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LIPID METABOLISM AND THE ONTOGENY OF ACYLCoA:CHOLESTEROL ACYLTRANSFERASE AND FATTY ACID-BINDING PROTEIN IN DEVELOPING EMBRYOS AND POST-HATCH TURKEYS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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The Ohio State University
1996

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ABSTRACT

To gain insight into lipid utilization during embryonic development of turkey poults, experiments were conducted to study: 1) lipid and fatty acid distribution and disappearance from the yolk sac; 2) the ontogeny of acylCoA:cholesterol acyltransferase (ACAT) activity and fatty acid binding protein (FABP) in the yolk sac membrane and liver of both turkey embryos and newly hatched poults.

Yolk sac lipids are the primary source of energy for avian embryos and are most extensively utilized during the latter stages of incubation. Enrichment of the maternal (hen) diet with medium chain fatty acids by adding coconut oil changed the yolk lipid fatty acid composition. It resulted in increased incorporation of C 14:0 fatty acids into fresh egg yolk and yolk sac of developing embryos.

There were no significant differences in total lipid disappearance from the yolk sacs of embryos from two genetic turkey lines (RBC2 and F lines). Fatty acid profile data showed that selection for growth did not influence fatty acid incorporation into yolk lipids.
Differences in fatty acid profiles from embryonic yolk sacs, however, may be a reflection of line differences in embryonic growth during latter stages of incubation when line differences in embryonic weight become apparent.

There were exceptionally high levels of ACAT activity in the yolk sac membrane and liver during embryonic development. The results suggest that the yolk sac membrane and liver play a significant role in embryonic cholesterol esterification and the subsequent packaging of lipids into lipoproteins for transport to other tissues. Total ACAT in intestine increased from embryonic day 16 through day 6 posthatch which suggests that the capacity for intestinal cholesterol esterification increases with stages of embryonic and posthatch development.

Turkey hepatic FABP was purified and a quantitative immunoblotting procedure was subsequently developed. Liver FABP increased with length of incubation and this increase was parallel to observed increases in liver lipid accumulation. A sharp increase in intestinal FABP just prior to hatch suggests that it is involved in dietary fatty acid utilization. FABP concentration in the yolk sac membrane plateaued between 16 and 19 days before declining through hatch (day 28). This suggests that FABP in the yolk sac membrane plays a role in lipid transfer from the yolk to the embryo via the yolk sac membrane.
To my mother and in memory of my father
I would like to thank Dr. Lilburn for serving as my major advisor. His guidance, encouragement, support throughout my graduate career at The Ohio State University are appreciated. Dr. Lilburn's insights into my research projects and suggestions for the preparation of this dissertation are also appreciated.

I would like to thank Dr. Wayne Bacon for serving in my graduate committee. Dr. Bacon's willingness to accommodate me in his laboratory, his encouragement, and help are appreciated.

I would like to thank Drs. David Latshaw and Karla Roehrig for serving in my graduate committee. The constant encouragement and guidance from Dr. Roehrig are appreciated.

My gratitude is extended to other faculty members, Drs. Donald Palmquist, Floyd Schanbacher, and Sandra Velleman for their willingness in sharing facilities.
I would like to thank Dr. Billy Hargis of Texas A&M for his gift, chicken liver FABP antisera, which had speeded up my research project.

The support from Dave Long, Donna Kinsey, Cindy Coy, John Nixon, Margaret Latta, George Barbour, Lisa Carl, Carolin Britt and Wanda Acord is greatly appreciated.

Sincere thanks are also given to the farm crew, Dennis Hartzler, Rick Neuhardt, Keith Patterson, Jack Sidle, and Mike Wengerd, for their assistance.

I would like to thank Graduate School for supporting my research by awarding me with the Graduate Student Alumni Research Award. I also wish to express my appreciation to the Chales E. Thorne Memorial Assistantship committee for sponsoring my last year of study. The financial support and facilities of the Ohio Agricultural Research and Development Center are appreciated.

I would like to thank my fellow graduate students, Todd Applegate, William Chan, Byung-Ryal Choi, Zhongxia Li, Michele McGuinness, Don Noble, Edwin Ngidi, Jingying Yang, and Jiangtao Zhu for their friendship, encouragement, and support.
I cannot say enough thanks to all my teachers, friends and families. Without their teaching, love and care, I could not have completed this degree.

Thanks are giving to my parents who had tried everything they could to provide their children a fair chance of getting education. Without their efforts and encouragement, I would not have the chance and courage to pursue this degree.

Special thanks are giving to my wife Jane. She has been the single most supporting, loving and caring person for me throughout my graduate study.

Finally, I would like to thank my god who always bless me, love me, and support me.
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Presentations and Abstracts:


Ding, S. T. and M. S. Lilburn, 1995. The fatty acid profile changes in different lipid classes during late embryonic development in two turkey lines. FASEB J. 9:A757.

Ding, S. T. and M. S. Lilburn, 1994. A comparison of lipid transfer during late embryonic development in randombred (RBC) and growth selected (F line) turkey strains. Poultry Sci. 73 (Suppl.):82.


FIELDS OF STUDY

Major Field: Poultry Science
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INTRODUCTION

Lipid and Fatty Acid Composition of Avian Egg Yolks

In chicken eggs, lipids comprise approximately 50% of total yolk dry matter (DM). The main components of yolk lipids are triacylglycerol (TG; 63% of total lipid, %TL) and phospholipids (PL, 29 %TL). Free cholesterol (FC), cholesteryl esters (CE), and free fatty acids (FFA) account for only 4.9%, 1.3%, and 0.9 %TL, respectively (Noble and Cocchi, 1990). Yolk TG account for approximately 90% of total embryonic energy needs and PL are incorporated in cellular membranes during development (Freeman and Vince, 1974).

The major fatty acids (FA) of TG in chicken egg yolks are oleate (C18:0; 46%), palmitate (C16:0; 25%), and linoleate (C18:2; 15%). Similar FA profiles are observed in CE but not, however, in PL (Noble, 1987).
The PL contains more arachidonic acid (C 20:4) and docosahexaenoic acid (C 22:6) and less oleic acid.

**Fatty Acid Utilisation in Avian Embryos**

During the first 15 days of incubation in chick embryos, lipid metabolism and transfer out of the yolk sac is considerably less than that observed during the latter stages of incubation (Noble and Moore, 1964). Most of the lipids absorbed from the yolk are TG and PL and de novo CE synthesis in the yolk sac membrane also increases during this latter stage of incubation. The increase in CE formation is the result of high ACAT activity in the embryonic yolk sac membrane (Noble et al., 1984; Shand et al., 1993).

The yolk sac membrane is a two-layer extra-embryonic membrane comprised of an outer mesoderm and an inner endoderm. Most nutrients absorbed from the yolk are taken up by the endoderm, transferred to the mesodermal blood vessels and then transported to the embryo. Lambson (1970) and Noble et al. (1988) used electron microscopy to study the incorporation of lipid droplets into yolk sac membrane cells. Yolk lipid droplets were first trapped within apical microvilli prior to enclosure within apical vesicles. The vesicles were subsequently transported away from the apical surface and temporarily stored within yolk sac membrane cells prior to further metabolism. Although
the primary mode of lipid absorption is via phagocytosis, the presence of lipase within the yolk suggests that lipid hydrolysis prior to absorption may also be occurring (Emmannalsson, 1951; Zacks, 1954; Lambson, 1970). The minimal amounts of mono- and diacylglycerol, free fatty acids, and lysophosphatides in yolk, however, suggest that if hydrolysis does occur it is minimal or that the products of hydrolysis are very rapidly absorbed into the yolk sac membrane (Noble and Moore, 1967ab).

In the aforementioned studies using electron microscopy, lipid droplets were observed in the endodermal cells of the yolk sac membrane (Noble and Cocchi, 1990) suggesting that during the process of lipid absorption from the yolk, the yolk sac membrane serves as a temporary storage tissue prior to lipid transfer into the embryonic circulation. Noble and Cocchi (1990) reported that lipid hydrolysis and resynthesis within the yolk sac membrane is an active process and that desaturation of C18:0 to C18:1 also occurs. These observations are all indicative of tremendous mass transport of FA within the endodermal cells of the yolk sac membrane.

Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) are the two primary PL within the chicken egg yolk. They comprise approximately 69% and 24% of total PL, respectively (Noble and Moore, 1965).
During the course of incubation, chicken embryos selectively absorb more PE than PC (Noble and Moore, 1965; 1967) which suggests that they selectively utilize PE (Noble and Moore, 1967ab) although the mechanism for this is still unknown.

The FA profile of yolk lipids change during the course of incubation and it was hypothesized that this was due to the changes in the utilization of different lipid classes during embryonic development (Donaldson, 1964). This hypothesis was subsequently shown to be true (Noble and Moore, 1964; Donaldson, 1967). However, a certain degree of FA modification within the yolk sac membrane may also be contributing to changes in FA profiles. Enzymes such as delta-9 desaturase, which catalyzes the desaturation of stearic acid to oleic acid, have been shown to be active within the yolk sac membrane and it has been hypothesized to contribute to the overall changes in FA profiles (Noble and Shand, 1985) although this hypothesis is not universally accepted (Donaldson and Fites, 1970).

The FA composition in residual yolk sac TG is fairly constant throughout embryonic development and the same is true for yolk sac PL, with the exception of docosahexaenoic acid (C22:6; Noble and Moore, 1964; 1965). It appears that C22:6 is associated with the PE fraction
of the yolk which is preferentially absorbed by embryonic tissues thus resulting in a net decrease in C 22:6 over the course of incubation (Noble and Moore, 1965).

Within the CE fraction, C 18:1 increases with length of incubation and at hatch may account for 74% of total FA within the CE fraction of the residual yolk sac (Noble and Moore, 1964).

The avian liver posthatch is critical to de novo lipid biosynthesis and overall lipid metabolism. In the avian embryo, the liver is also important to lipid metabolism, particularly during the latter stages of incubation (Noble et al., 1984; Escribino et al., 1988; Sansbury et al., 1989). In chick embryonic livers, TL (%DM) increases from 25.7 % on Day 15 to 45 % on Day 19 (Moore and Doran, 1962) and most of this is in the form of CE (64 %TL). From 15 days through hatch, there are only small increases in hepatic PL and TG (Noble and Moore, 1964).

The composition and FA profiles of hepatic lipids from chick embryos are different from that of yolk sac lipids. Oleic acid constitutes about 80 % of hepatic FA in the CE fraction of embryos from 15 to 21 days of incubation (Noble and Moore, 1964). This may be due to the high specificity of ACAT for oleic acid in the yolk sac membrane prior to transport to the embryo with
subsequent deposition in the liver (Sgoutas, 1970). Stearic, palmitic, and arachidonic acids are the primary FA in hepatic PL and each comprises 16 to 25% of total FA (Noble and Moore, 1967; Noble and Shand, 1985). In embryonic liver TG, however, oleic acid (33%), palmitic acid (23%), and C22:6 (16%) are the primary FA.

The level of dietary fat will not influence total yolk lipid concentration but different patterns of dietary FA may significantly change the FA profiles in yolk lipids and embryonic tissues (Jiang et al., 1991; Cherian and Sim, 1993). The incorporation of omega-3 fatty acids into the diet will increase the concentration of these FA in the yolk and tissues of newly hatched chicks (Cherian and Sim, 1991). Research has also shown that changes in the long chain fatty acid composition of diets fed to hens will also influence the incorporation of LCFA into yolk lipids (Feifenbaum and Fisher, 1959; Wheeler et al., 1959; Cough et al., 1973) and embryonic tissues (Cough et al., 1973; Vilchez et al., 1992). Cherian and Sim (1993) also concluded that incorporation of omega-3 fatty acids into diets fed to hens would also modify the cholesterol concentration of developing embryos. All of these changes may ultimately effect embryonic fatty acid metabolism and thereby influence overall embryonic development.
While there is extensive literature relative to FA metabolism in embryonic chicks, there is little research on the developing turkey poult. Christie and Moore (1972) reported that the lipid composition and fatty acid profile in TG was similar in chicken and turkey eggs although there were some species differences in fatty acid profiles (Christie and Moore, 1972). Cough (1973) reported that the long chain fatty acid profile of diets fed to hens could influence the FA profile in embryonic tissue TG but that FA accumulation in yolk lipids was similar between turkeys and chickens.

Medium chain fatty acids (MCFA) are more easily metabolized than LCFA because they do not require carnitine for transport into the mitochondrion (Babayan, 1987). Furuse et al. (1992) reported that diets supplemented with MCT (TG containing MCFA) resulted in decreased body weight compared with diets containing LCT (TG containing LCFA). This reduction of body weight gain, however, was the result of a significant reduction in feed intake. When adjustments were made for differences in intake, diets containing supplemental MCT (tricaprylin) did improve body weight gain and feed efficiency compared with LCT (corn oil; Mabayo et al., 1993). Chicks fed diets containing MCT also exhibited better protein
retention and increased protein utilization efficiency (protein retained/ protein intake). These data suggest that incorporation of MCT into embryonic lipids might enhance overall lipid metabolism during embryonic development.

**Fatty Acid Binding Protein**

The fatty acid-binding proteins (FABPs) are a group of small molecular weight proteins (12 to 15 Kd) which have been found in many tissues (Glatz et al., 1985). Levi et al. (1969) was the first to describe a small molecular weight protein, Z protein, in the rat hepatic cytoplasm which had an affinity for long chain fatty acids (LCFA). Ockner et al. (1972) went on to describe a cytosolic protein (12,000 M.W.) from the intestine with fatty acid binding capacity and referred to it as fatty acid binding protein. Since then, numerous reports have confirmed the existence of FABP's in many tissues and their possible roles in various aspects of lipid metabolism (Kaikaus et al., 1990; Glatz et al., 1993).

At least 8 distinct types of FABP have been characterized (Ockner et al., 1990). To reduce confusion, Glatz and van der Vusse (1990) proposed a naming formula, X-FABPY, where the prefix X denotes the tissue of origin or abundance and the suffix Y denotes the cellular
location of the protein. This formula has been adopted by many researchers.

1. Cellular Location of FABP

Through the use of light microscopy and immunohybridization, both hepatic (L-FABP) and intestinal FABP (I-FABP) have been shown to be cytosolic proteins (Shields et al., 1986). Liver L-FABP is found in the cytoplasm, endoplasmic reticulum, outer mitochondrial membrane and nuclei (Vinores et al., 1984; Bordewick et al., 1989). A family of FABP's have also been isolated from the plasma membrane (FABP-PM) in different mammalian tissues (Stremmel et al., 1985ab; Sorrentino et al., 1988). These FABP-PM are larger than the cytosolic proteins (M.W. 40 Kd) and are involved in LCFA uptake.

2. FABP Function

The definitive role for FABPs have not been determined but substantial evidence exists that in different tissues, FABP may have the following functions: 1) fatty acid uptake from plasma (Bass, 1985; Peeters et al., 1989) ; 2) directing of FA to different cellular compartments (Glatz et al. 1993) ; 3) protection of enzymes and cellular components from free fatty acid toxicity (Glatz and Veerkamp, 1985) ; 4) regulation of
enzymes involved in lipid metabolism (Bass, 1985; Glatz and Veerkamp, 1985; Greer and Hargis, 1992).

The facilitation of intracellular fatty acid transport by FABP was proposed by Ockner et al. (1972); Bass and Manning (1986) and Sweester et al., (1987). This hypothesis was based on the observation that L-FABP concentration correlated well with the rate of fatty acid uptake by hepatocytes. The observation that dietary clofibrate increased both the concentration of hepatic L-FABP and the uptake of LCFA supports this concept (Renaud et al., 1978). Theoretical testing (Tipping and Ketterer, 1981) and in vitro experimental results have lent further support to this hypothesis (Storch and Kleinfeld, 1986; McCormack and Brecher, 1987; Peeters et al., 1989; Storch, 1990; Stewart et al., 1991). Peeters et al. (1989) reported that using an equilibrium dialysis model, both H-FABP and L-FABP enhanced the transfer of LCFA from one monolayer compartment to another. Although experimental evidence for a specific role in transport of LCFA by FABP in vivo has not been reported, it has been shown recently that FABP in cardiomyocytes enhances FA cytosolic solubility 700-fold and increases 17-fold the FA flux from the plasma membrane to mitochondria. This in vitro data strongly supports a role for FABP in the
transport and targeting of fatty acids to sites of metabolic activity (Vork et al., 1993).

Although in vivo evidence has not been reported, there is indirect data suggesting a role for cytosolic FABP in targeting LCFA to different metabolic sites. McCormack and Brecher (1987) observed that L-FABP increased the transfer rate of LCFA across a polycarbonate membrane from a donor liposome compartment to a microsome compartment. Smith and Storch (1996) recently reported that adipocyte FABP bound both LCFA and unilayer membrane. The bond was the result of ionic interactions and was diminished by high salt concentrations and acetylation of lysine residues in the native protein. Further studies are needed in order to elucidate which amino acids are involved in ligand versus membrane binding.

It has been suggested that FABP protects cellular membranes from deterioration due to high concentrations of free fatty acids (Brenner, 1984). Both L-FABP and H-FABP reversed the inhibition of brain synaptosomal sodium-dependent amino acid uptake systems by free long chain unsaturated fatty acids (Rhoads et al., 1983; Bass et al., 1984). There are high concentrations of FABP in tissues which are active in FA metabolism (i.e. liver, heart, adipocyte, and intestine). The role of temporary FA sink
that FABP plays serves to decrease cytosolic free fatty acid toxicity.

Liver FABP has been shown in vitro to augment the activity of several enzymes involved in triacylglycerol and cholesteryl ester (CE) biosynthesis (Mishkin et al., 1975; O'Doherty and Kuksis, 1975; Wu-Rideout et al., 1976; Burnett et al., 1979; Greer and Hargis, 1992). It increases the activity of microsomal acyl-CoA glycerol-3-phosphate acyltransferase (Mishkin and Turcotte, 1974; Burnett et al., 1979), diacylglycerol acyltransferase (O’Dherty and Kuksis, 1975; Iritani et al., 1980); and acyl-CoA synthetase (Ockner and Manning, 1976; Burnette et al., 1979). L-FABP may also reverse the inhibition of acyl-CoA on acetyl-CoA carboxylase (Lunzer et al., 1977) and ACAT (Grinstead et al., 1983).

3. The Ontogeny of FABP

The expression of FABP in different tissues is different from an ontogeny perspective. In pigs, there is a two-fold increase in intestinal FABP in fetal intestine at 73 days of gestation compared with that of newborn piglets (Chi, 1993). Reinhart (1990) reported a high level of FABP activity in fetal liver and it remained constant through late gestation. Through the use of immunocytochemistry, Rubin et al. (1989) detected L-FABP
at 17 days of gestation in rat proximal intestine and found that the number of enterocytes producing L-FABP increased during the course of gestation. L-FABP was not detected until 19 days of gestation in the distal small intestine and as was mentioned before, L-FABP producing cells increased as gestation proceeded. Unlike L-FABP, I-FABP was not detected in the proximal small intestine until 18 days of gestation, yet the expression pattern of I-FABP in different enterocytes is similar to that of L-FABP (Rubin et al., 1989). The appearance of I-FABP in the distal small intestine is one to two days later than that in proximal small intestine. These observations suggest that different FABPs have different expression patterns within the same organ. Hepatic FABP has been reported to increase 20-fold from 5 days prior to birth through 45 days of age (Sheridan et al., 1987). Moreover, Gordon et al. (1985) found that both hepatic L-FABP mRNA and intestinal I-FABP mRNA (I: intestine) increased 3-fold during the first day of life and protein expression was 8 times higher just prior to weaning. The high levels of FABP observed in rats are associated with increased influxes of FA. In humans, fetal hepatic L-FABP can be detected at 7 weeks of gestation whereas L-FABP in the small intestine is not expressed until 23 weeks (Suzuki and Ono, 1988). Taken together, these observations
suggest that a given FABP may have different expression patterns in different organs combined with species differences.

In avian species, Katongole and March (1979) first reported the existence of an intestinal mucosal FABP with a M.W. < 12,400 the concentration of which was influenced by the level of dietary fat. Collins and Hargis (1989) characterized the distribution of FABP in the cytosol of liver, duodenum, myocardium, adipose tissue, and muscle of growing chickens. The avian proteins had M.W. similar to what was reported in mammals (14,000 to 15,000). Two forms of hepatic cytosolic FABP were isolated from chickens, each with a different pI (7.1 and 9.0; Scapin et al., 1988; Sewell et al., 1989). Within the intestine, FABP activity was reported to be low during the first 2 weeks of postnatal growth before increasing as birds got older (Katongole and March, 1980; Sell et al., 1986).

Sewell et al. (1989) reported that chicken L-FABP<sub>c</sub> (C, cytosolic) is similar to rat L-FABP<sub>c</sub> based on their structural homology, pI, and fatty acid binding affinities. The amino acid composition of chicken adipose FABP (A-FABP<sub>c</sub>) has been reported to have a high sequence homology with mammalian A-FABP<sub>c</sub> (Sams et al., 1990) which suggests that their function in adipose tissue of different species may be similar.
4. Quantitation of FABP

Several methods have been used to quantitate cytosolic FABP. Researchers have measured FABP activity by comigration of radiolabeled fatty acids with cytosolic samples on a gel filtration column (Katongole and March, 1980; Ockner et al., 1980). Fatty acid binding activity was subsequently determined from the amount of radioactivity in each FABP fraction. The sensitivity of this assay can be compromised, however, if there is any non-specific binding of the labelled fatty acid to the gel matrix. Morrow and Martin (1983) developed a ligand binding assay that quantitated FABP concentrations after gel filtration. This procedure is better than the comigration method yet the possibility still exists for non-specific fatty acid binding by non-FABP proteins.

Avanzati and Catala (1983) reported a procedure for partial purification of FABP using 70% ammonium sulfate precipitation to remove large proteins. This method was subsequently used as a first step in quantitating FABP activity (Dutta-Roy et al., 1988; Reinhart et al., 1992). The FABP activity in the partially purified fraction was determined using a radiolabeled ligand binding assay incorporating lipidex 1000 (Glatz and Veerkamp, 1983). This is a very simple procedure for determination of FABP activity, but incomplete removal of albumin and the
partial loss of FABP during the desalting phase of the assay on a PD-10 column are two disadvantages. Any remaining albumin can be removed by affinity chromatography using anti-albumin antibodies conjugated to a Sepharose column (Glatz et al., 1984).

Any immunochemical procedures used in quantifying FABP would require either monoclonal or polyclonal antisera. Ockner and Manning (1974) used a radial immunodiffusion assay (Mancini et al., 1963) to quantitate FABP content (100 to 125 ng) in a partially purified cytosolic suspension of rat intestinal mucosa, and this method has also been used to measure FABP levels in other tissues (Ockner et al., 1982; Bass et al., 1985). Fournier and Rahim (1985) described a Rocket immunoelectrophoresis procedure for the quantification of FABP concentration. Crisman et al. (1987) developed an enzyme-linked immunosorbent assay procedure (ELISA) to quantitate heart FABP, and new ELISA assays have subsequently been developed with the production of tissue specific FABP antisera (Paulussen et al., 1989; Ohkaru et al., 1994).

Immunoblotting techniques are also useful for the detection of FABP in a tissue and to quantify its concentration. Wilkinson and Wilton (1987) developed a quantitative immunoblotting procedure using 125I-labeled
protein A to bind to an antibody-FABP complex. An immunoblotting procedure will effectively separate FABP from other proteins so it is good for quantitation. The second phase colorimetric reaction has also been improved to the point where it is very sensitive and equally as important, non-radioactive.

In summary, ligand binding measures used to determine functional fatty acid binding protein activity may be compromised by other proteins which have the capacity for binding to fatty acids. On the other hand, immunochemical assays (i.e. immunoblots, ELISA) can be used to accurately quantify cellular FABP through the specificity of the antibody-antigen reaction. The combination of a fatty acid (ligand) binding assay with an immunochemical assay may help differentiate between the capacity for tissue fatty acid binding and the presence of a specific FABP within a given tissue or organ.

**AcylCoA:Cholesterol Acyltransferase (ACAT, EC 2.3.1.26)**

Cholesterol metabolism in mammalian tissues is regulated by three key enzymes: 1) 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase, EC 1.1.1.34); 2) ACAT; 3) cholesteryl ester hydrolase (Billheimer and Gillies, 1990). In avian embryos, cholesterol synthesis is relatively low in most tissues
with the highest activity found in the brain (Stokes et al., 1953; Goodridge, 1968). There is considerable cholesterol found in the yolk and this is the source of most of the embryonic cholesterol (Connor et al., 1969). The activity of cholesteryl ester hydroxylase (CEH) is not as great as ACAT in the chicken embryonic liver and yolk sac membrane (Shand et al., 1993, Shand et al., 1994), and this leads to a net accumulation of cholesteryl esters (CE). These serve as either a form of cholesterol storage in the cell or as a component of lipoproteins which are vehicles for cholesterol and lipid transport out of the cell (Spector et al., 1979; Noble et al., 1984).

ACAT was first detected in rat liver by Mukherjee et al. (1958). The enzyme catalyzes the formation of cholesteryl ester (CE) from acyl CoA and free cholesterol. The liver and intestine are two primary sites of ACAT activity, and it is located within the microsomal fraction (Spector et al., 1979; Norum et al., 1983). Microsomal ACAT from the rat liver has a high specificity for fatty acids with single cis double bond at C9 (oleic acid and palmitoleic acid; Sgoutas, 1970). Goodman et al. (1964) compared several acyl CoA substrates and found that cholesteryl ester formation rate occurred in the following order: oleyl- > palmityl- > stearyl- > linoleyl-CoA.
The developmental expression of ACAT is different across tissues. Liver ACAT activity in the rat is found to be very low before birth, increases during nursing (milk feeding) and peaks at 14 to 17 days of age (Little and Hahn, 1992; Smith et al., 1995). After the peak in ACAT activity, it declines commensurate with weaning and then increases again to the high level found in adults. Conversely, intestinal ACAT activity is high during embryonic development and then declines to a nondetecteable level by 14 days of age (Little and Hahn, 1992). In other words, hepatic ACAT activity is high when intestinal ACAT activity is at its nadir at approximately 14 days of age.

In chick embryos, cholesterol esterification occurs in the liver and yolk sac membrane as early as 13 days of incubation (Noble et al., 1984). Unlike in the rat liver, cholesterol esterification in the chick embryonic liver increases during the course of incubation. Hepatic ACAT activity peaks at 16 d of incubation and then decreases before hatch (Shand et al., 1994). The high CE content found in embryonic liver during the latter stages of development suggests that hepatic CE serves as a form of cholesterol storage during the latter stages of development (Noble et al., 1984).
Shand et al. (1993) reported that microsomal ACAT activity in the yolk sac membrane of chick embryos is high during the second phase of embryonic development (Day 9 to 20). A peak in enzyme activity is observed at approximately 16 days, and this coincides with the period of maximal transfer of yolk lipids through the yolk sac membrane and on into the embryonic circulation (Noble and Cocchi, 1990; Shand et al., 1993). CE is thought to be critical to the process of lipoprotein assembly in the yolk sac membrane for the transfer of lipid out of the yolk (Noble et al., 1984).

Many factors influence the activity of ACAT, both in vivo and in vitro. In rats, dietary fat and cholesterol supplementation increases ACAT activity in the intestine and liver (Norum et al., 1983). Hepatic cholesterol in rats can also be increased through a collective increase in dietary cholesterol, cholate, or mevalonolactone which increases ACAT activity (Erickson et al., 1980). Dietary cholesterol increases ACAT activity by increasing free cholesterol availability for the enzyme as well as by increasing sterol carrier protein-2, which has been shown to be an ACAT modulator (Kraemer et al., 1995). Liver microsomal ACAT activity is positively correlated with cholesterol concentration, supporting the hypothesis that microsomal cholesterol availability regulates ACAT
activity (Suckling and Stange, 1985; Field et al., 1987). In a combination of in vivo and in vitro experiments with rats, Field et al. (1987) concluded that the degree of fatty acid saturation in microsomal membranes effects the availability of cholesterol for ACAT which subsequently regulates its activity in both liver and intestine of rats. Alejandre et al. (1988), however, reported that dietary unsaturated FA reduced hepatic ACAT activity in chickens. Whether the differences are due to species or other factors needs to be further elucidated.

The addition of hypolipidemic factors (i.e. clofibrate, bezafibrate, and ciprofibrate) to the diets of rats decreases liver ACAT activity (Stahlberg et al., 1989). The microsomal cholesterol concentration was not affected by these factors so the authors speculated that they may have been directly inhibiting ACAT activity or decreasing enzyme protein synthesis.

Experimental Objectives

To better understand lipid metabolism and transfer processes in the developing embryo and early posthatch poults, this dissertation research was designed to accomplish the following:
1. Characterize lipid subclass and fatty acid profiles during the later stages of embryonic development and early
posthatch growth;
2. Determine the effect of diets high in MCFA on the fatty acid profiles of egg yolk and embryonic liver lipids;
3. Compare lipid transfer from the yolk sac into embryo in turkeys differing greatly in posthatch growth rate;
4. Study the ontogeny of ACAT activity in the yolk sac membrane, liver, and small intestine of turkey embryos during the second half of incubation and in neonatal pouls;
5. Purify liver FABP and develop a quantitative method to study the ontogeny of liver FABP in turkey embryos;
6. Investigate the ontogeny of FABP in the yolk sac membrane and small intestine in turkey embryos and in neonatal pouls.
References


Characterization of Changes in Yolk Sac and Liver Lipids During Embryonic and Early Posthatch Development of Turkey Poults

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ABSTRACT In two studies, changes in lipids and fatty acids from the yolk sac and liver of turkeys were determined during incubation and early postnatal growth. In Experiment 1, embryos were collected from Day 13 through 6 d posthatch. Embryos weighed 4.25 g at Day 13, 52.8 g at hatch (Day 28), and 104 g at 6 d. Total yolk lipid (grams per yolk sac) decreased from 9.48 g at Day 13 to 2.52 g at hatch (Day 28). Almost half (47%) of the yolk lipid decreased during the last week of incubation. In Experiment 2, embryos were collected daily from Day 22 through 2 d posthatch. Embryonic weight increased from 32.5 g at Day 22 to 55.8 g at hatch (Day 28), 3 g more than in Experiment 1. On Days 22 and 25, total yolk lipid (grams per yolk sac) was slightly less in Experiment 2 than in Experiment 1, but at hatch (Day 28) there were considerable differences (Experiment 1, 2.52 g; Experiment 2, 0.63 g), concomitant with increased hatch weight in Experiment 2. Liver DM percentage and ether extractable lipid increased as incubation proceeded. Oleic acid comprised the largest proportion of total yolk fatty acids and of liver fatty acids. There was a small but significant increase in yolk sac oleic acid (percentage of total fatty acids) between Day 22 (40%) and hatch (Day 28; 45.4%) and a much larger increase in liver oleic acid (46.6% to 56.5%). The absolute amount of all yolk sac fatty acids declined greatly during the second half of incubation through 6 d posthatch.

(Key words: turkey, poult, embryos, yolk lipid, fatty acids)

1996 Poultry Science 75:478-483

INTRODUCTION

Chick yolk lipid is composed primarily of triglyceride (TG; 72%) and phospholipid (PL; 22%) and is a major nutrient source for developing embryos (Romanoff, 1960). The yolk has been estimated to supply 90% of total embryonic caloric needs (Freeman and Vince, 1974). Noble and Moore (1964) reported that chick yolk contains approximately 6 g of lipid, 5 g of which are transferred to the developing embryo. A large portion of the lipid (2 g) is transferred to the chick embryo between 19 and 21 d of incubation. Concomitant with lipid transfer out of the yolk sac is a parallel increase in hepatic lipid, 70% of which is esterified cholesterol (Noble et al., 1984). Most of the cholesterol esterification occurs within the yolk sac membrane (Noble and Moore, 1967a; Noble et al., 1984) and cholesteryl oleate is the primary end product (Noble and Moore, 1964). Oleic acid makes up approximately 37% of total fatty acids in chick egg yolk (Noble and Cocchi, 1990).

Although the published research on embryonic lipid metabolism in chicks is extensive, there is little published information on turkey embryonic development. Escribano et al. (1988) reported that both the wet and dry weights of the yolk sac membrane and membrane total lipase activity peaked at 21 d in turkey embryos. Ding et al. (1995) compared different lipid classes in turkey embryos during late embryonic development and reported that total yolk lipid decreased 80% during the course of incubation. There are, however, no data presented on changes in total fatty acids during embryonic development of the turkey. The objective of the experiments reported herein was to characterize the changes that occur in yolk sac and liver total lipid and individual fatty acids during prenatal and early postnatal development in turkey poults.

MATERIALS AND METHODS

Experiment 1

Fertile turkey hatching eggs were purchased from Cuddy Farms, Danville, OH 43014. Fresh eggs were used for determination of yolk weight and yolk total lipid (Day 0). The eggs were individually weighed (mean weight 96.9...
TABLE 2.1. The embryonic development and lipid content changes in the yolk sac of turkey embryos. Experiment 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Embryo no.</th>
<th>Embryo weight (g)</th>
<th>Weight (DM) (%)</th>
<th>Lipid (% DM)</th>
<th>Total lipid (g/yolk sac)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>4.23</td>
<td>35.61</td>
<td>39.91</td>
<td>71.24</td>
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<td>13</td>
<td>5</td>
<td>9.02</td>
<td>27.04</td>
<td>68.30</td>
<td>70.28</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>19.30</td>
<td>22.53</td>
<td>52.06</td>
<td>66.24</td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>32.05</td>
<td>22.05</td>
<td>52.32</td>
<td>56.55</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>44.79</td>
<td>21.63</td>
<td>51.29</td>
<td>47.52</td>
</tr>
<tr>
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<td>5</td>
<td>52.81</td>
<td>10.74</td>
<td>52.86</td>
<td>42.46</td>
</tr>
<tr>
<td>P3</td>
<td>5</td>
<td>66.75</td>
<td>3.47</td>
<td>46.68</td>
<td>34.69</td>
</tr>
<tr>
<td>P6</td>
<td>5</td>
<td>104.53</td>
<td>0.35</td>
<td>40.68</td>
<td>0.05</td>
</tr>
<tr>
<td>SEM</td>
<td>5</td>
<td>4.72</td>
<td>2.68</td>
<td>2.65</td>
<td>7.02</td>
</tr>
</tbody>
</table>

Age effect: 0.0001  0.0001  0.0128  0.0001  0.0001

1Day 0 = fresh egg; 13 to 28 = 13 to 28 d of incubation; and P3 and P6 = 3 and 6 d posthatch.
2On Day 0, embryo no. = egg no., = no measurement, and yolk sac = yolk sac membrane plus yolk.
3Probabilities less than 0.05 indicate significant incubation time effect.

Experiment 2

Four hundred turkey hatching eggs were purchased from Cuddy Farms. The eggs were individually weighed (mean weight 93 g) and set in a Petersime incubator. From Day 13 of incubation through 6 d posthatch, five embryos and poults were killed every 3rd d for determination of embryo weight, yolk sac weight (including yolk sac membrane and yolk contents), and yolk sac total lipid. After hatch, all poults were reared in heated battery brooders with ad libitum access to feed and water.

RESULTS

Experiment 1

Turkey embryos increased in weight from 4.23 g at Day 13 of incubation to 52.81 g at hatch (28 d) and 104.53 g on Day 6 posthatch (Table 1). At Day 0, the yolk weighed 28.83 g and total yolk sac weight (yolk plus membrane) was 35.61 g at Day 13. There was a decline in total yolk sac weight as incubation days increased. At hatch, the yolk sac weighed 10.76 g and this decreased to 0.35 g by 6 d
posthatch. Total yolk sac DM decreased from Day 0 to 13 and increased thereafter through Day 19, after which it reached a plateau.

Yolk sac lipid (percentage dry matter) decreased from 71.24% at Day 0 to 42.46% at hatch (Table 1). Total yolk lipid (grams per yolk sac) declined from 10.95 g in the fresh yolk to 2.52 g at hatch and was essentially gone by 6 d posthatch (0.05 g). The greatest proportion of lipid disappearance occurred after Day 19 of incubation.

Total yolk sac fatty acids (percentage of total lipid) stayed relatively constant at 70 to 77% of total yolk lipid over the entire course of incubation and decreased significantly after hatch (Table 2). The concentrations of palmitic (C16:0) and palmitoleic acids (C16:1) were fairly constant through Day 25 and then decreased significantly. Palmitoleic acid accounted for a small percentage of total fatty acids, whereas palmitic acid represented more than 25% of total yolk sac fatty acids during incubation. The concentrations of stearic (C18:0) and linoleic acids (C18:2) were fairly constant from Day 13 through 25 of incubation. The C18:0 and C18:2 acids decreased from 10.26 and 15.23%, respectively, at hatch to 7.92 and 10.24% at 6 d posthatch. There was little or no linolenic acid (C18:3) present in the yolk sac. The concentration of arachidonic acid (C20:4) was low but consistent during incubation and increased after hatch. Of the individual fatty acids, oleic acid (C18:1) was found in the greatest amount. Its concentration was fairly constant through Day 22 of incubation (40%) and then increased linearly through 6 d posthatch (54.68%). Quantitatively, the amount of each fatty acid declined through incubation and posthatch development (Table 2). On Day 6 posthatch, only 25 mg of total fatty acids remained in the yolk sac.

**Experiment 2**

There were significant, positive correlations (0.42 to 0.82, P ≤ 0.05) between egg weight and embryo weight (without yolk sac) between Day 22 and 27 of incubation (data not shown). Embryonic weight increased linearly between Day 22 and 27 but there was a slight decline in mean BW between Day 27 and hatch (Table 3). Holding of poulters in the hatchers for 2 d resulted in a further 16% decline in poult weight. The weight of the yolk sac decreased significantly after Day 24 continuing through 2 d posthatch, whereas liver weight increased almost threefold from Day 22 through hatch.

There was a significant decline in yolk sac DM and lipid (percentage DM) during the last week of incubation. Yolk sac total lipid (percentage DM) was fairly consistent from Day 26 through 2 d posthatch.

Liver weight increased from 0.63 g at Day 22 of incubation to 1.78 g at hatch. Liver DM percentage increased linearly from Day 22 through 2 d posthatch. Ether extractable lipid (percentage DM) peaked at Day 26 of incubation and then declined slightly through hatch. There were no changes between values at hatch and at 2 d posthatch.

Serum TG concentration declined significantly between Day 27 and hatch (Day 28) and there was a further drop between 1 and 2 d posthatch (Table 4). The concentration of individual fatty acids in the yolk sac expressed as a percentage of total fatty acids is
TABLE 2. The embryonic development and lipid content changes in turkeys, Experiment 2

<table>
<thead>
<tr>
<th>Day</th>
<th>Embryo no.</th>
<th>Embryo weight (g)</th>
<th>Weight (g/yolk sac)</th>
<th>Weight (g)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>30</td>
<td>32.50</td>
<td>55.64</td>
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<td>23</td>
<td>33</td>
<td>38.56</td>
<td>52.07</td>
<td>3.04</td>
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</tr>
<tr>
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<td>37</td>
<td>43.79</td>
<td>46.03</td>
<td>1.60</td>
<td>1.60</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
<td>50.30</td>
<td>39.73</td>
<td>4.87</td>
<td>4.87</td>
</tr>
<tr>
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<td>27</td>
<td>35</td>
<td>59.50</td>
<td>37.48</td>
<td>7.01</td>
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<tr>
<td>28</td>
<td>30</td>
<td>55.62</td>
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<td>3.50</td>
</tr>
<tr>
<td>P1</td>
<td>10</td>
<td>54.40</td>
<td>43.63</td>
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<td>3.50</td>
</tr>
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<td>P2</td>
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<td>4.46</td>
<td>2.47</td>
<td>3.64</td>
<td>0.45</td>
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</tr>
</tbody>
</table>

Age effect: 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001

1 Day 0 = fresh egg; 27 to 28 = 27 to 28 d of incubation; and P1 and P2 = 1 and 2 d posthatch.
1 Probabilities less than 0.05 indicate significant incubation time effect.

Presented in Table 5, total fatty acids (percentage of DM) decreased significantly as incubation proceeded and this paralleled the decline in ether extractable lipids. The concentration of palmitic acid (C16:0) was fairly constant through Day 26 before it started to decline. Palmitoleic acid (C16:1) levels were fairly consistent between Day 23 and 27 before declining through 2 d posthatch. The concentrations of C16:0 and C16:2 were also fairly constant from Day 22 through hatch (Day 28). Oleic acid represented a large proportion of total yolk sac fatty acids (40 to 41%) from Day 22 through 26, after which there was a significant increase through 2 d posthatch.

The increase in liver total fatty acid concentrations paralleled the increase in ether extractable total lipid over the latter stages of incubation (Table 6). There was a decline in the concentrations of C16:0 and C18:0 from 9.30 and 12.18%, respectively, at Day 22 to 4.99 and 6.78% at 2 d posthatch. The hepatic concentration of oleic acid (C18:1) increased from 46.6% at Day 22 to 58.24% at 2 d posthatch.

**DISCUSSION**

The positive correlation between egg weight and embryo weight supports previous observations that heavier eggs yield heavier poults (Bray, 1965; Moran and Reinhart, 1981; Moran, 1990). Embryo weights on Day 22 were similar in both experiments and yolk sac total lipids were also similar at Day 22 in both studies. In Experiment 2, however, embryo weight at Day 25 and poult weight at hatch (Day 28) were considerably heavier than weights at similar stages of development in Experiment 1. This result is the opposite to what was observed for yolk sac total lipid, i.e., the faster growing embryos had the lowest amounts of yolk lipid at hatch. This variability in yolk lipid reserves at hatch (Day 28) raises a question as to the quantitative importance of residual yolk lipid as a nutrient source for use by poults during early postnatal life. Chamblee et al. (1992) compared deuctomized (yolk sac surgically ablated) and intact chicks and concluded that residual yolk sac reserves, lipid in particular, were critical for initial growth in broiler chicks. Noble and Oggunyemi (1989) reported, however, that within 2 d posthatch, there was a 50% reduction in yolk lipid (0.8 g) and only 50% of this was in the form of TG. This rapid depletion in yolk lipid supports a hypothesis that quantitatively, residual yolk sac lipid is a minor nutrient source for newly hatched poultry but qualitatively may be an important source of PL precursors.

The increase in yolk lipid C18:1 concentration during the latter stages of incubation is probably a function of increasing cholesteryl oleate accumulation. The cholesteryl ester fraction in yolk sacs from newly hatched chicks was found to be high in oleic acid (Noble and Moore, 1964) and recent data with turkey embryos also shows increased cholesteryl ester levels in older embryos (Ding et al., 1995). There was a reduction in the levels of C16:0, C18:0, and C18:2 after hatch, which may be a reflection of faster TG and PL depletion from the yolk sac compared with cholesteryl esters (Ding et al., 1995). In chicks, C16:0, C18:0, and C18:2 are the major constituent compared deuctomized (yolk sac surgically ablated) and intact chicks and concluded that residual yolk sac reserves, lipid in particular, were critical for initial growth in broiler chicks. Noble and Oggunyemi (1989) reported, however, that within 2 d posthatch, there was a 50% reduction in yolk lipid (0.8 g) and only 50% of this was in the form of TG. This rapid depletion in yolk lipid supports a hypothesis that quantitatively, residual yolk sac lipid is a minor nutrient source for newly hatched poultry but qualitatively may be an important source of PL precursors.

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**DISCUSSION**

The positive correlation between egg weight and embryo weight supports previous observations that heavier eggs yield heavier poults (Bray, 1965; Moran and Reinhart, 1981; Moran, 1990). Embryo weights on Day 22 were similar in both experiments and yolk sac total lipids were also similar at Day 22 in both studies. In Experiment 2, however, embryo weight at Day 25 and poult weight at hatch (Day 28) were considerably heavier than weights at similar stages of development in Experiment 1. This result is the opposite to what was observed for yolk sac total lipid, i.e., the faster growing embryos had the lowest amounts of yolk lipid at hatch. This variability in yolk lipid reserves at hatch (Day 28) raises a question as to the quantitative importance of residual yolk lipid as a nutrient source for use by poults during early postnatal life. Chamblee et al. (1992) compared deuctomized (yolk sac surgically ablated) and intact chicks and concluded that residual yolk sac reserves, lipid in particular, were critical for initial growth in broiler chicks. Noble and Oggunyemi (1989) reported, however, that within 2 d posthatch, there was a 50% reduction in yolk lipid (0.8 g) and only 50% of this was in the form of TG. This rapid depletion in yolk lipid supports a hypothesis that quantitatively, residual yolk sac lipid is a minor nutrient source for newly hatched poultry but qualitatively may be an important source of PL precursors.

The increase in yolk lipid C18:1 concentration during the latter stages of incubation is probably a function of increasing cholesteryl oleate accumulation. The cholesteryl ester fraction in yolk sacs from newly hatched chicks was found to be high in oleic acid (Noble and Moore, 1964) and recent data with turkey embryos also shows increased cholesteryl ester levels in older embryos (Ding et al., 1995). There was a reduction in the levels of C16:0, C18:0, and C18:2, after hatch, which may be a reflection of faster TG and PL depletion from the yolk sac compared with cholesteryl esters (Ding et al., 1995). In chicks, C16:0, C18:0, and C18:2 are the major constituent.
TABLE 2.3. The fatty acid composition of the yolk sac in turkey embryos during the last week of incubation and 2 d posthatch, Experiment 2

<table>
<thead>
<tr>
<th>Day</th>
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<th>C16:1</th>
<th>C18:0</th>
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Age effect: 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001
Probability3 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001

1Day: 22 to 28 = 13 to 28 d of incubation; and P1 and P2 = 1 and 2 d posthatch.
2TFA = total fatty acid content, expressed as percentage of dry matter.
3Probabilities less than 0.05 indicate significant incubation time effect.

Fatty acids in yolk lipid TG and PL (Noble and Moore, 1964). The accumulation of liver lipid, particularly cholesteryl esters, is similar to that reported previously in pouls (Ding et al., 1995) and chicks (Moore and Doran, 1962; Noble and Moore, 1964; Noble, 1987). The observed decline in C16:0 concentration is opposite to that reported for hepatic fatty acids in chicks (Noble and Moore, 1964). The decline in C18:0 and concomitant increase in C18:1 concentration may be a reflection of species differences in the activity of 6-9 desaturase. This enzyme has been reported to be active during the latter stages of incubation in chicks (Noble and Shand, 1985), although there is not universal agreement on the significance or presence of this particular enzyme (Donaldson and Fites, 1970; Donaldson and Mueller, 1971; Donaldson, 1981). Increased hepatic C18:1 may also be a reflection of selective accumulation of cholesteryl esters high in C18:1 coming from the yolk sac membrane (Noble and Moore, 1964; Noble et al., 1984). Arachidonic acid (C20:4) levels were similar to what has been reported for chicks based on calculations for yolk sac PL and PL fatty acid profiles reported by Noble and Moore (1967 b,c).

In summary, the results from the present study show that yolk sac lipids and fatty acids are rapidly depleted during the latter stages of incubation. The data suggest that yolk sac lipids and fatty acids are important nutrients for developing turkey embryos, although quantitatively, lipids in the residual yolk sac may not represent a major energy reserve for newly hatched pouls.

TABLE 2.4. The fatty acid composition of the liver in turkey embryos during the last week of incubation and 2 d posthatch, Experiment 2

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<th>C18:0</th>
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Age effect: 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001
Probability3 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001 0.0001

1Day: 22 to 28 = 13 to 28 d of incubation; and P1 and P2 = 1 and 2 d posthatch.
2TFA = total fatty acid content, expressed as percentage of dry matter.
3Probabilities less than 0.05 indicate significant incubation time effect.
ACKNOWLEDGMENTS

The authors wish to thank Donna Kinsey and Lisa Carl for their technical assistance.

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Ding, S. T., K. E. Nestor, and M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. Poultry Sci. 74:374-382.


CHAPTER 3

THE EFFECT OF DIETARY MEDIUM CHAIN FATTY ACID (MCFA) ON THE FATTY ACID COMPOSITION OF YOLK AND LIVER LIPIDS IN TURKEY EMBRYOS

Abstract

Turkey hens were fed either a control diet (14:0, 1.1 %; 16:0, 16.8 %; 18:1, 23 %; 18:2, 48.7 %) or a diet containing 5% coconut oil (COCO) enriched with MCFA (12:0, 22.6 %; 14:0, 10.8 %; 16:0, 12.5 %; 18:1, 14.8 %; 18:2, 24.6 %). After 10 d on the diets, fresh eggs were collected for yolk lipid and FA determination. An additional 60 to 95 eggs were incubated and the FA profiles of the neutral lipid (NL) and phospholipid (PL) fractions of yolk sac and liver lipids were determined. The NL fraction of the yolk sac from control eggs contained less 12:0 (0 vs 0.49 %) and 14:0 (0.7 % vs 4.6 %) and more 18:1 (41.3 % vs 37.5 %). The phospholipid fraction from both treatments contained < 1 % 14:0 and there were < 2 % differences between treatments in other FA concentrations. The hepatic NL fraction from both
treatments contained < 1% 14:0 and only 18:1 (Control = 59.9%; COCO = 56.62%) showed > 1% difference between treatments. There were no dietary effects on the FA profile of hepatic PL. The presence of only minimal quantities of MCFA in hepatic NL and PL suggests that absorbed yolk sac MCFA are extensively metabolized during embryonic development.

**Introduction**

It was estimated that yolk lipid fatty acids provide more than 90% of the total energy requirement for developing avian embryos (Freeman, 1974). Research has indicated that maternal dietary long chain fatty acid composition affected the incorporation of LCFA into yolk (Feifenbaum and Fisher, 1959; Wheeler et al., 1959; Cough et al., 1973). If fatty acid profiles of fresh egg yolk can be altered by dietary modification, such changes may subsequently influence embryonic fatty acid metabolism. Donaldson (1967), Couch et al. (1973), and Vilchez et al. (1992) demonstrated that the fatty acid composition of the maternal diet will change the fatty acid profile of avian embryos. Cherian and Sim (1993) also reported that the omega-3 fatty acid incorporation into the diet of hens modified the omega-3 fatty acid content and cholesterol content of tissues in developing embryos.
Medium chain fatty acids (MCFA, 6:0 to 12:0) are more easily metabolized than other long chain fatty acids due to their carnitine independent transport into hepatic mitochondria (Babayan, 1987). MCFA also may also have beneficial effects on overall growth in young chicks (Mabayo et al., 1993).

The purpose of this experiment was to determine the effect of MCFA enrichment of the diet of turkey hens on fresh egg yolk fatty acid profiles and the accumulation of MCFA in turkey embryonic tissues during the latter stages of incubation.

**Materials and methods**

Turkey hens from a randombred line (RBC2) and a subline selected for 16-week body weight (Ding et al., 1995) were fed either a control diet (CTRL) or a diet enriched with MCFA coming from 5% coconut oil (COCO; Table 3.1). The dietary fatty acid compositions are shown in Table 3.2. The diets were fed for 10 d after which, 20 fresh eggs from each diet and line combination were collected for yolk lipid and FA analysis. An additional 65 to 95 fertile eggs from each line and diet were incubated and the FA profile changes during embryonic development were measured. During the last week of incubation, 6 to 20 embryos from each line and treatment were collected every other day to determine the
**TABLE 3.1. Experimental diets.**

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<th>Ingredients and composition</th>
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<tr>
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<tr>
<td>Coconut oil</td>
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<td>.500</td>
</tr>
<tr>
<td>Trace mineral premix'</td>
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<td>.100</td>
</tr>
</tbody>
</table>

**Calculated composition**

| Crude protein (%)                          | 17.4  | 16.81 |
| ME, Kcal/kg                                | 2728  | 2948  |

1The selenium premix contains 200 mg Se/kg premix.
2The vitamin premix contributed the following per kg of diet: vitamin A, 8,745 IU; cholecalciferol, 3,745 IU; vitamin E, 60 U; vitamin K (menadione sodium bisulfite), 2.91 mg; thiamine HCl, 2.2mg; riboflavin, 6.6 mg; niacin, 99 mg; pantothenic acid, 15.4 mg; folic acid, 1.2 mg;
Table 3.1. continued.
pyridoxine, 2.2 mg; biotin, .165 mg; vitamin B$_{12}$, 15 ug;
ethoxyquin, 113.5 mg.

The trace mineral premix contributed the following per kg of diet: zinc oxide (72% Zn), 147 mg; manganous oxide (55% Mn), 152 mg; copper sulfate (25% Cu), 35 mg; ferrous sulfate monohydrate (31% Fe), 72 mg; potassium iodide, 1.5 mg.
Table 3.2. Lipid Content and Fatty Acid Composition of The Diets¹.

<table>
<thead>
<tr>
<th>Fatty acid²</th>
<th>CTRL³</th>
<th>COCO⁴</th>
<th>% total fatty acid</th>
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</table>

Lipid content, % DM 4.93 8.05

¹All data are based on laboratory analysis results.²8:0 = Caprylic acid; 10:0 = capric acid; 12:0 = lauric acid; 14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = linolenic acid; 22:6 = docosahexaenoic acid. ³CTRL = control diet. ⁴COCO = medium chain fatty acid enriched diet; 5% coconut oil was added.
lipid subclass and FA composition of the yolk sac and liver. Fresh yolk, total yolk sac (including yolk content and yolk sac membrane), and liver were lyopholyzed and stored at - 70 C prior to the determination of lipid and fatty acid composition.

The lipid content of all samples were extracted following the procedures of Folch et al. (1957) as modified by Marchesselli and Bazan (1990). In short, a chloroform:methanol mixture (2:1) was used to extract lipids and all samples were sonicated twice for 30 minutes to facilitate lipid extraction. All procedures were conducted under nitrogen gas to minimize FA peroxidation. Lipid extracts (25 mg) were further separated into neutral lipid (NL) and phospholipid (PL) fractions according to the procedures of Bacon et al. (1982) modified by Ding et al. (1995). The fatty acid composition of the NL and PL fractions were determined by gas chromatography as described by Sukhija and Palmquist (1988) with some modifications. Fatty acids (less than 15 mg) were methylated by 1.5 mL 5% methanolic HCl solution in 90 C water bath for 2 hours. Two mg nonadecanoic acid (C19:0) in 1 mL benzene was added to each sample as an internal standard prior to methylation. After methylation, 2.5 mL of 6% K2CO3 was added to neutralize the solution. Methylated fatty acids were extracted with hexane. Fatty acids were quantified by
Table 3.3. The effect of dietary fatty acid profiles on the neutral lipid fatty acid changes in yolk sacs of developing embryos.

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14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; and TFA = total fatty acid. RBC2 = randombred control line; F = subline of RBC2 selected for increased 16-wk body weight. CTRL = control diet; COCO = medium chain fatty acid enhanced diet by addition of 5% coconut oil. *0 = fresh egg; 22 to 28 = 22 to 28 days of incubation.
automatic gas chromatography (Hewlett-Packard 5890) using a SP-2340 fused silica capillary column. The temperature was programmed from 160 C to 180 C at 3 C per minute increment.

All data were analyzed by analysis of variance (SAS, 1986). Line (F; RBC2), diet (CTRL; COCO), and age (d of incubation) as the main effects tested. The percentage data were arc sine transformed prior to statistical analysis, but the original values are presented with the ANOVA results from transformed data.

Results

Yolk Sac NL

There were significant line differences in the relative concentrations of all FA measured (Table 3). The F line had significantly higher 18:0 and 18:1 but lesser relative levels of the other measured FA. There was a significant decline in the concentration of all FA with length of incubation with the exception of 18:1 which increased and 18:2 which did not change. The supplemental coconut oil resulted in a significant increase in 12:0 and 14:0 concentrations and declines in 18:0 and 18:1, respectively. An examination of the total FA per yolk sac data, however, suggests that 16:0 is also significantly increased by coconut oil supplementation. The total FA per yolk sac data also gives a clearer picture of what is happening with
individual FA during incubation. The RBC2 yolk sacs clearly have increased 16:0, 16:1, and 18:2 and decreased 18:0 with no differences in 18:1.

**Yolk Sac PL FA**

The relative concentration data suggests small but significant changes in yolk sac PL FA profiles between lines but the absolute concentrations per yolk sac suggest no significant line effects (Table 4). Supplemental coconut oil did increase both the relative and absolute concentrations of 14:0 (P ≤ .01). There were no significant effects of coconut oil on the relative concentrations of 16:0 and 18:0 but the absolute amounts of each FA was increased by coconut oil supplementation. The relative concentration of 18:1 was increased by coconut oil supplementation but there were no effects on absolute amounts whereas both the relative and absolute concentrations of 18:2 were increased. The absolute quantities of the polyunsaturated FA 20:4 and 22:6 were almost identical in the yolk sac PL from both dietary groups.

In fresh eggs and through 28 days of incubation, there were minimal changes in the relative concentrations of most FA with the exception of 22:6 which decreased considerably.
Table 3.4. The effect of dietary fatty acid profiles on the phospholipid fatty acid changes in yolk sacs of developing embryos.

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14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 20:4 = arachidonic acid; 22:6 = docosahexaenoic acid; and TFA = total fatty acid.

RBC2 = randombred control line; F = subline of RBC2 selected for increased 16-wk body weight. CTRL = control diet; COCO = medium chain fatty acid enhanced diet by addition of 5% coconut oil.
Table 3.5. The effect of dietary fatty acid profiles on the neutral lipid fatty acid changes in livers of developing embryos.

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% TFA

mg/liver
| Line | Diet | Age | .233 | .069 | .528 | .014 | .107 | .640 | .002 | .738 | .910 | .524 | .369 | .566 | .746 | .053 |
|------|------|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|

1\(^{14:0} = \text{myristic acid}; 16:0 = \text{palmitic acid}; 16:1 = \text{palmitoic acid}; 18:0 = \text{stearic acid}; 18:1 = \text{oleic acid}; 18:2 = \text{linoleic acid}; 22:6 = \text{docosahexaenoic acid}; \text{and TFA} = \text{total fatty acid}.  

\(^{2}\text{RBC2} = \text{randombred control line; } F = \text{subline of RBC2 selected for increased 16-wk body weight.}

\(^{3}\text{CTRL} = \text{control diet; COCO} = \text{medium chain fatty acid enhanced diet by addition of 5 \% coconut oil.}

\(^{4}22 \text{ to } 28 = 22 \text{ to } 28 \text{ days of incubation.}

\(^{5}\text{Table 6. The effect of dietary fatty acid profiles on the phospholipid fatty acid changes in livers of developing embryos.} 0 = \text{fresh egg; } 22 \text{ to } 28 = 22 \text{ to } 28 \text{ days of incubation.}
The absolute concentrations of all PL FA decreased significantly, however, throughout the course of incubation.

Liver NL

There was no 12:0 detected in hepatic NL of turkey embryos. The F line livers had significantly decreased relative concentrations of 14:0, 16:1, and 18:2 and this was also true for absolute differences in these FA (Table 5). The relative concentrations of 18:0 and 22:6 were increased in F line hepatic NL but the absolute quantities of these FA were not different between lines. Coconut oil supplementation significantly increased both the relative and absolute concentrations of 14:0. The relative concentrations of 16:0 (decrease), 18:1 (decrease), and 18:2 (increase) were all effected by the coconut oil but there were no significant carryover effects on the absolute concentration of any FA other than 14:0.

The relative concentrations of 16:0 and 22:6 declined with length of incubation whereas 18:1 increased. From a quantitative standpoint, there were significant increases in the concentrations of all FA over the course of incubation although the greatest quantitative change was in the level of 18:1, the concentration of which increased from 6 to over 60 mg between 22 and 28 d.
Table 3.6. The effect of dietary fatty acid profiles on the phospholipid fatty acid changes in livers of developing embryos.

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Table 3.6. continued

| Line*Diet*Age | .970 | .889 | .911 | .178 | .895 | .173 | .659 | .593 | .461 | .705 | .544 | .085 |

Table 3.6.

14:0 = myristic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 20:4 = arachidonic acid; 22:6 = docosahexaenoic acid; and TFA = total fatty acid.

2RBC2 = randombred control line; F = subline of RBC2 selected for increased 16-wk body weight.

3CTRL = control diet; COCO = medium chain fatty acid enhanced diet by addition of 5% coconut oil.

422 to 28 = 22 to 28 days of incubation.
Liver PL

There were no measurable concentrations of 12:0 or 14:0 in hepatic PL (Table 6). There were small but significant line effects on the relative concentrations of 16:0, 18:1, 20:4, and 22:6 but only 18:1 was both quantitatively and proportionately lower in F line hepatic PL. Supplemental coconut oil significantly increased the relative concentration of 18:2 but had no effect on the quantitative amounts whereas only the concentrations of 18:1 and 22:6 were quantitatively lower due to coconut oil supplementation. Between 22 and 28 days of incubation, 18:1 was the only FA whose relative concentration did not increase or decrease whereas quantitatively, there was a significant increase in all FA during the same time period.

Discussion

The addition of 5% coconut oil in diets for turkey breeder hens effectively increased the levels of 12:0 and 14:0 in the yolk of unincubated eggs. The concentration of 12:0 (22.6 % TFA) was almost twice that of 14:0 (10.7 % TFA) in the test diet yet the incorporation of 12:0 into yolk lipids was very low. This suggests that there is either extensive metabolism of 12:0 by the maternal liver (Bitman et al., 1983; Babayan, 1987) or it is elongated to longer
chain fatty acids leaving only a small amount available for incorporation into yolk lipids. Oleic acid (18:1) represents the largest single class of FA, almost 40% TFA, in the fresh egg and it also represents the class of FA which was both proportionately and quantitatively decreased by supplemental coconut oil. Its status as the predominant yolk lipid FA make it a logical choice for mass displacement by other dietary FA. Fisher (1959) and Wheeler et al. (1959) have previously reported that increased dietary linoleic acid (18:2) increased the incorporation of 18:2 primarily at the expense of oleic acid (18:1). There is other data in the literature which also shows the ease with which yolk FA can be changed by dietary lipid manipulation (Isaacks et al., 1964; Jiang et al., 1991; and Cherian and Sim, 1993).

The NL fraction in the turkey yolk sac comprises approximately 80% of total lipids (Ding et al., 1995) and is the primary energy source for developing embryos (Freeman, 1974). The large decline in yolk sac NL 12:0 and 14:0 are indicative of their utilization by turkey embryos. The shorter chain MCFA can be oxidized without the need for carnitine transport into the mitochondrion (Babayan, 1987) and thus makes it a potentially more efficient source of energy. The low level of MCFA incorporated into yolk lipids
in the present study would probably be of little benefit to the developing embryo.

Quantitatively, the yolk sac from RBC2 embryos had higher concentrations of 12:0, 14:0, 16:0, 16:1, and 18:2 compared with the F line and this may be a reflection of the faster growth rate and hence, greater amount of lipid being absorbed by F line embryos during incubation. This confirms earlier observations on differences in embryonic growth between the two lines (Ding et al., 1995).

The COCO treatment increased only the 14:0 content in the yolk PL and no effect on MCFA content. It is well studied that the dietary fatty acid composition has different effects on different fractions of lipids. Jiang et al. (1991) observed that a high oleic acid diet did not increase oleic acid in the PL of yolk although it increased the oleic acid content in the NL. High 18:2 and 18:3, on the other hand, increased the 18:2 and 18:3 in both TG and PL of yolk (Jiang et al., 1991; Vilchez et al., 1992). The high MCFA diet for turkey hens resulted in no MCFA incorporated into yolk PL, indicating that MCFA is not being used to synthesize PL in turkey hens.

No MCFA was detected in the hepatic NL of the COCO fed embryos, indicating that the MCFA from yolk have been metabolized and little are stored in liver. There was, however, a very high 18:1 content in the hepatic NL.
Similar results have been reported in turkey (Chapter 5) and chick embryos (Noble and Moore, 1964). NL in turkey embryonic liver is primarily comprised of CE (Ding et al., 1995). Accumulation of high 18:1 containing CE in liver (Noble and Moore, 1964; Noble et al. 1984; Ding et al., 1995) may be the reason of the high 18:1 in liver lipids.

The maternal MCFA does not affect the fatty acid profiles of hepatic PL except to increase slightly the C18:2 concentration. The results seem to indicate that there is high specificity in incorporating fatty acids into hepatic PL. Others were able to change the hepatic PL fatty acid profiles in embryos by incorporating 18:2 and 18:3 in maternal diets (Vilchez et al., 1992).

In conclusion, enrichment of dietary MCFA by adding coconut oil changes the yolk lipid fatty acid composition. The major effect is on the fatty acid profiles in NL. The incorporation of MCFA into yolk lipids through maternal diets is limited. But the yolk lipid MCFA is readily utilized by turkey embryos because its disappearance rate from yolk sac is faster than other long chain fatty acids during the latter stages of embryonic development.

Acknowledgment

The authors wish to thank Donna Kinsey, Cindy Coy, and George Barbour for their assistance.
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acid distribution in egg oil as influenced by type and level
CHAPTER 4

The Concentration of Different Lipid Classes During Late Embryonic Development in a Randombred Turkey Population and a Subline Selected for Increased Body Weight at Sixteen Weeks of Age

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ABSTRACT Lipid changes in the yolk sac of the embryo were studied in a randombred population of turkeys (RBC2) and a subline of the RBC2 selected for increased BW at 16 wk (F line). Comparisons of yolk sac and embryonic development were made between 22 d of incubation and hatch (23 d). Pouls from the F line had heavier yolk sacs from 24 to 28 d and yolk free body weight was also heavier at hatch (61.1 vs 52.8 g). Yolk sac lipid (percentage of DM) declined faster in F line embryos (69 to 39%) compared with the RBC2 line (62 to 48%). In both lines, embryonic liver dry matter and lipids (percentage of DM) were similar. Yolk sac neutral lipids increased from 22 to 28 d (70 to 80% total lipid) and there was a concomitant decline in phospholipids (30 to 20%). The direction of the changes was similar for embryonic liver lipid. At 26 and 28 d, there were significantly increased neutral lipids (94 vs 88%) and decreased phospholipids (6 vs 12%) in RBC2 compared with F line embryonic livers. The concentration of cholesterol esters (percentage of total lipid) increased in the yolk sac and embryonic liver during the course of incubation. At 26 and 28 d, livers from RBC2 embryos had increased cholesterol ester concentration compared with livers from the F line. These results suggest that selection for body weight at 16 wk of age has not changed total lipid content of the yolk, but there was a significant decline in the proportion of triacylglycerol (percentage of total lipid) and diacylglycerol (percentage of total lipid) in the F line during embryonic development. Selection also resulted in significantly increased hepatic total lipid in F line embryos but significantly decreased the proportion of hepatic neutral lipid.

(Key words: turkey, embryo, lipid, yolk, liver)

INTRODUCTION

Yolk lipids are primarily composed of triacylglycerol (TG) (63%) and phospholipid (29%; Noble and Cocchi, 1990). They provide more than 90% of embryonic energy requirements (Freeman and Vince, 1974) and embryonic membrane constituents, respectively. The transfer of different yolk lipid fractions to the chick embryo during incubation has been studied extensively (Noble and Moore, 1964, 1965, 1967; Noble et al., 1984). Noble and Moore (1964) reported that in embryonic chicks, TG and phospholipids were the main classes of lipids transferred out of the yolk sac and their transfer paralleled the overall decrease in total yolk lipid during the last 7 d of incubation. During the last 2 d of incubation, there was a significant increase in embryonic liver lipid and this was due
primarily to the accumulation of esterified cholesterol [70% of total lipid, (TL); Noble et al., 1984]. In turkey embryos, the utilization of lipids from the yolk sac by the embryo is also very rapid during the latter stages of incubation (Ding and Lilburn, 1993). There has been little quantification, however, of which lipid classes are important to and involved in lipid movement from the yolk sac to the embryo.

Lilburn and Nestor (1991) characterized age-associated changes in carcass development in a randombred population of turkeys (RBC2) and a subline of the RBC2 line selected for increased BW at 16 wk of age (F line). Body weight at hatch is significantly heavier in F line pouls (Anthony et al., 1991; Lilburn and Nestor, 1991; Christensen et al., 1993), although overall reproductive performance is reduced compared with the RBC2 line (Nestor, 1984). Embryonic development and yolk lipid utilization comparisons between the two lines have not been reported, however. The objectives of this experiment were to compare changes in the concentration of different lipid classes during embryonic development in the two lines and to quantify which classes of lipids changed to the greatest extent in the yolk sac and liver during the late prenatal period.

MATERIALS AND METHODS

Forty eggs each from the RBC2 and F lines were used to determine egg composition. From 22 d of incubation through hatch (28 d), 10 to 20 embryos from each line were collected daily. The embryos were decapitated, the yolk sac and liver were excised and weighed, and embryonic weight without the yolk sac was recorded. The yolk sacs and livers were lyophilized to determine the DM content. The lipid concentration of the dried yolk sacs and livers was determined according to the procedures of Folch et al. (1957) as modified by Marcheselli and Bazan (1990). In short, the extraction solvent was a 2:1 mixture of chloroform:methanol and each sample was sonicated for 30 min twice to facilitate complete lipid extraction. During all the lipid extractions, samples were kept under nitrogen gas to prevent fatty acid peroxidation. All the extracts were then stored at -20 C until further analysis. Lipid extracts (25 mg) were applied to a silica chromatography column (500 mg Silica). Four washes with 5-mL aliquots of diethyl ether and four washes with 5-mL aliquots of methanol were used to elute the neutral lipid (NL) and phospholipid fractions, respectively. The NL fraction was then further separated into TG, cholesterol ester (CE), diacylglycerol (DG), and free cholesterol (FC) fractions according to the HPLC procedure described by Bacon et al. (1982). The only modification was that 20 mL of 6% methyl t-butyl ether in hexane was used to elute out the TG fraction. A pure mixture of known lipids, including tripalmitin, glyceryl-1, 3-dipalmitin, glyceryl-1-monopalmitin, cholesteryl palmitate, and cholesterol, and a blank were used as standards and all quantitative measures were gravimetric.

The data were analyzed by two-way analysis of variance using the General Linear Models procedure of SAS* (SAS Institute, 1986). The main effects were line, age (days of incubation), and the interaction of line by age. All the percentage data were subjected to arc sine transformation before statistical analysis. However, original values were presented with the ANOVA results of transformed data.

RESULTS

The composition of eggs from the two lines are shown in Table 1. Eggs from F line hens were heavier and had increased absolute and relative albumen weight and increased shell weight. There were no line effects on absolute yolk weight, but the relative weights of the yolk and shell were greater in eggs from the RBC2 hens.

After 24 d of incubation, the weight of F line embryos was consistently greater than that of RBC2 embryos (Table 2). The weight of the residual yolk sac at 28 d was 34 and 28% of the weight of the fresh yolk.
Table 4.1. The egg composition of two lines of turkeys

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<th>RBC2 line</th>
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<td>Shell, g</td>
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<td>Shell-yolk, %</td>
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RBC2 = randombred control line; F = subline of the RBC2 line selected for increased 16-wk body weight.

Value = mean ± SE.

An asterisk indicates significant difference between two lines (P < .05).

in the F line and RBC2 eggs, respectively (Table 2). The greatest proportional decline in yolk sac weight occurred between 24 d and hatch. Yolk sac DM in RBC2 embryos remained fairly constant over the latter stages of incubation (Table 2). In the F line, however, there was a decline in yolk sac DM between 22 d and hatch. In fresh eggs and at 22 d of incubation, yolk sac lipid (percentage of DM) was increased in F line embryos (Table 2). From 24 to 28 d, however, RBC2 residual yolk sacs had increased lipid (percentage of DM) compared with the F line. In absolute terms, however, there were no line differences. The TL content of the yolk sac decreased from 6.6 to 1.9 g in the F line and from 6.7 to 2.1 g in the RBC2 line. As a result, approximately 20% of yolk TL (9.5 to 9.7 g in a fresh egg) remained at hatch.

Yolk sac neutral lipids, which encompass all lipid classes except phospholipids, increased from approximately 70 to 80% of TL over the course of incubation (Table 3). There were no line differences. Total phospholipids decreased from approximately 29 to 26% through 26 d of incubation followed by a major decrease from 26 to 28 d. There were no line effects. The CE content of the yolk sac increased from 6.6 to 16.64% and from 2.21 to 9.15% TL in the F line and RBC2 embryos, respectively. At hatch, the large differences between the F line (16.64% TL) and RBC2 (9.15% TL) contributed to an overall line effect (P < .069). The F line had considerably less yolk sac TG (percentage of TL) at 22 and 28 d, but there were no consistent line or age effects at other stages of incubation. In the F line, there were no consistent age-associated changes in yolk sac DG (percentage of TL), whereas in the RBC2 line DG increased at 26 and 28 d of incubation. At these latter two ages, DG was higher in the RBC2 yolk sac than in that of the F line. In absolute terms, however, all lipid classes except CE decreased in both lines as incubation proceeded (Table 3). There were no line differences in NL, PL, CE, and TG content during incubation.

There was a significant age-associated increase in embryonic liver weight from 22 to 28 d (Table 4). The F line embryos had consistently heavier livers than the RBC2 embryos. Liver DM increased significantly between 22 and 26 d with no change at 28 d (Table 4). There was a significant increase in embryonic liver lipid (percentage of DM) between 22 and 26 d, which paralleled the increase in liver DM (Table 4). At 26 and 28 d, there was an increase in liver lipid in F line poult compared with the RBC2 line.

In both lines, hepatic NL concentration (percentage of TL) increased significantly from 22 to 28 d (Table 5). At 26 and 28 d, there was increased hepatic NL concentration in RBC2 embryos compared with embryos of the F line. However, at 24, 26, and 28 d, the F line embryos had higher total hepatic NL (milligrams per liver) than the RBC2 line.

The CE fraction (percentage of TL) of the embryonic liver increased greatly between 22 and 26 d (Table 5). At 26 and 28 d, it represented approximately 75 and
### TABLE 4.2. The changes in embryonic weight, yolk sac weight,1 yolk sac DM, and yolk sac total lipid content in developing embryos from two turkey lines3

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</tr>
</tbody>
</table>

**SEM** 4.10 4.24 3.09 2.97 3.12 3.01 5.89 4.19 1.03 .65

**ANOVA results**

<table>
<thead>
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<th>Probabilities</th>
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</tr>
<tr>
<td>Day</td>
<td>.001 .001 .001</td>
</tr>
<tr>
<td>Line x day</td>
<td>.009 .009 .002</td>
</tr>
</tbody>
</table>

---

1Yolk sac: for embryos, it included yolk sac membrane and yolk content; for fresh egg, it was yolk.
2RBC2 = randombred control line; F = subline of RBC2 line selected for increased 16-wk body weight.
3Days: 0 = fresh egg, 22 to 28 = 22 to 28 d of incubation.
TABLE 4. The lipid composition of the yolk sac from different lines of turkeys

<table>
<thead>
<tr>
<th>Day³</th>
<th>Embryo number (n)</th>
<th>NL (%)</th>
<th>PL (%)</th>
<th>CE (%)</th>
<th>TG (%)</th>
<th>DG (%)</th>
<th>FC (%)</th>
</tr>
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<tr>
<td>0</td>
<td>20</td>
<td>69.62</td>
<td>71.21</td>
<td>30.38</td>
<td>28.79</td>
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<td>2.21</td>
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<td>22</td>
<td>10</td>
<td>71.14</td>
<td>74.00</td>
<td>28.86</td>
<td>26.00</td>
<td>3.89</td>
<td>2.95</td>
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<tr>
<td>24</td>
<td>10</td>
<td>73.60</td>
<td>73.01</td>
<td>26.40</td>
<td>26.99</td>
<td>5.65</td>
<td>5.96</td>
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<tr>
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<td>10</td>
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<td>73.73</td>
<td>26.15</td>
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<td>7.02</td>
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<td>20</td>
<td>78.64</td>
<td>79.98</td>
<td>21.36</td>
<td>20.02</td>
<td>16.64</td>
<td>9.15</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>2.67</td>
<td>2.83</td>
<td>2.68</td>
<td>2.83</td>
<td>3.58</td>
<td>1.98</td>
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</table>

ANOVA results

<table>
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<th>Line</th>
<th>Day</th>
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</tr>
</thead>
<tbody>
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<td>.001</td>
<td>.001</td>
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Probabilities

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<th>.001</th>
<th>.001</th>
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</table>

NL = neutral lipids; PL = phospholipids; CE = cholesteryl esters; TG = triacylglycerols; DG = diacylglycerols; and FC = free cholesterol.

JRBC2 = randombred control line; F = subline of RBC2 line selected for increased 16-wk body weight.

3Days: 0 = fresh egg and 22 to 28 = 22 to 28 d of incubation.

*On Day 0, embryo number represents egg number.
TABLE 4.4. The changes in liver weight, liver DM and liver total lipid content in developing embryos from two turkey lines

<table>
<thead>
<tr>
<th>Day</th>
<th>Embryo number (n)</th>
<th>Liver weight (g)</th>
<th>DM %</th>
<th>Lipid content (mg/liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>10</td>
<td>.58 .60</td>
<td>25.53</td>
<td>25.21</td>
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<tr>
<td>24</td>
<td>10</td>
<td>.91 .78</td>
<td>29.89</td>
<td>28.95</td>
</tr>
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<td>26</td>
<td>10</td>
<td>.98 .93</td>
<td>33.18</td>
<td>33.77</td>
</tr>
<tr>
<td>28</td>
<td>20</td>
<td>1.64 1.47</td>
<td>32.48</td>
<td>33.14</td>
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</table>

SEM .18 .18 .77 1.30 1.92 2.40 12.78 11.04

ANOVA results
Line .002 .830 .029 .001
Day .001 .001 .001 .001
Line x day .206 .468 .171 .120

<table>
<thead>
<tr>
<th>Probabilities</th>
</tr>
</thead>
</table>

RBC2 = randombred control line; F = subline of RBC2 line selected for increased 16-wk body weight.

3Days: 22 to 28 = 22 to 28 d of incubation.

80% of the TL in livers from F line and RBC2 embryos, respectively. In both lines, hepatic phospholipid concentration decreased significantly between 22 and 28 d. At 26 and 28 d, the F line (12% TL) had increased hepatic phospholipid compared with the RBC2 line (4 to 5% TL).

The minor lipids of the embryonic liver are the TG, DG, and FC fractions. Hepatic TG decreased from 6.89 to 2.21% TL in the F line compared with 3.92 to 1.50% TL in the RBC2 embryos (Table 5). The line differences approached significance (P ≤ .061). The hepatic DG and FC (percentage of TL) stayed relatively constant from 22 to 28 d and there were no consistent line differences.

In absolute terms, hepatic NL, CE, DG, and FC in both lines and PL in F line increased as incubation proceeded. The F line embryos had higher (P ≤ .05) hepatic NL, PL, CE, and TG content than the RBC2 line during the last 4 d of development (Table 5).

DISCUSSION

The increased albumen content of F line eggs resulted in a concomitant reduction in yolk percentage, similar to what was reported by Nestor et al. (1982). Reidy et al. (1994) also reported that eggs from a heavy turkey line had a lower percentage of yolk than eggs from a lighter line. The increased hatch weight of F line poults is consistent with the reports of Anthony et al. (1991).

At hatch, approximately 80% of yolk TL have been absorbed by the developing turkey embryo and the greatest proportion (63%) is transported out of the yolk sac during the last 6 d of incubation. This large increase in lipid uptake suggests that by the latter stages of incubation, the embryo has developed an active lipid transport system. Many enzymes associated with lipid transport out of the yolk into the embryo have been reported to have their maximal activity during this same time period. Escribano et al. (1988) reported that lipase activity (EC 3.1.1.3) in the yolk sac membrane is maximized before Day 21 of incubation. The activity of acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) in yolk sac membrane and liver and lipoprotein lipase (EC 3.1.1.34) in embryonic adipose tissue and heart are also high during this period of development (Noble et al., 1984; Shand et al., 1993; Speake et al., 1993). The yolk sac residual lipid is minimal at hatch and, as such, probably does not represent a significant source of energy for newly hatched poults.

The increased NL content of yolk sac is primarily a reflection of increased CE content. These results are similar to what has been reported for chicken embryos (Noble and Moore, 1964, 1967). This is probably due to a combination of in-
### TABLE 1. The lipid composition\(^1\) of embryonic liver from different lines\(^2\) of turkeys

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>Embryo number</th>
<th>NL F RBC2</th>
<th>PL F RBC2</th>
<th>CE F RBC2</th>
<th>TG F RBC2</th>
<th>DG F RBC2</th>
<th>FC F RBC2</th>
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<tr>
<td></td>
<td>(n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>10</td>
<td>71.80</td>
<td>70.66</td>
<td>28.20</td>
<td>57.15</td>
<td>6.89</td>
<td>3.92</td>
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<tr>
<td>24</td>
<td>10</td>
<td>81.69</td>
<td>78.39</td>
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<td>6.44</td>
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<td>87.79</td>
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**ANOVA results**

<table>
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<th>Line x day</th>
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<td>.649</td>
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<th>CE F RBC2</th>
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<th>DG F RBC2</th>
<th>FC F RBC2</th>
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<td>187.45</td>
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<td>3.05</td>
<td>10.19</td>
<td>11.24</td>
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**ANOVA results**

<table>
<thead>
<tr>
<th>Line</th>
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<th>Line x day</th>
<th>Probabilities</th>
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<td>Line x day</td>
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<td>.659</td>
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\(^1\) NL = neutral lipids; PL = phospholipids; CE = cholesteryl esters; TG = triacylglycerols; DG = diacylglycerols; and FC = free cholesterol.

\(^2\) RBC2 = randombred control line; F = subline of RBC2 line selected for increased 16-wk body weight.

\(^3\) Days: 22 to 28 = 22 to 28 of incubation.
creased ACAT and decreased activity of CE hydrolase in the embryonic yolk sac membrane (Noble et al., 1984; Shand et al., 1993). The reduction in yolk sac free cholesterol may also be a reflection of the high activity of these enzymes. Cholesterol ester formation in the yolk sac membrane has been proposed to play an important role in lipid transfer from the yolk sac membrane into the embryo (Noble et al., 1984).

The significant increase in embryonic liver size and lipid accumulation is similar to what was reported in embryos from commercial chicks and turkeys (Noble and Cocchi, 1990; Ding and Lilburn, 1993). This is primarily the result of increased hepatic NL, which are a mixture of CE, TG, DG, and FC. The significant increase in hepatic CE accounts for the greatest proportion of increased hepatic lipid. It may be the result of both increased CE transfer out of the yolk sac into the embryo and increased CE synthesis by the liver. In chickens, hepatic cholesterol esterification has been reported to be very active near hatch (Noble et al., 1984). It might serve as an energy source or for bile synthesis during the late embryonic and early posthatch developmental periods.

The significant, proportional reduction in liver TG over the last 4 d of incubation is similar to what has been reported for chicken embryos (Noble and Moore, 1964, 1967). This reduction in hepatic TG is not necessarily a reflection of decreased enzymatic synthetic capability (Sansbury et al., 1989), but rather the much higher, proportional rate of CE accumulation.

The large decline in the relative concentration of hepatic PL is consistent with the chick embryo data (Noble and Moore, 1967). Quantitatively, however, total liver PL stayed the same at 28 compared with 22 d (RBC2) or increased (F line). A clear rationale for the observed genetic line differences is not apparent but it may be related to genetic differences in phospholipid biosynthesis (Budowski et al., 1961). It is probably not a function of reduced phospholipid transport out of the yolk sac, because the reduction in yolk sac phospholipid with length of incubation was similar in both lines.

In summary, this report shows that turkey embryos utilize large quantities of lipid during the latter stages of incubation. Although there are no differences in TL disappearance from yolk sacs of turkey embryos from the two lines, the yolk sac from F line embryos contain proportionally less TG and the livers accumulate more lipid (NL, PL, CE, and TG) than livers from RBC2 embryos. This suggests that selection for increased body weight at 16 wk of age results in changes in lipid metabolism during the latter stages embryonic development.

ACKNOWLEDGMENTS

The authors wish to thank Wayne Bacon for his technical contributions in lipid fractionation and also the technical assistance of David Long, Cindy Coy, and John Nixon.

REFERENCES


CHAPTER 5

CHANGES IN FATTY ACID PROFILES IN DIFFERENT LIPID CLASSES DURING LATE DEVELOPMENT OF TURKEY EMBRYOS FROM TWO DIFFERENT LINES

ABSTRACT

Fatty acid (FA) profiles in embryonic yolk sacs and livers were studied in embryos from a randombred turkey line (RBC2) and a line selected for body weight at 16 wk (F line). Fatty acid profiles of fresh yolk lipids were similar in both lines. During incubation, F line embryos had increased oleic acid (C 18:1) and decreased linoleic acid (C 18:2) in yolk sac triglyceride (TG) and phospholipid (PL) compared with RBC2 embryos. In both lines, the oleic (C 18:1) acid content of yolk sac cholesteryl esters (CE) increased from 58 to 63% during the last 6 d of incubation. From 22 to 28 d of incubation, there was a constant oleic acid concentration
in hepatic CE and it was > 60% of total hepatic CE FA. As incubation proceeded, palmitic (C 16:0) and oleic (C 18:1) acids in hepatic TG decreased from 27 to 16% and 37 to 34%, respectively. Triglyceride stearic acid (C 18:0) increased from 12% at Day 22 to 32% of total FA at hatch (Day 28) in RBC2 embryos. The results suggest that more C 18:0 than C 16:0 and C 18:1 are incorporated into hepatic TG toward hatch. In hepatic PL, arachidonic acid (C 20:4) and docosahexaenoic acid (C 22:6) decreased while both C 16:0 and C 18:0 increased from Days 22 to 28. On Days 26 and 28, F line embryos had higher C 22:6 and C 20:4 in PL than the RBC2. These results suggest that selection for increased BW does not change yolk FA profiles but may change the proportional incorporation of different FA into embryonic lipids.

(Key Words: turkey, incubation, embryo, lipid, fatty acids)
INTRODUCTION

Yolk lipid provides about 90% of embryonic energy requirements (Freeman and Vince, 1974). In turkey embryos, yolk sac lipids and fatty acids (FA) are utilized rapidly during the last week of incubation (Ding and Lilburn, 1995).

From 23 to 28 d of incubation, embryos from a turkey line selected for BW at 16 wk (F line) grow faster than those from a randombred control line (RBC2; Ding et al., 1995). Ding et al. (1995) also described the changes that occurred in different lipid classes isolated from embryonic yolk sacs and livers during this latter stage of incubation. Yolk sac triglyceride (TG) and phospholipid (PL) fractions decreased while the cholesteryl ester (CE) fraction increased. F line embryos accumulated more hepatic lipid (% DM) than RBC2 embryos with the greatest proportion of hepatic lipid in both lines being accounted for by the CE fraction. F line poult's had proportionally less hepatic CE than RBC2 embryos (74.0% vs. 81.5%) but quantitatively, hepatic CE was still slightly higher in F line embryos. Cholesteryl ester accumulation in the liver is similar to what has been reported for chicks (Moore and Doran, 1962; Noble and Moore, 1964).
Different lipid classes with varying fatty acid profiles are utilized differently during the course of incubation in chick embryos (Noble and Moore, 1964; Donaldson, 1967). This experiment was conducted to evaluate the effect of selection for 16 wk BW on yolk FA profiles and the developmental aspects of FA profile changes during late embryonic development.

MATERIALS AND METHODS

Twenty fresh eggs from the RBC2 and F lines were used as Day 0 samples. Fertile eggs were set and 10 to 20 embryos from each line were collected at 22, 24, 26, and 28 d (hatch) of incubation. All embryos and poult's were killed by decapitation. The yolk sacs (membrane plus yolk material) and livers were collected, lyophilized and stored at -20°C until further analysis. Lipids were extracted from all samples based on the procedures of Folch et al. (1957) as modified by Marcheselli and Bazan (1990). In short, a mixture of chloroform/methanol (2:1, v/v) was used for lipid extraction in combination with sonication for 30 minutes (2X) to facilitate complete lipid extraction. All samples were kept under N₂ gas during the extraction. Lipid extracts (25 mg) from fresh yolks, yolk sacs, and livers were applied to a silica
chromatography column\(^1\) (500 mg). Diethyl ether was used to elute neutral lipids and methanol was used to elute phospholipids (Ding \textit{et al.}, 1995). The HPLC procedures of Bacon \textit{et al.} (1982) as modified by Ding \textit{et al.} (1995) were used to separate neutral lipids (NL) into TG, CE, and other lipid fractions.

Fatty acid compositions of different lipid fractions were determined by the direct methylation procedures described by Sukhija and Palmquist (1988). Fatty acids were methylated by a methanolic HCl solution in a 90\(^\circ\) C water bath for 2 h. Nonadecanoic acid (19:0) was used as an internal standard. Fatty acids were quantified by automatic gas chromatography (Hewlett-Packard 5890) using an SP-2340 fused silica capillary column\(^2\) (0.32 mm X 3 m). Temperature was programmed from 160 to 180\(^\circ\) C at 3\(^\circ\) C/minute. Nitrogen gas was used as the carrier gas at a flow rate of 1 mL/minute. Fatty acid results are reported as a percent of total fatty acids.

All data were analyzed by two-way analysis of variance using the General Linear Models procedures of SAS\(^\circledR\) (SAS Institute, 1986). Line and age (days of incubation) were the main effects tested.

\(^1\)Catalog number 309250; Alltech Associates, Inc., Deerfield, IL 60015.

\(^2\)Supelco, Inc., Bellefonte, PA.
Table 5.1. The changes of triacylglycerol fatty acids\(^1\) in embryonic yolk sac of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0</th>
<th>C 16:1</th>
<th>C 18:0</th>
<th>C 18:1</th>
<th>C 18:2</th>
<th>C 22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F RBC2</td>
<td>F RBC2</td>
<td>F RBC2</td>
<td>F RBC2</td>
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<td>3.95</td>
<td>4.53</td>
<td>6.30</td>
<td>5.27</td>
</tr>
<tr>
<td>22</td>
<td>28.03</td>
<td>29.52</td>
<td>4.28</td>
<td>4.58</td>
<td>6.89</td>
<td>5.68</td>
</tr>
<tr>
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<td>28.51</td>
<td>28.42</td>
<td>3.89</td>
<td>3.29</td>
<td>6.87</td>
<td>6.64</td>
</tr>
<tr>
<td>26</td>
<td>28.00</td>
<td>27.70</td>
<td>3.97</td>
<td>3.04</td>
<td>7.15</td>
<td>7.16</td>
</tr>
<tr>
<td>28</td>
<td>27.03</td>
<td>27.61</td>
<td>3.78</td>
<td>3.07</td>
<td>8.36</td>
<td>6.92</td>
</tr>
<tr>
<td>Pooled SE</td>
<td>.59</td>
<td>.87</td>
<td>.50</td>
<td>.43</td>
<td>.76</td>
<td>.67</td>
</tr>
</tbody>
</table>

ANOVA results

| Line     | .1164  | .0289  | .0004  | .0001  | .0001  | .0001  |
| Age      | .0002  | .0001  | .0001  | .0244  | .2706  | .0039  |
| Line*Age | .0706  | .0098  | .1190  | .5721  | .0049  | .7345  |

\(^1\)C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; and C 22:6 = docosahexaenoic acid. \(^2\)RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight. \(^3\)Days: 0 = fresh egg and 22 to 28 = 22 to 28 days of incubation.
RESULTS and DISCUSSION

Yolk Sac Triglyceride

In both genetic lines, oleic acid (C 18:1) was the predominant FA in Day 0 TG (42 to 43%; Table 1) followed by palmitic (C 16:0; 28%) and linoleic acids (C 18:2; 17%). The predominance of C 18:1 and C 16:0 are similar to what has been reported for chicks (Noble and Moore, 1964; Donaldson, 1967). There were no line differences in Day 0 C 16:0 and this was also true for later stages of incubation. The concentrations of C 18:1 and C 18:2 in Day 0 yolk and in yolk sacs during incubation are similar to the values of Noble and Moore (1964) but are considerably lower (C 18:1) and higher (C 18:2), respectively, than the values reported by Donaldson (1967). Yolk sac TG from F line embryos had increased C 18:1 and decreased C 18:2 compared with RBC2 embryos (P ≤ .0001) from 22 to 28 d. Whether this is a reflection of differences in embryonic development is not known. Some membrane PL and some eicosanoids require C 18:2 (Watkins, 1991) and increased F line embryo growth from 22 to 28 d (Ding et al., 1995) may influence the uptake of selected FA by embryos from the two lines.

The TG in Day 0 yolks from RBC2 eggs had increased palmitoleic acid (C 16:1) but at 24, 26, and 28 d it was
increased in yolk sac TG from F line embryos. The line
differences, while significant (P ≤ .028), were so small
as to question their biological relevance. Day 0 yolk and
yolk sac TG from F line embryos were higher in stearic
acid (C 18:0) compared with RBC2 embryos (P ≤ .0004) and
in both lines, there was a significant increase with
length of incubation (P ≤ .001). Donaldson (1967) also
reported an increase in C 18:0 with length of incubation
whereas Noble and Moore (1964) reported a decrease.
Decosohexaenoic (C 22:6) was present in only trace amounts
in Day 0 yolk or in yolk sacs during incubation and this
is similar to the data of Noble and Moore (1964).

Yolk Sac Phospholipids

There was a more uniform distribution of FA in Day 0
PL compared with TG (C 16:0, 27%; C 18:0, 19%; C 18:1,
24%; C 18:2, 15 to 16%; Table 2). FA profiles in Day 0
yolk PL were similar in both lines with the exception of
arachadonic acid (20:4) which was higher in RBC2 yolk PL.
The predominant FA profiles in Day 0 PL were similar to
what was reported for chick yolk PL by Noble and Moore
(1964) and Donaldson (1967) with the exception of C 18:1.
In the report by Donaldson (1967), concentrations of C
18:1 in Day 0 PL were two-fold greater than those reported
here and by Noble and Moore (1964). Donaldson (1967) had
Table 5.2. The changes of phospholipid fatty acids\(^1\) in embryonic yolk sac of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0</th>
<th>C 18:0</th>
<th>C 18:1</th>
<th>C 18:2</th>
<th>C 20:4</th>
<th>C 22:6</th>
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ANOVA Probabilities

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<td>Line*Age</td>
<td>.1889</td>
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\(^1\)C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; C 20:4 = arachidonic acid; and C 22:6 = decosohexaenoic acid. \(^2\)RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight. \(^3\)Days: 0 = fresh egg and 22 to 28 = 22 to 28 days of incubation.
also reported much higher concentrations of C 18:1 in Day 0 yolk TG. From 22 through 28 d, F line yolk sac PL had increased C 18:0, C 18:1, C 20:4 and decreased C 18:2 compared with the RBC2. With the exception of C 18:2, there were significant increases or decreases in the proportional concentrations of the other primary FA with length of incubation but again, these changes were small and their biological significance questionable. Two exceptions to this would be C 18:0 which increased 4 to 5% in both lines and C 22:6 which decreased from over 5% to less than 2% of PL FA. This is similar to what was reported by Noble and Moore (1964). Noble and Moore (1965; 1967) suggested that because C 22:6 is highly associated with the phosphatidyl ethanolamine (PE) fraction, decreasing C 22:6 concentrations may be a reflection of preferential absorption and incorporation of PE into embryonic tissues. There was less C 18:2 and more C 20:4 in the yolk sac PL of F line embryos compared with the RBC2 during incubation. This may be a continuance of the FA differences observed in Day 0 PL. Alternatively, the differences in FA may reflect genetic differences in the incorporation of FA into PL subclasses as suggested by Christie and Moore (1972).
Table 5.3. The changes of cholesteryl ester fatty acids\(^1\) in embryonic yolk sac of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0</th>
<th>C 18:0</th>
<th>C 18:1</th>
<th>C 18:2</th>
<th>C 22:6</th>
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<tr>
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<td>F  RBC2</td>
<td>F  RBC2</td>
<td>F  RBC2</td>
<td>F  RBC2</td>
<td>F  RBC2</td>
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<tr>
<td>22</td>
<td>5.90 5.65</td>
<td>4.71 3.55</td>
<td>58.43 58.42</td>
<td>10.55 11.60</td>
<td>1.24 2.65</td>
</tr>
<tr>
<td>24</td>
<td>5.18 5.90</td>
<td>4.35 3.79</td>
<td>57.99 58.69</td>
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<td>2.81 2.65</td>
</tr>
<tr>
<td>26</td>
<td>5.49 5.15</td>
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<td>62.06 59.23</td>
<td>10.04 11.06</td>
<td>2.93 2.52</td>
</tr>
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<td>28</td>
<td>4.98 5.52</td>
<td>4.84 4.53</td>
<td>63.21 60.07</td>
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<td>2.93 2.70</td>
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<td>Pooled SE</td>
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<td>1.30 1.55</td>
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\(^1\)C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; and C 22:6 = decosohexaenoic acid. \(^2\)RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight. \(^3\)Days: 22 to 28 = 22 to 28 days of incubation.
Yolk Sac Cholesterol Esters

Only a small amount of CE exists in Day 0 yolk (<1% total lipid, Ding et al., 1995) so the FA profile from Day 0 is not presented. Beginning on Day 22, the primary FA (Table 3) were C 18:1 (58%) and C 18:2 (10%). This is similar to the chick data reported by Noble and Moore (1964). The concentrations of C 18:1 and C 18:2 were slightly higher and lower, respectively, in F line yolk sacs compared with the RBC2 at 26 and 28 d. The possibility exists that F line embryos preferentially absorb CE with increased C 18:2 as opposed to C 18:1 because of faster embryonic development during the last week of incubation. In the F line, there was an approximate 5% increase in CE 18:1 from Day 22 to 28 compared with a much smaller increase (<2%) in the RBC2. There were no significant line or age effects on CE 16:0 from Day 22 to Day 28. Overall, there were less C 18:2 and more C 18:1 in yolk sac TG, PL, and CE in F line embryos compared with the RBC2 embryos suggesting that selection has changed the pattern of FA utilization during embryonic development.

Liver Triglyceride

Hepatic TG is a minor lipid class in embryonic livers during the latter stages of incubation. Ding et al.
Table 5.4. The changes of triacylglycerol fatty acids\(^1\) in embryonic livers of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0 F</th>
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<th>C 18:0 F</th>
<th>RBC2</th>
<th>C 18:1 F</th>
<th>RBC2</th>
<th>C 18:2 F</th>
<th>RBC2</th>
<th>C 22:6 F</th>
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<td>26.65</td>
<td>11.97</td>
<td>12.03</td>
<td>36.74</td>
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<td>14.66</td>
<td>9.64</td>
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<tr>
<td>26</td>
<td>23.85</td>
<td>25.55</td>
<td>13.83</td>
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<td>33.94</td>
<td>10.54</td>
<td>9.02</td>
<td>6.88</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>21.95</td>
<td>15.48</td>
<td>18.64</td>
<td>32.22</td>
<td>34.27</td>
<td>28.03</td>
<td>10.30</td>
<td>8.44</td>
<td>1.17</td>
<td>.38</td>
</tr>
<tr>
<td>Pooled SE</td>
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<td>1.74</td>
<td>6.56</td>
<td>2.85</td>
<td>2.55</td>
<td>1.66</td>
<td>1.90</td>
<td>1.49</td>
<td>1.26</td>
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ANCOVA results

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</tr>
<tr>
<td>Line*Age</td>
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</tbody>
</table>

\(^1\) C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; and C 22:6 = decosohexaenoic acid. \(^2\) RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight. \(^3\) Days: 22 to 28 = 22 to 28 days of incubation. - : undetectable.
reported that at hatch, hepatic TG was only 2% of total lipid in F line and RBC2 livers. There was lower C 18:1 and higher C 18:0 concentrations in hepatic TG as compared with yolk sac TG. These observations are in agreement with the chick embryo data (Noble and Moore, 1964). The reason for the sudden decline in C 22:6 and increased C 18:0 in RBC2 embryos on Days 26 and 28 of incubation is unknown. Ding et al. (1995) observed that after Day 24, there was no net increase in hepatic TG in either of the two turkey lines. The observed changes in hepatic TG fatty acid profiles, therefore, are indications of considerable TG hydrolysis and resynthesis within the liver.

On Day 22, the primary FA within hepatic TG were C 16:0 (27%), C 18:0 (12%), C 18:1 (35 to 36%) and C 18:2 (12 to 15%; Table 4). The C 18:1 values are in line with 15 to 17 d values for chick hepatic TG (Noble and Moore, 1964) but the concentrations of C 16:0, C 18:0, and C 18:2 are all increased in turkey hepatic TG. The concentration of C 22:6, however, was over two-fold higher in chick hepatic TG compared with the poult. In both species, yolk sac TG 22:6 concentrations were minimal, and the increased concentration in chick hepatic TG suggests that a greater capacity for FA desaturation may exist in chick embryo hepatocytes compared with the poult embryo.
There were no significant differences between F line and RBC2 hepatic TG in the concentration of either C 16:0 and C 18:2 from Days 22 to 28. Hepatic TG in the F line had greatly decreased C 18:0 and increased C 18:1 at Day 26 and 28 compared with the RBC2. As incubation proceeded, C 16:0 and C 18:2 in hepatic TG decreased in both lines. This is in contrast to the data of Noble and Moore (1964) where the concentration of these FA was fairly constant over the latter stages of incubation. The concentration of C 18:0 also increased to a far greater extent in the poult hepatic TG compared with the chick as reported by Noble and Moore (1964).

Liver Phospholipids

Across both lines, the major FA in hepatic PL was C 18:0 (28%; Table 5) followed by a fairly even distribution of C 16:0 (18%), C 20:4 (17%), C 18:2 (14%), and C 22:6 (14%). The level of C 18:1 (8%) in hepatic PL was low compared with its concentration in other hepatic lipid classes. Hepatic PL from F line embryos was higher in C 20:4 compared with RBC2 embryos throughout the final week of incubation but the differences were particularly great at 26 and 28 d. F line hepatic PL also had considerably higher C 22:6 concentrations at 26 and 28 d compared with the RBC2 whereas the concentrations of C 16:0, C 18:0, and
Table 5.5. The changes of phospholipid fatty acids\(^1\) in embryonic livers of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0 F RBC2</th>
<th>C 18:0 F RBC2</th>
<th>C 18:1 F RBC2</th>
<th>C 18:2 F RBC2</th>
<th>C 20:4 F RBC2</th>
<th>C 22:6 F RBC2</th>
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</thead>
<tbody>
<tr>
<td>22</td>
<td>18.68 17.85</td>
<td>28.17 27.47</td>
<td>7.36 7.92</td>
<td>13.96 15.75</td>
<td>18.60 15.75</td>
<td>13.23 14.74</td>
</tr>
<tr>
<td>26</td>
<td>18.21 21.71</td>
<td>27.44 38.70</td>
<td>7.90 11.14</td>
<td>13.96 16.90</td>
<td>17.07 8.37</td>
<td>15.41 4.50</td>
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<tr>
<td>Pooled SE</td>
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<td>.63 2.15</td>
<td>1.64 .66</td>
<td>.65 .71</td>
<td>1.13 1.18</td>
<td>1.34 2.00</td>
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ANOVA results

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<tr>
<td>Line*Age</td>
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</table>

\(^{1}\)C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; C 20:4 = arachidonic acid; and C 22:6 = decosohexaenoic acid.  
\(^{2}\)RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight.  
\(^{3}\)Days: 22 to 28 = 22 to 28 days of incubation.
C 18:2 were lower on Days 26 and 28. F line embryonic liver has been reported to have increased PL at 26 and 28 d but decreased neutral lipid (NL) compared with the RBC2 (Ding et al., 1995). The respective differences in longer versus shorter chain FA at these latter incubation ages could therefore be a partial reflection of line differences in lipid classes. There were no significant effects of length of incubation on the concentrations of C 18:1 or C 18:2. In RBC2 hepatic PL, the concentrations of C 16:0 and C 18:0 increased from 22 d through hatch (Day 28) whereas the concentrations of C 20:4 and C 22:6 decreased from 22 to 26 d. Again, this could be a reflection of the significant increase in NL and decreased PL observed in this line between the latter two ages (Ding et al., 1995).

Hepatic PL had different FA profiles than yolk sac PL. Embryonic liver PL had increased C 18:0, C 22:6 and C 20:4 compared with the yolk sac as a percentage of total fatty acids. This may be due to a selective absorption of PL subclasses with different FA profiles as suggested by Noble and Moore (1965).

Liver Cholesterol Ester

There were no significant line differences in C 16:0, C 18:0, or C 18:1 in hepatic CE (Table 6). In both lines,
Table 5.6. The changes of cholesteryl ester fatty acids\(^1\) in embryonic livers of two turkey lines\(^2\) during development.

<table>
<thead>
<tr>
<th>Day(^3)</th>
<th>C 16:0</th>
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<th>C 18:1</th>
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<td>65.31</td>
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<td>3.58</td>
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<tr>
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<td>3.22</td>
<td>3.86</td>
<td>3.70</td>
<td>64.79</td>
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ANOVA probabilities

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</thead>
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<tr>
<td>Probabilities</td>
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<td>.4882</td>
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\(^1\)C 16:0 = palmitic acid; C 18:0 = stearic acid; C 18:1 = oleic acid; C 18:2 = linoleic acid; and C 22:6 = docosahexaenoic acid. \(^2\)RBC2 = randombred control line and F = subline of RBC2 selected for increased 16-wk body weight. \(^3\)Days: 22 to 28 = 22 to 28 days of incubation.
C 18:1 was > 60% of total FA in the CE fraction during the last week of incubation. This supports the observations of Noble and Moore (1964). Although there were some statistical age effects on hepatic CE FA profiles, the changes in most FA were not consistent. The FA profile in hepatic CE was similar to that of the yolk sac and this has been previously reported for chick embryos by Noble (1987). It has been speculated that hepatic CE is primarily synthesized in the yolk sac membrane prior to transportation to the liver via an embryonically derived lipoprotein transport system (Noble et al., 1984). The absence of major line differences in hepatic CE FA suggests that differences in embryonic growth during the last week of incubation has not qualitatively influenced lipoprotein synthesis and lipid transport.

In summary, the concentrations of the major FA were similar in fresh F line and RBC2 yolk lipids (Day 0). This suggests that selection for growth in the F line has not changed FA incorporation into yolk lipids. Differences in FA profiles observed in the present experiment may be a reflection of line differences in embryonic growth rate during the latter stages of incubation. Thus, selection for increased BW may in fact change the overall incorporation of FA into embryonic lipids.
ACKNOWLEDGMENTS

The authors wish to thank Wayne Bacon for his technical contributions and the technical assistance of Donna Kinsey, Dave Long, and Lisa Carl.

REFERENCES


Ding, S. T., K. E. Nestor, and M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. Poultry Sci. 74:374-382.


CHAPTER 6

THE DEVELOPMENTAL EXPRESSION OF ACYLCoA:CHOLESTEROL
ACYLTRANSFERASE IN THE YOLK SAC MEMBRANE, LIVER AND
INTESTINE OF DEVELOPING EMBRYOS AND POSTHATCH TURKEYS

Abstract

AcylCoA:cholesterol acyltransferase (ACAT) catalyzes the formation of cholesteryl esters (CE) from free cholesterol and fatty acyl-CoA. This experiment was conducted to study the ontogeny of ACAT activity in the yolk sac membrane, liver and intestine during embryonic development and early posthatch growth of turkeys. ACAT activity was measured on tissue samples collected at 3 d intervals beginning on embryonic day 13 (ED 13) through 6 d posthatch (PD6). ACAT activity (pmol/mg protein/min) in the yolk sac membrane increased from 840 pmol at ED 13 to 2497 pmol at ED 22 and subsequently declined to a very low level by PD 3. The high level of enzyme activity at ED 22
is concomitant with the large quantity of CE formed within the yolk sac membrane at this developmental age. Liver ACAT activity increased from 60 pmol at ED 13 to 242-243 pmol at ED 25 and PD 3 followed by a decline to 130 pmol by PD 6. This mirrored the peak in hepatic CE concentration. This suggests that even during incubation, the liver plays a significant role in the packaging of lipids for transport to other tissues. Intestinal ACAT activity increased from 14 pmol (ED 16) to 44 pmol (ED 25) and then declined to 23 pmol by hatch (ED 28). There was no further decline through PD 6. Total ACAT activity (pmol/intestine/min) increased, however, from ED 16 through PD 6. This suggests that the total capacity for cholesterol esterification increases during the course of incubation and shortly after hatching.

Introduction

It is well documented that yolk lipids are the primary energy source for developing avian embryos. These lipids are initially taken up into the yolk sac membrane endoderm, reassembled into new lipoproteins and subsequently transferred to the embryo (Noble and Cocchi, 1990). Cholesterol esters (CE) represent the predominant subclass of lipids synthesized during the course of incubation, particularly during the latter stages (Noble
et al., 1984; Shand et al., 1993, 1994; Ding et al., 1995). Newly synthesized CE in the yolk sac membrane is likely involved in the packaging of lipids into lipoproteins for transport out of the yolk sac into embryonic circulation (Noble et al., 1984; Shand, et al., 1993). The embryonic liver in chicks and poulets also accumulates CE during the latter stages of incubation (Noble et al., 1984; Ding et al., 1995). This may serve as a source of cholesterol for the developing embryo or newly hatched chick or turkey.

The enzyme acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes the formation of cholesteryl esters (CE) from free cholesterol and fatty acyl-CoA. The resulting CE serves as a form of temporary cholesterol storage and is also incorporated into developing lipoproteins. As was alluded to for the yolk sac membrane, CE incorporation into lipoproteins is a vehicle for the transport of fatty acids and cholesterol out of the liver and small intestine in mammals (Spector et al., 1979; Suckling and Stange, 1985; Billheimer and Gillies, 1990). ACAT activity has been detected in the brain and liver of embryonic chicks (Marco et al., 1986; Shand et al., 1994) and yolk sac membrane of chick embryos (Noble et al., 1984; Shand et al., 1993). In the latter report (Shand et al., 1993), microsomal ACAT activity in the yolk sac membrane was
very high during the latter stages of chick embryonic
development coincident with the period of significant
transfer of lipid out of the yolk. Shand et al. (1994)
reported that in embryonic chick liver, ACAT activity
peaked at 16 D of incubation, concomitant with low levels
of cholesterol ester hydroxylase activity.

While the expression of ACAT activity in the yolk
sac membrane and liver of embryonic chicks been
characterized, there is no information on developing
poults nor information relative to ACAT activity during
the early stages of posthatch growth. Intestinal ACAT is
involved in the packaging and transportation of lipids to
other organs and in the overall regulation of cholesterol
utilization in rats (Norum et al., 1983), but there is no
information on its activity in the avian intestine. The
objective of this study, therefore, was to measure ACAT
activity in the yolk sac membrane, liver, and intestine
of turkeys during different stages of embryonic and early
posthatch growth.

**Materials and Methods**

Samples of the yolk sac membrane, liver and intestine
were taken from turkey embryos and poults at 3 day
intervals beginning on embryonic day 13 (ED 13) through 6
d posthatch (PD 6). Tissue samples were washed
immediately in ice-cold saline (.88%) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Four replicate samples were collected from each developmental age and each sample represented pooled tissue from 6 to 16 embryos or poults. All samples were stored at -70 C until analysis.

Two grams of frozen tissue were homogenized in 4 mL of 10 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM PMSF and 1 mM DTT with 3 strokes of a Teflon homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 10,000 X G for 15 min at 4 C. The upper fat layer and pellet were discarded and the supernatant was further centrifuged at 59,000 X G for 2 hours at 4 C. The pellet was dissolved in 2 mL phosphate buffer (10 mM, pH 7.4) and centrifuged again at 59,000 X G for 2 hours at 4 C. The pellet was collected and dissolved in 2 mL phosphate buffer for ACAT, protein, and cholesterol analysis.

Protein concentration was determined by the procedure of Bradford (1976) using bovine serum albumin (BSA) as the standard. Microsomal cholesterol concentration was determined using the method of Gamble et al. (1978). The standard curve ranged from 0.1 to 0.8 ug cholesterol. Subcellular enzyme markers pyrophosphatase, glucose-6-phosphatase, and succinate dehydrogenase were measured to
ensure that the microsomal fraction was not contaminated with other subcellular fractions.

**Determination of ACAT activity**

ACAT activity was measured using the procedure of Gavey et al. (1981) with the following modification. The reaction solution contained 10 mM potassium phosphate buffer (pH 7.4), 0.5 mM ATP, 1 mM DTT, microsomal protein, 1.2 % BSA and a mixture of phosphatase inhibitors (10 mM KF, 10 mM Na pyrophosphate, and 0.2 mM Na orthovanadate). The reaction was started with the addition of 15 μM oleoyl CoA and 20 nM [1,2-3H] cholesterol. A mixture of chloroform and methanol (2:1) was used to stop the reaction and to extract lipids (Folch et al., 1957). Newly synthesized cholesteryl esters were separated from free cholesterol on a Si chromatography column (500 mg Si, Alltech, IL) with 4 mL toluene. The separations of both pure cholesterol from CE and radiolabelled CE from cholesterol were used to test the separation technique. The recovery of CE was greater than 98 % using this procedure. The concentration of endogenous cholesterol was measured and used as a dilution factor prior to the addition of labeled cholesterol substrate.

Serial dilutions of microsomal protein and a time course study were used to determine the optimal assay conditions for ACAT activity from each tissue. The
optimal conditions for the yolk sac membrane was 2.5 min with a microsomal protein concentration at 0.125 mg/mL at 37 °C. The optimal conditions for both the liver and intestine were 20 min with microsomal protein concentrations of 0.5 mg/mL and 0.25 mg/mL, respectively.

**STATISTICAL ANALYSIS**

All data were analyzed by one-way analysis of variance using the General Linear Models procedures of SAS (SAS Inc., 1986). Age was the main effect tested. The correlation between microsomal cholesterol and ACAT was analyzed using the PROC CORR procedure of SAS.

**Results**

ACAT specific activity (pmol/mg protein/min) in the yolk sac membrane increased from 840 pmol at ED 13 to 2497 pmol at ED 22 before declining significantly to 200 pmol by PD 3 (Figure 6.1). Total ACAT activity (pmol/min/gm yolk sac membrane) showed the same type of developmental pattern, a rapid increase from ED 13 to ED 22 followed by a significant decline through PD 3. The concentration of microsomal cholesterol (µg/mg microsomal protein) increased from 215 µg at ED 13 to a plateau of 320 µg between ED 19 and 22 before declining to 53 µg at PD 3 (Table 1.1). The correlation between ACAT activity
TABLE 6.1. The microsomal cholesterol concentration in the liver, intestine, and yolk sac membrane of turkeys

<table>
<thead>
<tr>
<th>Variable¹</th>
<th>Liver</th>
<th>Intestine</th>
<th>yolk sac membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol concentration, ug/mg microsomal protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED 13</td>
<td>21.03+0.79d</td>
<td>-²</td>
<td>215.25+13.72b</td>
</tr>
<tr>
<td>ED 16</td>
<td>24.87+2.13cd</td>
<td>46.67+5.49e</td>
<td>256.10+10.05b</td>
</tr>
<tr>
<td>ED 19</td>
<td>33.71+1.92c</td>
<td>66.79+2.89bc</td>
<td>320.04+30.16a</td>
</tr>
<tr>
<td>ED 22</td>
<td>52.06+4.29b</td>
<td>74.15+3.07ab</td>
<td>320.64+17.14a</td>
</tr>
<tr>
<td>ED 25</td>
<td>75.26+3.51a</td>
<td>81.64+4.03a</td>
<td>234.79+19.34b</td>
</tr>
<tr>
<td>ED 28</td>
<td>60.14+5.40b</td>
<td>60.22+2.03cd</td>
<td>245.45+16.25b</td>
</tr>
<tr>
<td>PD 3</td>
<td>58.20+4.29b</td>
<td>51.75+3.27de</td>
<td>53.03+2.80c</td>
</tr>
<tr>
<td>PD 6</td>
<td>52.05+3.12b</td>
<td>35.64+7.48f</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Values in the same row with no common superscript differ significantly (P<.05).
 ED= embryonic day; PD= day posthatch.
²-= data not available.
Figure 6.1. Ontogeny of yolk sac membrane ACAT during incubation and early postnatal growth in turkeys. ED 13 to PD 3 represents embryonic day 13 to 3 days posthatch.
and microsomal cholesterol concentration in the yolk sac membrane was positive and significant \( (r = 0.88, P \leq 0.01) \).

The specific activity of hepatic ACAT (pmol/mg protein/min) increased from 60 pmol at ED 13 to 243 pmol at ED 25 and stayed high through PD3 before declining to 180 pmol at PD 6 (Figure 6.2). Total hepatic ACAT activity (pmol/min/liver) showed a progressive increase from 36 pmol at ED 13 to 9442 pmol at PD 6, a 264-fold increase. Microsomal cholesterol increased from 21 ug/mg protein at ED 13 to 75 ug at ED 25 before declining to 52 ug by PD 6 (Table 1.1). The correlation between hepatic ACAT activity and microsomal cholesterol concentration was also positive and significant \( (r = 0.92, P \leq 0.01) \).

Intestinal ACAT specific activity increased from 14 pmol/mg protein/min at ED 16 to 44 pmol at ED 25 and then declined to 23 pmol at hatch (ED 28; Fig. 6.3). There was a small but significant increase to 34 pmol between hatch and ED 6. Total intestinal activity (pmol/min/intestine), however, increased continually between ED 16 and PD 6, a 323-fold increase. Intestinal cholesterol concentration increased from 47 ug/mg protein to 83 ug between ED 16 and ED 25 and progressively declined to 36 ug by PD 6. The correlation between ACAT activity and microsomal cholesterol concentration was
Figure 6.2. Ontogeny of ACAT activity in the liver of developing embryos and early postnatal turkeys. ED 13 to PD 6 represents embryonic day 13 to 6 days posthatch.
Figure 6.3. Ontogeny of ACAT activity in the intestine of turkeys during embryonic and early postnatal growth. ED 13 to PD 6 represents embryonic day 13 to 6 days posthatch.
positive and significant ($r = .48; \ P \leq .01$) but of less magnitude than that observed for the yolk sac membrane and liver.

**Discussion**

It has been well documented that through the yolk sac membrane, a large quantity of lipid is taken up and transferred to embryonic chicks and poults (Noble and Cocchi, 1990; Ding et al., 1995). The process of lipid transfer from the yolk to the embryo involves active hydrolysis and resynthesis of lipids within the yolk sac membrane (Noble and Cocchi, 1990; Speak et al., 1993). Shand et al. (1993; 1994) reported high levels of ACAT in both the yolk sac membrane and liver of chick embryos. A similar situation is reported herein for turkey poult embryos together with the observation that there is also considerable ACAT activity in the embryonic intestine.

Over the course of the last 10 days of incubation, ACAT activity in yolk sac membrane peaked at ED 22 and this is at a proportionally similar developmental stage as that reported for chick embryos by Shand et al. (1993). It has been proposed that CE plays an important role in lipoprotein formation which is essential for yolk lipid transfer out of the yolk sac membrane and into embryonic circulation (Noble et al., 1984; Shand et al., 1993). Ding et al. (1995) reported that only minimal amounts of
CE are found in unincubated turkey egg yolks so the large preponderance of CE found in the yolk sac membrane is the end result of significant ACAT activity within the tissue.

One function of the CE formed or deposited in the liver is to serve as a cholesterol reservoir for perinatal turkeys. ACAT activity in the liver peaked between ED 25 and PD 3. This is concomitant with the tail end of yolk lipid transport out of the yolk sac but coincident with a period of considerable FA mobilization from the liver immediately after hatch (Ding et al., 1995). This supports a role for the liver during both the embryonic and early post-natal developmental periods. The reduction in hepatic ACAT specific activity at PD 6 may be a function of a greater proportional increase in non-ACAT microsomal protein together with decreased yolk cholesterol and dietary cholesterol to serve a precursors, although the total hepatic capacity for cholesterol esterification continues to increase. Shand et al. (1994) reported that hepatic ACAT activity in chick embryos also increased through the second half of incubation before dropping off just before hatch. Species differences in microsomal cholesterol availability may contribute to the observed differences in ACAT activity. In both species, microsomal cholesterol drops off near hatch, but the drop is considerably greater in chick embryos (Shand et al.,
1994) than in turkey embryos. The pattern of hepatic ACAT activity in prenatal turkeys is very different than that observed in rats, which has minimal enzyme activity prior to birth (Little and Hahn, 1992; Smith et al., 1995). These differences are largely due to species related differences in hepatic lipid metabolism, i.e. avian embryonic livers are exposed to considerable yolk cholesterol from lipoprotein remnants (Yafei and Noble, 1990; Ding et al., 1995) whereas in rats, ACAT is induced by high levels of milk fat during the early stages of life (Little and Hahn, 1992). Cholesterol availability has been proposed to be the major regulator of ACAT enzyme in many tissues (Billheimer and Gillies, 1990; Chang et al., 1993; Cheng et al., 1995). The correlation data from the present study and other reports (Suckling and Stange, 1985; Field et al., 1987; Shand et al., 1994) support the hypothesis that ACAT activity is greatly influenced by microsomal cholesterol availability (Erickson et al., 1980; Shand et al., 1994; Kraemer et al., 1995).

Intestinal ACAT is responsible for the formation of CE which is incorporated into intestinal lipoproteins and is intimately involved in dietary cholesterol and lipid utilization in mammals (Field et al., 1990). The only source of lipid for the avian embryo is from the yolk so ACAT activity would not be associated with the metabolism
of dietary lipids. Furthermore, there is no apoB detected in the chick embryonic intestine (Nadin-Davis, et al., 1980), and such apo-lipoprotein is found in portomicrons and is associated with lipid absorption and transport from the intestine. Thus, it would be expected that embryonic intestinal ACAT activity in turkey embryos would be very low but the results were just the opposite. Instead, intestinal ACAT activity increased to a high level at ED 22 and 25. One possible explanation is the role of intestinal ACAT in reducing blood cholesterol during the latter stages of development. This is the period during which embryos are actively transferring yolk lipids including cholesterol to the embryo and this may contribute to relatively high blood cholesterol concentrations (Speake et al., 1993). Suckling and Stange (1985) also suggested that ACAT is important to the process of reducing free cholesterol for the prevention of cell membrane disruption in mammals.

At hatch, ACAT activity decreased concomitant with a decrease in the concentration of microsomal cholesterol in the intestines. This observation lends support to the concept that ACAT activity is actively regulated by substrate availability (Billheimer and Gillies, 1990). Since this is the first report on the ontogeny of intestinal ACAT in prenatal and neonatal poultry, there is
no data available from which comparisons can be made. Overall, ACAT activity increased through PD 6 even while microsomal cholesterol concentrations were decreasing, particularly between hatch and PD 6. This suggest that enzyme synthesis or the presence of putative regulators (i.e. sterol carrier protein-2; Kraemer et al., 1995) may be regulating intestinal ACAT acticity in neonatal turkeys. The increase in total intestinal ACAT activity would secure the CE needs for lipoprotein formation and lipid transport from the intestine to peripheral tissues.

References


Ding, S. T., K. E. Nestor, and M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks od age. Poultry Sci. 74:374-382.


CHAPTER 7

The Development of an Immunoblotting Assay for the Quantification of Liver Fatty Acid-Binding Protein (FABP) during Embryonic and Early Posthatch Development of Turkeys

Abstract

Liver cytosolic fatty acid binding protein (FABP) was purified to be used as a standard in quantitating FABP. An immunoblotting procedure combined with computer scanning and color density analysis was developed to study the ontogeny of liver cytosolic FABP during embryonic and early posthatch development of turkeys. Activity of liver cytosolic FABP also was determined indirectly through a combination of gel filtration purification and subsequent binding of radiolabelled oleic acid. A trace amount of FABP was detected on Day 13 of incubation. The specific activity (ng/ mg of cytosolic protein) of liver FABP increased as incubation proceeded, exhibited an initial
peak on Day 22, declined on Day 25, and then increased through hatch (Day 28) and early posthatch development. Specific activity of liver FABP increased 12-fold between embryonic day 13 and 6 d posthatch whereas total activity (ng/liver) increased from 946 ng on Day 13 of incubation to $1.01 \times 10^6$ units by 6 d posthatch, a 1067-fold increase. The results from both analytical procedures were similar. This suggests that the immunoblotting procedure can be used to quantitate FABP content. The observed increases in FABP activity were parallel with increases in liver lipid accumulation during the later stages of embryonic development and early postnatal growth. These results suggest that liver FABP may be involved in hepatocyte fatty acid transport and metabolism during embryonic development and early postnatal growth.

Introduction

Cytosolic fatty acid binding proteins (FABP) are small proteins which have a high binding affinity for long chain fatty acids (LCFA). FABP's are involved in intracellular fatty acid movement (Tipping and Ketterer, 1981) and for protection of enzymes from the toxic effects of free fatty acids (Glatz and Veerkamp, 1985). While new, physiologic functions of FABP's need to be further elucidated, their role in the modulation of enzyme
activity and signal transduction already has been reported (Grinstead et al., 1983; Greer and Hargis, 1992; Glatz et al., 1993).

Several methods have been used for quantifying FABP concentration and activity. Binding of labeled LCFA after ammonium sulfate precipitation (Dutta-Roy et al. 1988) and comigration of cytosolic protein with radioactive LCFA on a gel filtration column (Ockner et al. 1972) are two procedures which have been used to quantitate FABP activity. Competitive, non-specific fatty acid binding by albumin and other proteins are disadvantages of the former method. Radiolabeled LCFA binding to the gel filtration matrix might decrease the sensitivity of the latter method. More sensitive procedures, i.e., immunochemical quantification (Ockner et al., 1982; Bass et al., 1985), radioimmunoassay (Fleischner et al. 1975) and ELISA (Paulussen et al., 1989; Ohkaru et al., 1994) are other methods which have been used to determine FABP concentration in different animal tissues. The specificity of antibody-antigen reactions makes immunoblotting a good method for the detection and quantification of FABP’s (Ockner et al., 1982; Epstein et al., 1994). One objective of the current experiments was to develop a procedure by which immunoblotting could be used in conjunction with computer scanning and color
intensity analysis software to quantify FABP without contamination from albumin and other proteins.

Both mammalian and chicken liver FABP have been well characterized. Rat liver FABP is approximately 14 kD with a pI of 8.1 (Ockner et al., 1974; Ockner et al., 1982; Glatz et al., 1984). Scapin et al. (1988) purified a basic form of chicken liver FABP (16.5 kD; pI 9.0). Sewel et al. (1989) also characterized a chicken liver cytosolic FABP (14 kD; pI 7.0).

There are numerous studies on the developmental expression of mammalian liver FABP. In rats, hepatic FABP activity increased 20-fold from 5 d prior to birth through 45 days of age (Sheridan et al., 1987). Gordon et al., (1985) reported that liver FABP mRNA levels increased at one day of age but did not change during early life. In pigs, liver FABP activity remains constant from 73 d of pregnancy through birth (114 d) but then decreases through 7 days of age (Reinhart, 1990; Chi, 1993). No data of the ontogeny of liver FABP in avian species has been reported. Turkey embryos metabolize large quantities of fatty acids (Ding and Lilburn, 1995) and fatty acid metabolism is associated with the presence of FABP (Glatz et al., 1993). Therefore, liver cytosolic FABP may play an important role in fatty acid transport and metabolism by turkey embryos.
This study was conducted to investigate the ontogeny of liver FABP activity during embryonic development and early postnatal growth in turkeys. Two methods were compared for the quantification of liver FABP activity, immunoblotting and gel filtration purification followed by radioligand binding.

**Materials and Methods**

Livers were taken from embryos and posthatch poults at 3 d intervals from embryonic day (ED) 13 of incubation through 6 d posthatch (PD). Livers excised from 8 to 16 individual embryos or poults were pooled into one sample, and 4 replicate samples were collected from each stage of development. The liver samples were immediately washed in ice-cold saline (0.88 % NaCl) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). All samples were subsequently frozen in liquid nitrogen immediately after dissection. Embryo weight and liver weight were recorded. The pooled samples were stored at -70 C until further analysis. Pooled samples were ground into a powder and homogenized in a 2 X volume of 10 mM phosphate buffer (pH 7.4) containing 0.2 mM PMSF and 1 mM DTT. The samples were homogenized with 3 strokes of a Teflon homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged
at 10,000 X G for 15 min. The upper fat layer and pellet were discarded. The supernatant was further centrifuged at 59,000 X G for 2 hours. The upper fat layer was again discarded and the supernatant was collected for FABP and protein analysis. Protein concentration was determined according to Bradford (1976) with bovine serum albumin (BSA) as the standard. Subcellular enzyme markers pyrophosphatase, glucose-6-phosphatase, and succinate dehydrogenase were measured to ensure that the cytosol was not contaminated with debris from other subcellular fractions.

Purification of liver FABP

Liver cytosol from PD 6 poult was treated with 70% ammonium sulfate at 4 C (Avanzati and Catala, 1983), centrifuged at 4,000 X g, and supernatant was desalted using a PD-10 column (Pharmacia, Piscatway NJ). The collected FABP fraction was then submitted to gel filtration on a Sephadex G-75 column (1.75 X 75 cm), and fractions were collected at 3 mL/fraction to analyze protein concentration and FABP activity (ligand binding assay described below). Fractions with high FABP activity (no. 24 to 32) were pooled, concentrated to 4 mL, and dialyzed against Tris buffer (30 mM, pH 9.0) overnight. The solution was then applied to a DE 52 anion exchange
column (1.75 X 25 cm) and first washed with 40 mL of 30 mM Tris buffer (pH 7.4), followed by 60 mL of a 0 to 100 mM NaCl gradient containing Tris buffer. Finally, 40 mL of 500 mM NaCl containing Tris buffer was used to wash out all proteins. The flow rate was 0.5 mL/minute, and 2.5 mL fraction were collected. Fractions containing FABP were combined. The existence of FABP of samples from each step was confirmed by western blotting analysis.

**Determination of Liver Cytosolic FABP**

The immunoblotting procedure was a modification of the method of Burnette (1981). Hepatic cytosolic protein (25 ug) from each of the 8 developmental stages together with an internal standard (6.25 ug of cytosolic protein from PD 6 poults) and a sample containing colored molecular weight markers (Sigma, St. Louis, MO 63178) were applied to a sodium dodecyl sulfate (SDS)/polyacrylamide gel (12.5 % acrylamide). All samples were run in triplicate. The gel was run for 2 h at 100 V using a dual vertical minigel apparatus (Harvard, Cambridge, MA 02138).

After electrophoresis, the gels were equilibrated in a transfer buffer (192 mM glycine; 25 mM Tris base; 20 % methanol) for 15 minutes. Proteins were subsequently transferred to nitrocellulose membranes (0.2 um, Schleicher and Schuell, Keene, NH, USA) using a Hoefer
electrophoretic transfer unit (Hoefer, San Francisco, CA 94107) set at 150 mA for 3 h. After the transfer, the membrane was dried and blocked for 30 minutes with 3% BSA. The membrane was then incubated for an additional 30 minutes with a 1:8000 dilution of primary antibody specific for chick liver FABP (Collins and Hargis, 1989). The primary antibody crossreacted with liver cytosolic FABP from both turkey embryos and posthatch poults (Figure 7.1). After each immunoreaction, membranes were washed three times for 5 minutes with Tris-Buffered Saline-Tween solution (TBST; 20 mM Tris base with .05% Tween-20; pH 7.50). Membranes were subsequently incubated for 30 minutes with a second antibody conjugated with alkaline phosphatase (1:10,000 rabbit antigoat IgG; Sigma, St. Louis, MO 63178). This was followed by 3 washes (each 5 minutes) in TBST and two subsequent brief washes with Tris-Buffered saline solution (pH 7.5) to remove Tween-20. Color development was done in a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) (Promega, Madison, WI 53711) for 3 minutes. The reaction was stopped by rinsing the membrane with Tris buffer containing 10 mM EDTA (pH 7.0).

The procedure used for color intensity determination was a modification of the procedure described by Velleman (1995). The blot was scanned with a Hewlett Packard
scanner using DeskScan™ II Version 2.0 software. The image was analyzed subsequently by Sigmascan (Jandel Scientific, San Rafael, CA, USA) to determine the color intensity of each band. The color intensity of each band was measured twice and a background intensity for each band was deducted from the average of the two measurements. A standard curve was developed from serial dilution of purified liver FABP (9 to 147 ng/lane). The color intensity of each FABP band was reported as "pixel" values and the background readings subtracted from each reading. The results of a typical western blot and standard curve are shown in Figure 7.2a. The FABP content of each sample was calculated using the data from the standard curve. Cytosolic protein (6.25 ug) from PD 6 livers was applied to each gel to serve as an internal standard. The intensity value of each sample was adjusted according to the internal standard to reduce variation among different sample runs.

**Functional Analysis of Liver FABP Activity**

The FABP activity of liver cytosols were determined using the procedure of Morrow and Martin (1983) with following modification. One mL of liver cytosol was prepared as described previously and applied to a Sephadex G-75 gel filtration column (1.75 X 75 cm; 1 ml/min; 3
mL/fraction; Pharmacia, Piscataway, NJ 08855-1327) to separate FABP from other, larger proteins. Fractions containing FABP were collected and pooled. The separation of larger from smaller proteins was confirmed by monitoring the separation of turkey serum albumin (TSA), a major contaminant with high fatty acid binding activity, from cytochrome C, a protein of the same approximate size as FABP. The pooled fractions used for FABP determination were free of TSA. The existence of liver FABP in these fractions was positively identified by western blot analysis as previously described. Fractions corresponding to FABP molecular weight were pooled for each sample. The FABP activity was low in embryonic liver so all samples were concentrated 2.5 X by lyophilization. FABP activity was subsequently determined via the radiolabel binding procedure of Glatz et al. (1984) using Lipidex 5000. The FABP activity was estimated as DPM/mg cytosolic protein and DPM/organ.

Statistics

Linearity of the calibration curves was tested using the PROC REG procedure of SAS (1986). A T test was used to test for parallelism between the two standard curves generated from the serial dilutions. The ontogeny data were analyzed by one-way analysis of variance using the
General Linear Models Procedure of SAS (1986). Age was the main effect tested.

**Results**

The body weight of embryos increased from 4.16 grams at ED 13 to 52.31 grams at hatch (ED 28) and from 71.6 grams at PD 3 to 104.1 grams at PD 6 (Table 7.1). The liver weighed 67 mg at ED 13, 1.57 grams at hatch (ED 28) and 4.12 grams at PD 6.

Liver FABP was purified from 6-day-old turkey poults. Figure 7.2 shows the protein concentration, FABP activity, and FABP presence in the fractions of gel filtration column. The FABP activity associated with small molecular weight proteins (second band, Figure 7.2a) was pooled for subsequent ion exchange chromatography. Samples of the first pregradient peak (fraction no. 12 and 13) of the ion exchange column was observed to have a single protein at approximate 14 kD and existence of FABP was confirmed by a western blotting using goat anti-chick L-FABP antisera (Figure 7.3). These fractions were pooled and used for development of the standard curve for FABP determination.

The standard curve was linear (P < 0.05) with R values of .983 (Figure 7.4ab). The ontogeny of liver FABP as visualized in a typical western blot is shown in Figure 7.5a. The ontogeny of liver FABP as determined by
Figure 7.1. Crossreactivity of goat anti-chick liver FABP antiserum with hepatic cytosol samples from broiler chicks (4 wk), turkey poults (6 days) and turkey embryos (Day 22). Hepatic cytosol aliquots (20 ug protein) were subjected to SDS-PAGE (12.5%) and proteins were subsequently transferred to a nitrocellulose membrane for western blot analysis. MW is molecular weight marker. The hepatic cytosols from chickens, turkey poults, and turkey embryos all had crossreactivity with the antiserum. The FABP activity was observed at the molecular weight of about 14 Kd.
Table 7.1. The changes of body and liver weight during embryonic development and early postnatal growth of turkeys.

<table>
<thead>
<tr>
<th>Day</th>
<th>embryo no.</th>
<th>Body weight</th>
<th>liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED 13</td>
<td>32</td>
<td>4.16 ± .02</td>
<td>.067 ± .002</td>
</tr>
<tr>
<td>ED 16</td>
<td>64</td>
<td>9.59 ± .17</td>
<td>.18 ± .003</td>
</tr>
<tr>
<td>ED 19</td>
<td>64</td>
<td>19.44 ± .34</td>
<td>.34 ± .003</td>
</tr>
<tr>
<td>ED 22</td>
<td>32</td>
<td>29.44 ± 1.27</td>
<td>.59 ± .010</td>
</tr>
<tr>
<td>ED 25</td>
<td>32</td>
<td>42.94 ± .66</td>
<td>.78 ± .020</td>
</tr>
<tr>
<td>ED 28</td>
<td>32</td>
<td>52.31 ± 1.14</td>
<td>1.57 ± .050</td>
</tr>
<tr>
<td>PD 3</td>
<td>24</td>
<td>71.56 ± 1.75</td>
<td>2.69 ± .100</td>
</tr>
<tr>
<td>PD 6</td>
<td>24</td>
<td>104.12 ± 2.18</td>
<td>4.12 ± .110</td>
</tr>
</tbody>
</table>

Probability

| Age effect | .0001 | .0001 |

1 Ed 13 to 28 = embryonic day 13 to 28; ED 28 is the hatching day for turkeys; and PD 3 to 6 = 3 to 6 day posthatch.
immunoblotting and expressed in ng calculated from the FABP standard is shown in Figure 7.5b. There was a significant age effect (P < .0001) for both specific activity (ng per mg cytosolic protein) and total activity (ng per liver). There was a trace amount of liver FABP detected on ED 13. Specific activity increased as embryonic development proceeded, reached an initial peak on ED 22, decreased on ED 25 and then increased through PD6. There was overall, a 12-fold increase in liver FABP specific activity between ED 13 and PD 6. Total liver activity increased continually from 946 ng at ED 13 to 1.01 X 10^6 units at PD 6, a 1067-fold change. The ontogeny of liver functional FABP activity determined by ^14C oleic acid ligand analysis following gel filtration was similar to the immunoblot results (Figure 7.6). Specific activity (DPM/mg cytosolic protein) increased between ED 16 and ED 22, decreased at ED 25 and then increased dramatically through PD 6. Overall, specific activity of liver cytosolic FABP increased 10-fold between ED 16 to PD 6. Total activity (DPM/liver) increased 286-fold between ED 16 and PD 6.

Discussion

The purified liver FABP had a similar molecular weight (14 Kd) as compared with chicken L-FABP (14.5 Kd) and it has strong crossreactivity to the antisera raised
Figure 7.2. Liver cytosol was treated with 70% ammonium sulfate solution and desalted by PD 10 column before it was applied on a Sephadex G-75 column (1.75 X 75 cm). Fractions eluted with 10 mM phosphate buffer (pH 7.4) were collected at 1 mL/minute and 3 mL/fraction. (a) Protein conc. and FABP activity of collected fractions. (b) Proteins was detected on SDS-PAGE gel by silver staining. MW: molecular size markers; 24-32: pooled samples from fraction no. 24 to 32. (c) Western blot analysis of partially purified liver FABP. Crossreactivity of the protein with chick L-FABP antibody was observed.
Figure 7.3. Purification of L-FABP. Fractions with FABP activity (#24 to 32 from Figure 7.2) were pooled and applied to DE 52 anion exchange column and washed with 30 mM Tris (pH 7.4). (a) Protein concentration and FABP activity of fractions. (b) Samples of the first FABP peak (Fractions no. 12 and 13) was observed to have a single band of protein at approximate 14 Kd. (c) The existence of FABP was confirmed by a western blotting using Goat anti-chick L-FABP as primary antibody.
Figure 7.4. The standard curve of liver FABP. (a) Western blots of serial dilutions of turkey liver cytosolic FABP separated by SDS-PAGE on 12.5% acrylamide gels. Proteins were transferred to nitrocellular membranes and FABP detected using a goat anti-chick liver FABP primary antiserum and rabbit anti-goat IgG conjugated with alkaline phosphatase as the secondary. (b) The color intensity value determined by computer scanning and density analysis by SigmaScan. The curve was significantly linear (P<0.01) with r = 0.983.
Figure 7.5. (a) The ontogeny of liver cytosolic FABP as visualized in a typical western blot. ED13 to ED25 = embryonic days 13 to 25; H = hatch; and PD3 to PD6 = posthatch days 3 to 6. Each lane contained 25 ug of total liver cytosolic proteins. (b) Ontogeny of liver FABP during embryonic and early postnatal growth in turkeys using western blot analysis. Color intensity of each band was scanned and analyzed with HP DeskscanTM II Version 2.0 software and SigmaScan, respectively. The standard curve of purified FABP was used.
against chicken L-FABP. The immunoblot procedure described herein was useful for both visualizing changes in liver FABP concentration during development and in quantitating those differences. Moreover, a quantitative immunoblotting method avoids the problems of sample contamination with albumin and other proteins which may also bind LCFA (Avanzati and Catala, 1983; Paulussen et al., 1986; Dutta-Roy et al., 1988). The specificity of the antibody-antigen reaction enhanced the accuracy of the assay, and assay variability was reduced by including samples from each developmental stages on the same blot. Similar results were observed with both the immunoblotting method and the ligand binding assay which further supports the validity of the new immunoblotting procedure. Immunoblotting also has the attraction of being colorometric instead of radioactive.

In the present study, the specific concentration of liver FABP increased throughout development until just prior to hatch (ED 25) and then continued to increase posthatch. This developmental pattern is somewhat different from what has been reported in other species. In swine, hepatic FABP is high in fetal liver and then decreases during the first week after birth (Reinhart, 1990; Chi, 1993). Sheridan et al. (1987) reported that liver FABP was 20-fold higher in fetal liver than adult
Figure 7.6. Ontogeny of liver FABP activity during incubation and early postnatal growth in turkeys. Liver cytosols were subjected to a Sephadex G-75 gel filtration column (1.75 X 75 cm) eluted with 0.01 M phosphate buffer (pH 7.4; 4 C, 60 mL/hr). Fractions corresponding to an eluant volume of 72 to 99 mL were pooled for FABP activity determination. $^{14}$C-oleic acid was the ligand used for FABP activity determination.
rats. Paulussen et al. (1986) reported no change in liver FABP during early postnatal development. The samples used by Paulussen et al. (1986) may have various levels of alpha-fetoprotein and this may have limited their ability to measure developmental differences in liver FABP (Sheridan et al., 1987; Paulussen et al., 1989). The species differences probably reflect differences in fatty acid metabolism during the latter stages of embryonic development through early postnatal growth. The observed increases in liver FABP during latter stages of embryonic development and early postnatal growth correlated well with the documented increases in liver lipid and the active movement of fatty acids between the yolk sac and embryo (Ding et al., 1995; Ding and Lilburn, 1995). This suggests that FABP may play an important role in cytosolic fatty acid translocation in turkey embryos. In chick livers, enzymes involved in lipid metabolism increase during the latter stages of embryonic development (Linares et al., 1993; Shand et al., 1994). Moreover, Greer and Hargis (1992) reported that chick liver FABP stimulated the activity of acyl CoA:cholesterol acyltransferase (ACAT), an important enzyme involved in the esterification of fatty acids to cholesterol (Shand et al., 1994). Ding and Lilburn (1995) reported that lipid subclass concentration and fatty acid profiles in turkeys
are similar to what has been reported for chicks. Therefore, that FABP would also play a similar role in turkey embryos. The reason for the unexpected decline in liver FABP specific activity at ED 25 is not apparent at this time.

The increased activity of liver FABP from hatch to PD 6 is reflective of major physiologic fluxes in liver lipid during this period. Ding and Lilburn (1995) reported that at hatch the turkey liver is high in CE. Fatty acids hydrolyzed from CE are utilized by newly hatched chicks (Noble and Cocchi, 1990), and the same is probably true for poults. Liver FABP may serve as both a temporary sink for hydrolyzed fatty acids and a transport vehicle to sites of B-oxidation.

In conclusion, an immunoblotting procedure combined with computer scanning and color intensity analysis has been developed for the quantitation of liver FABP in turkey embryos and newly hatched poults. The results were similar to those obtained with the standard ligand binding assay. The ontogeny of liver FABP concentration is consistent with observed increases in liver lipid and fatty acid metabolism.
Acknowledgment

The authors want to thank Billy Hargis, Texas A & M University, for providing the chick liver FABP antisera used in the assay. We would also like to thank Ms. Michele McGuinness for her assistance in helping with the color density measurement.

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Ding, S. T., K. E. Nestor, And M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. Poultry Sci. 74:374-382.


Abstract

Cytosolic fatty acid-binding proteins (FABP) are small proteins with a high binding affinity for long chain fatty acids. Experiments were conducted to confirm the presence of FABP in turkey intestine and yolk sac membrane during embryonic and posthatch development. FABP in the intestine was determined with an immunoblotting assay using antisera developed against chicken liver FABP (CL-FABP). FABP activity in the intestine and yolk sac membrane were also measured with a ligand binding assay. CL-FABP cross reactivity in the intestine was not detected until hatching (Day 28). FABP activity (ng/mg cytosolic protein) increased 39% during the first 3 days posthatch
and did not increase any further at 6 days posthatch. Total activity (ng/intestine), however, increased more than ten-fold through 6 days posthatch. FABP activity as determined with a ligand binding assay confirmed that prior to hatch, there was little FABP activity. However, the specific activity (dpm/mg cytosolic protein) was the highest at hatching and slightly decreased thereafter. The sharp rise in intestinal FABP activity just prior to hatch suggests that FABP may be needed for utilization of dietary fatty acids. FABP specific activity (dpm/mg cytosolic protein) in yolk sac membrane increased from embryonic day (ED) 13 to a plateau at ED 16 and 19 and then declined thereafter. Total activity (dpm/yolk sac membrane), however, reached a plateau at ED 22 and then declined to a low level through 3 days post-hatch. The correlation between the presence of FABP and lipid metabolism in the yolk sac membrane during the latter stages of embryonic development suggest that FABP may play a role in lipid transfer from the yolk to the developing embryo via the yolk sac membrane.

Introduction

Cytosolic fatty acid binding proteins (FABP) are small proteins (12 to 15 kd) with high binding affinities
for long chain fatty acids (Ockner et al., 1972; Glatz and Veerkamp, 1985; Collins and Hargis, 1989). In rats, it has been reported that there are tissue specific FABPs, (i.e. intestinal, I-FABP; liver, L-FABP) (Ockner and Manning, 1982; Bass et al., 1985; Shields et al., 1986). FABP concentration can be influenced by factors such as sex (Bass et al., 1985), developmental stage (Gordon et al., 1985; Rubin et al., 1989), dietary fat and hypolipidic drugs (Bass et al., 1985). Intestinal FABP may be involved in fatty acid absorption and utilization (Ockner et al., 1972; Ockner and Manning, 1982).

Katongole and March (1979) reported the presence of FABP in the intestine of chickens. The concentration of intestinal FABP was low during the first week of age (Katangole and March, 1980). The low activity of I-FABP may be a factor contributing to the limited capacity for fat absorption in young chicks. In the rat fetus, both L-FABP and I-FABP are expressed in the intestine as early as 18 days of gestation, with L-FABP expression appearing earlier than I-FABP (Rubin et al., 1989).

Active fatty acid metabolism in the yolk sac membrane of chick embryos has been reported during the latter stages of incubation (Noble and Cochi, 1990). The largest proportion of lipid movement from the yolk sac into the developing turkey embryo occurs during the last
week of incubation (Ding et al., 1995; Ding and Lilburn, 1996). There have been no studies which have looked for FABP presence in the yolk sac membrane or intestine of turkey embryos.

The procedure which combined immunoblotting and image analysis to quantitate L-FABP in turkey embryonic liver was stated in previous chapter. This procedure has the advantage of avoiding non-specific binding of labelled fatty acids by non-FABP proteins which is always possible in traditional ligand binding assays. Using this new procedure in conjunction with a ligand binding assay, the current experiment was conducted to quantitate the ontogeny of FABP activity in the intestine and yolk sac membrane during embryonic development and early postnatal life in turkeys.

**Materials and Methods**

Intestine and yolk sac samples were taken from embryos and posthatch poults at 3 d intervals from embryonic day (ED) 13 through 6 d posthatch (PD). Tissues from 6 to 16 individual embryos or poults were pooled into one sample and 4 replicate samples were collected from each stage of development. The intestine and yolk sac membrane samples were immediately washed in ice-cold
saline (0.88 % NaCl) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Collected tissues were frozen in liquid nitrogen immediately after dissection. The weight of the intestine and yolk sac membrane were recorded. The samples were stored at -70 C until further analysis. Pooled samples were ground into a powder and homogenized in a 2 X volume of 10 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM PMSF and 1 mM DTT. The samples were homogenized with 3 strokes of a Teflon homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 10,000 X G for 15 min at 4 C. The upper fat layer and pellet were discarded. The supernatant was further centrifuged at 59,000 X G for 2 hours at 4 C. The upper fat layer was again discarded and the supernatant was collected for FABP and protein analysis. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard. Subcellular enzyme markers pyrophosphatase, glucose-6-phosphatase, and succinate dehydrogenase were measured to ensure that the cytosol was not contaminated with debris from other subcellular fractions.
**Determination of CL-FABP in intestine**

The presence of CL-FABP in the intestine was detected by a western blot analysis using antiserum developed against chicken liver L-FABP (Collins and Hargis, 1989). The immuno-quantitation method was described in previous chapter. In short, the immunoblotting procedure was a modification of the method of Burnette (1981). Intestinal cytosolic protein (25 ug) from each of the 8 developmental stages together with an internal standard (12.5 ug of cytosolic protein from PD 6 poults) and a sample containing colored molecular weight markers (Sigma, St. Louis, MO 63178) were applied to a sodium dodecyl sulfate (SDS)/polyacrylamide gel (12.5 % acrylamide). All samples were measured in triplicate. Proteins were electrophoretically separated at 100 V for 2 h and then transferred to nitrocellulose membranes (0.2 um, Schleicher and Schuell, Keene, NH, USA) using a Hoefer electrophoretic transfer unit set at 150 mA for 3 h (Hoefer, San Francisco, CA 94107). Antibody specific for chick L-FABP (1:8,000; Collins and Hargis, 1989) was used as the primary antibody. Secondary antibody conjugated with alkaline phosphatase (1:10,000 rabbit antigoat IgG) was purchased from Sigma (St. Louis, MO 63178). Color development was done in a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT).
(Promega, Madison, WI 53711). The blot was scanned with a Hewlett Packard scanner using DeskScan™ II Version 2.0 software following the procedure of Velleman (1995). The image was analyzed by SigmaScan (Jandel Scientific, San Rafael, CA, USA) to determine the color intensity of each band. A standard curve was developed from purified turkey liver cytosolic FABP. FABP concentrations for the calibration were reported as "pixel" values and background readings were deducted from each reading. The FABP concentration in each sample was calculated using the calibration curve. The intensity value of each sample was adjusted according to the internal standard (6.26 ug of PD 6 intestinal cytosolic protein) to reduce variation among the different sample runs.

**FABP Binding Activities in intestine and yolk sac membrane**

FABP activity was determined using the procedure of Morrow and Martin (1983) as modified by Ding (Chapter 7). In brief, one mL of cytosol was prepared as described previously and applied to a Sephadex G-75 gel filtration column (1.75 X 75 cm; 1 mL/min; Pharmacia, Piscataway, NJ 08855-1327) to separate FABP from other, larger proteins. Fractions containing FABP were collected and pooled. The separation of larger from smaller proteins was confirmed.
<table>
<thead>
<tr>
<th>Day</th>
<th>embryo no.</th>
<th>Intestine weight</th>
<th>Yolk sac membrane weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED 13</td>
<td>32</td>
<td>-2</td>
<td>1.76 ± .13</td>
</tr>
<tr>
<td>ED 16</td>
<td>64</td>
<td>.06 ± .01</td>
<td>2.74 ± .16</td>
</tr>
<tr>
<td>ED 19</td>
<td>64</td>
<td>.21 ± .01</td>
<td>5.52 ± .48</td>
</tr>
<tr>
<td>ED 22</td>
<td>32</td>
<td>.45 ± .01</td>
<td>6.56 ± .24</td>
</tr>
<tr>
<td>ED 25</td>
<td>32</td>
<td>.52 ± .04</td>
<td>3.85 ± .33</td>
</tr>
<tr>
<td>ED 28</td>
<td>32</td>
<td>1.18 ± .03</td>
<td>3.18 ± .14</td>
</tr>
<tr>
<td>PD 3</td>
<td>24</td>
<td>3.64 ± .16</td>
<td>1.07 ± .08</td>
</tr>
<tr>
<td>PD 6</td>
<td>24</td>
<td>6.72 ± .46</td>
<td>-</td>
</tr>
</tbody>
</table>

**Probability**

| Age effect | .0001 | .0001 |

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1 Ed 13 to 28 = embryonic day 13 to 28; ED 28 is the hatching day for turkeys; and PD 3 to 6 = 3 to 6 day posthatch.

2 - = not available.
Figure 8.1. Western blots of serial dilutions of turkey liver cytosolic FABP separated by SDS-PAGE on 12.5% acrylamide gels. Proteins were transferred to nitrocellulose membranes and FABP detected using a goat anti-chick liver FABP primary anti-serum and rabbit anti-goat IgG conjugated with alkaline phosphatase as the secondary antibody. A plot of western blot color intensity estimates of liver cytosolic FABP. Color intensities were estimated using a Hewlett Packard scanner and SigmaScan software. Intensities are reported in "pixel" units/band and the background counted values were subtracted from each band. The standard curve was linear (P<0.05) with r=0.9934.
Figure 8.2. Ontogeny of L-FABP in intestine during embryonic and early postnatal growth in turkeys using western blot analysis. Aliquots of turkey intestine cytosol (25 ug protein) from different ages of poult and embryos, and an internal standard, 6.25 ug intestine cytosolic protein of Day 6 poult, were subjected to SDS-PAGE (12.5 %) for western blot analysis. Goat anti-chicken L-FABP antiserum was the primary antibody. Secondary antibody conjugated with alkaline phosphatase was used. Color intensity of each band was scanned and analyzed with HP DeskScanTM II Version 2.0 software and SigmaScan, respectively. Color density of immunoblottings from each stage of development was expressed in "pixels". The serial dilution values (Figure 1) was used as a standard curve.
by monitoring the separation of turkey serum albumin (TSA), a major potential contaminant with high binding affinity for fatty acids, from cytochrome C, a protein of the same approximate size as FABP. The pooled fractions used for FABP determination were free of TSA. The existence of an FABP protein which cross-reacted with CL-FABP antisera was positively identified by western blot analysis (data not shown). The collected fractions were pooled for each sample. FABP activity was subsequently determined using the ligand binding procedure of Glatz et al. (1984). The ligand was \(^{14}\text{C}-\text{Oleic acid} and FABP activity was estimated as both dpm/mg cytosolic protein and dpm/organ.

**Statistics**

Linearity of the calibration curve was tested using the PROC REG procedure of SAS (1986). The ontogeny data were analyzed by one-way analysis of variance using the General Linear Models Procedure of SAS (1986).

**Results**

The intestine weighed 60 mg at ED 16, 1.18 g at hatch (ED 28) and 6.72 g at PD 6 (Table 1). The yolk sac membrane weight increased from 1.76 g at ED 13 to a
Figure 8.3. Ontogeny of intestine FABP during incubation and early postnatal growth in turkeys. Intestine cytosols were subjected to a Sephadex G-75 gel filtration column (1.75 X 75 cm) eluted with 0.01 M phosphate buffer (pH 7.4; 4 C, 60 mL/hr). Fractions corresponding to an eluant volume of 75 to 99 mL were pooled for FABP activity determination. $^{14}$C oleic acid was the ligand used for FABP activity determination.
Figure 8.4. Ontogeny of yolk sac membrane FABP during incubation and early postnatal growth in turkeys. Yolk sac membrane cytosols were subjected to a Sephadex G-75 gel filtration column (1.75 X 75 cm) eluted with 0.01 M phosphate buffer (pH 7.4; 4 C, 60 mL/hr). Fractions corresponding to an eluant volume of 75 to 99 mL were pooled for FABP activity determination. $^{14}$C oleic acid was the ligand used for FABP activity determination.
plateau of 6.56 g at ED 22 and then decreased to 1.07 g at PD 3. By PD 6, the yolk sac membrane had essentially disappeared.

The standard curve was linear (P ≤ 0.05) with R value of 0.9934. The standard curve was used for further calculations. A typical curve showing the ontogeny of CL-FABP as determined by immunoblotting is shown in Figure 2. There was a significant age effect (P < 0.0001) for both specific activity (ng per mg cytosolic protein) and total activity (ng per intestine). Before hatch, no CL-FABP is detected in intestinal samples. Intestinal CL-FABP concentration increased from 730 ng/mg cytosolic protein at ED 28 to 1,023 ng/mg of cytosolic protein at PD 3 through PD 6. Overall, there was a 39% increase in intestinal CL-FABP specific activity between hatch and PD 3. Total activity increased continually from 13,823 ng at hatch to 145,599 ng at PD 6, a 10.5-fold change.

The ontogeny of intestinal FABP binding activity as determined via 14C- oleic acid ligand binding analysis after gel filtration was slightly different than the results from immunoblot assay (Figure 3). A small amount of FABP activity was detected in the embryonic intestine between ED16 and ED25, followed by an 85-fold increase in FABP activity (dpm/mg cytosolic protein) between ED 25 and ED 28. Specific activity (dpm/mg cytosolic protein)
decreased from 76,631 dpm/mg cytosolic protein at ED 28 to 58,453 dpm/mg cytosolic protein at PD 6. Overall, the specific activity of intestinal FABP decreased 24% between ED 28 to PD 6 whereas total activity (dpm/intestine) increased 5.8-fold during the same time period.

No CL-FABP was detected in samples of embryonic yolk sac membrane cytosol (data not shown). The ontogeny of yolk sac membrane FABP activity as determined by ligand binding assay is shown in Figure 4. There was a significant age effect (P < .0001) for both specific activity (dpm per mg cytosolic protein) and total activity (dpm per yolk sac membrane). Specific activity increased from 25,699 dpm/mg cytosolic protein at ED 13 to 33,108 dpm/mg of cytosolic protein at ED 16 and remained constant through ED 19. There was a further decline through ED 22 and no further changes through the remaining sampling ages. Total activity increased continually from 731,229 dpm/yolk sac membrane at ED 13 to 4,827,886 dpm/yolk sac membrane at ED 22, a 6.6-fold change. This was followed by a steady decline in total activity through PD 3.
Discussion

Intestine FABP

The immunoblotting assay incorporating chick liver FABP antisera specifically quantitates CL-FABP or a similar protein whereas the ligand binding assay estimates FABP activity based on its affinity for oleic acid. In the intestine, the changes in CL-FABP concentration are different from the FABP activity estimated from ligand binding assay during early posthatch development, suggesting that more than one type of FABP may exist in embryonic and early posthatch turkey intestine and these are expressed at different times during development. In the rat, two distinct proteins, I-FABP and L-FABP, are present in the intestine. Their expression is regulated by different mechanisms (Bass et al., 1985) and their appearance in the intestine also occurs at different stages of development (Rubin, et al., 1989). These findings support a hypothesis that there might also be at least two forms of FABP in the intestine of poults.

Katongole and March (1980) observed constant fatty acid binding activity during the first week of age in chick intestine whereas Sell et al. (1986) reported that fatty acid binding activity in intestinal mucosa increased when two week old poults were compared with newly hatched poults. In the present study, specific activity of
intestinal FABP either increased or decreased slightly between hatch and PD3 depending upon the assay used. Total intestinal FABP activity increased dramatically, however, during the first 6 d posthatch suggesting that this protein(s) does play a role in intestinal fatty acid metabolism in young poults.

The lack of CL-FABP expression in the intestine during the first 25 days of incubation is different compared with liver FABP expression in the embryonic liver which is high by 22 d of incubation (Chapter 7). The developmental pattern of embryonic intestinal FABP activity is also different from that of other species. In swine, FABP activity in intestinal mucosa is high four days before birth and then increases three-fold through 7 d of age (Reinhart et al., 1990). In the rat, L-FABP is first detectable on day 17 of gestation and increases further as development proceeds (Rubin et al., 1989). These differences are most likely a reflection of species differences in fatty acid metabolism during embryonic development.

In avian species, the embryonic intestine is still actively differentiating during the last 4 days of incubation (Moog, 1950; Richardson et al., 1955; Bellware and Betz, 1970). The observation in the present study that intestinal FABP activity increases dramatically
between ED 25 and hatch supports the earlier observations that significant intestinal development occurs during the last 3 d of incubation. The results also suggest that there is an immediate need for FABP(s) for the absorption and utilization of dietary fatty acids commensurate with hatching and the onset of feeding.

**Yolk Sac Membrane**

FABP activity in the yolk sac membrane was detected as early as ED 13 in turkeys. Total FABP activity in the yolk sac membrane was high from ED 19 to ED 28, which is the period during which lipids are rapidly mobilized from the yolk and utilized by growing embryos (Ding and Lilburn, 1995). The results of the ligand binding assay suggest that there is FABP activity in the yolk sac membrane and it seems probable that it would be involved in the lipid transfer from the yolk to the embryo. The types of FABP(s) present in the yolk sac membrane is yet to be determined.

In conclusion, an immunoblotting procedure combined with computer scanning and image analysis was used to quantitate of CL-FABP in the intestine of embryonic and newly hatched poults. The large increase detected in intestinal FABP activity at hatch suggests that these proteins are needed for the absorption and utilization of
dietary fatty acids. The correlation between the appearance of FABP activity in the yolk sac membrane and lipid transfer from the yolk to the embryo during the latter stages of incubation suggests that FABP(s) may play an important role in lipid transfer from the yolk to the developing embryo.

Acknowledgment

The authors want to thank Billy Hargis, Texas A & M University, for providing the chick L-FABP antisera used in the assay.

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Ding, S. T., K. E. Nestor, And M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. Poultry Sci. 74:374-382.


CHAPTER 9

SUMMARY AND CONCLUSIONS

This dissertation determined lipid subclass and fatty acid profile changes in the yolk sac and liver of developing embryos and quantitated the ontogeny of ACAT and FABP in yolk sac membrane, liver, and intestine in developing turkey embryos and posthatch poults.

Lipid metabolism in developing turkey embryos was very active and yolk sac lipids and fatty acids were rapidly depleted, particularly during the latter stages of embryonic development (20 to 28 days). The data confirm the hypothesis that yolk sac lipids and fatty acids are important nutrients for developing turkey embryos, although quantitatively, lipids in residual yolk sac may not represent a major energy reserve for newly hatched poults. Enrichment of the diet with medium chain fatty acids (MCFA) from supplemental coconut oil changed the yolk lipid fatty acid composition. The biggest change was
an increase in C 14:0 incorporation into fresh yolk and embryonic yolk sac neutral lipids.

In the experiments with embryos from the two genetic lines, there were no significant differences in total lipid uptake from the yolk sac but yolk sacs from F line embryos contained less triacylglycerol and liver lipid accumulation was greater than that from RBC2 embryos. This suggests that selection for increased body weight at 16 wk of age results in changes in lipid metabolism during the latter stages of embryonic development. Fatty acid profile data showed that selection for growth in the F line has not changed FA incorporation into yolk lipids. Differences in the FA profiles of lipid subclasses, however, may be a reflection of line differences in embryonic growth rate during the latter stages of incubation. Thus, selection for increased BW may in fact change the overall incorporation of FA into embryonic lipids.

Microsomal AcylCoA:cholesterol acyltransferase (ACAT) activity was studied in samples taken from the yolk sac membrane, liver, and intestine of turkey embryos. A very high level of ACAT activity in the yolk sac membrane during the latter stages of embryonic development supports the observation of increased cholesterol ester concentration during the same time period. There was also
a high level of ACAT activity in the embryonic liver which suggests that even during embryonic development, the liver plays a significant role in the packaging of lipids for transport to other tissues. The total ACAT activity in intestine increased from embryonic day 16 through posthatch day 6 suggesting that the intestine contributes to overall lipid metabolism well in advance of hatching and the actual consumption of feed.

Liver fatty acid-binding protein (FABP) was purified from turkey liver and was used as a standard for a quantitative immunoblotting assay to determine the ontogeny of liver FABP in turkeys. Liver FABP increased as incubation proceeded and this paralleled increases in hepatic lipids. These results suggest that liver FABP may be involved in hepatocyte fatty acid transport and metabolism during embryonic development and early postnatal growth. There was a sharp rise in intestinal FABP activity just prior hatch which may be reflective of the involvement of FABP in the utilization of dietary fatty acids. FABP specific activity in the yolk sac membrane increased from embryonic day 13 to a plateau between embryonic days 16 and 19 followed by a decline. Total yolk sac membrane activity, however, plateaued at ED 22 prior to declining to a low level through 3 days posthatch. The correlation between FABP activity and
lipid metabolism in the yolk sac membrane during the latter stages of embryonic development suggests that FABP plays a role in lipid transfer from the yolk to the developing embryo via the yolk sac membrane.
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