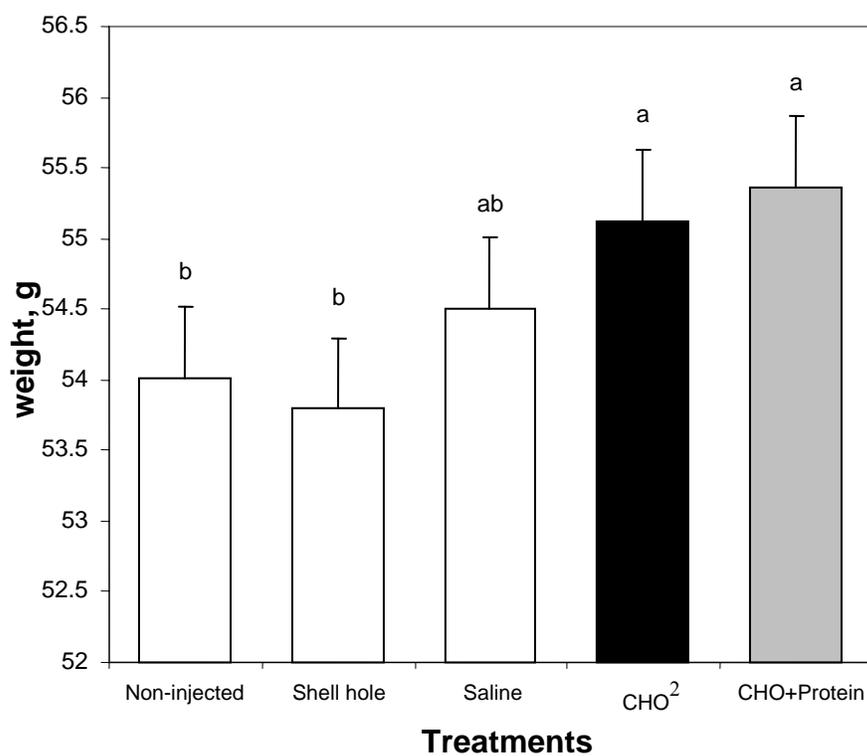


Figure 5.4. Effect of *in ovo* feeding treatment at 23 days of incubation on body weight turkey poults at hatch.¹

¹Values are means of *ca.* 10 poults per treatment.

²CHO= carbohydrates.

^{a,b}Bars with different letters differ significantly (P<0.05)

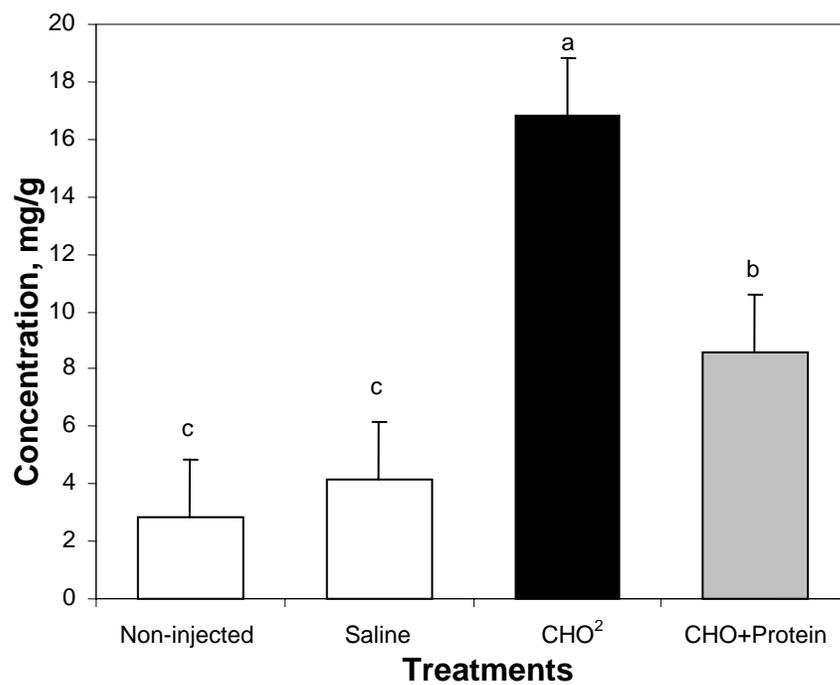


Figure 5.5. Effect of *in ovo* feeding treatment at 23 days of incubation on hatching muscle glycogen concentration of -fed turkey poults at hatch.

¹Values are means of *ca.* 10 sampled poults per treatment.

²CHO= carbohydrates.

^{a,b,c}Bars with different letters differ significantly ($P < 0.05$)

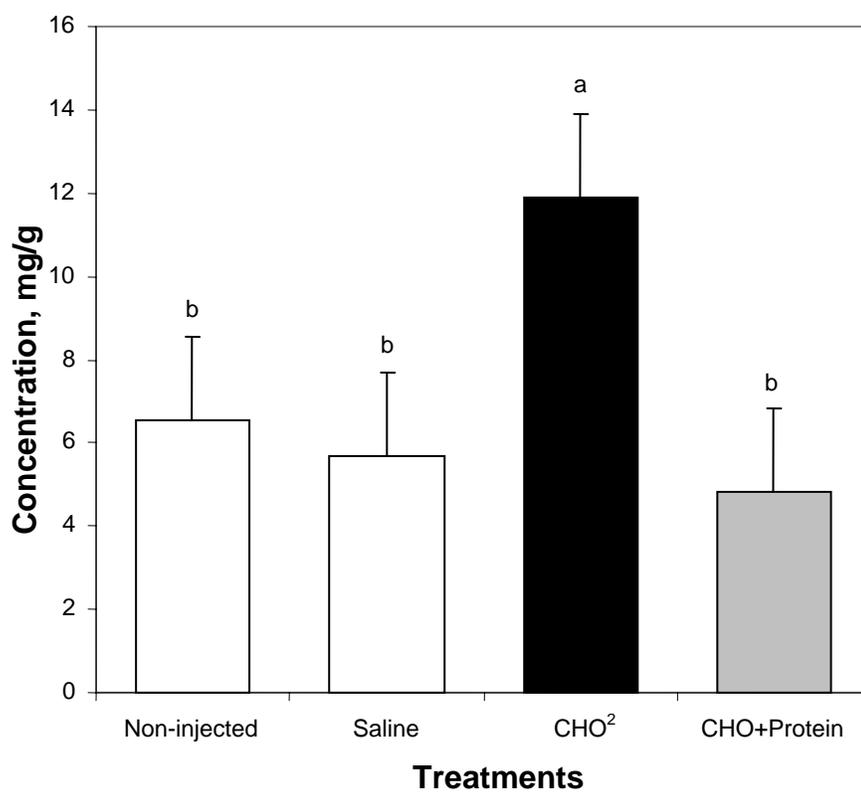


Figure 5.6. Effect of *in ovo* feeding treatment at 23 days of incubation on liver glycogen concentration of turkey poults at hatch.¹

¹Values are means of *ca.* 10 sampled poults per treatment.

²CHO= carbohydrates.

^{a,b}Bars with different letters differ significantly ($P < 0.05$)

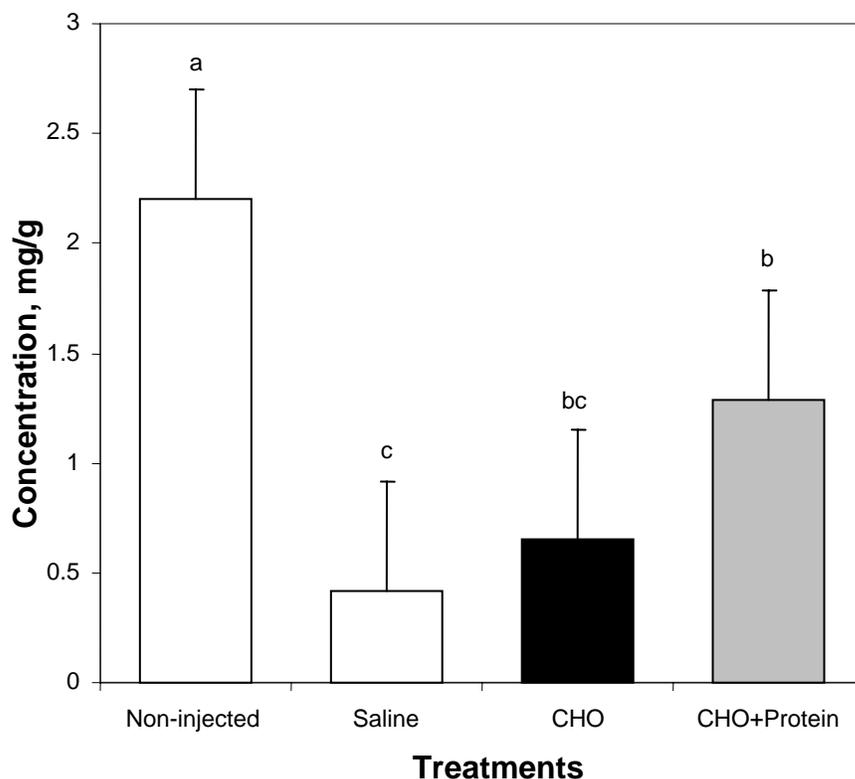


Figure 5.7. Effect of *in ovo* feeding treatment at 23 days of incubation on pectoral muscle glycogen concentration of turkey poults at hatch.

¹Values are means of *ca.* 10 sampled poults per treatment.

²CHO= carbohydrates.

^{a,b,c}Bars with different letters differ significantly ($P < 0.05$)

Discussion

During the early stages of incubation, avian embryos depend on glucose as their energy source (Romanoff, 1967; Moran, 2007), then they switch to fatty acids once choriallantoic respiration is established (Donaldson and Christensen, 1991; Moran, 2007). According to Christensen et al. (1996), yolk fatty acids supply 90% of nutrients for tissue growth, but when the embryo reaches its maximum size, oxygen supply also reaches a plateau stage initiating a cascade of events to prepare the embryo for emergence (Christensen et al., 1996; Christensen et al., 1999). At this point, the amniotic fluid is orally consumed (Moran, 2007), providing substrate for gluconeogenesis (Donaldson and

Christensen, 1991; Donaldson, 1995), so glycogen can be stored in liver and muscle tissues (Christensen et al., 1996; Christensen et al., 1999; Christensen et al., 2003; Uni et al., 2005). These glycogen reserves will be the embryo's sole energy supply during hatching (Romanoff, 1967; Donaldson, 1995; Moran, 2007), since the choriallantois is detached from the shell during pipping, limiting oxygen supply and lipid catabolism (Moran, 2007). Even after hatch, gluconeogenesis and glycogenolysis will continue to be the poult's glucose source until feed is consumed (Donaldson and Christensen, 1991; Keirs et al., 2002; Peebles et al., 2006; Moran, 2007). The liver is especially important because it is the embryo's largest glycogen reserve (Romanoff, 1967), and in contrast to muscle, the liver has all the dephosphorylation enzymes necessary to mobilize glucose to other tissues (Matthews and Holde, 1990). This means that glucose derived from muscle glycogen can only be used locally, while liver glucose can be mobilized for use in other body tissues.

In the process of glycogen accumulation, glucose is taken in greater amounts by tissues that are active during hatching, like the hatching muscle, pectoral muscle and the heart. The *complexus* muscle (hatching muscle), located in the back of the neck right below the skull, is responsible for pulling the head back so the beak can break the membranes and the shell (John et al., 1987; Moran, 2007). This muscle grows larger in size right before internal pipping, and shrinks after a couple days post-hatch (John et al., 1987). It swells due to water and glycogen granules accumulation, and most of its muscle fibers are exclusively glycolytic (John et al., 1987; Moran, 2007).

Stress conditions, such as low oxygen levels and high temperatures, during late incubation can cause embryos to hatch earlier. Embryonic distress may have occurred

among the NC and CHP embryos in this study, these treatment groups had a higher number of internal pipped embryos at 25E than the other treatments. The NC treated group may be distressed because of limited glycogen reserves to sustain anaerobic metabolism, whereas the CHP embryos may have been distressed by increased nitrogen metabolism or by soy anti-nutritional factors. Even though HSP product composition does not show specific composition of carbohydrates, it states that the level is high (Kerry Bio-Science, 2007), so common soy anti-nutritional factors, such as oligosaccharides and lignin, may be present. Adding nutrients to the amnion by IOF influenced the size and [GLY] of the hatching muscle at 25E (Figures 5.1 and 5.2). NC and SC control groups had [GLY] similar to levels reported by John et al. (1987), while CHP and CHO increased [GLY] 1 and 2 fold, respectively. The same treatments that were pipping earlier at 25E (NC and CHP, Table 5.1) also had bigger hatching muscles (Figure 5.1), confirming the key role of this tissue on pipping. Incubation distress has been observed to speed hatching muscle development and accelerate hatching in turkeys.

The IOF formulation apparently influenced energy partitioning in the late-term embryo. Feeding CHO alone reduced hatching muscle size (Figure 5.1) and [GLY] as compared to NC (Figure 5.2), probably because these embryos directed more resources to liver glycogen stores (Figure 5.3). Combining protein with carbohydrates in the IOF solution (CHP) did not change hatching muscle size (Figure 5.1), perhaps because more amino acids were available for protein synthesis. Evidently, IOF solutions containing caloric substrates affect energy partitioning prior to pipping and hatching because embryos fed CHO or CHP had less hatching muscle [GLY] (Figure 5.2) and more liver [GLY] at 25E and NC and SC (Figure 5.3). Hepatic [GLY] of poults at hatch is 12-15

mg/g (Donaldson and Christensen, 1991; Donaldson, 1995; Christensen et al., 1996), which was similar to the 12 mg/g found in this study for the CHO fed group, while the other treatment groups had only half of that amount. Apparently, the embryos in this study had lower energy reserves than poultz studied by Donaldson and Christensen.

The SC treatment was intended to serve as a positive control treatment to test the effect of the IOF base solution, .4% saline. The SC treatment was not significantly different from the NC treatment for all measured parameters except for pectoral muscle [GLY]. Because the Pectoral muscle [GLY] was significantly lower among the SC treatment group than the NC treatment, *in ovo* feeding a saline solution evidently affected breast tissue metabolism and thus cannot be considered as sham-injection control relative to the CHO and CHP treatments.

Liver glycogen levels were clearly enhanced before pipping by feeding CHO and CHP (Figure 5.3), confirming our hypothesis that embryos can use IOF nutrients to complement innate energy sources. This advantage in energy status was why IOF poultz were heavier than NC and HC at hatch (Figure 5.4). Remaining [GLY] in hatching muscle and liver were higher for CHO fed poultz than for other treatments (Figures 5.5 and 5.6), indicating a surplus of glycogen. Combining protein and carbohydrates in the IOF solution (CHP) reduced the remaining [GLY] in the hatching muscle and liver after hatch in comparison with the IOF solution containing CHO alone (Figures 5.5 and 5.6), which may be again due to higher energy cost of protein metabolism. Hatching muscle and liver [GLY] of the SC treatment group did not differ the NC treatment group at hatch (Figures 5.5 and 5.6), but [GLY] remaining in pectoral muscle was reduced to a quarter of NC, and half of CHP levels (Figure 5.7). Lower levels of remaining [GLY] in pectoral

muscle were also observed in all other IOF treatments at hatch as compared to NC (Figure 5.7), which indicates that they used whatever glycogen stores they had in this tissue. In contrast to the IOF treatments, the NC treatment group had higher amounts of energy trapped as glycogen in pectoral muscle, which was unavailable to other tissues.

Based in these results it seems that IOF triggered better energy partition, favoring liver over muscle. Pectoral muscle [GLY] of IOF poults varied between 0.4-1.2 mg/g, which agreed with the 0.7 mg glycogen/g pectoral muscle observed by Christensen et al., (1999). In contrast, the pectoral muscle [GLY] of the NC treatment group at hatch was over 2 mg/g, higher that observed by Christensen et al., (1999). This difference in pectoral muscle [GLY] between studies may be associated with the differences in genetic strain of poults used. Christensen et al. (1999) observed embryos from unique genetic lines selected either for egg production or body weight, whereas a commercial genetic line⁷ was used in the present study. Therefore, genetic differences may be responsible for increased glycogen deposition in pectoral muscle found in this study, exacerbating the effects of limited energy reserves by trapping glycogen in the muscle.

The HC group was not different from NC group for any of the parameters studied until hatch; thus any benefit of IOF on poults at hatch was due to the addition of *in ovo* nutrients, and not due to extra air exchange through the injection site. However, weight gain during the first week post-hatch was enhanced by the HC treatment (Table 5.3), disqualifying this treatment as a control for IOF studies.

Treatment differences in BW at hatch disappeared after 7 days post-hatch (Table 5.3). There may be two possible reasons why weight differences at hatch were not carried on later in life: 1) The caloric content of the IOF solutions was enough to help embryos

⁷ Hybrid turkeys, Inc., Kitchener, Ontario, Canada

hatch, but it inhibited post-hatch gluconeogenesis, affecting glucose supply; and 2) Poults subjected to IOF may require different nutrition, management, and brooding conditions to retain advantages found at hatch. This is indicated by lower weight gain of CHO fed poults during the first week post-hatch, as compared to controls (Table 5.3). Based on the results of this study, IOF carbohydrates and protein altered energy partitioning during the perinatal period, favoring glycogen stores in liver over muscle. Just punching a hole in the shell or IOF saline should not be considered as positive or sham control groups of IOF study because the former change weight gain and the later changed pectoral muscle glycogen stores as compared to the non-injected controls. More studies are necessary to detail the impact of *in ovo* feeding carbohydrate and protein on metabolism, and to determine why differences at hatch dissipate within a few days after hatch.

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Chapter 6

“Late-term turkey embryo survey of amnion fluid proprieties and its implication to *in ovo* feeding studies”

Abstract

Three observational studies of similar design were conducted during the later stages of embryonic development on each of the three major commercial turkey strains used by the industry (BUT, NIC and HYB). The eggs used on each of these experiments were from first-cycle turkey breeder flocks in their 12-13th week of lay. The objective was to document the changes that occur in the amnion fluid volume and osmolality from 20 to 24 days of incubation (E). This data was then used to determine the proportion of amnion relative to egg mass, and the rate of amnion consumption by viable embryos. Amnion fluid volume relative to egg mass decreased with day of incubation in all three strains. Although NIC and HYB embryos had similar amounts of amnion fluid (11 mL/egg) at 20E, HYB turkeys had the greatest amniotic fluid volume relative to egg mass. Consumption rate of amniotic fluid was not consistent among turkey strains until after 21E. Considering all three strains, amnion consumption followed a linear trend with consumption rate of about 0.0012 mL/hr/g egg, or 0.10 mL/hr for a typical 85g turkey egg. This information was useful to determine amnion volume capacity, maximum safe injection volume for *in ovo* studies (1.5 mL), best injection time (22-23E), and osmolality constraints of solutions that can be administered *in ovo* (570 mOsm maximum, 450 mOsm ideal).

Introduction

Successful avian embryonic development is essential to poultry industry profitability. Although artificial incubation has been a standard practice for over 50 years, few improvements in hatchability has occurred during last 20 years (Schaal and Cherian, 2007). Despite incredible advances in genetic potential for growth (Hammerstedt, 1999), no changes were observed in incubation time, thus incubation now accounts for an increasing percent (30-40 %) of the total development time of the bird (Hulet, 2007). Many physiological processes that take place inside the egg during incubation remain unknown (Romanoff, 1960; Vleck, 1991). Research work on new technologies like *in ovo* vaccination (Johnston et al., 1997) and *in ovo* feeding (Uni and Ferket, 2003; Ferket et al., 2005) have revealed that more information on late-term embryo development is needed. Particularly more information is needed related to amnion fluid volume and its consumption by the embryo and the osmotic limits of amnion and embryo during late-term development. Such information is especially needed for turkeys, since this has become a more important poultry production sector (Schaal and Cherian, 2007) and hatchability and poult quality problems are so prevalent (Christensen et al., 2001; Christensen et al., 2003). This paper reviews the literature on water compartments of the egg, and amnion formation and function during the last third of the incubation period. This paper will also report on a study of amnion properties and its consumption by the developing turkey embryo, which can help determine practical *in ovo* feeding constraints.

The role of water during incubation of avian eggs

In contrast to many reptilian eggs that can exchange mass with the environment through a liquid phase, avian eggs depend upon gas phase mass exchange. Since inorganic solutes can not leave the egg, the embryo must compensate for variability in water content by distribution of solute pools (Vleck, 1991). The avian egg shell structure allows for water loss to the environment but not to gain water from its surroundings during incubation. The amount of water lost during incubation will depend on incubation temperature, humidity and ventilation associated to egg shell conductance characteristics, all of which are not controlled by the developing embryo (Amos, 1991). Metabolic water is the only way to add water to the system. Organic molecules can be oxidized to CO₂ and water, with CO₂ diffusing through the shell while water is added to the pool of water within the egg (Vleck, 1991). Water loss plays a necessary part on incubation. Since egg internal space is fixed by shell rigidity, water space is replaced by air in the air cell at the blunt end of the egg. The air in the air cell is important feature by the time of internal pipping when pulmonary respiration starts. Unlike mammals, where the lungs fill up immediately at birth, the bird' lungs and air sacs become gas-filled gradually during the perinatal period (Amos, 1991). To be able to shut down choriallantoic circulation before hatch, the respiratory system must be inflated beforehand with air available inside the egg (air cell). According to Amos (1991), too little water loss may prevent full inflation of the respiratory system due to insufficient air in the air cell. Excessive water loss on the other hand must be compensated by embryonic adaptive physiology, or the embryo may die.

About 75% of the water in the egg is initially in the albumen (Amos, 1991), which is 85% to 90% water (Vleck, 1991). The water content of yolk is much lower than albumen due to its lipid richness, varying between 43% and 66% water, and it is kept

constant throughout development (Vleck, 1991). As the germinal disc expands, the embryo starts changing water distribution by translocating water from the albumen to form a layer of sub-embryonic fluid between itself and the yolk. The volume of sub-embryonic fluid decreases until the embryo is enclosed by the expanding amniotic membrane, which is filled with fluid (Vleck, 1991). At first, the amniotic sac is in contact to the embryo, but it is carried away from the body as the clear amniotic fluid accumulates. The origin of the amniotic fluid seems to be transudation from blood vessels of the *area pellucida* (Romanoff, 1960).

The volume of amniotic fluid in several species of birds was studied by Romanoff and Hayward, (1943). Changes in volume by percent of incubation period are illustrated in Figure 6.1. From all species studied, turkey eggs showed the highest peak of amnion volume (8 mL) for an average 85g egg. The turkey embryo exhibits two distinct peaks in amnion volume during incubation: one at 50% and another at 80% of incubation. Maximum volume is reached at the second peak, which corresponds to 22.5E, then rapidly decreases toward hatch (28E). The lowering in volume observed between peaks (57% of incubation) coincides with the highest peak of allantoic fluid accumulation (Romanoff and Hayward, 1943). The great rise in volume that creates the second peak happens as a consequence of albumen invading the amniotic cavity when both egg compartments are joined during the last third of the incubation period (Romanoff, 1960), and by movement of water from the allantois into the amnion (Vleck, 1991). Disappearance of amniotic fluid is due to its oral consumption by the embryo in preparation for hatching (Romanoff, 1960; Moran, 2007), and it finally decreases to near zero by the time the embryo starts internal pipping (Romanoff, 1960).

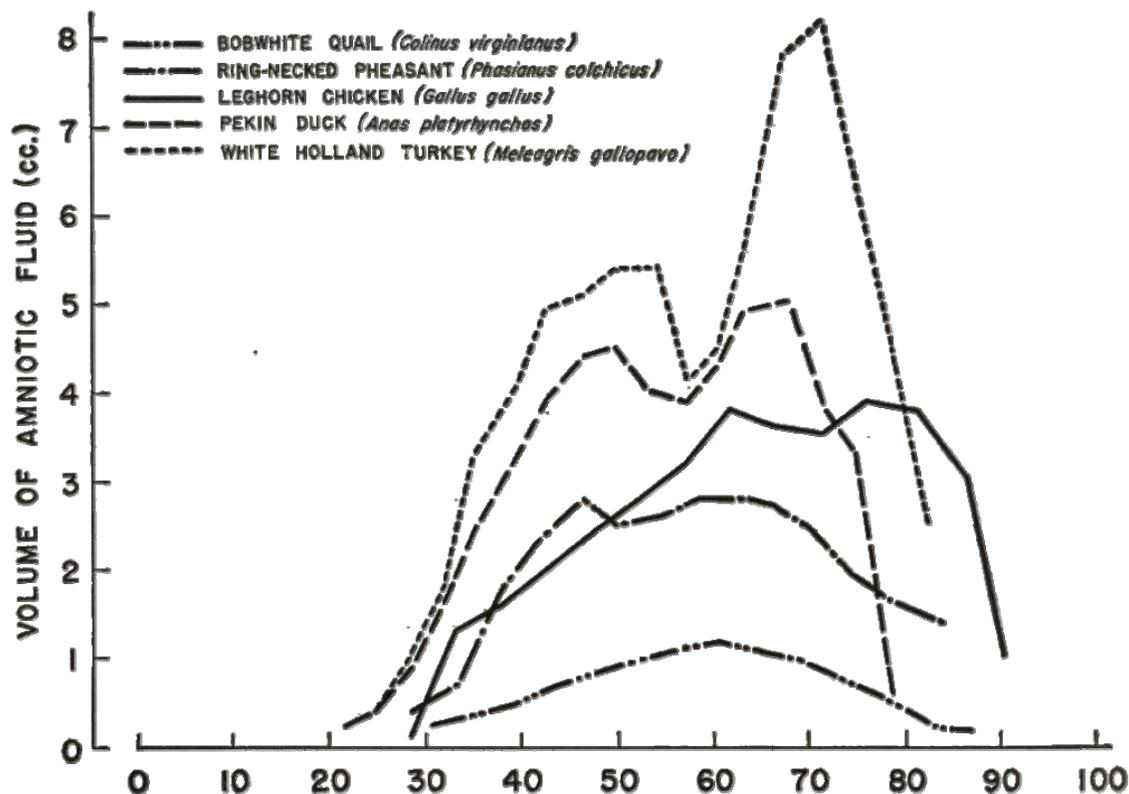


Figure 6.1. Changes in volume of amniotic fluid of avian species. (Adapted from Romanoff and Hayward, 1943).

The self sustaining capsule that is the egg can fulfill its task because it carries plenty of water (Gray, 1926). Although it is true that the egg contains enough water, embryonic survival is possible only thanks to its amazing capacity to deal with great variation in egg water content, such as no adult vertebrate could tolerate (Vleck, 1991).

Active exchange of water and solutes occurs between all of the fluid compartments within the egg. According to Vleck (1991), formation of the sub-embryonic fluid and amnion depend on sodium concentration in those cavities, where sodium coming from the albumen through the amnion membrane is followed by water movement in the same direction.

The allantois, which is a membrane-defined cavity linked to the hind gut, performs two important roles: 1) it is the embryonic respiratory system; and 2) it serves as a repository of waste from the kidneys (Romanoff, 1960). As the embryo develops, the amount of nitrogenous waste accumulated in the allantois also increases, reaching its peak volume shortly after the middle of the incubation period. At that point, it reaches a volume corresponding to 8-10% of the original egg weight (Romanoff and Hayward, 1943).

The allantoic membrane can actively transport sodium against both electrical and concentration gradients. According to Hoyt (1979), this is an important feature because the allantois, containing large amounts of fluid and being normally hyposmotic as compared to the embryo, is the perfect osmoregulatory organ. Water resorption from the allantois naturally occurs late in incubation, but as investigated by Hoyt (1979), the embryo osmoregulates by adjusting water and solute exchange with the allantois. If osmolality of the embryo or amnion increases due to excessive water losses, sodium is pumped in that direction so water from the allantois will follow it. In the event of little water loss during incubation, the allantoic fluid will be reabsorbed only before internal pipping; so the allantoic fluid will be part of the amnion consumed by the embryo.

Hoyt (1979) conducted a study varying incubation humidity. He was able to create egg water losses varying from 4 to 16% of initial egg mass, but water content and dry matter of the yolk and amnion remained unchanged (Hoyt, 1979). Thus if egg weight was reduced but yolk and amnion remained unchanged, embryonic water content must have changed. According to Vleck (1991) avian embryos can change tissue hydration (less than 5%) without compromising tissue dry matter. In contrast, reptile embryos

change tissue synthesis without altering tissue water content (Vleck, 1991). Thus, avian hatchling weight can vary because of the amount of water in their tissues, whereas reptilian hatchlings reduce size by utilizing less yolk, which can be left with the shell at hatch.

Amniotic fluid influence on hatch

Correct formation and utilization of the amnion can be affected by several conditions. Other than incubation humidity, other factors like incubation temperature and egg turning can alter amnion characteristics.

Temperature affects the speed of embryonic development (Lourens, 2005). It is possible that because eggs incubated in higher temperatures mature faster, they will reach maximum amnion volume earlier, losing less water and leaving more water to be drained from other egg compartments into the amnion. In contrast, low incubation temperatures increases incubation time (Mortela, 2006), so it is possible that these eggs would have lost more water by the time amnion reaches its maximum volume. Research is necessary to confirm these hypotheses.

Improper egg turning or no turning will prevent the embryo from regulating water movement between egg compartments. It also reduces protein accumulation in the amniotic fluid, and decreases thyroid hormone levels (Tona et al., 2003; Tona et al., 2005). Non-turned eggs will have reduced amnion fluid volume (Romanoff, 1960), and they will have leftover albumen by hatch, while the embryo hatches in a dehydrated state and they are smaller due to unused albumen nutrients and water (Tona et al., 2005).

Proper accumulation of amniotic fluid is not only important for embryonic hydration but also because albumen proteins will be the substrate used for

gluconeogenesis so glycogen can be stored prior to hatch (Donaldson, 1995). Stored glycogen is the main energy source to fuel the hatching process and sustain the poult until feed intake initiation (Donaldson and Christensen, 1991; Foye et al., 2006). Anything that results in amnion reduction will also result in smaller glycogen reserves, causing embryos to struggle to hatch due to lack of energy. If embryos deplete their energy stores they may die during internal or external pipping stages. Extensive embryonic mortality is usually observed in turkeys between pipping and emergence (Christensen et al., 1993; Foye et al., 2006). Although some embryos may hatch, they have very low energy status and they are weak and dehydrated. This condition can be aggravated in poultry commercial operations by long holding periods and servicing stress (Donaldson et al., 1991). Weak and dehydrated poult are very common in turkey operations, resulting in higher mortality during brooding (Christensen et al., 2003). In the field these poult are known as “starve-outs” and “flip-overs”, which are characterized by poult that are too weak to eat and to turn back on their feet (Noble et al., 1999; Christensen et al., 2003).

The study of amniotic volume done by Romanoff and Hayward (1943) (Figure 6.1) was done with White Holland turkeys, which is no longer an economically important breed. Today the main turkey breeds utilized by the industry are Nicholas, Hybrid and British United Turkeys (BUT). Because the growth performance conformation of commercial turkeys of today is very different from the commercial turkeys 60 years ago (Havenstein et al., 2007), amnion volume and utilization are most likely different also.

As part of their preliminary IOF studies, Uni and Ferket (2003) determined amniotic fluid volume, pH and osmolality of Hibryd turkey eggs from 21E to 27E (Table

6.1). Based on the changes in amniotic fluid volume, the embryos finished consuming the amnion between 23E and 24E. The lower initial volume they observed at 21E (Table 6.1), as compared to the 8 mL reported by Romanoff and Hayward (1943), may be an indication that Hybrid embryos have already started amnion consumption when the study started. Moreover, the pH values observed by Uni and Ferket (2003) were generally lower than the values observed by Romanoff and Hayward (1943), who observed a decreasing trend in pH towards the end of incubation, from pH 7.6 at 14E to 7.0 at 24E. Apparently, the embryos observed by Uni and Ferket (2003) were near the end of amnion consumption (Table 6.1). These marked differences between the observations of Romanoff and Hayward (1943) and Uni and Ferket, (2003) validate the necessity for more studies on changes in embryonic anatomy among turkey strains. Therefore, a study of amnion volume, its physiological characteristics, and how it is consumed by the developing embryos was done on the three major commercial turkey strains (Hybrid, Nicholas and BUT). This information will be used to determine the constraints for *in ovo* feeding turkey eggs.

Table 6.1. Average volume, pH and osmolality of the amniotic fluid from Hybrid turkey egg from 21 to 24 days of incubation (Uni and Ferket, 2003).¹

Parameter	Days of incubation (E)			
	21E	22E	23E	24E
Volume (mL)	3.5	2.0	1.0	0.0
pH	6.5	6.5	7.3	-
Osmolality (mOsm)	322	322	318	-

¹Values are mean of 10 sampled embryos.

Turkey embryo and amnion survey

Materials and Methods

Separate experiments were conducted for each strain of turkeys, BUT, Nicholas and Hybrid, represented subsequently as BUT, NIC and HYB, respectively.

In experiment 1, 250 turkey eggs were obtained at 20E from a commercial hatchery¹, and incubated at the North Carolina State University Turkey Research Unit² in a Jamesway 252 incubator, set to 37.5C and 60% humidity. The eggs came from first-cycle BUT breeder hens in the 13th week of lay. Upon arrival, the eggs were candled to remove infertile eggs and dead embryos, and then they were divided into 5 groups of 25 eggs with similar weight distribution, ranging from 70 to 95g. Starting at 20E, 25 eggs were sampled every day at the same time until amniotic fluid could not be found. The eggs were carefully opened at the blunt end using surgical scissors, going through the air cell, piercing the allantoic membranes so the embryo could be removed from the shell, and placed with its attachments on a Petri dish, making sure that yolk sac and amnion membranes remained intact. The embryos were euthanized by cervical dislocation, and the amniotic fluid was aspirated using a graduated syringe and 23 gauge needles through the amniotic membrane. The embryo along with all other egg contents was weighed after removal from the shell. The amnion fluid was dispensed into a 10 mL graduated test tube from where volume was recorded, and its osmolality was determined using a Micro Osmometer³, and reported in milliosmoles (mOsm).

¹ Prestage Farms, Clinton, NC

² Lake Wheeler Rd, Raleigh, NC

³ Model 3300, Advanced Instruments, Inc., Norwood, MA

In experiment 2, 250 eggs from NIC were transported from the same hatchery¹ at 18E, weighed and divided into groups in the same manner as in experiment 1. Eggs from first-cycle hens in 12th week of lay ranged in weights from 70 to 95g. The eggs were incubated and sampled daily as in experiment 1, except each egg compartment was measured separately. Amnion and allantoic fluids volume were measured, as well as yolk sac weight detached from the embryo. Embryo free body weight (YFBW) was determined without any other attachments.

Amnion fluid and embryo serum osmolality was determined. To obtain embryo serum, the euthanized embryos without any attachments were individually ground to a homogenous mixture using commercial blender⁴. A 10g representative aliquot of this embryo homogenate was centrifuged at 1000 rpm for 5 minutes and the supernatant serum transferred to a new tube for osmolality determination. All osmotic measurements were performed using the same osmometer used in experiment 1.

Experiment 3 was conducted at the hatchery at Cold Springs Farm, Ltd.⁵ using eggs from a first-cycle flock of HYB turkey breeder hens in their 13th week of lay. At 18E, 200 viable eggs were weighted and distributed into 5 groups with similar weight distribution, ranging in weights from 60 to 95g. A group of 25 eggs was sampled every day starting on 20E as in experiment 1. Amnion fluid was aspirated, and to measure its volume and the embryo with all egg contents (except the shell) was weighted. Osmolality was not measured in this trial since an osmometer was not available on site, and also because osmolality of HYB strain have already been reported by Uni and Ferket (2003) (Table

⁴ Waring Commercial, Model 51BL31, Torrington, CT

⁵ Thamesford, Ontario, Canada

6.1). Amnion fluid as a percent of egg weight was calculated to give an idea of proportion of egg corresponding to amnion in each day of incubation.

Statistical Analysis

The experimental design in all three experiments was completely randomized. The data was analyzed by ANOVA using the general linear models procedure (proc GLM) of SAS (SAS Institute, 2004). Means were separated by least-square means test (ls means). Regression analysis was performed on data when considered appropriate.

Results

The effect of embryonic development on embryo weight, amnion fluid volume, and amnion fluid osmolality for BUT, NIC and HYB is presented on Tables 6.2, 6.3, 6.4 and 6.5, respectively. Egg weight was not significantly affected by day of incubation (Tables 6.2, 6.3 and 6.5) in all 3 strains. Even though a slight decrease in egg weight is expected due to moisture loss, the differences and sample sizes were not big enough to detect statistical significance. BUT and HYB embryos finished consuming amniotic fluid at 24E (Tables 6.2 and 6.5), while NIC amnion fluid could not be measured passed 23E (Table 6.3). Embryos from BUT did not show significant increase in weight during the period studied (Table 6.2), which may indicate that BUT embryos approached their full size by the time the experimental observations started. Amnion volume of BUT eggs significantly decreased from about 7.8 mL at 20E to near zero at 24E (Table 6.2), while osmolality of amnion fluid decreased about 10 mOsm in the same period (Table 6.2).

In experiment 2, NIC embryo size increased towards hatch, indicating that embryos of this strain only reach their full size at 23E (Table 6.3). All other egg fluids (yolk, allantois and amnion) decreased in volume during the period studied for NIC eggs

(Tables 6.3 and 6.4). Amnion fluid and embryo serum osmolality decreased at 21E, as compared to all other time points (Table 6.4). Embryos from HYB also increase in size during the studied period (Table 6.5). Amnion fluid volume and its proportion of egg weight decreased from 21E until it was almost totally consumed by 24E (Table 6.5).

Table 6.2. Effect of embryonic stage of development of BUT turkeys on embryo weight, amnion fluid volume, and amnion fluid osmolality.¹

Day of incubation	Egg (g)	Embryo ² (g)	Amnion (mL)			Amnion Osmolality (mOsm)		
			Mean	Min	Max	Mean	Min	Max
20E	83.24±5.54	64.01±5.13	7.752 ^a ±2.40	3.50	12.0	303 ^a ±13.8	292	346
21E	84.37±5.99	64.40±5.22	7.429 ^a ±2.30	2.25	13.1	296 ^a ±6.3	286	312
22E	83.29±6.93	63.89±4.17	3.498 ^b ±1.980	0.40	8.0	289 ^b ±2.6	284	295
23E	83.42±7.00	67.55±5.08	1.925 ^c ±1.79	0.20	6.7	287 ^b ±2.6	283	293
24E	83.96±7.17	68.59±4.58	0.248 ^d ±0.31	0	0.8	292 ^a ±3.4 ³	288	296
P-value								
	0.969	0.15	0.0001	-	-	0.0001	-	-

^{a,b,c,d}Means followed by different superscripted letters in the same column are statistically different (P<0.01).

¹Values are mean ± standard error of 25 sampled eggs per day of incubation.

²Embryo weight includes all egg contents including amnion, but without the shell

³Mean osmolality of amnion on 24E was based only on 4 replicate samples, which had sufficient quantity for measurement

Table 6.3. Effect of embryonic stage of development of NIC turkeys on embryo weight, yolk sac weight, and allantoic fluid volume.¹

Day of incubation	Egg (g)	Embryo ² (g)	Yolk sac (g)	Allantoic fluid (mL)
20E	85.58±6.21	27.13 ^c ±2.18	23.58 ^a ±2.28	4.43 ^a ±1.89
21E	84.64±6.00	30.12 ^{bc} ±2.18	23.37 ^a ±2.37	4.11 ^a ±2.04
22E	84.60±5.67	35.83 ^b ±3.58	22.68 ^a ±1.83	4.06 ^a ±1.72
23E	84.27±5.81	41.94 ^a ±3.75	22.04 ^b ±1.74	3.04 ^b ±1.63
P-value				
	0.877	0.0001	0.045	0.049

^{a,b}Means followed by different superscripted letters in the same column are statistically different (P<0.01).

¹Values are means ± standard error of 25 eggs sampled per day of incubation.

²Embryo weight does not include any other egg contents

Table 6.4. Effect of embryonic stage of development of NIC turkeys on amniotic fluid volume, amniotic fluid osmolality and embryonic osmolality.¹

Day of incubation	Amniotic fluid (mL)			Amniotic fluid osmolality (mOsm)			Embryonic osmolality (mOsm)		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
20E	10.96 ^a ±2.35	7.0	18.0	299.52 ^a ±6.51	288	309	310.30 ^a ±5.95	297	319
21E	10.70 ^{a±} 2.83	17.5	6.5	216.20 ^b ±4.99	208	227	234.78 ^b ±5.35	225	247
22E	6.20 ^b ±3.58	2.5	14.5	291.46 ^a ±10.11	283	335	304.68 ^a ±4.06	295	312
23E	1.44 ^c ±1.28	0.0	4.0	294.56 ^a ±9.92	279	319	317.68 ^a ±11.31	298	343
P-value									
	0.0001	-	-	0.0001	-	-	0.0001	-	-

^{a,b,c}Means followed by different superscripted letters in the same column are statistically different (P<0.01).

¹Values are means ± standard error of 25 eggs sampled per day of incubation.

Table 6.5. Effect of embryonic stage of development of HYB turkeys on embryo weight and amnion fluid volume.¹

Day of incubation	Egg (g)	Embryo ¹ (g)	Amnion (mL)			Amnion, % of Egg Wt.		
			Mean	Min	Max	Mean	Min	Max
20E	76.9±6.60	57.2 ^b ±5.27	10.4 ^a ±2.24	7.5	14.0	13.5 ^a ±2.32	10.0	18.4
21E	77.4±5.13	59.4 ^b ±4.94	8.03 ^b ±1.23	5.1	10.5	10.4 ^b ±1.37	7.9	13.3
22E	77.6±5.04	62.3 ^{ab} ±6.76	5.28 ^b ±1.70	2.2	8.0	6.8 ^b ±2.14	3.0	10.7
23E	78.6±5.34	66.1 ^a ±5.73	1.37 ^c ±1.19	0.1	5.4	1.7 ^c ±1.37	0.01	5.57
24E	77.4±5.39	66.0 ^a ±5.92	0.25 ^c ±0.19	0.0	0.6	0.7 ^c ±0.02	0.0	0.1
P-value								
	0.859	0.0001	0.0001	-	-	0.0001	-	-

^{a,b,c}Means followed by different superscripted letters in the same column are statistically different (P<0.01).

¹Values are means ± standard error of 25 eggs sampled per day of incubation.

Discussion

Amnion fluid volume of 20E BUT embryos ranged from a maximum of 12 mL to a minimum of 3.5 mL, with an average of 7.7 mL. The amnion fluid volume did not decrease significantly until after 21E. The embryos imbibed the amnion fluid at a rate of about 0.16 mL/hr from 21E to 22E and then at slower a rate of 0.07 mL/hr from 22E until 24E when negligible amnion fluid remained (Table 6.2). Because amnion volume is a function of egg or embryo size, the changes in amnion fluid volume during the later stages of embryonic development is best expressed relative to egg weight (Figure 6.2). The amnion comprised about 9% of the total egg weight at 20-21E, which agrees with the proportion described by Romanoff and Hayward (1943) at the time of the second and maximum peak of amnion volume. During the following 24 hr period, over half of the amnion was consumed. Indeed, some of the 22E eggs had as little as 0.4 mL, while others had as much as 8 mL (Table 6.2). Apparently by 23E, most of the embryos had consumed either all or nearly all of the amniotic fluid. At 22E, some embryos may only begin imbibing the amnion while at 24E most of the embryos have completed the process of consuming the amnion and begun to pip into the air cell.

According to the results of this survey, the best time to inject an IOF solution in BUT eggs would be between 22E and 24E, with an optimum injection time at 23E. As the embryo consumes the amniotic fluid and his body size increases, the capacity to accept the IOF solution also changes. Assuming the maximum capacity of about 9 mL of amnion fluid, as observed in over 80% of the eggs at 20E, all eggs have a capacity to accept a maximum volume of 1 mL of IOF solution at 22E. By 23E, the embryos consumed at least 25% of the amnion, but its body size increased only 5%, leaving a

capacity of at least 20% of the initial amniotic volume at 20E. Thus, the 23E BUT embryo should be able to accept at least 1.55 mL ($7.75 \text{ mL} \times 0.20$) of IOF solution without compromise in an egg weighing an average of 83g (about 1.85% of total egg mass). Based on BUT amnion disappearance data, the embryo consumes the amnion at a rate of 0.07 mL/hr at 23E, so 1.55 mL of IOF solution would be fully consumed within 21 hr, well before internal pipping starts. Although the capacity of the amnion sac at 24E may exceed 2mL of IOF solution, the embryos would likely be unable to consume it all before internal pipping begins.

Osmolality of the amniotic fluid is an important factor that controls the fluid balance of the embryo (Romanoff 1960; Vleck, 1991). If the osmolality of the amnion exceeds the osmolality of the body fluids of the embryo, the embryo will dehydrate and may be unable to proceed with the hatching process. For that reason the fluid of the allantois can be moved to and from the amniotic sac by pumping sodium to keep amnion fluid osmolality fairly unchanged during incubation (Hoyt, 1979). The osmolality of the amniotic fluid from BUT eggs ranged from 292 mOsm to 346 mOsm with an average of about 300 mOsm at 20E (Table 6.2), then decreasing to 285 mOsm by 23E and then back to 292 at 24E. Even though this variation (3.3%) was statistically significant, it still confirms Hoyt's observation of small changes in amnion osmolality.

In experiment 2, embryonic weight significantly increased relative to egg mass by a polynomial function ($y=0.895x^2-32.391x+321.53$, $R=0.9992$) by day of incubation (x). Amnion volume of 20E NIC embryos averaged about 11 mL (Table 6.4) and did not decrease in volume until after 21E, as among BUT embryos observed in experiment 1. This peak volume (18 mL maximum) is much greater than amnion volumes reported in

past literature (Romanoff and Hayward, 1943; Romanoff, 1960; Romanoff, 1967; Hoyt, 1979; Vleck, 1991), but it could be explained by an association of bigger egg size and strain characteristics. Nevertheless it is an important strain difference to consider, since it can influence embryonic survival and poult quality. Following the same logic as discussed above for experiment 1, NIC embryos would have to consume amniotic fluid faster and grow more during these later stages of development to hatch at the same time as BUT embryos. Indeed, NIC embryos consumed about 40% of its amniotic fluid by 22E (Table 6.4), and grew about 18% (Table 6.3), leaving a capacity of about 20% of the amnion realized at 20E-21E. Thus, NIC embryos should be able to accept at least 2 mL (11×0.20) of IOF solution without compromise in an average egg weighting 85g (2.3% of total egg mass). Based on data on Table 6.4, NIC embryos consumed the amnion at a rate of 0.13 mL/hr at 22E, so they would take 16 hours to consume 2 mL of IOF solution. Even if IOF would be administered at 23E, the NIC embryos would still be able to consume it before pipping into the air cell. However, it must be noted that in this trial NIC embryos finished consuming the amniotic fluid by 23E, so IOF may have to be performed earlier than 23E for NIC embryos to receive it at the same physiological time of amnion consumption as BUT.

Amnion fluid osmolality of NIC embryos ranged from 216 to 299 mOsm, which corresponds to a 28% variation in the studied period. This greater variation in amnion fluid osmolality observed among NIC than BUT embryos may indicate NIC embryos manage osmotic balance differently. The lower amnion osmolality (21E) was observed among NIC embryos around the time of highest allantoic fluid volume (20-22E), which in this case was also the point of lowest embryonic serum osmolality (21E), and then

osmolality of both egg compartments increased concomitant with allantoic fluid decrease (Tables 6.3 and 6.4). This apparent correlation between osmolality and water movement between egg compartments agrees with Vleck (1991) who proposed that the embryos must create an osmotic gradient to drain allantoic fluids prior to hatch, even when egg water is abundant.

The maximum osmolality limit of the IOF solution can be estimated mathematically based on amnion volume limits determined herein, and the osmolality values of amniotic fluid and embryo serum. In the case of BUT eggs, where embryo serum osmolality was not determined, the maximum observed amnion osmolality of 346 mOsm was assumed to be also the maximum possible serum osmolality value. An equation was derived based on the idea that amnion osmolality must be less than embryo serum osmolality to prevent embryo dehydration. Amnion fluid osmolality is due to a proportion of residual fluid and IOF solution osmolality. The equation produced to estimate IOF solution osmolality was:

$$\text{IOFO} < [\text{EO}(\text{AV}_x + \text{IOFV}_x) - \text{AV}_x(\text{AO}_x)] / \text{IOFV}_x \text{ (Equation 1),}$$

where: IOFO = *in ovo* feeding solution osmolality; EO = embryo blood serum osmolality; AV_x = residual amnion fluid volume at day x; AO₂₃ = osmolality of residual amnion fluid at day x; and IOFV_x = maximum volume of *in ovo* feeding solution that can be administered at day x.

Using the equation 1 above, BUT IOFO must be less than 427 mOsm for an IOF solution to be safely injected into the amnion of a 23E embryo, assuming embryo blood serum of 346 mOsm, maximum volume of 1.5 mL, and remaining 2 mL amnion fluid of 285 mOsm. The same equation applied for NIC eggs injecting 1.5 mL of IOF solution at

22E, with EO = 310 mOsm, EO = 7.5 mL, AV = 6 mL, and AO = 245 mOsm, will give an IOFO value that should be less than 570 mOsm.

As observed among NIC embryos, the weight of HYB embryos significantly increased with embryonic age (Table 6.5). Amnion volume was greatest at 20E and decreased linearly thereafter at a rate of 0.10 mL/hr. In contrast to the other 2 strains that were observed to begin consuming the amniotic fluid after 21E, the HYB embryos started consuming the amnion after 20E (Table 6.5). HYB embryos are known by the industry to develop at a faster rate and have shorter incubation period than BUT and NIC embryos. Apparently, HYB embryos started consuming the amniotic fluid earlier completely consumed the amnion fluid by 24E, as observed in NIC embryos (Table 6.5). This observation may explain why Uni and Ferket (2003) found lower amnion fluid volumes at 21E (Table 6.1) than herein observed for BUT and NIC. Based on the data observed in experiment 3, the optimum time to administer an IOF solution for HYB embryos would be between 22E and the first half of 23E. Thus following the same discussion logic as used in experiment 1, the maximum capacity of the amnion for HYB eggs is assumed to be 10 mL, as observed at 20E (Table 6.5). By 22E the embryos consumed at least 30% of its amniotic fluid and increased body weight by about 15%, leaving a capacity of about 15% of its total amnion capacity realized at 20E. Thus, 22E HYB embryos should be able to accept at least 1.5 mL (10×0.15) of IOF solution without compromise in an average egg weighting 77 g (1.9% of total egg mass). With the embryo consuming the amnion at a rate of 0.10 mL/hr at 22E, 2 mL of IOF solution would be fully consumed within 15 hr, which is before internal pipping starts at 23E.

There were marked differences in egg size between breeds, and egg size influences embryo weights and amnion volume. Therefore amnion volume must be expressed relative to egg mass to properly compare amnion consumption patterns among the different turkey strains. The results of amnion volume adjusted for egg size of all three strains is presented on Figure 6.2. The HYB (BUT) embryos had the highest amnion volume relative to egg size among the three strains, and that they were already consuming the amnion at 20E, while the other 2 strains began consuming the amnion only after 21E. Amnion consumption is best predicted as a function of hours of incubation (Figure 6.2). Therefore, the best time for IOF solution administration would be between 520 and 560 hr (22-23E) of incubation, independent of strain. The maximum volume that can be injected without risk to any of the breeds would be 1.5 mL and optimum IOF solution osmolality would be 450 mOsm and not higher than 570 mOsm.

More research needs to be done to confirm the impact of amnion volume and consumption patterns on hatchability and poult quality of the different commercial turkey strains. The volume and osmolality constraints for IOF administration determined herein also needs to be confirmed by further experimentation. The relationship between water movement among egg compartments and the embryo should also be established by measuring solute (sodium) movement between egg compartments.

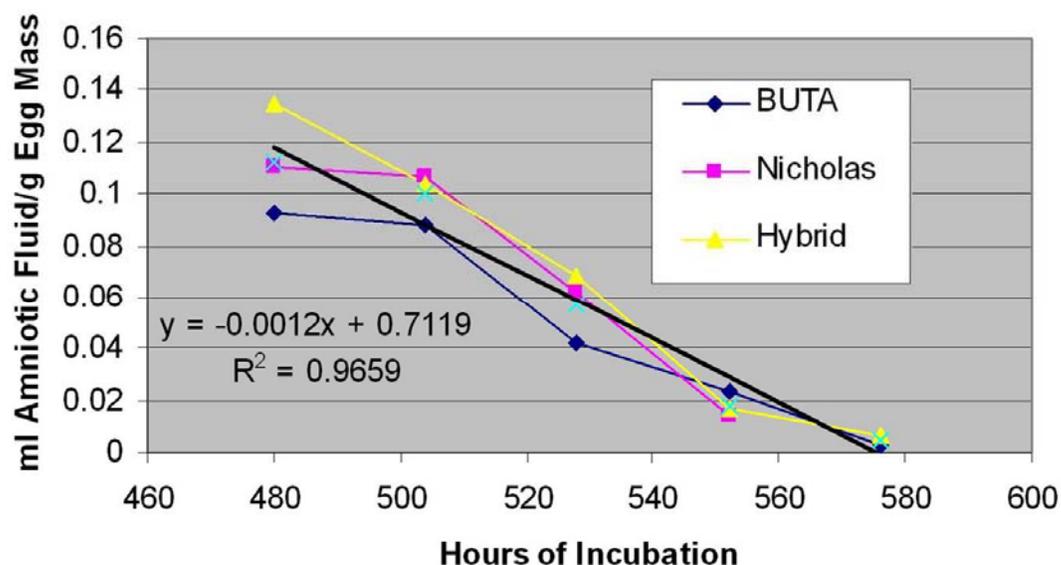


Figure 6.2. Effect of embryonic stage (age) of development of turkeys from different strains on amnion fluid volume relative to total egg mass (mL amniotic fluid/g egg mass)

Conclusion

Amnion accumulation and consumption, as well as its characteristics were affected by genetic selection in the last 60 years. Amnion volume peaked at 20 days of incubation and was consumed by the embryos around 21E at a rate of 0.10 mL/hour. Amnion fluid and embryo serum osmolality averaged around 300 mOsm. Osmolality limit of IOF solution can exceed amnion osmolality, but the amnion plus IOF solution osmolality can not exceed higher the observed embryonic serum osmolality. When corrected for egg mass, amnion fluid consumption pattern was similar for all three major turkey breeds commercially available. Breed differences may be considered when injecting turkey eggs with solutions into the amnion, with general recommendations being maximum volume of 1.5 mL at 23 days of incubation and osmolality of 450 mOsm.

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Chapter 7

“Survey of energy metabolism and intestinal development gene expression in late-term turkey embryos using microarrays”

Abstract

Microarray technology was used to identify differences in gene expression of key genes of the metabolism of turkey embryos from 20 days of incubation (E) until hatch. The objective of this study was to map development of turkey embryos incubated under standard conditions. The information from this study could be used to better understand changes and shifts in metabolism that happens during the perinatal period and to serve as a baseline for future incubation and *in ovo* manipulation studies. A customized two-color focused oligonucleotide microarray was designed to annotate 96 chicken gene sequences. This array was utilized to study turkey embryo gene expression of liver, pectoral muscle, hatching muscle, and duodenum. The turkey gene sequences successfully hybridized to chicken sequence probes in all four tissues. Gene expression data was utilized to determine gene expression patterns for all genes that significantly changed in the studied period. The gene expression patterns in the liver showed that the embryo switches from lipid to carbohydrate metabolism at 22E. Both enzymes responsible for glycogen synthesis and glycogen degradation follow the same pattern gene expression in liver and muscle. Genes of enzymes associated with carbohydrate digestion in the intestine begin to be expressed 48hrs before hatch, while nutrient transporter gene expression peaked at hatch. Based on these data gluconeogenic compounds may be good candidates to be fed *in ovo* after 22E, but digestion capacity of complex carbohydrates may not be developed

by the time the embryo consumes the amniotic fluid. These results will also be useful as baseline data for future research involving turkey embryo development.

Introduction

The importance of poultry production has been growing tremendously. In 2006 the United States alone produced 10.9 billion broiler chickens and 261.9 million turkeys (USDA, 2007). This progress is based on fast application of current and new technologies (Smith, 2006). The turkey industry alone has grown 70% since 1985 (Schaal and Cherian, 2007). To ensure continuous success and sustainability, it is necessary to identify and solve problems that affect production efficiency and animal welfare. One problem that affects both production efficiency and animal welfare in the turkey industry is poor hatchability and poult quality, which has not improved much during the last 20 years (Schaal and Cherian, 2007).

High embryonic mortality during the late stages on incubation has been identified, to occur between internal pipping and egg emergence (Christensen et al., 1993; Foye, 2005). Little is known about what metabolic perturbations cause late-term embryonic mortality. However, since the completion of the chicken genome (International Chicken Genome Sequencing Consortium, 2004) new doors of possibilities has opened to study avian biology (Lamont, 2006), particularly during periods of early development. Even though turkey sequences are not available yet for most genes, there is a chance that both species, chicken and turkeys, will be similar enough so chicken target sequences could hybridize to turkey probes. Investigation of gene expression during critical periods of development could accelerate the identification of metabolic distress points that affect early growth performance and viability. Previous molecular approach methods were very

time consuming, expensive and subject to many causes of variation (Harris, 2000; Wiseman, 2002; Yadetie, et al., 2004).

The advent of microarrays revolutionizes the understanding of biological systems (Garosi et al., 2005). Microarray is a technique that permits studying thousands of genes in a single experiment, or even the entire genome at once (Harris, 2000; Spielbauer and Stahl, 2005). This method consists in having the desired sequences of genes fixed on a surface, usually a glass slide or chip, and then hybridizing them with complementary cDNA obtained from the samples to be tested. The single stranded cDNA sample sequences are labeled with a fluorescent dye (target) and during hybridization they base-pair to DNA sequence on the array (probe) if complementary to each other (Spielbauer and Stahl, 2005). Fluorescence is then measured through laser scanning, so image color intensity can be used to compare samples (Harris, 2000). The higher the amount of hybridized labeled sample attached to the probe, the higher the color intensity for that spot on the array. The high throughput is possible because microarrays are based on miniaturization of gene printing patterns that can only be produced by automation. Whole genome arrays have their sequences synthesized on the array surface, while focused arrays can have cDNA sequences synthesized and then printed on the surface as spots. Focused arrays have become very popular in academic studies because they can be customized as desired and are not as costly as whole genome arrays (Spielbauer and Stahl, 2005). If the objective of the study is to compare gene expression of samples from different conditions, they can be labeled with different fluorescent dyes and then hybridized together on the same array. The most popular of these methods is the two-color arrays, where two samples, one labeled with Cy3 (cyanide 3-NHS ester) and the

other with Cy5 (cyanide 5-NHS ester), are being compared (Harris, 2000; Spielbauer and Stahl, 2005). Two-color arrays are scanned with two separate laser settings, one for each cydye, so color intensity differences between the two conditions can be compared (Gibson, 2003; Garosi et al., 2005).

The objective of this study was to produce a customized focused array containing key genes of metabolic importance based on chicken genome information, and utilize it to map the development of turkey embryos during the critical period from internal pipping to hatch. This information could be used to better understand normal development and to identify changes that occur during developmental perturbations or during various interventions during incubation or *in ovo*.

Materials and methods

Incubation and sampling

Two hundred fertilized turkey eggs from the same breed were obtained from a commercial hatchery¹. Upon arrival the eggs were weighted and divided into groups with similar weight distribution. The eggs were incubated in a Jamesway 252 incubator, set to 37.8C of temperature and 60% humidity. A group of 25 eggs were sampled at 20, 22, 24 and 26 days of incubation (E) and at day of hatch (28E), respectively. Each egg was opened at the blunt end of the egg using surgical scissors, and then the embryo was extracted from the shell and euthanized by cervical dislocation. Each embryo had liver, duodenum, pectoral muscle and hatching muscle removed (500 samples total), dissected

¹ Prestage Farms, Clinton, NC

to be 200 mg in weight and immediately placed on labeled 7 mL scintillation vials² previously filled with 2 mL of RNA Later³ kept at room temperature. Tissue samples were stored at -20C until processed for RNA extraction. The remaining of the liver and muscle samples were immediately place on ice and stored at -20C for posterior glycogen determination. All sampling procedures were performed in a clean laboratory facility with surfaces and instruments wiped with RNaseZap⁴ decontamination wipes. To cross validate gene expression data with poult energy status, liver, pectoral muscle and hatching muscle samples were also used to determine glycogen concentration [GLY], using the modified iodine binding method described by Foye et al. (2006).

RNA extraction and quality control

The tubes containing tissue samples were thawed and 100g of tissue was weighted and placed on a 2 mL microcentrifuge tube filled with 1 mL of TRI Reagent⁵. Muscle sample tubes were also filled with 0.3 mL of 0.1 mm glass beads to improve disintegration. The tubes were shaken in a 45 tube block using a Mini-Beadbeater-96⁶ for 1 minute or until tissue samples were completely dissolved. When more than one cycle was needed, the block was placed on ice for a minute in between cycles to avoid sample overheating. The tubes contained beads had the homogenate transferred to a new tube. Each tube received 300 µl of chloroform and then vortexed for 10 seconds. Homogenate tubes were centrifuged at 12,000g for 5 minutes at 4C to separate phases. The top aqueous phase was carefully transferred to a 1.5 mL microcentrifuge. Each tube received

² Fisher Scientific, Pittsburg, PA

³ Ambion, Inc., Austin, TX

⁴ Ambion, Inc., Austin, TX

⁵ Molecular Research Center, Inc., Cincinnati, OH

⁶ Biospec, Inc., Bartlesville, OK

500 μ l of molecular biology grade 100% ethanol, inverted 5 times and then placed in a -20C freezer for 30 minutes. After that, the samples were centrifuged at 12,000g for 15 minutes at 4C to form RNA pellets. The supernatant was discarded and the RNA pellets was washed with 500 μ l of 75% ethanol and centrifuged again at 12,000g for 10 min at 4C. The ethanol was discarded and the pellets were let to dry for 5 minutes under the hood. Air dried pellets were resuspended in 30 μ l of nuclease-free water. Extract RNA was quantified using a ND-1000 spectrophotometer⁷ and RNA integrity was verified by electrophoresis on 1.5% agarose gel. The same amount of RNA from 6 samples from same tissue and day of incubation were pooled together in one tube and adjusted to 0.5 μ g/ μ l of concentration. Tubes containing RNA were stored in a -80C freezer when not being manipulated and kept on ice during manipulation.

Microarray manufacturing

A focused microarray was designed to contain specifically chosen long oligonucleotides, and it was customized for gene high replication number. Seventy base-pair oligonucleotides (oligos) were designed for 90 unique gene sequences selected from the chicken genome (Chicken Genome Database, 2004), including 41 genes from carbohydrate metabolism (glycolysis, gluconeogenesis, TCA and pentose phosphate pathways), 13 genes from glycogenesis and glycogenolysis pathways, 11 genes from lipid metabolism, 18 genes from hormone related metabolism and 7 intestinal enzymes and nutrient transporters. The oligos were manufactured by Operon Biotechnologies, Inc.⁸. The synthesized oligos were added to a 384 well plate where the oligo present in each well position was recorded. To the 90 oligo sequences present on the plate, 6 more

⁷ NanoDrop Technologies, Inc., Wilmington, DE

⁸ Operon Biotechnologies, Inc., Germantown, MD

wells were used for 2 housekeeping genes (GAPDH and chEF2), one plant gene (atCAB2-arapdopsis), one bacterial gene (chBACT) and turkey genomic DNA (2 wells), to serve as internal controls, totalizing 96 genes in the array⁹. The oligos were dried and resuspended in array spotting solution, and then printed (spotted) on UltraGAPS™ Amino-Silane Coated Slides¹⁰ to produce all the slides used in this study. The slides were printed on a VersArray Chipwriter Compact Arrayer¹¹ using 1 pin.

A Cy5 test scanned image of the spotted array can be seen on Figure 7.1. Each used well on the plate was spotted twice (side-by-side) and repeated 4 times on each slide, totalizing 8 spots per gene per slide (technical replicates). The array printer software generated a spot ID file used to produce the annotation file for the array. After printed, the slides were let to dry in the printer for 24 hours and then crosslinked on a ultraviolet oven set to 6000 x 100 $\mu\text{J}/\text{cm}^2$ (UVP, Inc., Upland, CA), and then stored on a dust-free dehumidifier chamber until used.

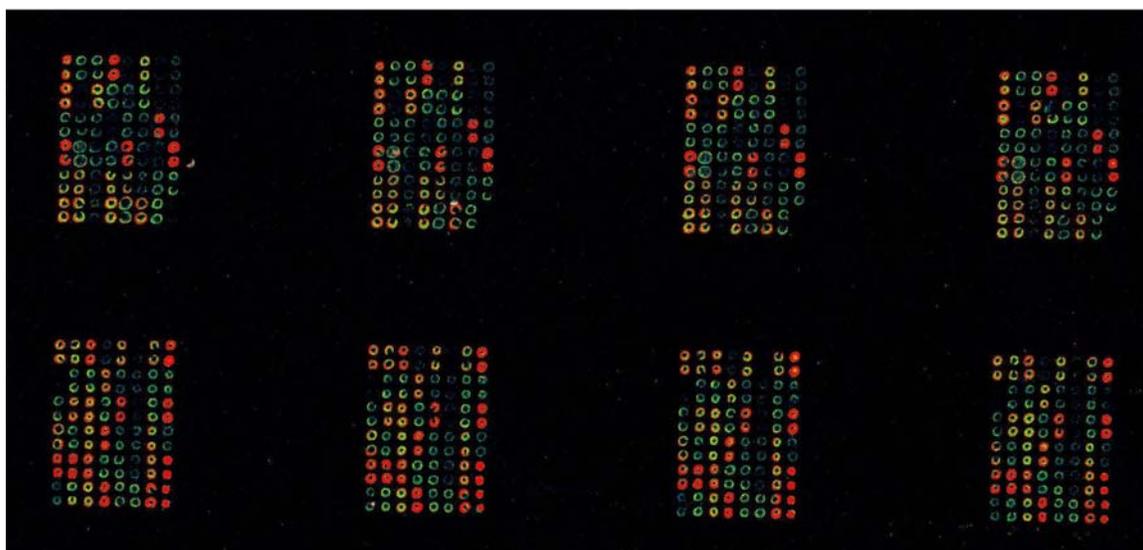


Figure 7.1. Scanned image of a Cy5 sample using the microarray produced in this study

⁹ For complete list of genes on the array consult appendix 3

¹⁰ Corning, Inc., Acton, MA

¹¹ Biorad, Inc., Waterloo, Ontario, Canada

Sample labeling

Tissue RNA pooled samples were used to produce cydye labeled cDNA, using the indirect labeling technique. ChipShot™ Indirect Labeling and Clean-up System¹² kits were used to produce aminoallyl-cDNA, which was labeled either with Cy3 or Cy5 fluorescent dyes¹³ according to the experimental design. Samples from each day of incubation were labeled twice with each cydye using different pools of RNA. All laboratory protocols were followed as recommended by the kits' manufacturer manual.

Hybridization and image scanning

Pre-hybridization and hybridization of array slides were performed utilizing the Pronto Plus! Microarray Hybridization Kit¹⁴. The labeled cDNA from two distinct time point samples were mixed, dried and resuspended in hybridization solution. The cDNA mix was then applied to pre-hybridized slide covered with a pre-cleaned glass coverslip¹⁵, and hybridized overnight. The same procedure was repeated with all pair of samples according to the experimental design, assuring the samples hybridized together were also labeled together. Microarray slides were scanned on a ScanArray G_X PLUS Microarray Scanner¹⁶ set to 65% laser power. All sample and slide manipulation from labeling until slide scanning was performed while protecting them from light.

¹² Promega, Madison, WI

¹³ Amersham Biosciences Corp., Piscataway, NJ

¹⁴ Corning, Inc., Acton, MA

¹⁵ Lifterslip, Portsmouth, NH

¹⁶ PerkinElmer Life and Analytical Sciences, Shelton, CT

Array data processing and statistical analysis

The experimental design was a complete interwoven loop design (Garosi et al., 2005). Generated data files were converted into image intensity raw data files for each slide-dye combination using ScanAlyze Software (Eisen et al., 1998). Raw data files were joined, transformed to a \log_2 base and analyzed in JMP Genomics (SAS Institute, 2007). The \log_2 -transformed data for all spot measures were subjected to loess normalization according to the model,

$$Y = \mu + A + Dye + (A \times Dye) + e,$$

where μ is the sample mean, A is the effect of the array, Dye is the effect of dye, $(A \times Dye)$ is the array-dye interaction (channel effect), and e is the stochastic error. The residuals from this model were analyzed by mixed ANOVA according to the gene-specific model,

$$Y = \mu + E + Dye + Hyb + e,$$

with day of incubation (E) and cydye (Dye) as fixed effects, hybridization batch (Hyb) as a random effect. Mean intensities were compared using False Discovery Rate (FDR) at $P < 0.01$. Results were used to produce clustering plots and parallel plots.

Embryonic growth and glycogen concentration statistical analysis

Embryonic growth and [GLY] followed the same experimental design, which was completely randomized. Data was analyzed by fit model procedure in JMP (SAS Institute, 2005), according to the model,

$$Y = \mu + E + e,$$

where day of incubation (E) was the fixed effect. Treatment means were compared by Tukey's test ($P < 0.05$) and regression analysis modeling.

Results and Discussion

Embryonic development and energy status

The weight of embryos and sampled tissues were plotted and illustrated Figures 7.2, 7.3, 7.4, 7.5 and 7.6. Even though quadratic or exponential curves could statistically fit the data in some of these plots, linear regression curves were used instead because they made better biological sense to help identify different rates of growth. Embryonic growth increased linearly from 20E until hatch at a rate of more than 11g/day (Figure 7.2). This fast rate of growth was expected since it is the last stage of growth for the developing turkey embryo. During the same period, the yolk sac is used as a nutrient source, so it is disappearing at a rate of 2.62g/day in an opposite manner of embryo weight (Figure 7.3). Liver and pectoral muscle tissues weights increased linearly from 20E until hatch (Figure 7.4). However, the duodenum and hatching muscle had distinct phases of growth (Figures 7.5 and 7.6). Duodenum mass increased at a rate of 0.0084 g/day from 20E until 26E, when it's mass increased 7 fold from 26E until hatch (Figure 7.5). This sudden change in duodenum growth rate around 26E indicates that this is when gut maturation accelerates so it will be functional by hatching, as observed in chick embryos by Uni et al. (2003).

The hatching muscle growth was divided in 3 distinct phases: 20-24E, 24-26E and 26-28E. The first phase, describing hatching muscle growth before preparation for pipping starts had a growth rate of 0.045 g/day. Between 24-26E, hatching muscle growth rate increased 4 fold in preparation for internal pipping; but after 26E, it virtually stopped growing probably because it reached its full size (Figure 7.6). General increase in tissue

growth around the time of internal pipping is part of embryo's preparation to hatch. During this period the embryo also accumulates nutrients into tissues (coming from other egg structures) and general organ maturation is promoted as described by Moran (2007). The hatching muscle development observed in this study follows a pattern previously described for chicken embryos (Romanoff, 1960; John et al., 1987; Moran, 2007).

The results of glycogen analysis of liver, pectoral muscle and hatching muscle are presented on Figures 7.7, 7.8 and 7.9, respectively. These data were submitted to regression analysis starting from 22E, which is the peak of glycogen accumulation, until hatch. [GLY] data from 20E was included in the plots to show that maximum glycogen storage has not yet happened. Glycogen utilization could be described as linear in all studied tissues, decreasing at a rate of 1.27 mg/day in the liver, 0.0847 mg/day in pectoral muscle, and 0.2076 mg/day in the hatching muscle (Figures 7.7, 7.8 and 7.9, respectively). According to these data, liver and pectoral muscle reached their peak of [GLY] between 20 and 22E (Figures 7.7 and 7.8), while the hatching muscle peaked later, between 24 and 26E (Figure 7.9). The later peak of [GLY] in hatching muscle indicates that this tissue had not yet reached its full size until 26E (Figure 7.6). The linear decrease in [GLY] in breast and hatching muscles indicates that these tissues demanded continuous supply of energy, what can be associated to muscle contraction during pipping and hatching.

Foye (2005) compared the accumulation of glycogen and its use in these tissues by the avian embryo to an athlete that prepares to run a marathon. The rate of glycogen utilization is greatest in the liver, which was 6 fold above hatching muscle and 15 fold higher than pectoral muscle, followed by hatching muscle, which was 2.5 fold higher

than in pectoral muscle. These differences in glycogen use rate suggest that liver must supply glucose to maintain energy homeostasis in the whole organism (Krebs, 1972), and by intense activity of hatching muscle during pipping and hatching as compared to pectoral muscle participation in emergence from the shell. By combining the data from tissue growth and energy accumulation as glycogen, an intricate pattern of physiological events can be seen. It is then clear why embryos are so sensitive to adverse conditions during this critical period of development. If tissue growth, energy stores or energy use are compromised, the embryo may not be able to emerge from the shell and it will die in the process.

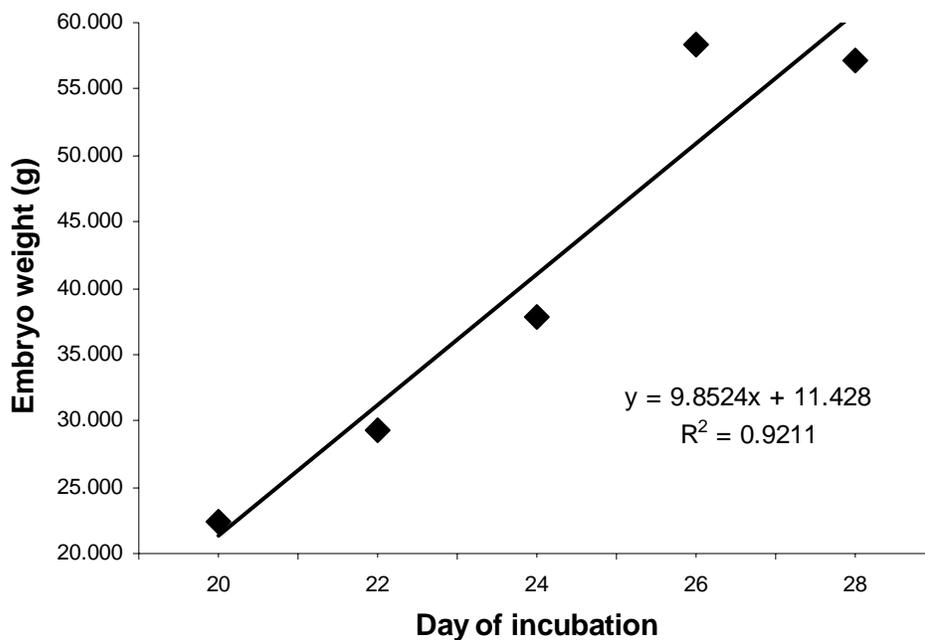


Figure 7.2. Turkey embryo growth during the last week of incubation

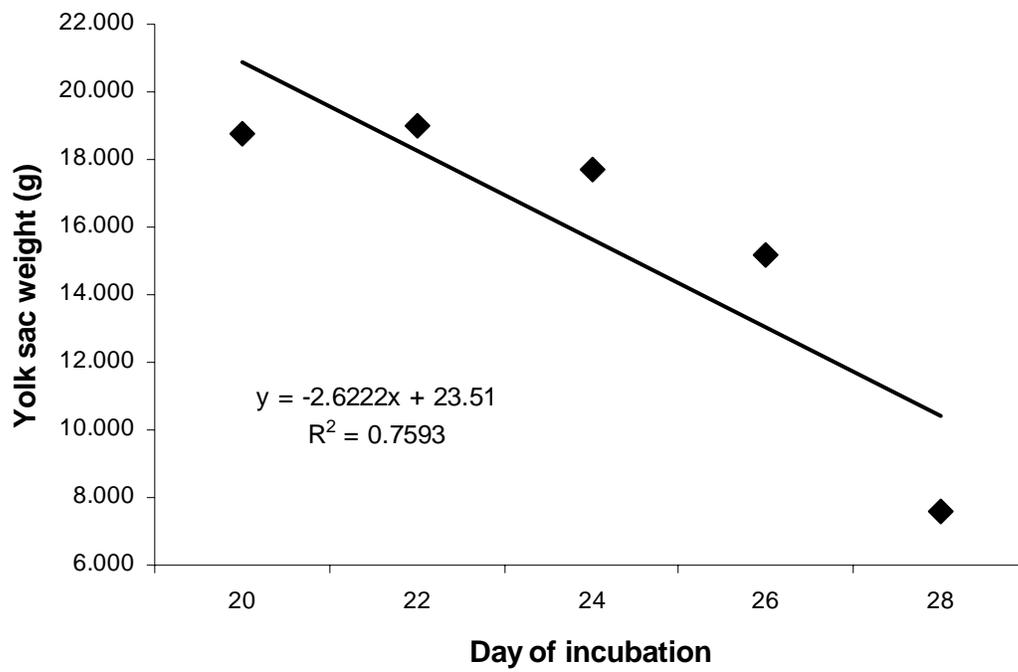


Figure 7.3. Turkey embryo yolk sac weight during the last week of incubation

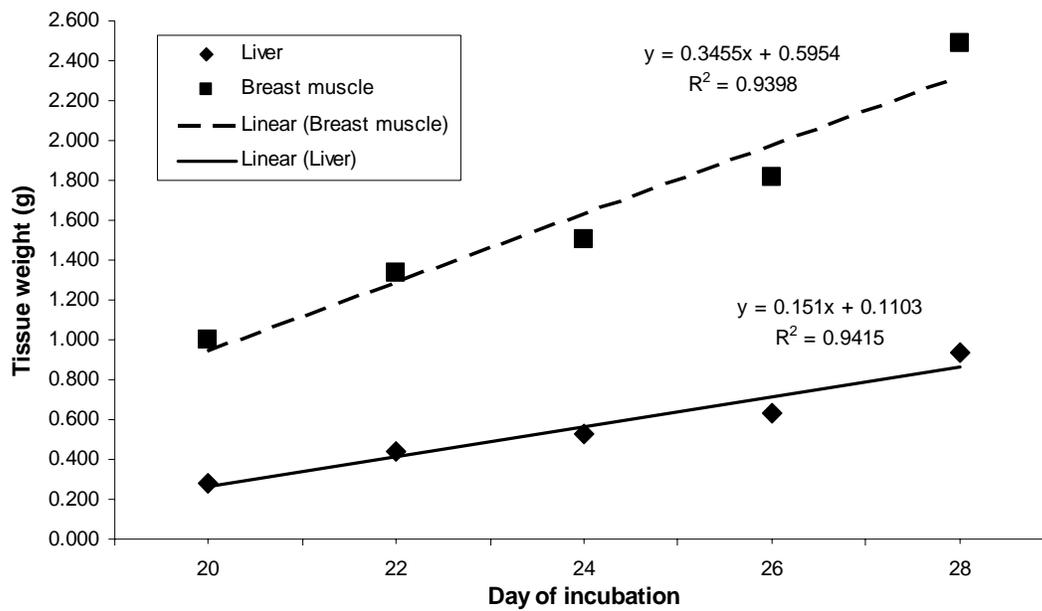


Figure 7.4. Turkey embryo tissue development during the last week of incubation

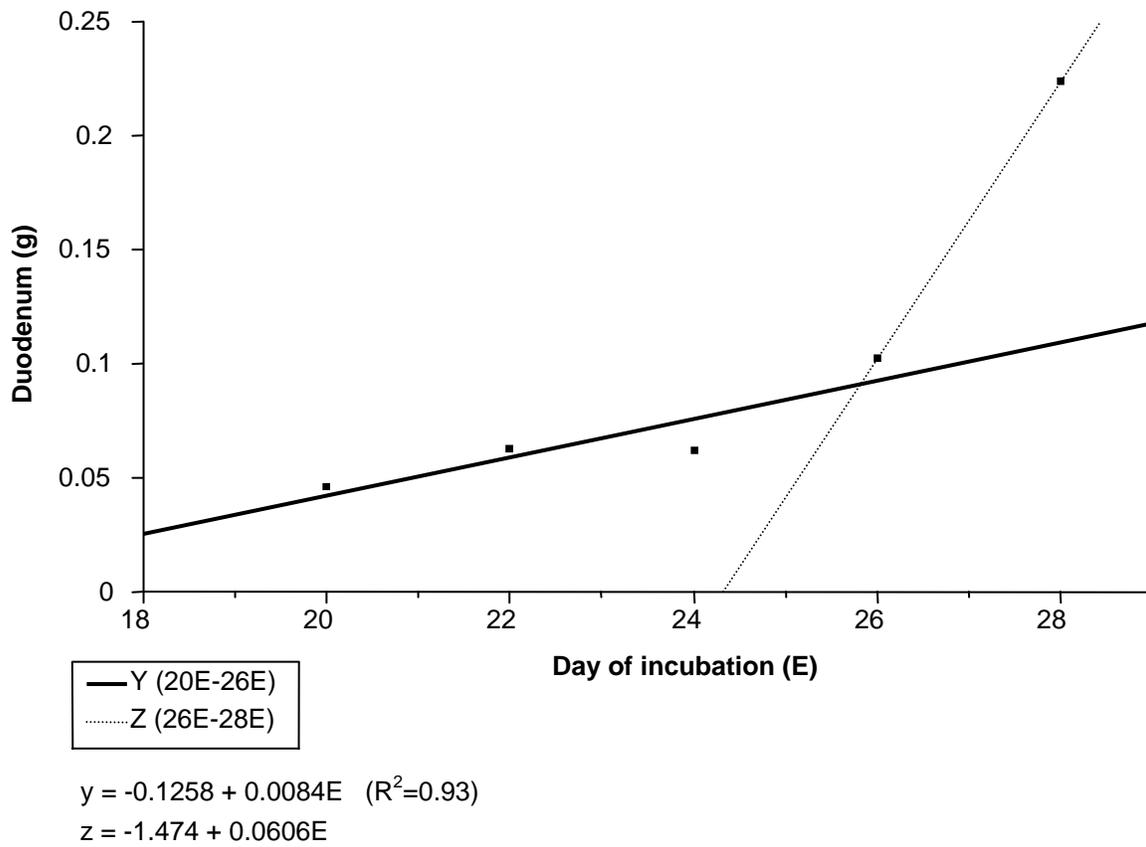


Figure 7.5. Changes in turkey embryo duodenum mass during the last week of incubation

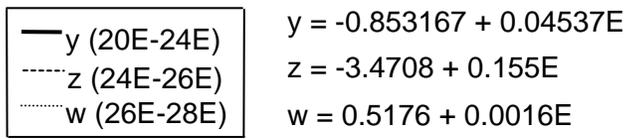
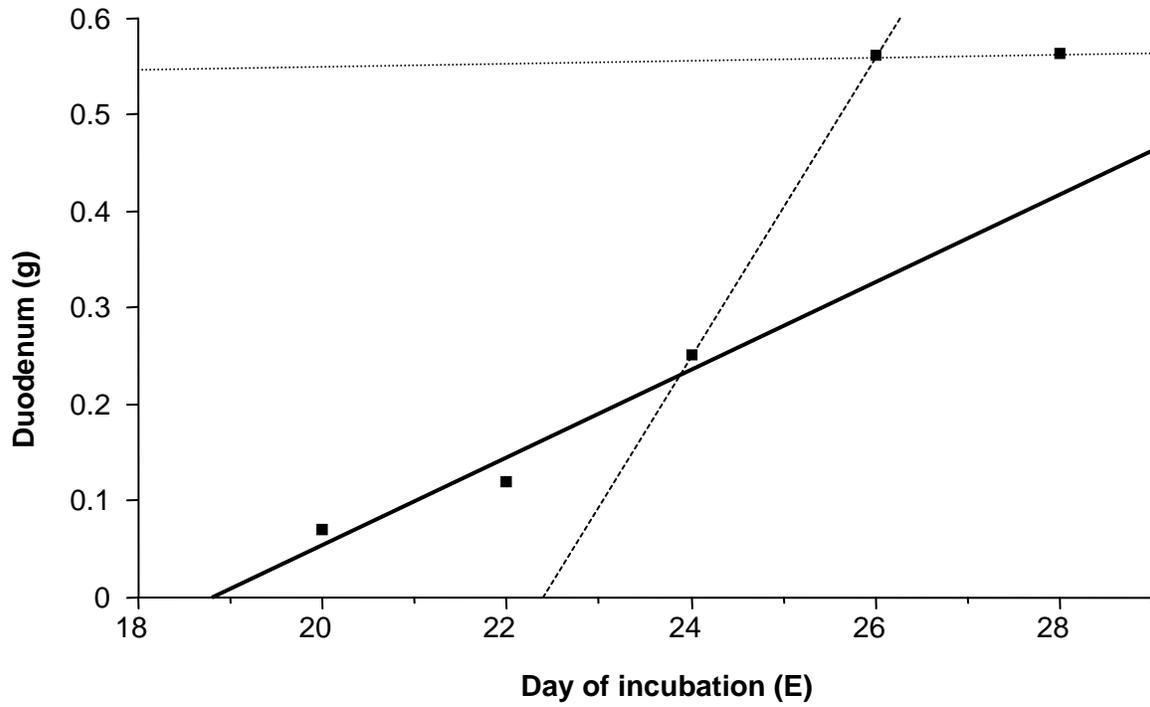
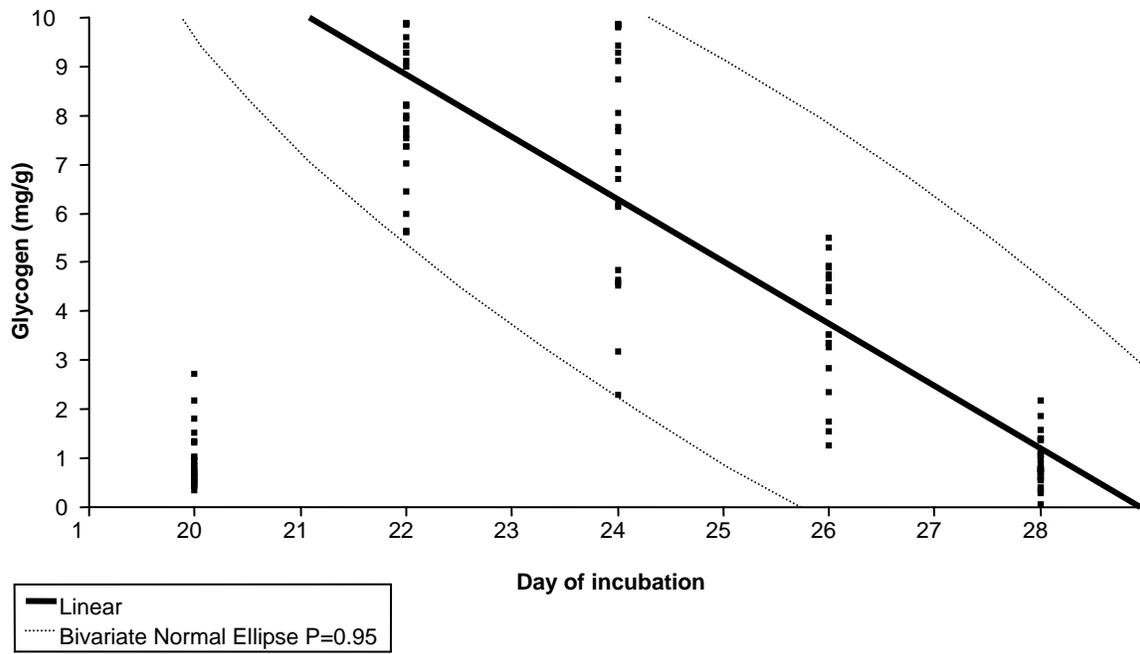
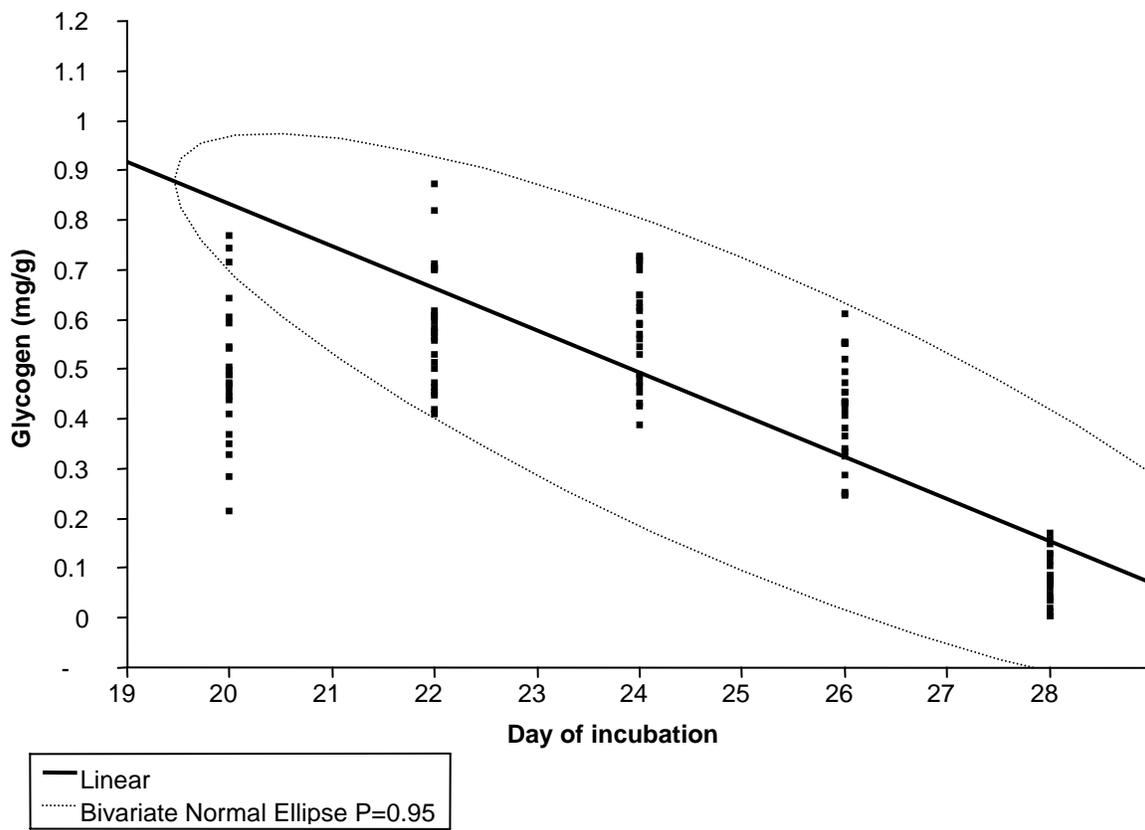


Figure 7.6. Turkey embryo hatching muscle growth during the last week of incubation



Glyc = 36.78 - 1.27E ($R^2 = 0.75$); Correlation = -0.86 ($P < 0.0001$)

Figure 7.7. Turkey embryo liver glycogen concentration during the last week of incubation



Glyc = 2.527 - 0.0847E ($R^2=0.70$), correlation = -0.83 ($P<0.0001$)

Figure 7.8. Turkey embryo pectoral muscle glycogen concentration during the last week of incubation

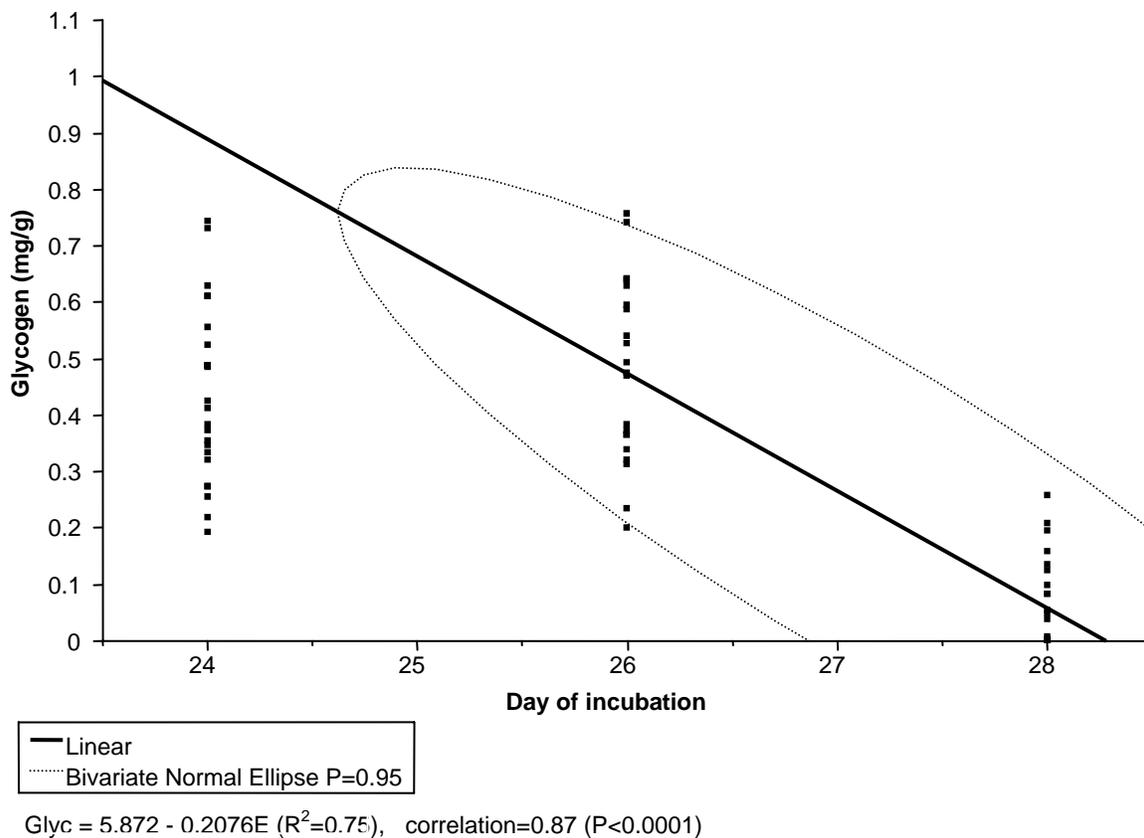


Figure 7.9. Turkey embryo hatching muscle glycogen concentration during the last week of incubation

Microarrays

Array construction was found to be successful, although several “doughnut-shaped spots (Figure 7.1) caused by less than perfect amounts of salt in the resuspended oligos had occurred without interfering experimental results. This issue can be corrected in the future by better quality control of oligo plate preparation. Labeled cDNA quality control showed good cydye incorporation and cDNA spectrophotometry 260/280 ratios above 1.8.

Cluster Analysis

Genes, whose expressions were significantly different in at least one time point from 20E to hatch, were included in the cluster analysis. The results of the cluster analysis of liver, pectoral muscle, hatching muscle, and duodenum are presented on Figures 7.10, 7.12, 7.14 and 7.16, respectively. The list of significant genes appears on the figures left column, the color map can be seen in the middle and the clustering is shown on the right of the figures. The expression patterns are grouped by their similarity along days of incubation, which appear on the very bottom of the color map. The color and its intensity represents the level of expression of that gene at the specific embryonic age, blue indicating lower expression or down regulation and red meaning higher expression or up-regulation. Based on the clustering, the liver genes were divided into 6 clusters of similar expression patterns, and the genes of other tissues were divided into 4 clusters each. The clusters in each tissue were color coded and identified by a lower case letter starting with “a” from top to bottom (right hand side of figures).

The mean expression of each cluster in liver, pectoral muscle, hatching muscle and duodenum are plotted on Figures 7.11, 7.13, 7.15 and 7.17, respectively. The tissue with the largest number of genes significantly affected by embryonic age was the duodenum with 85 significant genes, followed by the liver with 60 genes and then pectoral muscle (53 genes), and hatching muscle (51 genes).

One important observation is that usual housekeeping genes, chGAPDH and chEF2, can change significantly between days due to high rate of development. Also, due to high sensitivity of this array, even very small changes in color intensity can be significant for genes with very consistent signals. For example, a gene with constant zero

color intensity (or background intensity) can be significant if an intensity higher than zero spikes in one time point. This is the case when atCAB2-arapdopsis and chBACT, which are not present in animal tissues, showed as significant in some clusters (Figures 7.12, 7.14 and 7.16). The same thing happens when a gene seems to be expressed in a tissue where it is not expected to be expressed, like alpha-amylase in liver and muscle tissues (Figures 7.10 and 7.12). This inherent error can occur by using a false discovery rate (FDR) of 0.01, as one false positive or negative gene every one hundred genes is expected. A general discussion of each cluster by tissue will be presented next.

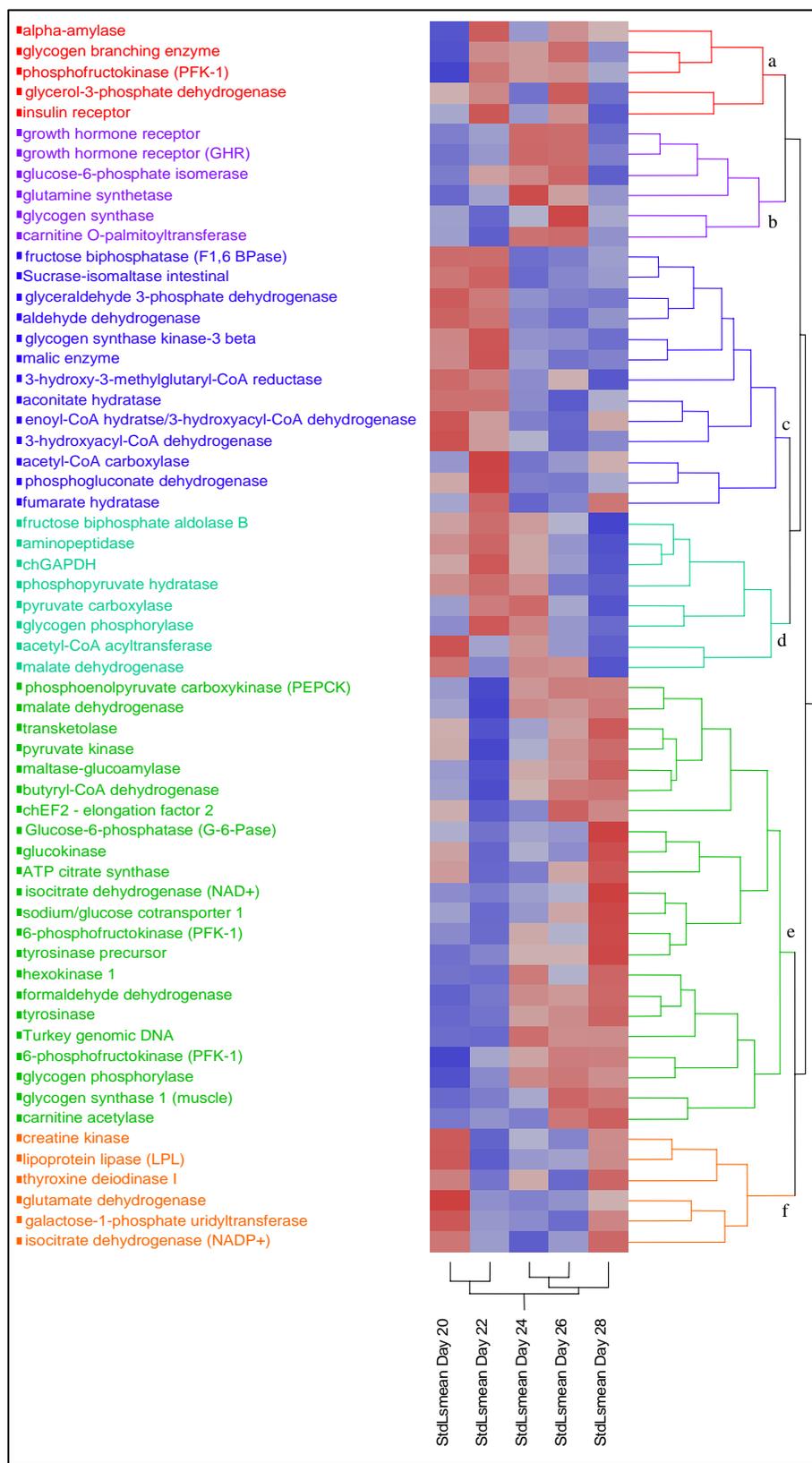


Figure 7.10. Clustering of genes significantly affected by time in embryonic liver

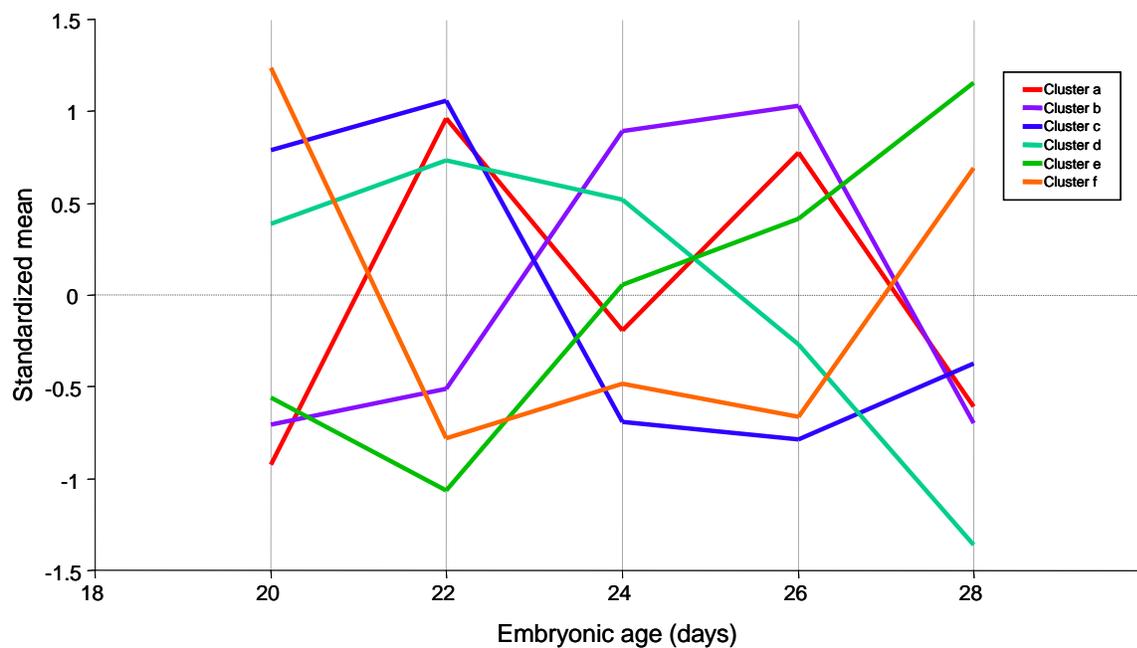


Figure 7.11. Parallel plots of cluster analysis for turkey embryo liver

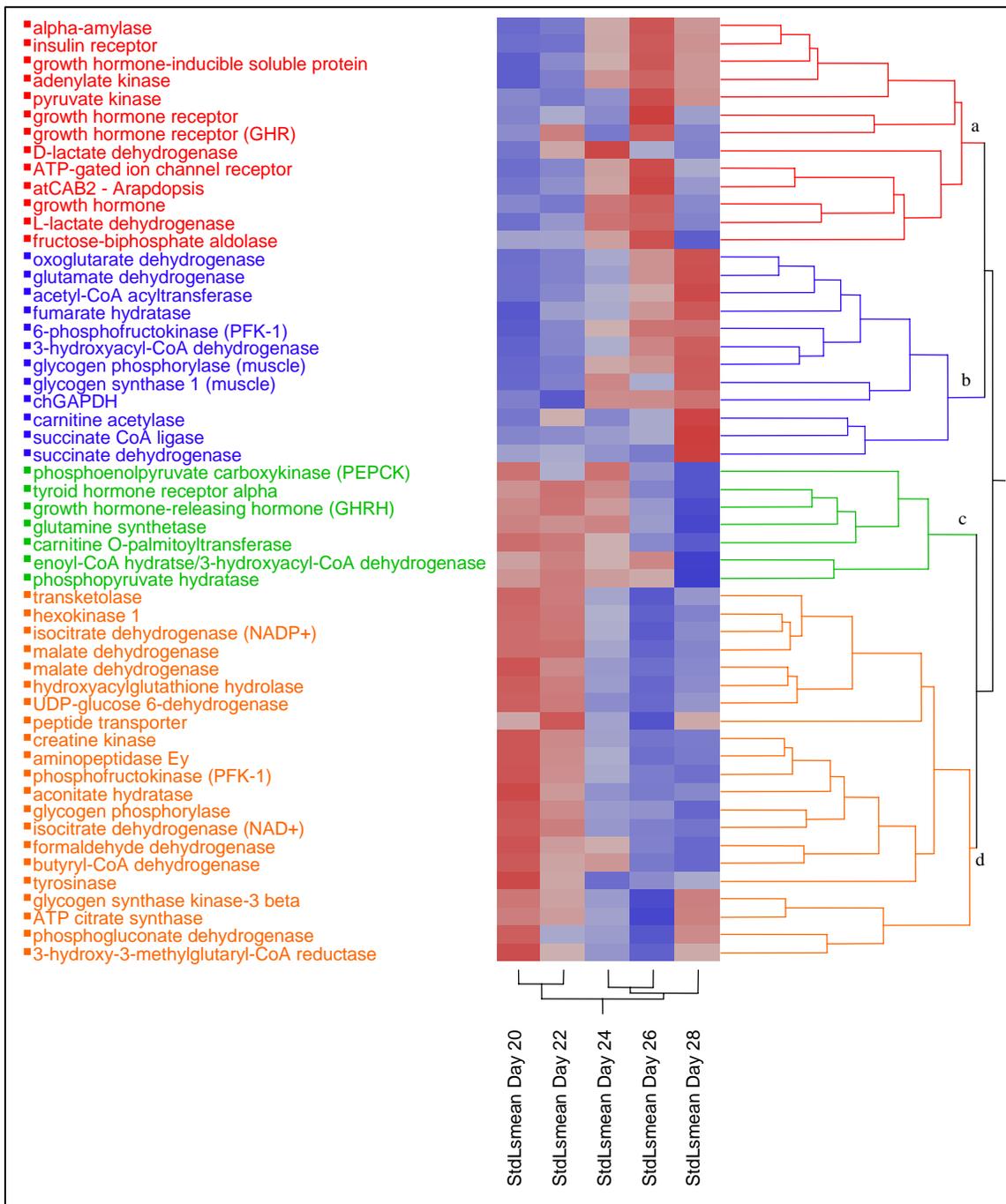


Figure 7.12. Clustering of genes significantly affected by time in embryonic pectoral muscle

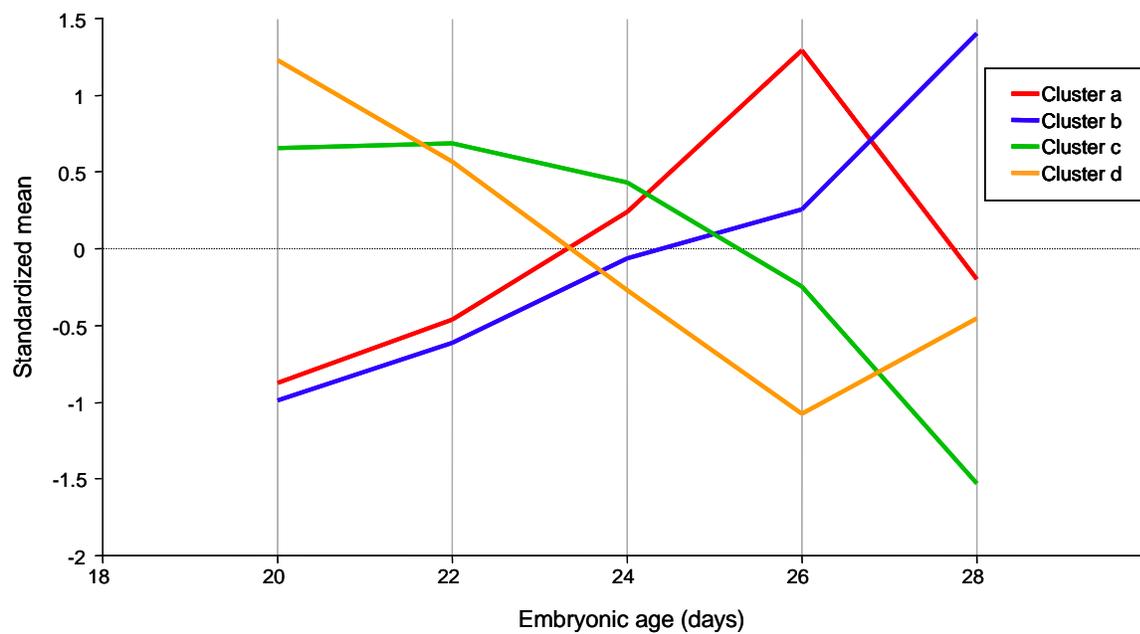


Figure 7.13. Parallel plots of cluster analysis for turkey embryo pectoral muscle

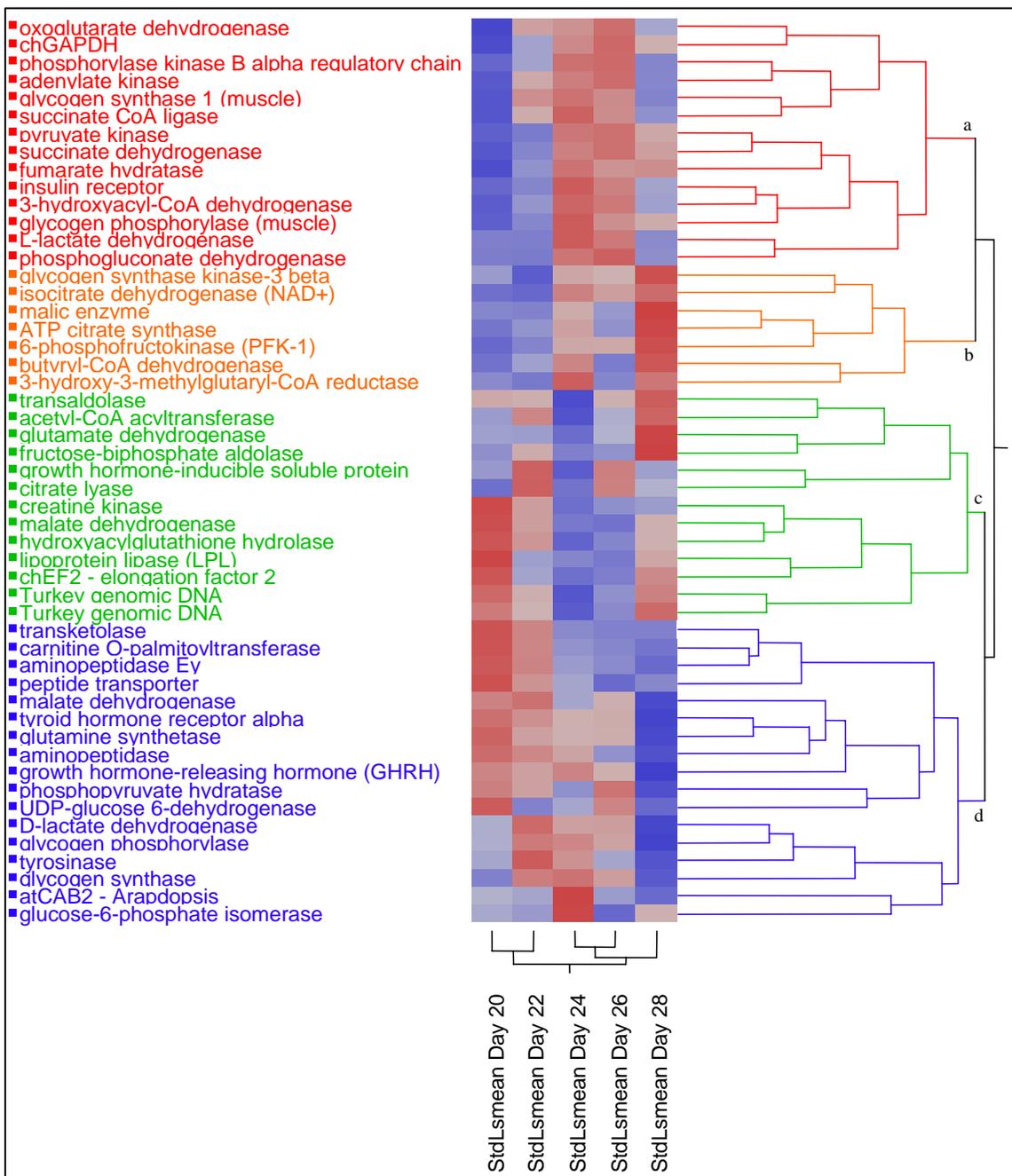


Figure 7.14. Clustering of genes significantly affected by time in embryonic hatching muscle

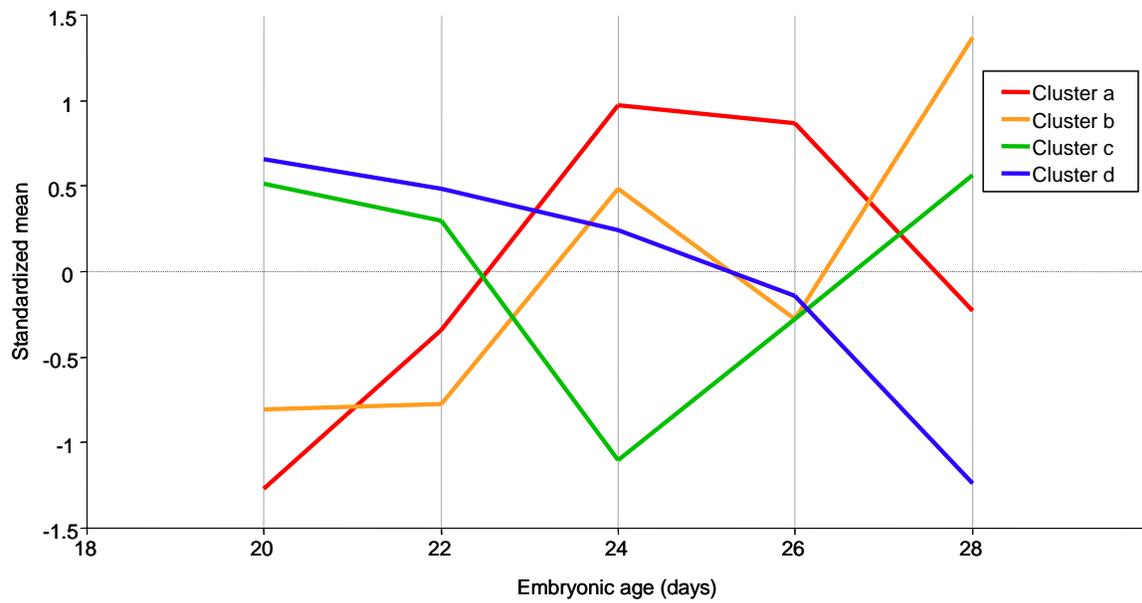


Figure 7.15. Parallel plots of cluster analysis fro turkey embryo hatching muscle

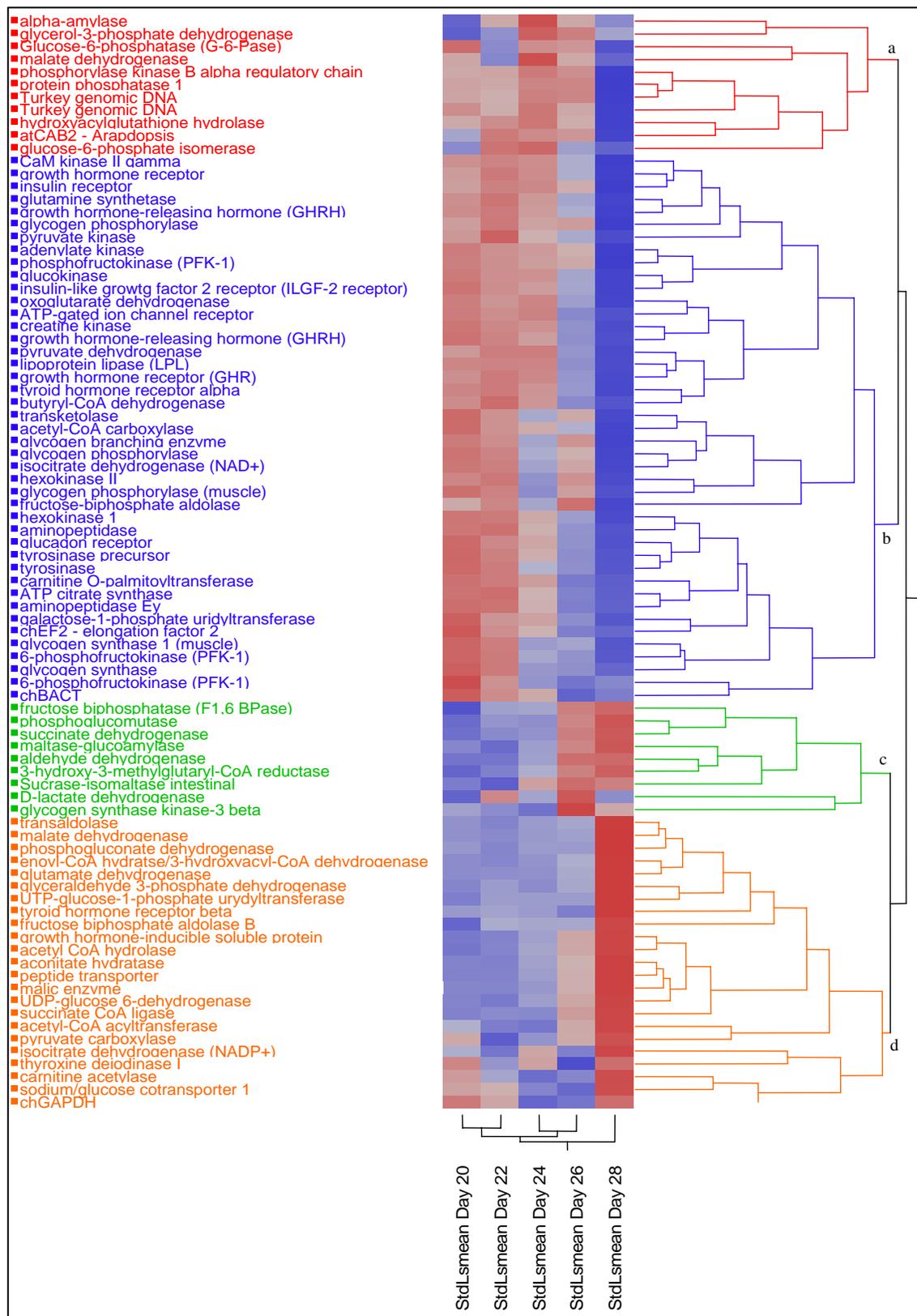


Figure 7.16. Clustering of genes significantly affected by time in embryonic duodenum

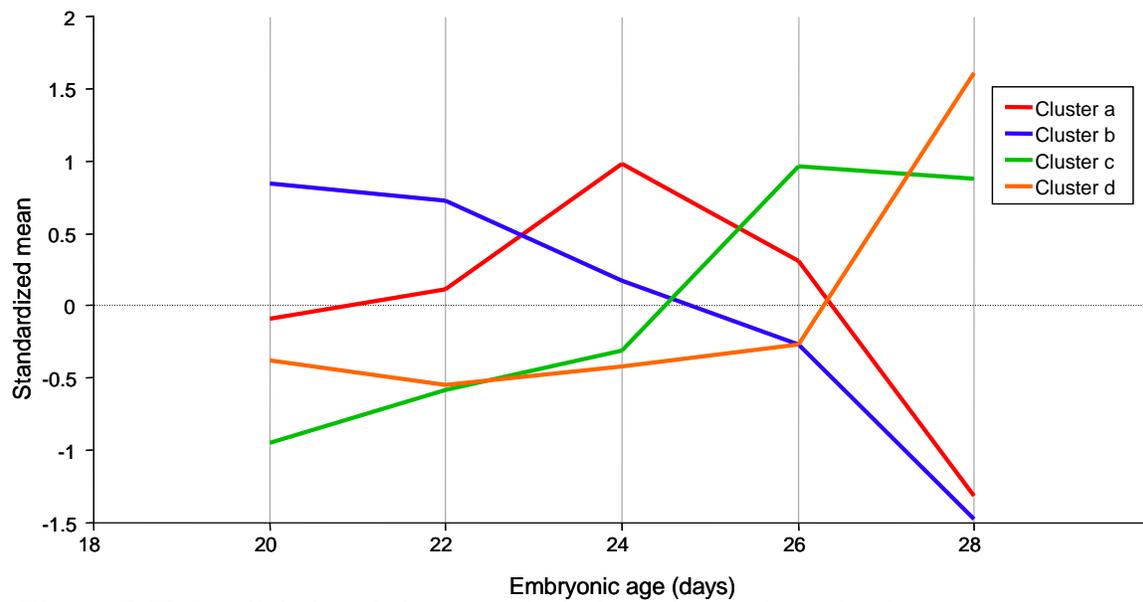


Figure 7.17. Parallel plot of cluster analysis for turkey embryo duodenum

Liver

According to the results, the liver had 60 genes whose gene expression significantly changed during the last week of embryonic development. This large number of genes was expected because the liver is the most metabolic active organ (Krebs, 1972), and even more during this stage of development when the embryo is preparing for its life outside the shell. The complexity of liver metabolism is also reflected in the number of clusters, being the tissue with the most clusters (six). The liver is known for being the organ with the most enzymes and able to perform almost any pathway that exists in the animal (Kietzmann and Jungermann, 1997). During the last days of incubation, two main hepatic functions can be highlighted: storage of energy as glycogen for hatching (Uni and Ferket, 2004) and transfer of many nutrients from other egg compartments to the liver, especially large amounts of cholesterol from the yolk sac (Moran, 2007).

The liver parallel plot of the six clusters can be seen on Figure 7.11. The first observation is paired clusters with almost perfect opposite patterns, like cluster “a” is opposite of “f”, “b” is opposite of “c” and “d is the opposite of “e”. This observation facilitates understanding of gene functions by associating their pattern of expression with their pathways.

Lipid metabolism

Identifying the stage of embryonic development when the embryo switches from lipid- to carbohydrate-based metabolism was one of the main objectives of this study. This change in metabolism has being suggested by several authors (Romanoff, 1967; Donaldson et al., 1991; Noy and Sklan, 1999; Uni and Ferket, 2004; Foye, 2005; Moran, 2007) to be based on the fact that fatty acid beta-oxidation must be limited by low oxygen

supply during the plateau and internal pipping stages, and also that high amounts of glycogen are stored and used during that same period. Based on the gene expression patterns observed in this study, fatty acid beta-oxidation is inhibited starting at 22E. This conclusion is based on the fact that enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, which are respectively responsible for the second and third steps of beta-oxidation, were down-regulated after 22E as illustrated in cluster “c” (blue) (Figure 7.11). Other indications of reduced lipid metabolism during this period are supported by the down-regulation of 3-hydroxy-methylglutaryl-CoA reductase (cholesterol synthesis) and acetyl-CoA carboxylase (first step of FA synthesis) being also in cluster “c”. The acetyl-CoA acetyltransferase gene, which is responsible for removing acetyl-CoA units from FA during degradation, is included in cluster “d” (light green), showing a decreasing pattern of expression towards hatch. The lipoprotein lipase enzyme (LPL), responsible for triacylglycerol hydrolysis into FA + glycerol during fat mobilization is specifically down regulated between 22-26E (Figure 7.11, cluster “f”, orange), as well as the thyroxine deiodinase enzyme, which converts T4 to T3, the active form of the hormone responsible for stimulating fat mobilization. These results indicate that the liver is not mobilizing fat during the plateau stage until external pipping occurs. One unexpected finding was that the long-chain fatty acid transport into the mitochondria seems to be up-regulated during the same period (22-26E) as indicated by carnitine O-palmitoyl transferase gene presence in cluster “b” (purple), even though another enzyme involved in the process, carnitine acetylase, is included in cluster “f” which has the opposite pattern (Figure 7.11). The gene expression profile observed in this study is evidence that fat metabolism in the turkey embryo is inhibited between 22-26E.

Glycogen metabolism

Liver glycogen metabolism is very important in poultry embryos during hatch. As discussed above, lipid metabolism is down-regulated during the plateau stage of oxygen consumption and pipping, so the embryo must depend on anaerobic metabolism of carbohydrates for energy. Amnion consumption provides substrate for the synthesis of glycogen that, must be stored before hatching. The liver is the main glycogen storing organ, being able to store 5% of its weight as glycogen as compared to 1% in muscle (Krebs, 1972). Glycogen reserves mobilized during hatching yield glucose that can be exported to other tissues, and reduced to lactate when oxygen is limiting.

The two main enzymes of glycogen metabolism are glycogen synthase and glycogen phosphorylase, which respectively synthesize and mobilized glycogen. The glycogen synthase gene was found to be up-regulated in the period between 24-26E (Figure 7.11, cluster “b” purple), as well as glucose-6-phosphate isomerase, which connects glycolysis to glycogen pathways when internal and external pipping may occur. Glycogen synthase kinase-3-beta, which inhibits glycogen synthase, is down-regulated starting from 22E through to hatch as demonstrated by cluster “c” (blue). The two glycogen phosphorylase isoforms showed up in distinct clusters, cluster “d” (light green) and “e” (dark green), which may indicate that one is important during internal pipping (cluster “d”) while the other is expressed after pipping (cluster “e”) (Figure 7.11). The other possible energy source, creatine, has its enzyme, creatine kinase, down-regulated during the period when glycogen metabolism is most active (22-26E) as it is included in cluster “f” (orange). Based on these results, liver glycogen synthesis and degradation

often occur simultaneously, but glycogen synthesis is predominant between 24-26E and glycogen degradation is predominant after 26E.

Glycolysis/gluconeogenesis

As the key enzymes that control flux favoring glycolysis or gluconeogenesis is studied, it is clear how complex this control is in the liver. These apparently antagonist pathways work together to favor flux of substrate towards other connecting pathways, like glycogenesis and glycogenolysis. For example, the enzyme at the first point of control of glycolysis, glucokinase, is up-regulated starting at 22E until hatch (cluster “e” dark green, Figure 7.11), although the opposing gluconeogenic enzyme, glucose-6-phosphatase, is also in the same cluster. Other example of contradictory regulation is PEPCK (point of control for gluconeogenesis) and pyruvate kinase (point of control for glycolysis), which are also in the same cluster (“e”). Instead of showing conflicting pathway controls, this observation illustrates the liver’s ability to move substrates up and down these pathways during this critical period of embryonic development.

There were 2 isoforms of PFK-1 on the array, one for muscle (cluster “e” green) and one for liver (cluster “a” red), but the later will be considered in this discussion. The liver isoform of PFK-1 appears in the same cluster as the gene for insulin receptor, which indicates that these two gene products may work together favoring glycolysis at 22E and 26E, as represented in the pattern of cluster “a” (red) (Figure 7.11).

The last enzyme considered as a significant point of regulation of gluconeogenesis was Fructose 1-6 biphosphatase, which was down-regulated after 22E until hatch (cluster “c” blue, Figure 7.11). Surprisingly, the embryo liver has enzymatic capacity to perform

either glycolysis or gluconeogenesis during late-term incubation, with glycolysis being favored only at 22E and 26E, as demonstrated by peaks of PFK-1 and insulin receptor.

In general, the gene expression results observed in the liver confirmed current knowledge of the liver's role in supporting the energy needs other tissues, by switching energy metabolism from substrates depending ample oxygen supply (i.e. lipids) to substrates of anaerobic metabolism (i.e. carbohydrates and protein) that fuel increased physical activity during hatching. The major contribution of these findings is to point exactly when these events are happening during late-term embryonic development.

Pectoral muscle

The parallel plot of the 4 clusters selected in pectoral muscle is illustrated by Figure 7.13. The plot show 2 distinct cluster groups, "a" and "b", that are down-regulated, and two cluster groups, "c" and "d" that are up-regulated. Clusters "c" and "d" seem to have opposite patterns with shifts occurring at 22E and around 27E (Figure 7.13). Clusters "a" and "b" showed similar expression from 20E until 26E, when their patterns became antagonistic to each other, with a shift occurring around 27E (Figure 7.13).

Growth

Three genes associated with growth characteristics were present in cluster "a" (red): growth hormone inducible-soluble protein, and 2 isoforms of the growth hormone receptor, indicating a peak in expression right before hatch at 26E (Figure 7.13). Only an up-regulation of growth hormone releasing hormone was in observed in cluster "c", which should not be relevant since this pituitary hormone should not be expressed in muscle. Thus pectoral muscle mass increased continually during the period of study (Figure 7.4), and gene expression of genes involved in growth indicated higher stimulus

at 26E. Continuous increase in muscle mass towards hatch may also be a consequence of increased tissue hydration in preparation for hatch (Vleck, 1991).

Glycogen metabolism

As observed in liver, genes of isoforms for glycogen synthesis and mobilization in pectoral muscle were present in the same cluster (“b” blue, Figure 7.13), indicating either simultaneous ability to synthesize and mobilize glycogen, or that both enzymes are present and their functionality depends in post-translational forms of control such as phosphorylation. As shown in Figure 7.8, pectoral muscle glycogen concentration decreased from 22E until hatch, so the actual amount of glycogen in the muscle at any given time may be a result of the balance between constant synthesis and degradation.

Energy utilization

Pectoral muscle lipid metabolism showed that 3 enzymes involved in fatty acid beta-oxidation, carnitine O-palmitoyl transferase, enoyl-CoA hydratase and thyroid hormone receptor, were being down-regulated after 24E until hatch (cluster “c” green), although 3 other enzymes of the same pathway were up-regulated after 26E (acetyl-CoA acyltransferase, 3-hydroxyacyl-CoA dehydrogenase and carnitine acetylase). Combining both patterns leads to the conclusion that fatty acid oxidation in pectoral muscle decreases after 22E, as observed in the liver, but it is active again after external pipping occurs and plenty of oxygen is supplied.

Glycolytic enzymes, hexokinase and phosphofructokinase-1, are in clusters with opposite patterns, indicating that even though pectoral muscle up-take of glucose was being reduced by less hexokinase gene expression (cluster “d” orange), glycolysis was active as demonstrated by increase PFK-1 gene expression (cluster “b”), probably

because of glucose coming into the pathway through glycogenolysis (Figure 7.8). Gluconeogenic enzyme PEPCK was being down-regulated towards hatch as indicated in cluster “c” (green), which may signify unavailability of gluconeogenic substrates coming from oxaloacetate (TCA cycle).

Most TCA cycle enzymes (citrate synthase, isocitrate dehydrogenase and malate dehydrogenase) were present in cluster “d”, indicating down-regulation from 20E until 26E, which was expected since limited oxygen supplies lead glycolysis to produce lactate instead of acetyl-CoA. Exceptions to this pattern were the two succinate enzymes, succinate-CoA ligase and succinate dehydrogenase, which were up-regulated by hatch (cluster “b” blue). This is interesting because branch-chain amino acid catabolism in muscle is common, where their carbon backbone enters the TCA through succinate. Thus, increased succinate metabolism may be an indication of pectoral muscle protein degradation at hatch.

Hatching muscle

The four clusters selected from the gene expression patterns in the hatching muscle are illustrated in Figure 7.15. As for the pectoral muscle, we can identify 2 clusters of up-regulated genes (“c” and “d”) and 2 clusters of down-regulated genes (“a” and “b”) at 20E. The only clusters with apparently opposite patterns are clusters “a” and “c”. Another interesting pattern observation is that clusters “a” and “d” showed similar parallel pattern starting at 24E until hatch, with genes being down-regulated during that period.

Growth

The expression of growth-related genes in hatching muscle that changed significantly during the prenatal period was the growth hormone-inducible soluble protein (cluster “c” green), which was down-regulated only at 24E, the growth hormone releasing hormone (cluster “d”), which is a pituitary gene not important in muscle, and the thyroid hormone receptor (cluster “d”), which showed down regulation of this gene after 26E. The growth curve of the hatching muscle illustrated by Figure 7.6, shows that full mass of the hatching muscle was achieved at 26E, which indicate that thyroid hormone, may play an important role in this tissue’s growth and maturation.

Glycogen metabolism

Once more, enzymes of glycogen synthesis and degradation appeared in the same cluster (“a” red, Figure 7.15), where higher expression occurred between 24E and 26E. Peak expression of the synthase kinase-3 beta and glucose-6-phosphate isomerase at 24E and at hatch (cluster “b” orange) indicate the embryo’s need to inhibit glycogen synthase by phosphorylation and to transport intermediates via the glycolysis pathway. Changes in hatching muscle glycogen concentration over time (Figure 7.9) indicates that glycogen begins to be mobilized after 26E, which may be a consequence of glycogen synthase inhibition by glycogen synthase kinase-3 beta. After studying gene expression patterns of glycogen metabolism enzymes in liver and muscle, it is clear that gene expression of enzymes related to synthesis and degradation are not enough to predict pathway flux because both enzymes seem to be highly expressed during late-term incubation, so flux is likely regulated by post-translational modifications.

Energy utilization

Hatching muscle energy utilization shows a unique array of enzymes gene expression, with the fatty acid beta-oxidation enzymes (3-hydroxyacyl-CoA dehydrogenase, glycolysis enzyme pyruvate kinase), and the succinate metabolism enzymes (succinate-CoA ligase and succinate dehydrogenase) all in the same cluster (“a” red), being up-regulated between 24E and 26E when energy demand of this tissue should be at its maxim. This is an indication that hatching muscle may be able to utilize simultaneously glycogen, fatty acids and amino acids as energy sources, which was not observed in any other tissue. Even the TCA cycle enzymes, citrate synthase and isocitrate dehydrogenase, were up-regulated at 24E (cluster “b” orange) when oxygen supply is limiting even though hatching muscle is working for internal pipping. The spike in gene expression of TCA enzymes at hatch was then expected since oxygen supply was restored upon external pipping..

Duodenum

Four gene clusters were plotted to identify for the time of up-regulation of carbohydrate and protein digestive enzymes and nutrient transporters (Figure 7.17). According to the cluster analysis, carbohydrate digestive enzymes maltase-glucoamylase and sucrase-isomaltase were first expressed at 26E (cluster “c”, green), while the amino peptidase enzymes were concurrently down-regulated. Even though there is no substrate to stimulate the expression of carbohydrate digestive enzymes, the intestine apparently is programmed to produce this enzymes right before hatch so the poult can digest external feed rich in carbohydrates. Similar pattern of expression of the sucrase-isomaltase gene was found by (Uni et al., 2003) in chicken embryos, where expression peaked 48 hours

prior to hatch. Although peptides present in the amniotic fluid justify amino peptidase gene expression as early as 20E, completion of amnion consumption around 24E may remove the stimulus for the expression of this enzyme. This AP expression pattern differed from the pattern found by (Uni et al., 2003) in chicken embryos, where the expression peaked only 48 hours before hatch (19E).

Nutrient transporters, sodium-glucose co-transporter-1 (SGLT-1) and peptide transporter-1 (pept-1) were grouped in cluster “d” (orange) showing sharp gene up-regulation at hatch (Figure 7.17). Van et al. (2005) observed studied PepT-1 gene expression in turkey embryo peaks between 27E and hatch in an identical pattern as observed in cluster “d” in this study. This agreement in observations confirms that the present array results are as precise as the Northern Blots. In comparison to the SGLT-1 expression pattern observed in chicken embryos by Uni et al. (2003), SGLT-1 gene expression in turkey embryos peak closer to hatch (28E) than in chicken embryos, which peak 48 hours prior to hatch. This difference in SGLT-1 expression may indicate that the turkey has delayed capacity to absorbing glucose relative to the chicken. The pattern of intestinal enzymes described here indicates gut maturation occurring in the 48 hrs prior to hatch. Moreover, the increase in duodenum mass illustrated by Figure 7.5 may be due to cell proliferation as well as maturation of existing cells, which agrees with the down-regulation of the growth inducing genes (growth hormone receptor, insulin-like growth factor-2 receptor and thyroid hormone receptor) observed herein after 26E (cluster “b”). Evidently, intestinal enzymes in both chicken and turkey embryos are produced before hatch so that they can be present by the time of hatch to digest incoming external feed (Uni et al., 2003).

One interesting observation was that turkey genomic DNA showed in the intestine in cluster “a” (red). This may be an indication of some DNA cross-contamination during RNA extraction. Moreover, more important is the fact that the pattern observed shows intensity being significantly reduced in the 48 hours previous to hatch. This reduction indicates that the ratio between DNA and RNA in intestinal cells was high during incubation, with prevalence of DNA. Right before hatch the ratio reversed due to high amounts of RNA produced through gene expression as a consequence of intense intestinal cell maturation and function as preparation for post-hatch life.

Conclusion

The production of a gene microarray to be utilized to study chicken and turkey gene expression was successful. Using this array it was possible to identify the pattern of expression of many key genes of turkey embryo metabolism and development. One major finding was the indication that the turkey embryo switches from lipid- to carbohydrate-based metabolism at 22 days of incubation, and that the glycogen metabolism related enzymes are produced in parallel in liver and muscle.

Based on intestinal gene expression patterns, carbohydrate digestive enzymes start being produced 48 hours prior to hatch, while nutrient transporter gene expression peaked at hatch. The approach used in this study could bring substantial contribution to current knowledge about late-term poultry embryo development.

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Chapter 8

“Effects of *in ovo* feeding on late-term turkey embryo growth, intestinal morphology, poult quality, energy status, and their association to differences in gene expression observed by microarrays”

Abstract

In ovo feeding (IOF) has been demonstrated to enhance the development of digestive capacity and energy status of late-term turkey embryos, but the effect of IOF on energy metabolism is not yet clearly understood. An experiment was designed to correlate the effect of IOF on the growth and energy status of turkey embryos from 22 days of incubation (E) until hatch with the expression of critical metabolic genes using microarrays and hatchling quality. Three hundred turkey eggs were divided into 2 groups with similar egg weight. At 24E one group of eggs was injected with a nutritive solution containing co-factors of metabolism. At 25E, 26E and 28E (day of hatch) sixteen eggs from each treatment (IOF and control) were sampled to measure embryo and yolk mass to calculate embryonic yolk-free body weight (YFBW). Liver, pectoral muscle, hatching muscle, duodenum and ileum were collected from the same embryos for microarray analysis. Liver, pectoral and hatching muscles were also sampled for glycogen analysis. Poult quality scores and relative shank asymmetry were calculated from 35 poults from each treatment at hatch. The IOF embryos were heavier and had less pectoral muscle glycogen than controls at 25E, but there were no treatment differences at hatch. IOF poults had less glycogen in the liver at hatch, but 15 points higher quality scores and more symmetric legs than control poults. Evaluation of gene expression by microarray revealed that IOF reduced energetic stress in the liver at 25E and 26E, enhanced hatching

muscle maturation, and reduced energy metabolism in the intestine of in comparison to controls. IOF of metabolic co-factors changed embryonic development, favoring skeleton maturation instead of intestinal metabolism, resulting in improved poult quality at hatch.

Introduction

Despite great advances in growth performance (Havenstein et al., 2007) turkey hatchability has not improved much above 80% of fertile during the last 20 years (Schaal and Cherian, 2007). Embryonic mortality during the last stages of incubation is a significant problem of poult production (Christensen et al., 1993; Foye, 2005), in addition to weak poults, starve-outs and flip-over poults (Noble et al., 1999; Christensen et al., 2003). These depressed conditions have been associated to poult low energy status (Donaldson, 1995; Christensen et al., 1999).

It has been documented that poultry embryos undergo several shifts in metabolism during incubation and early development (Romanoff, 1960; Romanoff, 1967; Moran, 2007), but little is known about when these metabolic shifts occur and how they can be manipulated to improve poult quality. A new technology called *in ovo* feeding (IOF) may provide a means to improve the nutrition and energy status of turkeys before, during, and after hatch (Uni and Ferket, 2004; Ferket et al., 2005). Turkey embryos that struggle to hatch are good candidates for IOF technology. *In ovo* feeding involves injecting a nutritive solution into the amnion, which will be orally consumed by the embryo before hatching (Uni and Ferket, 2003). IOF has been demonstrated to enhance energy status (Uni et al., 2005; Foye et al., 2006), promote gastrointestinal maturation through improvements in morphology and mucus production (Tako et al., 2004; Smirnov et al., 2006; Uni et al., 2006), and to alter gene expression of intestinal enzymes and

nutrient transporters (Tako et al., 2005; Foye et al., 2006). Because many of these changes are not always predictable, advanced molecular techniques and poult quality assessments may help explain the potential of IOF.

Microarrays were used in this study to identify changes in metabolism by gene expression after IOF of turkey embryos. Microarrays are powerful tools that capable of measuring thousands of mRNA populations in a single experimental sample (Wolfinger et al., 2001; Garosi et al., 2005). Using focused microarrays, slides or chips can be customized by robotically spotting large numbers of known gene sequences, and then hybridizing them with two cDNA populations labeled with different fluorescent dyes that can be scanned for color intensity and produce data for statistical analysis (Spielbauer and Stahl, 2005). This approach allows high throughput gene expression profile production that would not be impossible to obtain by other techniques, like real-time PCR or Northern Blot (Spielbauer and Stahl, 2005). Page et al. (2003) recommend the use of microarrays in nutritional studies because it can lead to major contributions to nutritional research.

Poult quality is usually assessed by measuring hatchability, chick vitality and uniformity (Boerjan, 2006). Poult quality assessment can also be very subjective, often involving the visual observation by an experienced hatchery manager. The need for more objective methods to assess the quality of hatchlings motivated different research groups to develop scoring methods. One method was an adaptation of the Apgar score for newborn children, the Pasgar score (Boerjan, 2002). This scoring method is based on morphological criteria like reflex, naval, legs, beak and yolk condition, where the chick starts with a score of 10 and then loses points for each abnormality observed (Boerjan,

2006). Another method was developed by Tona et al. (2003), in which physical conditions such as activity, feathering, eyes, leg conformation, aspect of naval area and yolk absorption are given different scoring weights, depending on their importance to the survival of the chick and the severity of any abnormality. Points are accumulated with maximum score of 100, which would be a perfect chick (Tona et al., 2003). These scoring systems may also be used to assess the quality of turkey poults.

Leg abnormalities are of concern in turkey production due to its high recurrence in fast growing heavy flocks (Hester et al., 1987). Many important leg integrity features are defined very early in life and may be a consequence of nutrient usage during incubation and brooding (Simsa and Ornan, 2007). A method to evaluate leg conformation in chicks and poults was proposed based on symmetry between both legs (Moller et al., 1995; Yalcin et al., 2003). Leg relative asymmetry (RA) can be calculated based on measurements performed on chick shank lengths at hatch (Yalcin et al., 2005). Perfect symmetry will be $RA = 0$, with values deviating from this number as asymmetry is found.

This experiment was conducted to evaluate the effects of IOF on embryonic development, energy status, gut maturation and poult quality, comparing differences in these parameters with changes in gene expression assessed by microarrays.

Materials and methods

Incubation and sampling

Three hundred fertilized turkey eggs from first-cycle Nicholas breeder hens at 7 weeks of lay were obtained from the a commercial farm¹ from the first egg collection (8 am) on the day they were laid. The eggs were transported to the North Carolina State University Turkey Research Unit², weighed and then divided into 6 groups of eggs with similar weight distribution (averaging 85.2g±15). The eggs were incubated in a Jamesway 252 incubator set to 37.8° C and 60% RH. At 20 days of incubation (E), all eggs were candled to remove infertile eggs and dead embryos, and each egg group was assigned to one of 6 treatments, corresponding to IOF or control eggs and three sampling times (25E, 26E and 28E). IOF treatment eggs were manually injected with 0.4 mL of nutritive solution containing metabolic co-factors³ from the blunt end of the egg into the amnion at 24E. Control eggs were kept outside the incubator for the same amount of time as IOF eggs (20 min). Sixteen eggs from each group (IOF and control) were sampled on days 25E, 26E and at day of hatch (28E). Sample eggs were opened at the blunt end of the egg using surgical scissors, and the embryos were extracted from the shell and euthanized by cervical dislocation. Subsequently, the embryos were separated from the yolk sac and had their individual weights recorded. Liver, duodenum, ileum, pectoral muscle, and hatching muscle were removed from each embryo (480 total), and about 200 mg samples of each tissue were immediately placed in labeled 7 mL scintillation vials

¹ Talley Farms, Stanfield, NC

² Lake Wheeler Field Laboratory, Raleigh, NC

³ proprietary formulation of Pfizer-Embrex, Durham, NC and North Carolina State University, Raleigh, NC (see Appendix 4, Table A4.2 for details)

(Fisher Scientific, Pittsburg, PA) containing 2 mL of RNA Later⁴ kept at room temperature. The remaining of liver and muscle tissues were immediately placed on ice, and then stored at -20C for subsequent glycogen analysis. RNA tissue samples were stored at -20C until extraction. All sampling procedures were performed in a clean laboratory facility with surfaces and instruments wiped with RNaseZap decontamination wipes⁴. To correlate gene expression data with poult energy status, liver, pectoral and hatching muscle samples were analyzed to determine glycogen concentration using the modified iodine binding method described by Foye et al. (2006).

RNA extraction and quality control

The tubes containing tissue samples were thawed and 100 mg of tissue was weighed and placed in a 2 mL microcentrifuge tube filled with 1 mL of TRI Reagent⁵. Muscle sample tubes were also filled with 0.3 mL of 0.1 mm glass beads to improve disintegration. The tubes were shaken in a 45 tube block using a Mini-Beadbeater-96⁶ for 1 minute or until tissue samples were completely dissolved. When more than one cycle was needed, the block was placed on ice for one minute to avoid sample overheating. Homogenates were transferred from the tubes containing beads to new tubes, and each of these tubes received 300 µl of chloroform and was vortexed for 10 seconds. These homogenate tubes were then centrifuged at 12,000g for 5 minutes at 4C to separate phases. The top aqueous phase was carefully transferred to a 1.5 mL microcentrifuge tube using a 1000 µl micropipette. Each tube received 500 µl of molecular biology grade 100% ethanol, inverted 5 times, and placed in a -20C freezer for 30 minutes. After that,

⁴ Ambion, Inc., Austin, TX

⁵ Molecular Research Center, Inc., Cincinnati, OH

⁶ Biospec, Inc., Bartlesville, OK

the samples were centrifuged at 12,000g for 15 minutes and 4C to form RNA pellets. The supernatant was discarded and the RNA pellets were washed with 500 μ l of 75% ethanol and centrifuged again at 12,000g for 10 min at 4C. The ethanol was discarded and the pellets were let to dry for 5 minutes under the hood. Air-dried pellets were resuspended in 30 μ l of nuclease-free water. Extracted RNAs were quantified using a ND-1000 spectrophotometer⁷ and RNA quality was assessed by running samples on an Experion Automated Electrophoresis Station⁸ using standard sensitivity chips. Four samples from the same tissue, treatment, and day of incubation were pooled together in one tube and adjusted to 0.5 μ g/ μ l of concentration. These tubes containing RNA were finally stored in a -80C freezer until assessed by microarray analysis.

Microarray manufacturing

A focused microarray customized for gene high replication number was designed to contain specifically chosen long oligonucleotides. Seventy base-pair oligonucleotides (oligos) were designed for 320 unique gene sequences selected from the chicken genome (International Chicken Sequencing Consortium, 2004), so the same array would serve several research groups at North Carolina State University. The gene list included 41 genes from carbohydrate metabolism (glycolysis, gluconeogenesis, TCA and pentose phosphate pathways), 13 genes from glycogenesis and glycogenolysis pathways, 11 genes from lipid metabolism, 18 genes from hormone related metabolism, 7 intestinal enzymes and nutrient transporter genes, 120 genes related to cardiovascular and blood system development, and 84 immunity related genes. The list also included 2 housekeeping genes (GAPDH and chEF2), one plant (atCAB2-arapdopsis) and one

⁷ NanoDrop Technologies, Inc., Wilmington, DE

⁸ Biorad Laboratories, Hercules, CA

bacterial gene (chBACT), and chicken and turkey genomic DNA, with the goal to serve as internal controls. Oligos were manufactured by Operon Biotechnologies, Inc.⁹ and obtained in a 384 well plate where each of 320¹⁰ wells corresponded to one gene and had their position documented. Plate oligos were resuspended in array spotting solution and printed (spotted) on UltraGAPS™ Amino-Silane Coated Slides¹¹ to produce all the slides used in this study. The slides were printed on a VersArray Chipwriter Compact Arrayer¹² using 4 pins.

A test scanned image of a slide hybridized with Syto 61 fluorescent dye can be seen on Figure 8.1. Each gene was spotted 3 times (side-by-side) in the same grid, and grids were replicated 4 times on each slide; so each gene was spotted a total of 12 times per array (technical replicates) (Figure 8.1). The array printer software generated a spot ID file used to produce the annotation file for this array. After printed, the slides were let to dry for 24 hours in the printer and then the oligos were attached to the slide surface on a CL-1000 ultraviolet crosslinker (UVP, Inc., Upland, CA) set to 6000 x 100 $\mu\text{J}/\text{cm}^2$. Slides were stored on a dust-free dehumidifier chamber until used.

⁹ Operon Biotechnologies, Inc., Germantown, MD

¹⁰ Complete list of genes printed on this array is available at Appendix 2

¹¹ Corning, Inc., Acton, MA

¹² Biorad, Inc., Waterloo, Ontario, Canada

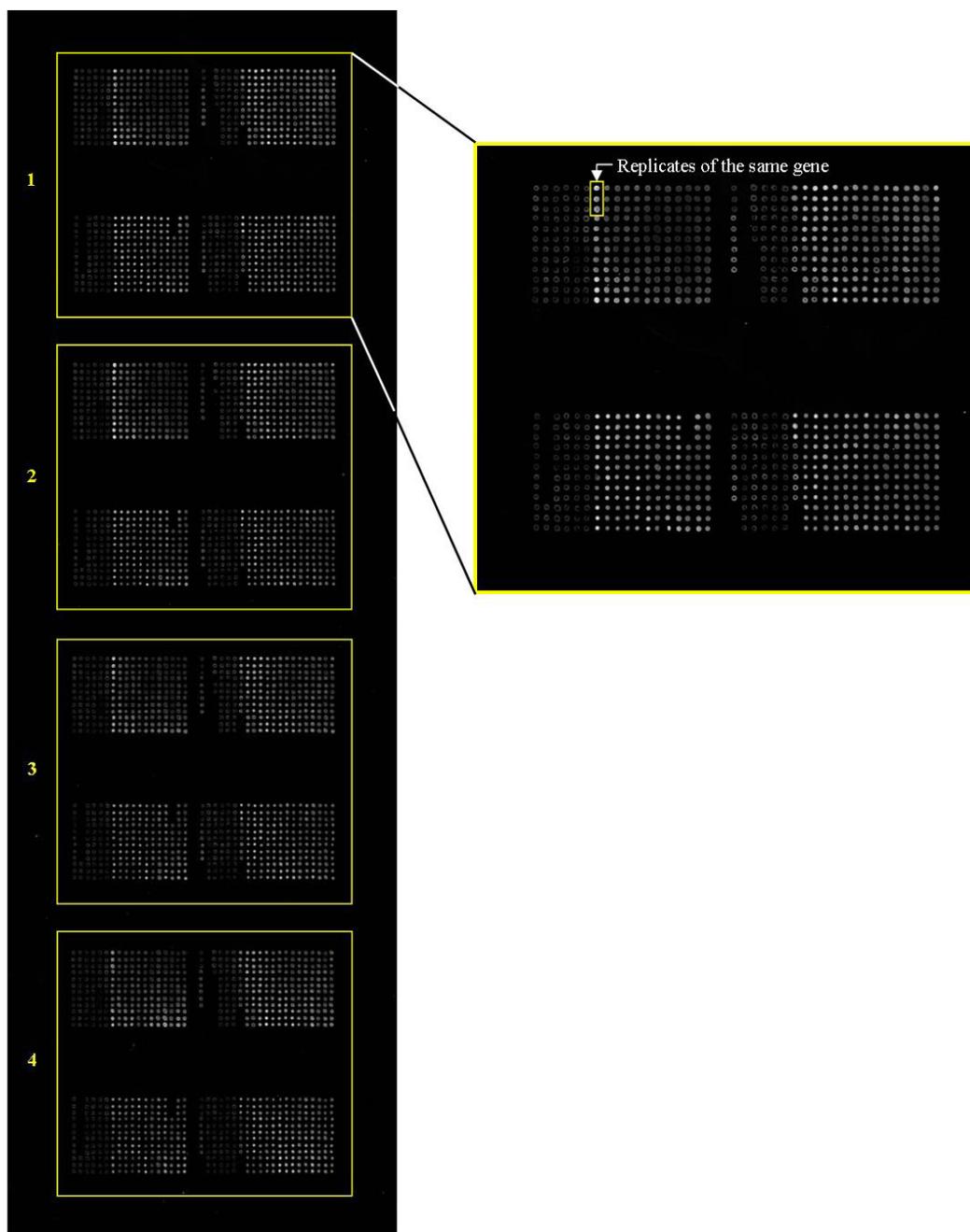


Figure 8.1. Scanned image of the “NCSU_JSARRAY_2” Focused Chicken Microarray slide stained with Syto 61 fluorescent dye. Image scanned on Cy5 channel 40% laser power. Yellow boxes show the 4 grid replicates and in detail the 3 gene replicates on a grid.

Sample labeling

Tissue RNA pooled samples were used to produce cydye labeled cDNA using the indirect labeling technique. The ChipShot™ Indirect Labeling and Clean-up System¹³ kit was used to produce aminoallyl-cDNA, which was labeled either with Cy3 or Cy5 fluorescent dye¹⁴ according to the experimental design. Samples from each treatment and day of incubation combination were labeled twice with each cydye using different pools of RNA (biological replicates). All laboratory protocols were followed as recommended by manufacturer manuals.

Hybridization and image scanning

Labeled cDNA from two distinct treatment samples were mixed, dried and resuspended in hybridization solution. The cDNA mix was then applied to a pre-hybridized slide covered with a pre-cleaned glass coverslip¹⁵, and hybridized overnight. The Pronto Plus! Microarray Hybridization Kit¹⁶ was used for hybridization, following their manual recommendations. The same procedure was repeated with all sample pairs according to the experimental design, caring that samples that were to be hybridized together were also labeled together. Microarray slides were scanned on a ScanArray G_X PLUS Microarray Scanner¹⁷ set to 65% laser power. All sample and slide manipulations from labeling until slide scanning were performed protecting them from light.

¹³ Promega, Madison, WI

¹⁴ Amersham Biosciences Corp., Piscataway, NJ

¹⁵ Lifterslip, Portsmouth, NH

¹⁶ Corning, Inc., Acton, MA

¹⁷ PerkinElmer Life and Analytical Sciences, Shelton, CT

Data processing, Normalization and statistical analysis

Generated data files were converted into image intensity raw data files for each slide and dye combination using ScanAlyze Software (Eisen et al., 1998). Raw data files were joined, transformed to a \log_2 base and analyzed by JMP Genomics (SAS Institute, 2007). The \log_2 -transformed data for all spot measures were subjected to lowess¹⁸ normalization. The experimental design was an complete interwoven loop design (Garosi et al., 2005).

Differences in gene expression between IOF and control poult

Array data was analyzed by mixed ANOVA according to the model:

$$Y = \mu + IOF + E + IOF * E + Dye + Hyb + e,$$

with *in ovo* feeding (IOF), day of incubation (E), *in ovo* feeding and day of incubation interaction (IOF*E), and cydye (Dye) as fixed effects. Hybridization batch (Hyb) was added as a random effect. \log_2 -transformed mean intensities were tested by False Discovery Rate (FDR) ($P < 0.01$) (Yang and Speed, 2003). Analysis output was used to create volcano plots.

Embryonic growth, glycogen concentration and poult quality statistical analysis

Embryonic growth, glycogen concentration, poult quality scores and leg RA had the same experimental design, which was completely randomized. The analysis was done by fit model procedure in JMP (SAS Institute. 2005), according to the model:

$$Y = \mu + IOF + Age + IOF \times Age + e,$$

¹⁸ locally weighted scatterplot smoothing

where *in ovo* feeding (IOF), age (Age) and IOF-Age interaction were fixed effects. Treatment means were compared by t-student test ($P < 0.05$).

Results and discussion

Embryonic and yolk sac weights were used to calculate embryonic yolk-free body weight (YFBW). There were no differences between IOF treatments on yolk weights at any of the studied days of incubation, with yolk averaging 14.84g (± 2.98), 13.32 (± 2.93) and 5.67g (± 2.24) at 25E, 26E and 28E (hatch), respectively. YFBW of embryos from IOF and control embryos were plotted and shown on Figure 8.2. IOF embryos had heavier YFBW than controls 24 hours after receiving IOF (25E), but they had minimal weight gain during the following 24 hours, resulting in similar mass as the controls at hatch. There was no need to correct embryonic mass by egg weight because egg weight distribution was randomized at 20E, so there were no statistical differences in egg weight between treatments. The apparent increase in YFBW followed by a period of attenuated growth rate of IOF poult could be due to several reasons, including insufficient nutrients to support growth or some uncontrolled issue with incubation. Because treatment differences in embryo size were observed at 25E, treatment differences in gene expression were also expected, as discussed later. At 25E, 28% of eggs exhibited internal-pipping, whereas at 26E 31% of the eggs achieved internal-pipping and 59% of eggs were external pipping. No treatment effects were observed on the proportion of pips, at either 25E or 26E.

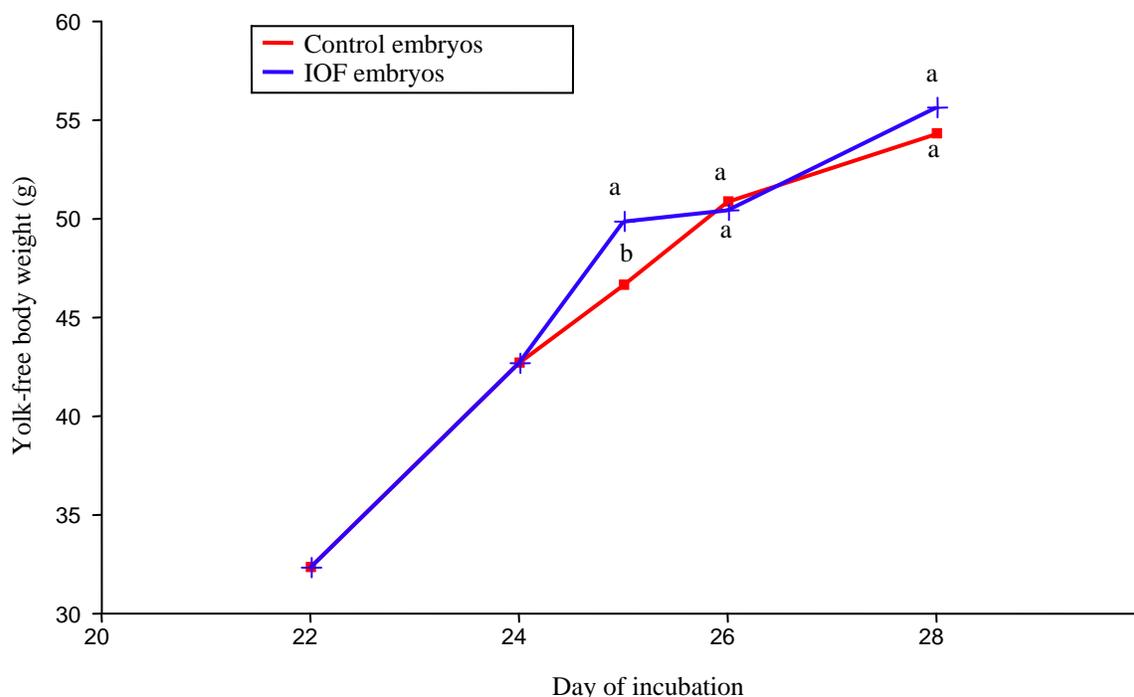


Figure 8.2. Embryonic development of turkey embryos from 22E until hatch expressed as yolk-free body weight (g).

^{a,b} Different letters in the same time point (vertically) indicate that means are significantly different ($P < 0.05$)

Glycogen concentration

Figures 8.3 and 8.4 illustrate the effect of IOF on glycogen concentration respectively in liver and pectoral muscle during the last 3 days of incubation. The IOF treatment increased liver glycogen concentration only at hatch (Figure 8.3). Apparently, the IOF poults used more of their liver glycogen reserves for hatching than the control poults. The ability to use glycogen reserves has been positively correlated to thyroid hormone levels. Christensen et al. (1996) observed embryos and hatchlings with low levels of T3 were not able to utilize their energy reserves even when liver and muscle glycogen was plentiful. Christensen et al. (2003) found that flip-over poults, considered to be hypoglycemic, had lower thyroxine levels than normal poults.

In pectoral muscle, glycogen reserves differed between IOF treatments only at 25E (Figure 8.4), with control embryos again showing higher glycogen concentrations than IOF embryos. Notice that the pattern of glycogen in pectoral muscle is similar to an inverted pattern of embryonic growth shown on Figure 8.2. These data together demonstrate that high growth rate (25E) coincides with lower pectoral muscle glycogen accumulation, which can be explained by the fact that IOF embryos were channeling energetic substrates for growth instead of storing them. Pectoral muscle glycogen also showed a plateau phase between 25E and 26E, with IOF embryos not utilizing muscle glycogen during the time they were not growing (Figure 8.2). Some physiological or incubation condition may have affected IOF embryos, keeping them from growing during this period, so differences between control and IOF embryos observed during incubation disappeared by the time of hatch.

Hatching muscle samples could only be analyzed for glycogen after 24E because this muscle was not big enough to provide adequate samples for RNA extraction and glycogen analysis prior to 24E. Hatching muscle glycogen did not differ between IOF treatments for any of the time points studied, averaging 5.7 mg/g at 24E, 7.1 mg/g at 25E, 7.3 mg/g at 26E, and 1.83 mg/g at hatch. These data indicate that hatching muscle finished accumulating glycogen around 25E and, as expected, it was rapidly utilized during the last 2 days of incubation when the embryo was pipping the shell.

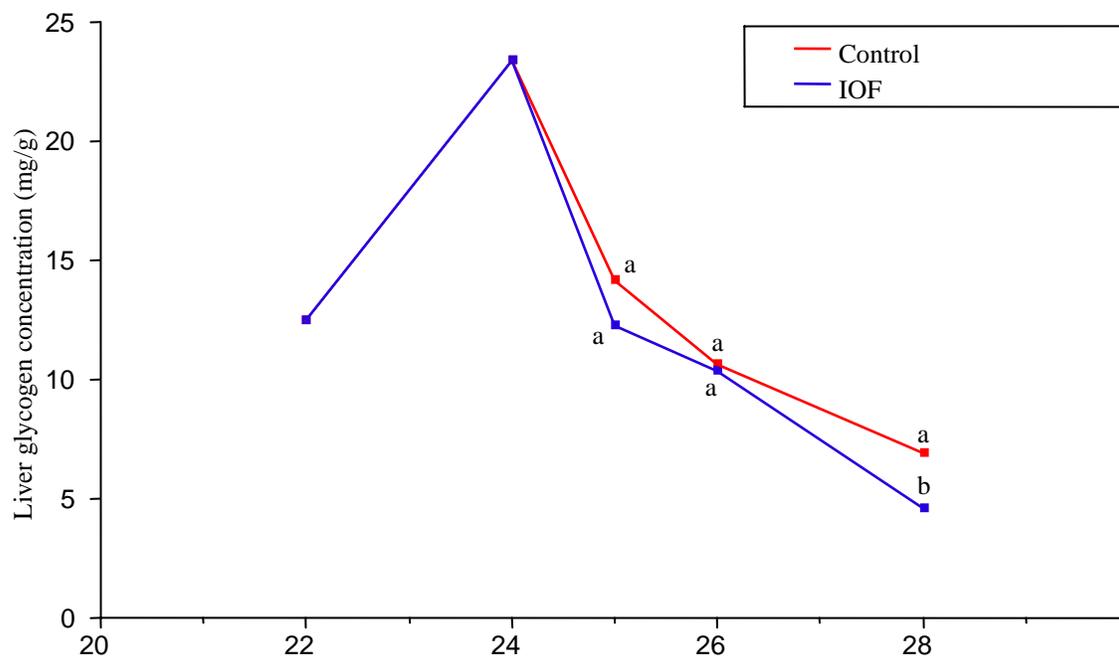


Figure 8.3. Liver glycogen concentration (mg/g) of turkey embryos from 20E until hatch.
^{a,b} Different letters in the same time point (vertically) indicate that means are significantly different (P<0.05)

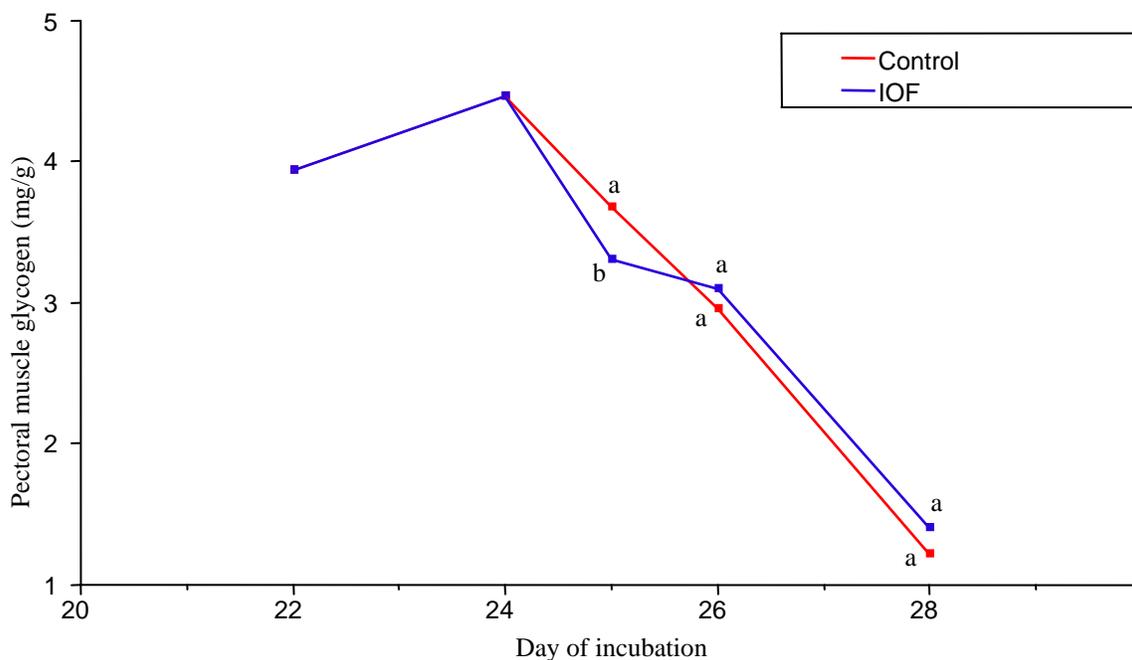


Figure 8.4. Pectoral muscle glycogen concentration (mg/g) of turkey embryos from 20E until hatch.

^{a,b}Different letters in the same time point (vertically) indicate that means are significantly different ($P < 0.05$)

Poult quality

Poult quality scores determined according to Tona et al. (2003) and leg shanks RA values are presented on Figures 8.5 and 8.6, respectively. IOF poults quality scores were 15 points higher than control poults scores (Figure 8.5). Apparently, the IOF poults are in better general condition to survive and grow than control poults. In a broiler chicken study, Tona et al. (2003) found that chicks with higher scores at hatch also showed significantly higher relative growth during the first week of life. Chicks that received low scores by a similar scoring method had higher mortality rate during the first week post-hatch (Boerjan, 2002). The IOF poults also had greater symmetry of shanks than control poults as indicated by low RA values.

According to Simsa and Ornan (2007), long bone formation in turkeys starts with cartilaginous elements that can be found at 11E. At 15E ossification of the skeletal elements starts but it is not completed until 7d post-hatch. During this period, ossification of the skeleton formed during incubation occurs simultaneously with fast bone growth at the growth plate (Simsa and Ornan, 2007). Calcification of the cartilaginous skeleton is promoted by dissolution of mammary knobs adjacent to the chorionallantois-shell membrane interface, mobilizing great amounts of calcium into circulation right before hatch. Even during the period from hatch until placement, ossification continues using calcium from calcium-enriched lipid granules present in the internalized yolk sac (Moran, 2007). Thus, symmetry of shanks may signify better nutrient utilization during cartilage formation and ossification *in ovo*, which will reduce the animal's chances to develop leg problems later in life. More studies need to be done to confirm this hypothesis.

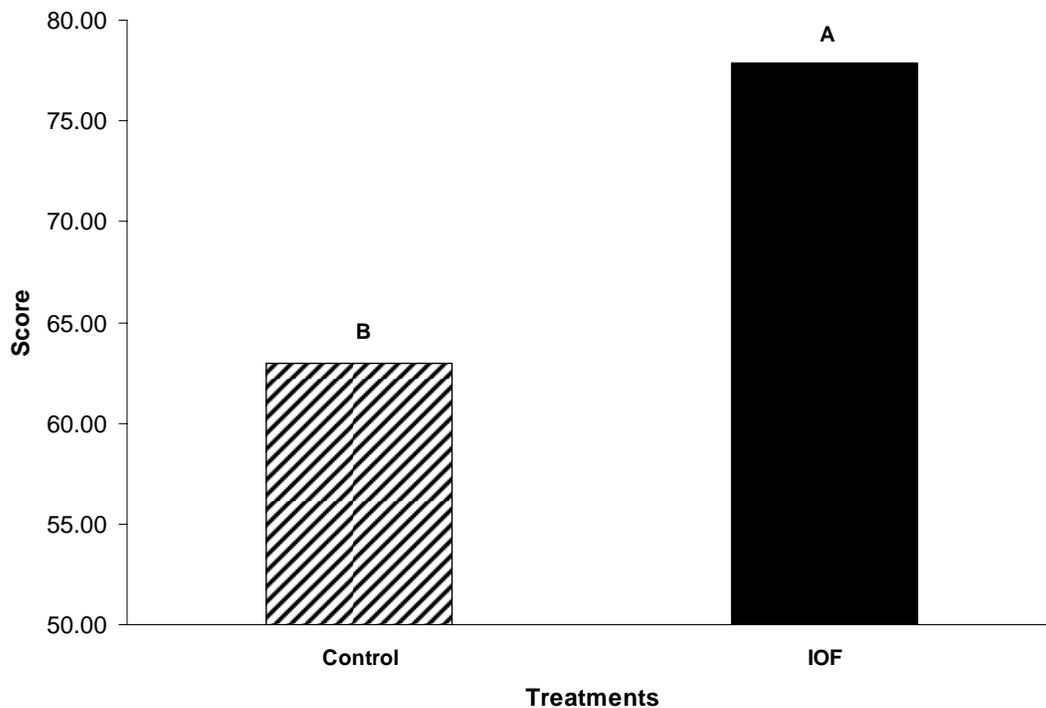


Figure 8.5. Poult quality scores of *in ovo* fed and control turkey poults at hatch.

^{A,B}Treatment bars with different letters are statistically different ($P < 0.05$)

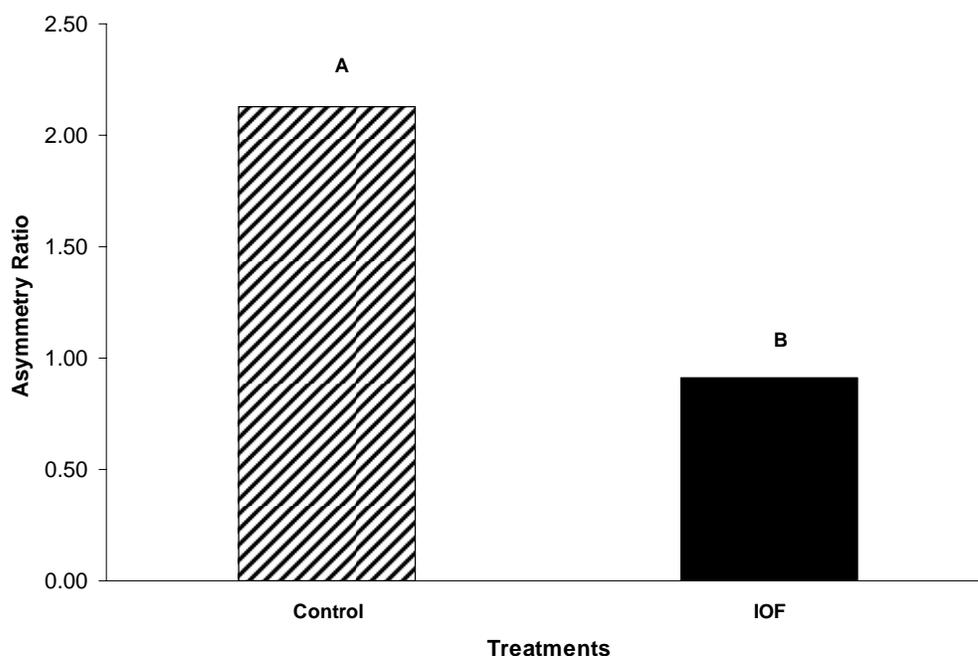


Figure 8.6. Leg shanks relative asymmetry of control and *in ovo* fed turkey poults at hatch.

^{A,B}Treatment bars with different letters are statistically different ($P < 0.05$)

Array results

All fixed effects on the analysis were significant for at least one gene. Therefore, only differences between IOF treatments for each time point (age) will be presented and discussed. Age differences were already the focus of a previous study (Chapter 6). Figure 8.7 presents the volcano plots containing genes that were significantly affected by IOF treatments on the same day in several tissues. The list of these genes and their fold difference between treatments are presented in Table 8.1.

Liver

At 25E (Figure 8.7, volcano plot A) the liver enzymes creatine kinase (A1) and transketolase (A2) are up-regulated in control embryos relative to IOF embryos. Creatine kinase activity in liver of embryos is usually minimal, but over-expression of creatine

kinase isozymes in the liver have been reported as a way to stabilize energy metabolism under low oxygen stress and after metabolic challenges (Meffert et al., 2005). Apparently, the control embryos were more energetically challenged at 25E than the IOF embryos. Transketolase is an enzyme that connects pentose phosphate pathway to glycolysis, usually to bring 5 carbon substrates (pentoses) to glucose (hexose). The higher expression of transketolase among control embryos at 25E may be an indication of their need to increase efficiency of glycolysis by gathering energetic substrates from other pathways. Evidently, the IOF treatment help the embryo deal with the energetic challenge at the time of internal pipping.

At 26E (Figure 8.7.7, volcano plot B), the glycolytic enzyme glyceraldehydes-3-phosphate (B3) was significantly up-regulated among IOF embryos relative to the controls (Table 8.1). The IOF embryos appeared to be able to move substrates up-and down the glycolysis pathway more effectively than control embryos. Gluconeogenesis is the major embryonic energetic pathway in the liver during pre-pipping embryonic energy storage, to produce glycogen for hatching (Donaldson, 1995; Keirs et al., 2002; Moran, 2007), and compounds will need to pass through liver this enzyme to produce glucose. The other affected enzyme gene was the heat-shock protein-1 (HSP1) (B4). This gene is expressed in situations of distress, including heat or cold embryo distress (Leandro et al., 2004). Heat shock protein gene expression is induced during heat and many other distress conditions in poultry (Givisiez et al., 2001; Rivera et al., 2005). The fact that HSP1 was more highly expressed in the livers of control embryo than IOF embryos (Table 8.1) indicating that the control embryos may have suffered greater distress during this period of incubation, which was alleviated by the IOF treatment.

Pectoral muscle

The only gene in pectoral muscle that was significantly affected at day of hatch (28E) by the IOF treatment was tropoelastin (5) (Figure 8.7, volcano plot C). This gene was more highly expressed in muscle of control embryo than in IOF embryos (Table 7.1). Tropoelastin is the precursor of elastin fibers that are part of muscle cell matrix. According to Parks et al. (1988), this gene is highly expressed during a specific stage of embryonic development, with lower levels of expression in mature animals. Higher expression on control embryos may indicate a distinct pattern on elastin formation on IOF embryos when compared to the controls. More research needs to be done to confirm this finding and to determine the consequences of this differential in tropoelastin gene expression.

Hatching muscle

Hatching muscle gene expression was affected by IOF at two time points. At 25E (Figure 8.7, volcano plot D), UDP-glucose dehydrogenase (6) was up-regulated in IOF embryos, indicating the production of glycosaminoglycans, involved in cell osmoregulation, causing extra-cellular space filling with water (Wormbase, 2007). This may indicate advanced hatching muscle maturation in IOF embryos, as hatching muscle hypertrophy must happen prior to hatch (John et al. 1987). Glycogen branching enzyme was the other gene that was up-regulated at 25E among control embryos (7). Up-regulation of this gene may lead to more branching of glycogen granules; however the results of glycogen analysis showed no actual difference in glycogen concentration between treatments.

At 26E (Figure 8.7, volcano plot E), the tyrosinase gene (8) was expressed at higher levels in the hatching muscle of IOF embryos than controls (Table 8.1). Tyrosinase enzyme activity has been shown to stabilize protein systems by forming additional covalent bonds between tyrosine residues in muscle fiber proteins (Lantto et al., 2007), so elevated expression of this gene may indicate enhanced muscle structure in the hatching muscle of IOF-treated embryos, and this response continued at 28E (Figure 8.7, volcano plot F) (Table 8.1).

The other gene significantly affected by IOF at 26E and 28E was HOXA3 (9 and 11, respectively). HOXA3 is a transcription factor whose gene expression is an indicator of whole tissue gene expression (Kameda et al., 2004). This gene was up-regulated in the hatching muscle of 26E control embryos, but it was up-regulated in hatching muscle of IOF embryos at hatch (28E). This difference in response between control- and IOF-treated embryos demonstrate the differences in hatching muscle general metabolism, where increased gene expression that occurs during hatching in control embryos only happened after hatch in IOF embryos. Thus the outcome of both treatments was the same as far as hatching muscle function. However, IOF treatment apparently increased hatching muscle hypertrophy and muscle fiber structuring at hatch, but it delayed the peak in general gene expression.

Duodenum

Intestinal tissues are expected to undergo many changes during late-term incubation in preparation for external feeding. Four genes in the duodenum were found to be expressed differently among control and IOF-treated embryos.

At 25E, there were two genes expressed differently between the two treatments (Figure 8.7, volcano plot G). Citrate synthase (12), the first gene of the tricarboxylic acid cycle (TCA), was expressed at higher level in the duodenum of IOF-treated embryos than control embryos. A similar response occurred for isocitrate dehydrogenase (13), the second enzyme of TCA cycle. Because both TCA cycle enzyme genes were up-regulated by IOF, the duodenum of IOF embryos were likely at a more advanced stage of development, enough to produce higher amounts of ATP than controls.

At 26E (Figure 8.7, volcano plot H), the aconitate hydratase gene (14) was more highly expressed in duodenum cells of control embryos than IOF-treated embryos. This gene is associated with the enzyme that connects the pentose phosphate pathway to the glycolysis pathway, and it is usually the rate limiting step in channeling compounds from one pathway to the other when gluconeogenic substrate is needed. Therefore, the up-regulation of aconitate hydratase may indicate some degree of energy shortage in duodenum cells of control embryos in comparison to the IOF-treated embryos. Thus, IOF treatment seems to have enhanced energy status of duodenum cells in embryos prior to hatch (25E and 26E).

Ileum

The ileum is the intestinal section where most of enteric digestion and absorption occurs, and it is also the tissue with the largest number of genes differentially expressed between the two treatments. Even though seven genes were differently expressed, they all occurred at 25E (or 24 hours after IOF was performed), all genes up-regulated among control embryos.

The control embryos exhibited gene expression related to intense energy utilization at 25E in comparison to the IOF-treated embryos. Up-regulation of aldehyde dehydrogenase (21) and pyruvate kinase (17) (both enzymes of glycolysis), 3-hydroxyacyl-CoA transferase (15) and carnitine O-palmitoyl transferase (20) (involved in beta-oxidation), and isocitrate dehydrogenase (18) (involved in the TCA cycle), are all indicators of energy utilization from glucose and fat. Other genes that were significantly up-regulated in control embryos included tyrosinase (19), indicating increased elastin crosslinking, and aminopeptidase (16), which indicates increased protein digestion.

In general, the IOF-treated embryos had a lower expression of genes related to energy utilization and metabolic distress. Perhaps increased expression of these genes in the ileum of IOF-treated embryos was not necessary because endogenous and injected nutrients had already passed through the ileum, whereas amniotic proteins were still present in the 25E control embryos. This hypothesis is supported by the fact that there were no differences in expression of these genes between the two treatments after 25E, and also IOF embryo duodenum energy metabolism was already up-regulated at the same time point (Figure 8.7, volcano plot G). This period of reduced gene expression in the ileum also coincides with the period when the YFBW of IOF embryos grew bigger than the controls (Figure 8.2), which is an indication that nutrient resources may have been invested in skeletal growth instead of intestinal function at 25E. Better distribution of nutrients at that time may be one of the reasons why IOF poult showed higher quality scores (Figure 8.5) and more symmetric legs (Figure 8.6).

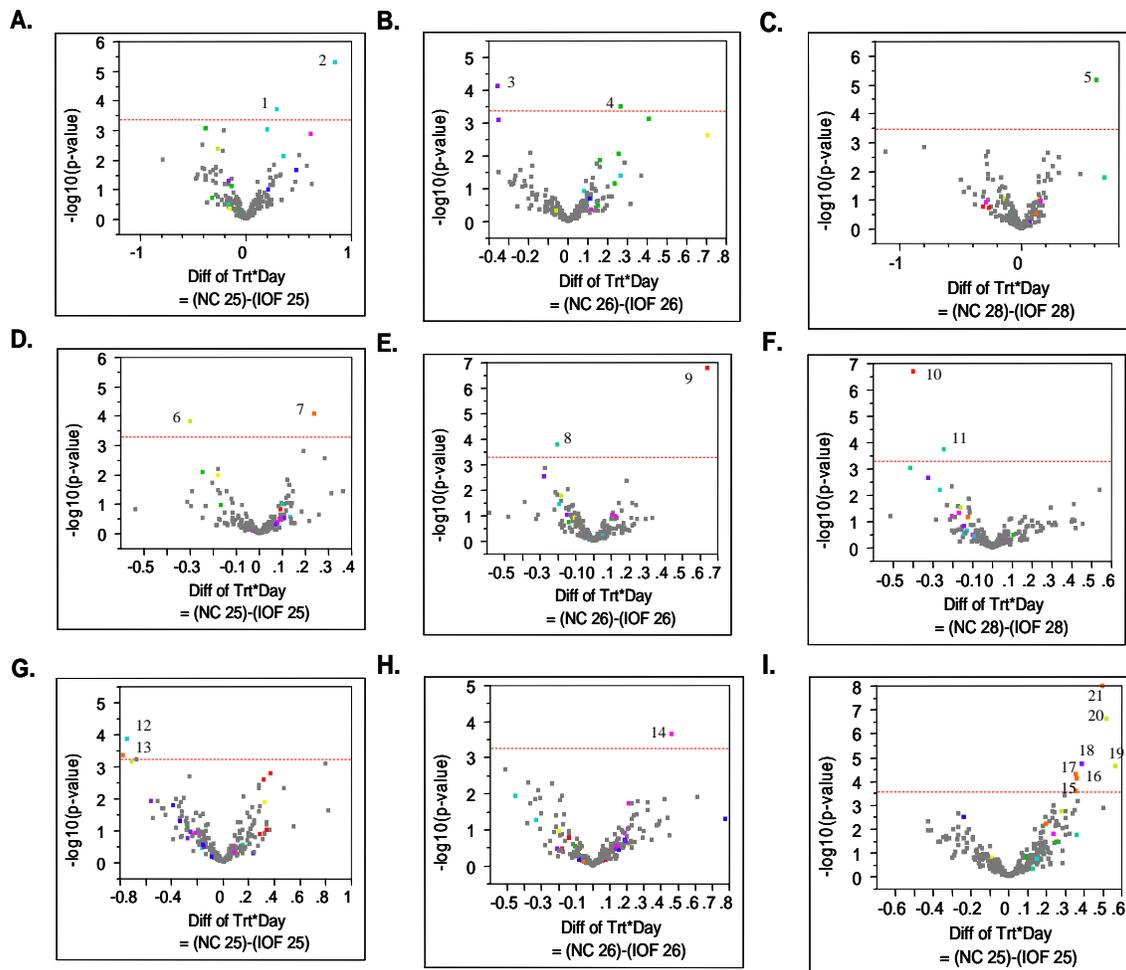


Figure 8.7. Volcano plots showing difference in gene expression between control (NC) and *in ovo* fed (IOF) turkey embryos. A. liver at 25E; B. liver at 26E; C. pectoral muscle at 28E (hatch); D. hatching muscle at 25E; E. hatching muscle at 26E; F. hatching muscle at 28E (hatch); G. duodenum at 25E; H. duodenum at 26E; I. ileum at 25E. Red dashed line corresponds to p-value cutoff; dots on the right side of the zero on the X axis are higher expressed on control embryos (NC); dots on the left of zero on X axis are higher expressed on IOF embryos. Each significant gene received a number. Gene number and correspondent gene ID and fold difference can be found in Table 1.

Table 8.1. Genes that were significantly affected¹ by *in ovo* feeding (IOF) in different turkey embryo tissues analyzed by microarrays.²

Tissue	Day of incubation	Gene	Treat.	*Fold diff.
Liver	25E	(1) creatine kinase	NC	0.8428
		(2) transketolase	NC	0.2951
	26E	(3) Glyceraldehyde-3P dehydrogenase	IOF	-0.3554
		(4) HSP1	NC	0.2640
Pectoral muscle	28E	(5) tropoelastin	NC	0.6189
Hatching muscle	25E	(6) UDP-glucose-6 dehydrogenase	IOF	-0.3012
		(7) Glycogen branching enzyme	NC	0.2377
	26E	(8) Tyrosinase	IOF	-0.2062
		(9) HOXA3	NC	0.6427
	28E	(10) Tyrosinase	IOF	-0.2427
		(11) HOXA 3	IOF	-0.4014
Duodenum	25E	(12) ATP citrate synthase	IOF	-0.7441
		(13) Isocitrate dehydrogenase	IOF	-0.7790
	26E	(14) Aconitate hydratase	NC	0.4635
Ileum	25E	(15) 3-hydroxyacyl-CoA transferase	NC	0.3583
		(16) Aminopeptidase	NC	0.3608
		(17) Pyruvate kinase	NC	0.3554
		(18) Isocitrate dehydrogenase	NC	0.3874
		(19) Tyrosinase	NC	0.5666
		(20) Carnitine O-palmitoyl transferase	NC	0.5206
		(21) Aldehyde dehydrogenase	NC	0.4953

¹(P<0.01)

²Treat. column identifies which treatment had highest expression of that gene; Day column identify which day the significant difference was found; Gene column identify which gene and its number on the volcano plot (Figure 7.7); Fold Diff. column corresponds to the fold difference on gene expression between treatments, being negative when IOF expression was higher and positive when control expression was higher.

Conclusion

In ovo feeding enhanced turkey embryo growth and glycogen storage and use in comparison to non-injected controls. These positive changes were confirmed by differences in the expression of several genes related to energy metabolism in liver, muscle, and intestinal tissues. Although these gene expression results by IOF did not yield heavier poults at hatch than the controls, they may be associated with improved poult quality and vigor at hatch, which may improve post-hatch survival and growth performance.

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Chapter 9

Conclusions

Historically the success of the poultry industry has been associated with genetic selection for increased rate of growth, feed conversion efficiency, and meat yield. As the age of commercial poultry to reach market age decreases, the period of incubation and brooding accounts for a greater portion of total production period. This genetic selection for increased growth performance favors the selection of animals that devote more resources on growth (i.e. altricial birds) than on survival (i.e. precocial birds) (Figure 9.1). As commercial poultry become more altricial, increased growth rate and production efficiency comes at the expense of weaker hatchlings that are more susceptible to high mortality during the late stages of incubation and early post-hatch development. This is especially true for turkey embryos, which spend considerable time in the plateau stage of oxygen consumption and pipping, and they are at great risk to low energy status during anaerobic metabolism. Improper incubation (E) conditions can aggravate these risks even further. In order to improve the energy status of these “altricial” hatchlings, early nutrition must be enhanced to facilitate the development of digestive capacity, thermoregulation, and immune system. *In ovo* feeding (IOF) was identified as a feasible and practical method to support the perinatal development of “altricial” turkeys so they can achieve their genetic potential for growth without compromise to survivability.

In this dissertation, chapters 2, 3, 4 and 5 demonstrated that *in ovo* feeding improved energy status of perinatal turkeys, but its effects on hatchability and performance were variable (Table 9.1). Therefore, greater understanding of the

physiology and metabolism of the late-term embryo was concluded to be necessary to advance *in ovo* feeding technology further.

First to be studied were the changes in amnion volume and embryonic fluid osmolality that occur during the late-term incubation of the three major breeds of commercial turkeys. These studies revealed that the embryos of all breeds consumed amniotic fluid at a rate of 0.10 ml/hr between 20E and 25E. Based on embryo fluid osmolality and amnion volume data, to the optimum IOF solution osmolality (460 mOsm) and volume (1.5 mL), and the optimum time of IOF solution delivery (between 22E and 23E) could be predicted.

The second step was to better understand metabolism of the late-term (20E to hatch) embryo by surveying gene expression of key enzymes associated with energy metabolism using a customized focused microarray. These results indicated that the embryos were switching from lipid to carbohydrate metabolism at 22E, confirmed metabolism associated with glycogen synthesis and degradation, and revealed that intestinal enzymes and nutrient transporters began to be up-regulated 48 hours prior to hatch. A summary of my compiled understanding on turkey embryo development from consulted literature and knowledge acquired through this research is illustrated on Figure 9.2.

Finally, with this information about the physiology and metabolism of the late-term embryo together with automated application of IOF solution delivery by the Inovoject[®] system¹, a new IOF solution was formulated to be injected at 24E, containing several metabolic co-factors. The embryonic development and hatchling quality of poults that were subjected to this new IOF treatment was compared to non-injected control

¹ Pfizer-Embrex, Inc., Research Triangle Park, Durham, NC

embryos. The embryos subjected to the IOF treatment had a distinct pattern of growth, which resulted in poults with higher quality scores and more symmetric legs at hatch. These observations were further confirmed by gene expression as accessed by microarray: the *in ovo*-fed embryos invested more resources to skeletal growth, while the control embryos devoted more metabolic resources to intestinal metabolism.

In conclusion, *in ovo* feeding of saline, β -hydroxy β -methylbutyrate (HMB) and co-factors (appendix 3, Table A3.2) will enhance the energy status of turkey embryos and advance their development, resulting in superior poult quality. Future *in ovo* feeding research should focus on identifying a protein or amino acids source that is more appropriate for *in ovo* feeding formulas, and then evaluate the effect of *in ovo* feeding with different early feeding strategies.

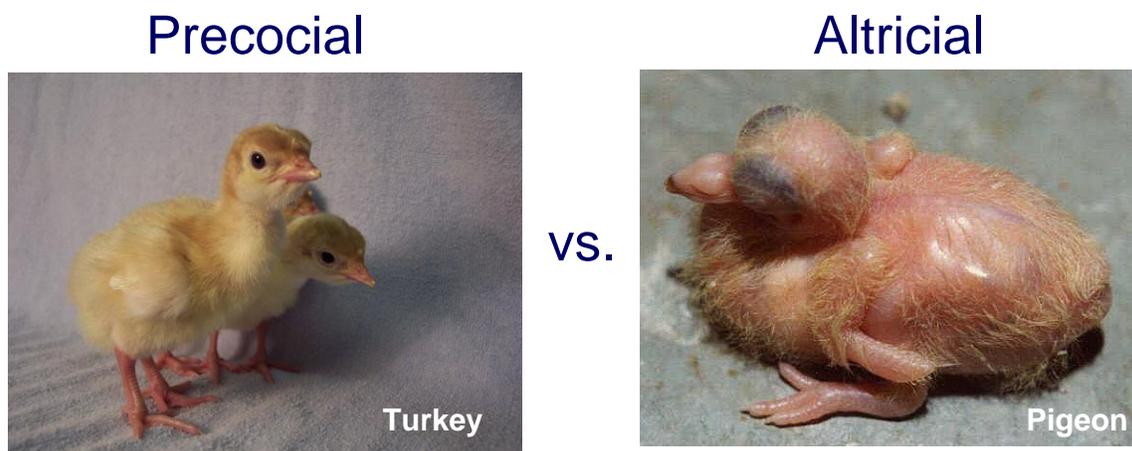


Figure 9.1. Comparison between precocial versus altricial birds at hatch. At right: turkey poults, which must be independent at hatch spending resources on survival; at left a pigeon hatchling, which depend on its parents to survive, so invests its resources on growth.

Table 9.1. Summary of research trials reported in chapters 2, 3, 4 and 5.

Trial		IOF formula	Hatchability	BW hatch	BW 7d	[GLY] at hatch
Chapter 2 (1)		CHO+HMB	+9%	ns	ns	-25%L
		CHO+8%HSP+HMB	-1.5%	+5%	ns	+30%L
		CHO+4%HSP+HMB	+5%	ns	ns	+30%L
Chapter 2 (2)		CHO+HMB	-2.3%	ns	ns	-
		CHO+8%HSP+HMB	-7%	ns	ns	-
		CHO+8%HSP+HMB+MET	-6%	+3%	ns	-
Chapter 3 (1)	1.5ml	Saline	+0.5%	ns	ns	+17%L, +53%HM
	IOF	CHO+8%HSP+HMB	-5%	ns	ns	+22%L, +65%HM
Chapter 3 (2)	1.0ml	Saline	+3%	ns	ns	-10%L, +72%HM
	IOF	CHO+8%HSP+HMB	+4%	ns	ns	+14%L, +142%HM
Chapter 4		Saline	+2%	ns	ns	0.0%L, 0.0% HM, -400%BM
		CHO+HMB	+2%	ns	ns	+85%L, +600%HM, +260% BM
		CHO+8%HSP+HMB	+2%	ns	ns	+0.0% L, +280% HM, -83% BM
Chapter 5	Hat 1	Saline	0.0	ns	ns	-65%BM
		CHO+EWP+HMB	-12%	ns	+6%	+185%BM
	Hat 2	Saline	0.0	ns	ns	-63%BM
		CHO+EWP+HMB	-16%	ns	ns	-87%BM

All data compared to non-injected control treatments

CHO=carbohydrates, HSP=hydrolyzed soy protein, HMB= β -hydroxy- β -methylbutyrate, EWP=egg white protein

*L=liver, HM=hatching muscle, BM=pectoral muscle

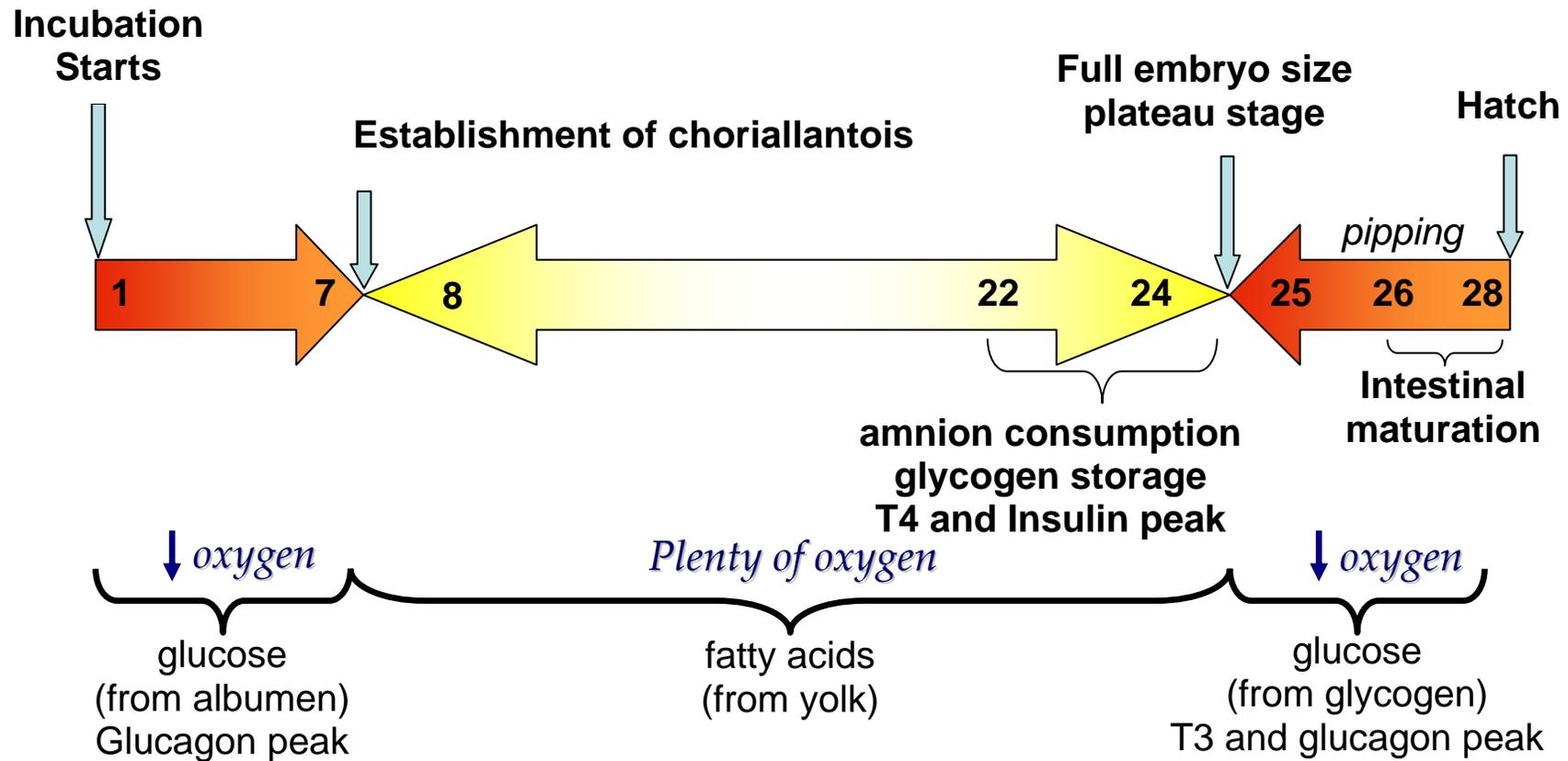


Figure 9.2. Summary of embryonic development stages emphasizing energy metabolism and intestinal development.

Appendices

Appendix 1

“The effect of various forms of administration of nutritive solutions on turkey performance”

Project justification or rationale

Previous research showed that productive performance of poultry embryos was improved after they were *in ovo* fed a combination of HMB and arginine (Foye, 2005). Some other methods of administration (Keers et al., 2002; Peebles et al., 2006) of the same solution could also have beneficial effects and be less expensive or more feasible in the industry setting. This trial will try to determine if the improvements seen with IOF can only be achieved by that method or also by other methods.

Objective

This experiment was designed to compare the efficacy of giving poult extra nutrients *in ovo* or post-hatch by several methods in order to identify the best delivery method that yields the greatest effect on early development.

Material and methods

Eight-hundred-forty BUT turkey eggs were obtained from a commercial hatchery at 20 days of incubation (E). The eggs were candled, weighted and divided into 5 groups of 105 eggs with similar weight distribution. Each egg group was assigned to one of five treatment groups: 1) *in ovo* injection from the top at 24E (ITP); 2) *in ovo* injection from the side at 22E (ISD); 3) non-injected controls (NC); 4) gavage at hatch; and 5) subcutaneous injection on the neck at hatch (INK). At 28E the hatch was pulled and hatchability and poult body weight was recorded for each treatment. Pectoral muscle, liver and yolk as a percent of embryo weight were calculated at hatch. Poults were sexed and placed in floor pens according to treatment X gender combination (14 poults/pen) at day of hatch or 24hrs later. The poults placed 24 hrs later remained in transport boxed under heating lamps in the brooding house. Once placed, treatments received feed and water *ad libitum*. Feed was formulated to attend or exceed NRC (1994) requirements. Poults were raised until 42d of age, and poults were individually weighted at 3, 7, 14, 21, 35 and 42d. The experimental protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee.

Results

Table A1.1. Hatchability, piped eggs and dead eggs¹

Treatment	Hatchability (%)	Piped (%)	Dead (%)
IOF top injected (ITP)	79.58	13.47	6.52
IOF side injected (ISD)	83.63	13.21	3.16
NC	83.38	10.68	5.93
Gavage	84.25	12.12	3.62
Neck Injected	80.67	15.32	4.00
SEM	3.273	2.602	1.523
p-value	0.8056	0.8575	0.6474

¹Values are mean of 12 replicate baskets.

Table A1.2. Body weight of turkey pouts at day of hatch¹

Treatment	Body Weight (g)
IOF top injected (ITP)	62.58 ^b
IOF side injected (ISD)	67.46 ^a
NC	61.60 ^b
Gavage	63.74 ^b
Neck Injected	62.40 ^b
SEM	1.482
p-value	0.0233

¹Values are mean of 175 poults.

^{a,b}Means with different upper case letter superscripts are significantly different (P<0.05)

Table A1.3. Body weight of turkey pouts at day of hatch, by gender.

Gender	Body weight (g)
Male	63.49
Female	63.63
SEM	0.939
p-value	0.9128

²Values are mean of 435 poults

Table A1.4 – Pectoral muscle, liver and yolk of turkeys at day of hatch^{1,2}

Treatment	Pectoral Muscle (%)	Liver (%)	Yolk (%)
IOF top injected (ITP)	2.66	2.14	10.80 ^a
IOF side injected (ISD)	2.51	2.36	8.23 ^b
NC	2.37	2.26	10.14 ^a
SEM	0.147	0.080	0.756
p-value	0.3699	0.1767	0.0611

¹results for pectoral muscle (without bones), liver and yolk, expressed as percentage of body weight.

²Values are mean of 10 samples

^{a,b}Means with different upper case letter superscripts in the same column are significantly different (P<0.05)

Table A1.5. Body weight of turkey poults from 3 to 42 days of age¹

Treatment	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
IOF top injected (ITP)	81.78	136.90	295.34	578.06	1039.75	1560.71	24441.47
IOF side injected (ISD)	82.73	138.48	303.73	585.53	1053.11	1589.07	2471.83
NC	82.44	138.39	297.56	582.76	1036.98	1586.22	2447.80
Gavage	82.98	139.32	298.77	583.79	1047.74	1578.48	2482.80
Neck Injected	81.68	136.93	301.18	590.73	1050.31	1593.94	2472.17
SEM	0.692	1.415	4.365	7.253	11.759	16.356	24.119
P-value	0.586	0.681	0.674	0.796	0.859	0.609	0.696

No differences between means were observed (P<0.05)

¹Values represent a mean of 70 poults.

Table A1.6. Body weight of turkeys placed at day of hatch and 24 hours later¹

Placement	Day 3	Day 7	Day 14	Day 35	Day 42
	(g)				
Day of hatch	84.97 ^A	142.42 ^A	305.33 ^a	1592.76	2479.98
24hrs holding	79.66 ^B	133.57 ^B	293.29 ^b	1570.61	2446.36
SEM	0.432	0.913	2.813	10.614	15.423
P-value	0.0001	0.0001	0.0025	0.1388	0.1220

¹Values are mean of 435 poults

^{a,b/A,B}Means with different upper letter superscripts in the same column are significantly different, lower case (P<0.05) and upper case (P<0.01)

Conclusion

The different forms of delivering the nutritive solution did not affect hatchability, but poultts that were injected from the side at 22E were heavier at hatch than all the other treatment groups. Embryos *in ovo* fed by side injection also had less yolk at hatch. There were no differences among treatments for body weight post-hatch. Poultts that had immediate access to feed and water were heavier and gained more weight than poultts that were held for 24 hours all the way to 42d of age.

In ovo feeding enhanced poult body weight at hatch and was identified as the most feasible method of delivering nutritive products that impact early development. Poultts held for 24 hours had inferior performance in comparison to those poultts give *ad libitum* access to feed and water right after hatch.

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Appendix 2

“The effect of osmolality and protein sources of nutrition solutions injected *in ovo*”

Project justification or rationale:

Several *in ovo* feeding solution components have been evaluated on the development of perinatal chickens and turkeys (Foye 2005; Smirnov et al 2006; Tako *et al.* 2004; Tako et al., 2005; Uni *et al.* 2005). However, optimum formulation of more complex IOF solutions requires knowledge about the osmolality limits for IOF applications using different ingredients. Therefore, a study was designed to determine the effect of *in ovo* feeding solution osmolality on hatchability of turkey poults.

Objectives

1. Study the optimum osmolality for *in ovo* feeding solutions.
2. Compare two sources of protein (egg white and whole egg).

Material and methods

Six-hundred fertile turkey eggs were obtained from a commercial hatchery. The eggs were weighted and divided into 8 groups of eggs with similar weight distribution. Six treatment groups of 50 eggs each were injected into the amnion with isocaloric solutions of dextrin and glucose formulated to 5 osmolality levels (200, 500, 800, 1100 and 1400 mOsm, corresponding to treatments 1 through 6, respectively). These 5 treatment groups of increasing osmolality were compared to non-injected eggs (NC). The last 2 groups of 50 eggs were *in ovo* fed solutions containing whole egg (24% Protomax + 0.1% HMB + 0.4% saline) (WEG) or egg white (24% egg white protein + 0.1% HMB + 0.4% NaCl) (EWP). The *in ovo* feeding protocol was performed at 23E. At day of hatch, hatchability and poult body weight was recorded for each treatment. At the end of the trial poults were euthanized by CO₂. The experimental protocol was approved by the North Carolina State University Institutional Animal Care and Use Committee.

Results

Table A2.1. Osmolality of Injected Solutions

Solution	Osmolality
T2 (250 mOsm)	253
T3 (550 mOsm)	542
T4 (850 mOsm)	863
T5 (1150 mOsm)	1176
T6 (1450 mOsm)	1455
T7 (Protomax)	448
T8 (Egg White)	665

Values are mean of triplicate readings¹

Hatchability

Table A2.2. Percent hatchability of turkey eggs *in ovo* fed with different solutions¹

Treatment	Hatchability (%)
T1 (control)	70.67 ^B
T2 (250 mOsm)	90.00 ^{A^B}
T3 (550 mOsm)	91.67 ^A
T4 (850 mOsm)	88.33 ^{AB}
T5 (1150 mOsm)	91.67 ^A
T6 (1450 mOsm)	81.67 ^{AB}
T7 (protomax)	26.00 ^C
T8 (Egg white)	76.67 ^{AB}
SEM	7.363
P-value	0.0002

^{A,B,C}Means within columns with different upper case superscript letters differ significantly (P<0.01)

¹Values are mean of triplicate hatching baskets

Table A2.3. Percent of turkey poults that piped the egg shell but did not hatch¹

Treatment	Piped Eggs (%)
T1 (control)	0.00
T2 (250 mOsm)	1.67
T3 (550 mOsm)	5.00
T4 (850 mOsm)	5.00
T5 (1150 mOsm)	0.00
T6 (1450 mOsm)	1.67
T7 (protomax)	54.00
T8 (Egg white)	10.00
SEM	6.036
P-value	0.2045

¹Values are mean of triplicate hatching baskets

¹ Micro osmometer Model 3300, Advanced Instruments, Inc., Norwood, MA

Body weight**Table A2.4.** Body weight of day old turkey poult *in ovo* fed with solutions with different protein sources

Treatment	Body weight Mean (g)
T7 (protomax)	62.59
T8 (Egg white)	63.71
SEM	1.239
P-value	0.5401

No difference was found between treatments

Table A2.5. Body weight of day old turkey poult *in ovo* fed with solutions with different osmolalities

Treatment	Body weight Mean (g)
T1 (control)	62.80 ^B
T2 (250 mOsm)	65.33 ^B
T3 (550 mOsm)	67.53 ^A
T4 (850 mOsm)	64.94 ^B
T5 (1150 mOsm)	64.76 ^B
T6 (1450 mOsm)	63.61 ^B
SEM	0.750
P-value	0.001

^{A,B}Means with different upper case letter superscripts in the same column are significantly different (P<0.01)

Regression:

Quadratic equation:

Table A2.6. Parameter estimates for quadratic regression of day old turkey poult

Parameter	Estimate	P-value
Intercept	63.39667050	<.0001
mOsm	0.00815645	0.0006
mOsm ²	-0.00000576	0.0002

$$BW=63.3966705+0.00815645*mOsm-0.00000576*mOsm^2$$

$$R^2=0.061725$$

Where:

BW = body weight

mOsm = osmolality of the solution

Best estimated body weight at **708.0 mOsm** (equation derivate)

QUADRATIC REGRESSION FOR OSMO HATCH

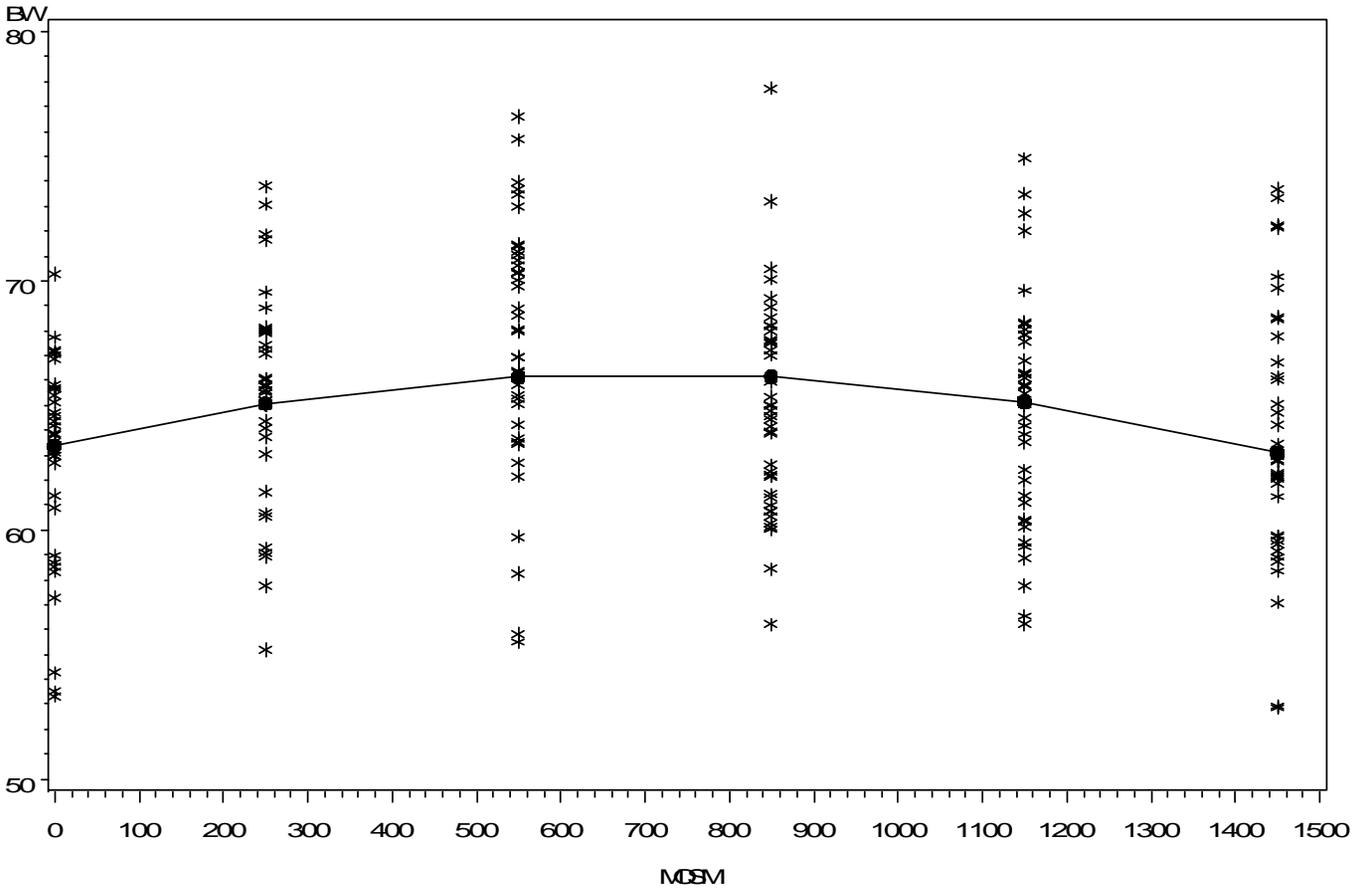


Figure A2.1. Regression analysis of body weight by osmolality of *in ovo* feeding solution

Sampled poult:**Day of Hatch****Table A2.7.** Body weight of day old turkey poult

Treatment	Body Weight (g)
NC	61.73b
IOF carbohydrates 550 mOsm	64.91a
IOF egg white protein	63.00b
SEM	1.206
P-value	0.0736

^{a,b}Means with different lower case letter superscripts in the same column are significantly different (P<0.05)

Table A2.8. Pectoral muscle of day old turkey poult as percent of body mass

Treatment	Pectoral muscle (%)
NC	2.85
IOF carbohydrates 550 mOsm	2.81
IOF egg white protein	2.86
SEM	0.111
P-value	0.929

No difference was found between treatments

Table A2.9. Yolk present in day old turkey poult as percent of body mass

Treatment	Yolk (%)
NC	8.21
IOF carbohydrates 550 mOsm	9.89
IOF egg white protein	9.10
SEM	0.7359
P-value	0.283

No difference was found between treatments

Egg Samples**Table A2.10.** Weight of turkey eggs sampled on different days of incubation

Day of incubation	Egg weight (g)
20E	85.58
21E	84.64
22E	84.60
23E	84.27
SEM	1.186
P-value	0.8766

No difference was found between treatments

Table A2.11. Allantois of turkey eggs sampled on different days of incubation as percent of egg weight

Day of incubation	Allantois (%)
20E	5.09 ^a
21E	4.87 ^a
22E	4.75 ^a
23E	3.56 ^b
SEM	0.365
P-value	0.049

^{a,b}Means with different lower case letter superscripts in the same column are significantly different (P<0.05)

Table A2.12. Turkey embryo mass from eggs sampled on different days of incubation as percent of egg weight

Day of incubation	Embryo (%)
20E	31.80 ^D
21E	35.76 ^C
22E	42.37 ^B
23E	49.91 ^A
SEM	0.733
P-value	0.0001

^{A,B,C,D}Means within columns with different upper case superscript letters differ significantly (P<0.01)

Table A2.13. Extra-embryonic fluid collected from turkey eggs sampled on different days of incubation as percent of egg weight

Day of incubation	Fluid (%)
20E	7.25 ^A
21E	5.22 ^B
22E	4.36 ^B
23E	3.82 ^B
SEM	0.534
P-value	0.0001

^{A,B}Means within columns with different upper case superscript letters differ significantly (P<0.01)

Table A2.14. Yolk of turkey eggs sampled on different days of incubation as percent of egg weight

Day of incubation	Yolk (%)
20E	27.64
21E	27.30
22E	26.89
23E	26.23
SEM	0.504
P-value	0.2318

No difference was found between treatments

Table A2.15. Osmolality of the amnion from turkey eggs sampled on different days of incubation

Day of incubation	Amnion Osmolality (mOsm)
20E	287.54 ^A
21E	216.20 ^B
22E	291.46 ^A
23E	200.30 ^B
SEM	15.326
P-value	0.0001

^{A,B}Means within columns with different upper case superscript letters differ significantly (P<0.01)

Table A2.16. Osmolality of turkey embryos sampled on different days of incubation

Day of incubation	Embryo Osmolality (mOsm)
20E	310.30 ^B
21E	234.78 ^D
22E	304.68 ^C
23E	317.68 ^A
SEM	1.444
P-value	0.0001

^{A,B,C,D}Means within columns with different upper case superscript letters differ significantly (P<0.01)

Table A2.17. Amnion volume of turkey embryos sampled on different days of incubation

Day of incubation	Amnion (ml)
20E	10.96 ^A
21E	10.70 ^B
22E	6.20 ^C
23E	1.43 ^D
SEM	0.526
P-value	0.0001

^{A,B,C,D}Means within columns with different upper case superscript letters differ significantly (P<0.01)

Conclusion

The optimum IOF solution osmolality based on body weight at hatch was determined to be 708 mOsm, but this level of osmolality may adversely affect hatchability. *In ovo* feeding of whole egg reduced hatchability by 70%, so it is not a viable ingredient for *in ovo* feeding formulation. Osmolality of amniotic fluid averaged 241.90 mOsm, while embryo osmolality averaged 291.86 mOsm. *In ovo* feeding osmolality combined with amniotic fluid osmolality should not exceed embryo osmolality, but this hypothesis must be confirmed by future investigation.

References

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Appendix 3

Table A3.1. Genes printed on NCSU_JArray_1

Well	TC	Gene
a1	TC186705	alpha-amylase
a2	TC186914	creatine kinase
a3	TC186925	hexokinase 1
a4	TC187124	fructose biphosphate aldolase B
a5	TC187356	formaldehyde dehydrogenase
a6	TC187369	pyruvate dehydrogenase
a7	TC187430	acetyl-CoA acyltransferase
a8	TC187511	citrate lyase
a9	TC187936	adenylate kinase
a10	TC188444	CaM kinase II gamma
a11	TC188637	phosphoenolpyruvate carboxykinase (PEPCK)
a12	TC188815	fructose biphosphatase (F1,6 BPase)
a13	TC188827	Glucose-6-phosphatase (G-6-Pase)
a14	TC188867	oxoglutarate dehydrogenase
a15	TC188910	transaldolase
a16	TC188929	growth hormone-inducible soluble protein
a17	TC189600	phosphoglucomutase
a18	TC189614	glycerol-3-phosphate dehydrogenase
a19	TC189690	transketolase
a20	TC190340	aconitate hydratase
a21	TC191346	glycogen branching enzyme
a22	TC192153	malate dehydrogenase
a23	TC192437	maltase-glucoamylase
a24	TC192696	phosphorylase kinase B alpha regulatory chain
b1	TC192763	hexokinase II
b2	TC193299	glutamate dehydrogenase
b3	TC193508	growth hormone receptor
b4	TC193519	phosphopyruvate hydratase
b5	TC193574	glucagon receptor
b6	TC194407	pyruvate carboxylase
b7	TC194518	acetyl-CoA carboxylase
b8	TC195362	glycogen synthase 1 (muscle)
b9	TC195364	glycogen synthase
b10	TC196585	D-lactate dehydrogenase
b11	TC196862	thyroid hormone receptor beta
b12	TC197415	thyroid hormone receptor alpha
b13	TC198192	acetate CoA ligase
b14	TC198738	ATP-gated ion channel receptor
b15	TC198849	malate dehydrogenase
b16	TC200175	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
b17	TC201283	glucokinase
b18	TC202032	insulin receptor tyrosine kinase
b19	TC202805	protein phosphatase 1

b20	TC206940	acetyl CoA hydrolase
b21	TC207102	Sucrase-isomaltase intestinal
b22	TC207111	glyceraldehyde 3-phosphate dehydrogenase
b23	TC207134	fructose-biphosphate aldolase
b24	TC207821	growth hormone
c1	TC207878	pyruvate kinase
c2	TC207931	L-lactate dehydrogenase
c3	TC208213	succinate CoA ligase
c4	TC208369	phosphogluconate dehydrogenase
c5	TC208477	aldehyde dehydrogenase
c6	TC208609	3-hydroxy-3-methylglutaryl-CoA reductase
c7	TC208805	3-hydroxyacyl-CoA dehydrogenase
c8	TC208909	UDP-glucose 6-dehydrogenase
c9	TC209183	isocitrate dehydrogenase (NAD ⁺)
c10	TC209295	glycogen phosphorylase
c11	TC209686	hydroxyacylglutathione hydrolase
c12	TC210057	fumarate hydratase
c13	TC210085	isocitrate dehydrogenase (NADP ⁺)
c14	TC210106	insulin receptor
c15	TC210122	glutamine synthetase
c16	TC210127	phosphofructokinase (PFK-1)
c17	TC210710	carnitine O-palmitoyl transferase
c18	TC210716	glycogen synthase kinase-3 beta
c19	TC211999	malic enzyme
c20	TC212313	butyryl-CoA dehydrogenase
c21	TC212504	carnitine acetylase
c22	TC212978	aminopeptidase
c23	TC213033	6-phosphofructokinase (PFK-1)
c24	TC213364	ATP citrate synthase
d1	TC213413	lipoprotein lipase (LPL)
d2	TC214310	galactose-1-phosphate uridylyltransferase
d3	TC214367	aminopeptidase Ey
d4	TC214722	insulin-like growth factor 2 receptor (ILGF-2 receptor)
d5	TC214754	peptide transporter
d6	TC215002	growth hormone-releasing hormone (GHRH)
d7	TC216336	UTP-glucose-1-phosphate urydylyltransferase
d8	TC216360	glucose-6-phosphate isomerase
d9	TC216375	sodium/glucose co-transporter 1
d10	TC217180	thyroxine deiodinase I
d11	TC219249	6-phosphofructokinase (PFK-1)
d12	TC219401	tyrosinase precursor
d13	TC221240	growth hormone-releasing hormone (GHRH)
d14	TC223014	glycogen phosphorylase (muscle)
d15	TC223171	growth hormone receptor (GHR)
d16	TC225543	succinate dehydrogenase
d17	TC225625	glycogen phosphorylase
d18	TC227576	tyrosinase
d19	TC186641	chGAPDH

d20	TC251311	atCAB2 - Arapdopsis
d21	TC207783	chEF2 - elongation factor 2
d22	TC201083	chBACT
d23	N/A	Turkey genomic DNA
d24	N/A	Turkey genomic DNA

Appendix 4

Table A4.1. Genes printed on NCSU_JSArray_2

well	TC	gene
d4	TC195766	IFN?
g3	TC227712	Tenascin precursor
g4	TC227805	Signal transducer and activator of transcription 3
g5	TC227960	signal transducer and activator of transcription 4
g17	TC230252	Interferon gamma precursor (IFN-gamma)
g22	TC230774	Putative CXCR1 isoform I and II
h3	TC236322	Insulin-like growth factor I precursor (IGF-I) (Somatomedin)
h4	TC239714	Interleukin-7
h9	TC243080	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog
h11	TC246235	JNK2 beta2 protein kinase
h14	TC258323	Interferon alpha-A precursor
h17	TC260683	Signal transducer and activator of transcription 5
h18	TC262334	Toll-like receptor 3
h20	TC264863	Interferon type B precursor
h22	TC273089	2' 5'-oligo adenylate synthetase A
i7	TC288734	Nuclear factor NF-kappa-B p100
i8	TC289521	Small inducible cytokine A2
i9	TC296939	Interleukin 12 receptor beta 2
i10	TC299780	Signal transducer and activator of transcription 2
l6	TC208609	3-hydroxy-3-methylglutaryl-CoA reductase
l7	TC208805	3-hydroxyacyl-CoA dehydrogenase
l23	TC213033	6-phosphofructokinase (PFK-1)
m11	TC219249	6-phosphofructokinase (PFK-1)
E17	TC211222	ACE
k13	TC198192	acetate CoA ligase
k20	TC206940	acetyl CoA hydrolase
j7	TC187430	acetyl-CoA acyltransferase
k7	TC194518	acetyl-CoA carboxylase
j20	TC190340	aconitate hydratase
d11	TC196698	ACTA2
j9	TC187936	adenylate kinase
d14	TC197933	AGTR1
l5	TC208477	aldehyde dehydrogenase
j1	TC186705	alpha-amylase
l22	TC212978	aminopeptidase
m3	TC214367	aminopeptidase Ey
E4	TC208792	ANF / ANP
c22	TC194431	Ang1
f2	TC214356	Ang2B
a1		ANKRD1
b21	TC186185	APLP2
f13	TC217958	AR

f3	TC214418	ARNTL
a2		ARNTL2
a3		ARPC1B
l24	TC213364	ATP citrate synthase
k14	TC198738	ATP-gated ion channel receptor
c7	TC189780	AZIN1
i20		BACT
m20		BACT
a4		BMPER
l20	TC212313	butyryl-CoA dehydrogenase
i22		CAB2
m22		CAB2
j10	TC188444	CaM kinase II gamma
l21	TC212504	carnitine acetylase
l17	TC210710	carnitine O-palmitoyl transferase
a5		CASQ2
i23		CHICK DNA
m23		CHICK DNA
j8	TC187511	citrate lyase
f5	TC214693	CMF1
a6		COUP-TFII
a7		cox
j2	TC186914	creatine kinase
a8		CTGF
a9		cTnT
g2	TC226344	CYGB
e9	TC209608	DCN
d24	TC206985	Desmin
a10		DIO2
k10	TC196585	D-lactate dehydrogenase
f24	TC225040	Dopamine beta-hydroxylase
c15	TC192318	ECE1
e23	TC213898	EDNRA
d7	TC196241	EDNRB2
i21		EF2
m21		EF2
f16	TC218455	EFNB2
f10	TC216565	EGFr
a11		EGR1
c19	TC193337	Elastin
e13	TC210338	Endoglin
a12		ENO1
f12	TC217613	eNOS
k16	TC200175	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
f1	TC214190	EPAS1
a13		FIGF
c8	TC189896	Fih1
a14		Flk1

a15		FLT1
j5	TC187356	formaldehyde dehydrogenase
j12	TC188815	fructose biphosphatase (F1,6 BPase)
j4	TC187124	fructose biphosphate aldolase B
k23	TC207134	fructose-biphosphate aldolase
a16		FSTL1
l12	TC210057	fumarate hydratase
m2	TC214310	galactose-1-phosphate uridylyltransferase
i19		GAPDH
m19		GAPDH
d23	TC206873	GATA-4
m13	TC221240	GHRHR
k5	TC193574	glucagon receptor
k17	TC201283	glucokinase
j13	TC188827	Glucose-6-phosphatase (G-6-Pase)
m8	TC216360	glucose-6-phosphate isomerase
k2	TC193299	glutamate dehydrogenase
l15	TC210122	glutamine synthetase
k22	TC207111	glyceraldehyde 3-phosphate dehydrogenase
j18	TC189614	glycerol-3-phosphate dehydrogenase
j21	TC191346	glycogen branching enzyme
l10	TC209295	glycogen phosphorylase
m17	TC225625	glycogen phosphorylase
m14	TC223014	glycogen phosphorylase (muscle)
k9	TC195364	glycogen synthase
k8	TC195362	glycogen synthase 1 (muscle)
l18	TC210716	glycogen synthase kinase-3 beta
a17		GPI
k24	TC207821	growth hormone
k3	TC193508	growth hormone receptor
m15	TC223171	growth hormone receptor (GHR)
j16	TC188929	growth hormone-inducible soluble protein
m6	TC215002	growth hormone-releasing hormone (GHRH)
a18		HA
f21	TC221573	HAND1
c20	TC193975	HAND2
e1	TC207660	HB alpha A
b22	TC186781	HB alpha D
c1	TC187403	HB rho
e2	TC207666	HBB
e6	TC209414	HBE
a19		HBZ
f6	TC215499	Hepatoma-derived growth factor
j3	TC186925	hexokinase 1
d10	TC196652	HIF1A
k1	TC192763	HK2
e11	TC209954	HMOX1
d17	TC198810	HOXA13

a20		HOXA3
d8	TC196302	HSPE1
l11	TC209686	hydroxyacylglutathione hydrolase
c24	TC194969	HYOU1
e21	TC213016	ID2
f7	TC216296	IER5
a21		IGF2BP3
f11	TC216581	IGFBP1
k18	TC202032	insulin receptor tyrosine kinase
m4	TC214722	insulin-like growth factor 2 receptor (ILGF-2 receptor)
a22		IRX1
a23		IRX2
d9	TC196427	IRX4
l9	TC209183	isocitrate dehydrogenase (NAD+)
l13	TC210085	isocitrate dehydrogenase (NADP+)
l14	TC210106	insulin receptor
a24		KCNA3
b1		KCND3
f15	TC218406	KCNG2
c17	TC192784	LBR
f23	TC224514	LEREPO4 protein - similar
m1	TC213413	lipoprotein lipase (LPL)
l2	TC207931	L-lactate dehydrogenase
j22	TC192153	malate dehydrogenase
k15	TC198849	malate dehydrogenase
l19	TC211999	malic enzyme
j23	TC192437	maltase-glucoamylase
d3	TC195226	Mitochondrial uncoupling protein
e3	TC207977	MLC2a
c3	TC188631	MTPN
c12	TC191332	MYBPC3
b2		MYH7
b3		MYH7B
b4		MYL4
e16	TC210862	MYL9
b5		NA
b6		NKX2-5
e7	TC209493	NOS2A
e8	TC209493	NOS2A
b7		NP
b8		NP959225
b9		NR2F2
b10		NS
e22	TC213495	NT5C3
j14	TC188867	oxoglutarate dehydrogenase
b11		PA
b12		PB1
b13		PB2

g1	TC225202	pCTnC1
c11	TC191022	PDGFA
b14		PDGFRA
b15		PDK1
m5	TC214754	peptide transporter
e19	TC211891	PHF14
b16		PHF20L1
j11	TC188637	phosphoenolpyruvate carboxykinase (PEPCK)
l16	TC210127	phosphofructokinase (PFK-1)
j17	TC189600	phosphoglucomutase
l4	TC208369	phosphogluconate dehydrogenase
k4	TC193519	phosphopyruvate hydratase
j24	TC192696	phosphorylase kinase B alpha regulatory chain
c10	TC190986	PLB
f22	TC224053	Preproendothelin-1
e12	TC210077	PRKAG2
d18	TC199407	Probable hemoglobin and hemoglobin-haptoglobin binding protein 3 precursor
k19	TC202805	protein phosphatase 1
b17		PTK2
k6	TC194407	pyruvate carboxylase
j6	TC187369	pyruvate dehydrogenase
l1	TC207878	pyruvate kinase
d22	TC205515	RAP1GDS1
d16	TC198637	RARB
b18		sEH
c9	TC189944	SERCA2
c14	TC191959	SLC2A1
c6	TC189636	SM22
b19		SOD1
b20		SOD2
m9	TC216375	sodium/glucose co-transporter 1
d1	TC194994	SOX4
b24	TC187113	SPARC
l3	TC208213	succinate CoA ligase
m16	TC225543	succinate dehydrogenase
k21	TC207102	Sucrase-isomaltase intestinal
d13	TC197879	TBX20
f17	TC218562	TBX5
i12		TGFBR1
c23	TC194887	TGFBR2
d19	TC199532	TH
k12	TC197415	THRA
k11	TC196862	THRB-1
m10	TC217180	thyroxine deiodinase I
i13		TNN2
i14		TNS1
c5	TC189578	TOP2B
j15	TC188910	transaldolase

j19	TC189690	transketolase
c18	TC193336	Tropoelastin
i24		TURKEY DNA
m24		TURKEY DNA
m18	TC227576	tyrosinase
m12	TC219401	tyrosinase precursor
l8	TC208909	UDP-glucose 6-dehydrogenase
m7	TC216336	UTP-glucose-1-phosphate urydyltransferase
i15		VEGF
i17		XIN
i16		YWHAB
i18		YWHAB
e5	TC209233	ZYX
i1	TC284742	IL-2 precursor
g21	TC230691	Interleukin-1beta
g19	TC230515	IL-8
g20	TC230515	IL-8
h23	TC274133	Gal-1 alpha
h24	TC279522	Gal-1 alpha
i3	TC286576	Gal-2
g15	TC229568	Q4JIM4
g9	TC228700	Q5ZKW4
g8	TC228683	Carnitine palmitoyl transferase I
h12	TC251657	CD3 glycoprotein
h15	TC259029	CD3 glycoprotein complete
h13	TC253132	Cytoskeletal beta actin
h19	TC264669	Bu-1b
h5	TC240827	Chemokine K203 precursor
h1	TC234083	Beta-actin
g12	TC228810	CCR
i4	TC287964	CCR5
i5	TC288248	CCR9
h10	TC243402	CNDP dipeptidase 2
g18	TC230261	CXCR4
h2	TC234344	FasL
h6	TC242243	GAPDH
h7	TC242243	GAPDH
f18	TC219402	GM-CSF
i11	TC301924	homologue to T-cell immunoglobulin and mucin domain containing 4
g14	TC229540	IFNAR2
i6	TC288597	IFNGR2
e20	TC212665	IL1
d12	TC197579	IL10
h16	TC259509	IL10RA
h8	TC242575	IL11RA
f19	TC221524	IL13
h21	TC272839	IL13RA2
f4	TC214674	IL15

g11	TC228787	IL17R
e15	TC210696	IL18
g24	TC232347	IL1R1
e24	TC214019	IL2
g23	TC230927	IL2RA
f20	TC221532	IL3
d6	TC196102	IL4
d20	TC200395	IL5
c21	TC194010	IL6
c4	TC188846	IRF1
f9	TC216539	IRF10
e14	TC210368	IRF2
c16	TC192542	IRF3
e18	TC211361	IRF4
b23	TC186941	IRF6
e10	TC209944	IRF8
d5	TC195947	Jak
c2	TC188064	KIF3A
d2	TC195106	MX
d15	TC198289	Rantes
g7	TC228391	RNaseL
i2	TC285720	similar to (Q9XYW2)
g16	TC229708	T-cell immunoglobulin
c13	TC191380	TLR1
g6	TC228249	TLR1
f14	TC218153	TLR2 -2
f8	TC216531	TLR2-1
d21	TC201541	TLR4
g10	TC228744	TLR5
g13	TC229100	TLR7

Table A4.2. Preparation of IOF formulation used in Chapter VIII (Array_2) (1L of IOF solution, pH=6.99, 301 mOsm)¹

Ingredient	Grams / L	Concentration
Water, 0.2 μ filtered	~990 ml	N/A
NaCl	4.0	0.4%
Ca-lactate	20.0	2.0%
Niacin	0.1	0.01%
HMB	2.0	0.2%
Zn-gluconate	1.0	0.1%
TYR-Na salt	1.5	0.15%
Pyridoxine	0.2	0.02%
Folic acid	0.02	0.002%
Biotin	0.1	0.01%
Preservative: GentaPoult®, 100mg/ml	0.30 ml	30 ppm
Amphotericin B, 0.25mg/ml	10 ml	2.5 ppm
Citric acid (1%)	~ 1.0 ml	

¹This information is proprietary of NCSU and Pfizer-Embrex