Characterization of the Ileal Lipid Binding Protein (FABP6) in Tissues Involved in Bile Acid and Steroid Metabolism in Poultry

Rosanne Mcquaid

Department of Animal Science
McGill University, Montreal
21111 Lakeshore Rd., Ste Anne De Bellevue, QC, H9X 3V9

December 2011

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

Table of Contents

List of Tables

List of Figures

Abstract

The Characterization of Ileal Lipid Binding Protein (FABP6) in Tissues Involved in Bile Acid and Steroid Metabolism in Poultry

Fatty acid binding proteins (FABPs) are a family of proteins involved in the transport of a wide variety of hydrophobic molecules within the cytosol of cells. Tissues with high levels of fatty acid (FA) metabolism such as adipocytes, intestine and liver have a correspondingly high levels of FABPs which correlate to the rate of cellular FA uptake and utilization. Moreover, many of the 11 FABP family members are mainly expressed in a tissue specific manner. For example, FABP6 is predominantly expressed in adult animals in the ileum where it is involved in intracellular transport of bile acids, however it has also been identified in the steroid hormone producing cells of the ovary and adrenal glands in mammals and zebrafish where it may participate in steroid metabolism. Since very few studies have been conducted in aves, the objective of the present study was to determine the tissue and cell specific distribution of FABP6 transcripts and protein through the use of PCR, sequence verification, immunoblotting, and immunohistochemical staining approaches in both female and male chickens and turkey embryos. The purpose of this study is to compose a comparative analysis between FABP6 gene expression in mammals and poultry, and identify the similarities between bile acid and steroid hormone metabolism that may further identify the role of this protein in cellular physiology.

Unlike mammals, FABP6 was not restricted to the distal region of the small intestine, but rather distributed across the intestine with a significant abundance in the ileum in the embryo, broilers, and layers. This was confirmed by immunohistochemical images that have a high reactivity of FABP6 in the epithelium of the ileum, with lower quantities found in the duodenum. This study is the first to identify the presence of FABP6 mRNA and protein in the testis, supporting the proposed overlapping function of FABP6 in bile acid and steroid hormone metabolism. Furthermore, FABP6 was examined in various stages of follicular development, being identified in all phases of follicular development, with the greatest abundance in the small yellow follicles. In mammals, FABP6 was identified strictly in the progesterone producing cells of the luteum; while in chickens FABP6 is found in both the granulosa and theca cells that are not limited to progesterone production.

Résumé

La caractérisation des protéines lipides iléale reliure (FABP6) dans les tissus intervenant dans le métabolisme des acides biliaires et la stéroïdogenèse chez la volaille

Les protéines de liaison des acides gras (FAPBs) sont une famille de protéines impliquées dans le transport d'une grande variété de molécules hydrophobes dans le cytosol des cellules. Les tissus avec des niveaux élevés d'acides gras (AG) du métabolisme tels que les adipocytes, l'intestin et le foie ont un niveau élevé de FABPs qui sont en corrélation avec le taux important d'absorption cellulaire et d'utilisation de FA. De plus, plusieurs des 11 membres de la famille FABP sont principalement exprimés dans un tissu spécifique. Par exemple, FABP6 est principalement exprimé chez les animaux adultes au niveau de l'iléon, où il est impliqué dans le transport intracellulaire des acides biliaires, mais il a également été identifié dans les cellules de l'ovaire et des glandes surrénales chez les mammifères et chez les poissons zèbres où il peut participer dans le métabolisme des stéroïdes. Étant donné le petit nombre d'études qui ont été menées chez Aves, l'objectif de la présente étude était de déterminer le tissu et la distribution de cellules spécifiques des transcrits de FABP6 et des protéines grâce à l'utilisation de la PCR, de la vérification séquentielle, de l'immunoblot et des approches à la fois dans la coloration immunohistochimique dans des embryons féminins et masculins de poulets et de dindes. Le but de cette étude est de composer une analyse comparative entre l'expression des gènes FABP6 chez les mammifères et chez la volaille, et d'identifier les similitudes entre les acides biliaires et le métabolisme des hormones stéroïdiennes qui peuvent également préciser le rôle de cette protéine dans la physiologie cellulaire.

Contrairement aux mammifères, FABP6 n'était pas limitée à la région distale de l'intestin grêle, mais plutôt répartis dans l'intestin avec une abondance significative dans l'iléon dans l'embryon, les poulets de chair, et les couches. Cela a été confirmé par des images immunohistochimiques qui ont une forte réactivité de FABP6 dans l'épithélium de l'iléon, avec des quantités plus faibles dans le duodénum. Cette étude est la première à identifier la présence d"ARNm de FABP6 ainsi que celle de la protéine dans le testicule, en soutenant la fonction proposée dans le chevauchement des acides biliaires FABP6 et du métabolisme des hormones stéroïdes. Par ailleurs, FABP6 a été examiné à divers stades de développement folliculaire et a été identifié dans toutes les phases du développement folliculaire, avec la plus grande abondance dans les petits follicules jaunes. Chez les mammifères, FABP6 a été strictement identifié dans les cellules du corps jaune produisant la progestérone, tandis que chez les poulets, FABP6 se trouve dans les

cellules de la granulosa et de la thèque qui ne sont pas limités à la production de progestérone.

Acknowledgements

First and foremost, I would like to thank Dr. David Zadworny who provided me with an opportunity to challenge my interests in science under his supervision. I will be eternally grateful for his immeasurable amount of support, patience, and guidance, knowledge and insightfully refreshing suggestions he provided throughout the completion of this thesis.

I would like to express my sincere gratitude to Dr. Gen Hiyama, my mentor, who has spent countless days teaching me the foundations of molecular biology and genetics techniques. I admire his motivation and passion for research, and am proud that we could be a successful team. I wish him the best in his future endeavours.

I wish to express my sincere thanks to Dr. Luis Agellon and his laboratory, for the use of his antibody and their enthusiasm for FABPs. Special thanks to Dr. Dana Praslickova for helping me process tissues for immunohistochemistry.

I appreciate my colleagues of the animal science department, especially Christine Lafleur, Daynanda Siddappa, Neerusha and Bushaningh Baurhoo for their encouragement and help throughout my project.

Thank you to my family and Mike for your constant love, support, and encouragement.

I dedicate this thesis to my father Kenneth James McQuaid, whom I will dearly miss and love forever and always (May 18, 1947-April 1, 2012).

Introduction

The fatty acid binding protein 6 (FABP6) is a part of a super family of intracellular lipid binding proteins. These proteins are thought to facilitate the uptake of fatty acids (Fas) and other hydrophobic molecules by modulating their solubility and transport within the cytoplasm. Most FABPs have been primarily linked to their affinity for long-chain fatty acids; however, FABP6 is capable of binding bile acids, a larger and more rigid ligand, due to the protein's slight difference in tertiary structure. FABP6 is thought to be involved in the control of intracellular transport of bile acids in the ileal epithelium, thereby having an effect on enterohepatic circulation; subsequently affecting the body's cholesterol homeostasis. The expression of FABP6 is tightly regulated by nuclear hormone receptors that act on the promoter region of FABP6. The Liver X Receptor (LXR) increases the abundance of FABP6 in the ileum in response to the concentration of cholesterol and cholesterol derivatives in the liver. The Farnesoid X Receptor (FXR) stimulates FABP6 transcription in the presence of bile acids in the ileum.

FABP6 has also been identified in steroid hormone producing tissues of the adrenal glands and ovaries. Steroid hormones have a similar biosynthetic pathway to bile acids, in that they are both metabolites of cholesterol by Cytochrome P-450 enzymatic pathways. The steroid biosynthetic pathway involves the movement of cholesterol derivatives between the mitochondria and the cytoplasm. The similarity in structure between bile acids and steroid hormones may suggest that steroid hormones are a suitable ligand for FABP6. Therefore, FABP6 may play an integral role in the intracellular transport of steroid hormones.

Although FABP6 tissue distribution and insights into the molecular function have been identified in mammals over the past two decades, very few studies have focused on poultry. Thus, it was only in 2007 that the

relationship of FABP6 to binding capacity of bile acids in poultry was first reported. The present study aims to characterize a profile of the FABP6 gene in poultry. Poultry's precocial development, digestive and reproductive systems are all different than the mammalian species. Thus, these differences may be associated with altered patterns of gene expression of FABP6 in poultry than in other species which may be useful for further elucidating the physiological roles in animals.

Fatty Acid Binding Proteins Literature Review

Fatty Acid Binding Proteins

Fatty acid binding proteins (FABP) were discovered in the early 1970s by Ockner et al., as a small protein in the cytosol of rat tissues that binds fatty acids [1, 2]. These intracellular fatty acid binding proteins (FABPs) are thought to modulate the solubility of fatty acids and other hydrophobic molecules in aqueous environments, mediating their metabolic trafficking within an internal cavity [3, 4]. Currently, 11 FABPs have been discovered in vertebrate species that are encoded by a conserved multigene family that have sequence identity of 20% to 70%. This family of genes encode ~14-15kDa proteins that non-covalently bind a hydrophobic ligand and have different tissue-specific expression patterns and functions [4]. .

All FABPs are identical in gene structure, consisting of four exons and three introns. Although the primary structure of FABP differs among the tissues, the tertiary structure remains very similar. The common structure is composed of ten β-strands linked by hydrogen bonds to form two-orthogonal β-sheets that shape a β-clam that is enclosed by two α-helices (Fig 1.). The structure creates an internal water-filled cavity lined with ~50% polar amino acids. Entry into the internal cavity is bordered between two of the β-strands that surround the portal domain. Two α-helices frame the portal domain with a N-terminal helix-turn-helix domain that functions as a membrane-high-affinity site. Once in the pocket, the ligand is bound to a carboxylate group coordinated by a tyrosine and two arginine residues. There are a number of conformational changes to coordinate the binding of the fatty acid and the protein to adapt their structures to fit together. The structure has high molecular stability and flexibility and is able to bind bulky and rigid molecules [5-8].

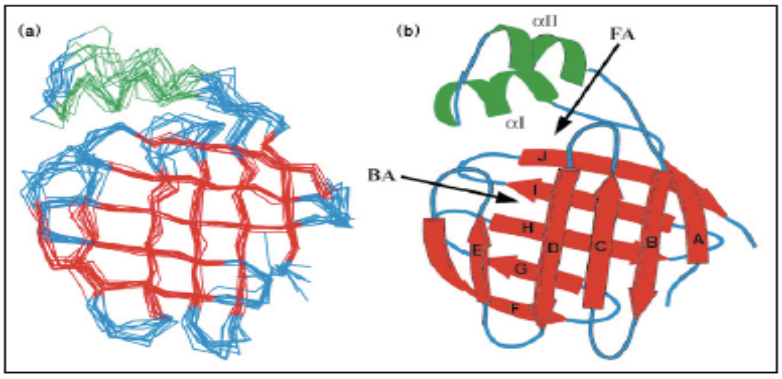

Figure 1. FABP tertiary structure of the Beta-Clam: 10 Beta Strands and 2 α-helices. FABP6 has both a bile acid and fatty acid binding sites as labeled by BA and FA, respectively [8]

Fatty acids are the primary ligands of these proteins, suggesting the participation of FABPs in the modulating lipid transport and metabolism. The presence of the FABPs in the intestine, liver and heart demonstrate a substantial effect on fatty acid flux [2]. The effect of FABPs on fatty acid uptake is variable based on FABP-type/cell-type. The potential roles of FABPs include: the uptake of fatty acids into the cell across the plasma membrane and the transfer of fatty acids to sub-cellular organelles; protecting cellular proteins and structures from detergent effects by sequestering fatty acids in the cytosolic pool; mediating fatty acid transport; and maintaining the fluxes of fatty acids within cells. Fatty acids have a poor solubility in aqueous media. FABPs could be essential in regulating of enzymatic activities by acting as a carrier to control transport of fatty acids to specific intracellular locations for metabolism. Furthermore, FABPs may modulate cell growth and differentiation through the transport of fatty acids to the nucleus to activate gene specific transcription [9, 10].

Most FABPs have primarily been linked to their affinity for long-chain fatty acids. The Liver-FABP (FABP1) is also capable of binding to other acyl ligands and Ileal lipid binding protein (FABP6) primarily binds to bile acids and potentially steroid hormones [2, 8].

FABP6

Fatty Acid Binding Protein 6 (other names include gastrotropin, porcine ileal polypeptide, Ileal lipid binding protein, and I-15P) was originally identified as Gastrotropin. It was first isolated from the porcine gut in 1984 by Wilder et al, who proposed that it functioned as an ileal polypeptide with a stimulatory effect on parietal cell acid production [11]. Further studies suggested a dose dependent effect upon the gastric acid secretion in pig gastric mucosal slices and a stimulatory effect of FABP6 on growth of primary mixed cell cultures of the gastrointestinal epithelium [12]. However, a direct effect on parietal cell function was difficult to reconcile since FABP6 was not detectable in goblet or crypt cells [7, 13, 14]

Walz et al, (1988) identified the primary structure of gastrotropin (FABP6) to be similar to that of fatty acid binding proteins. The binding of FABP6 to bile salts and bilirubin is indicative of the mechanism of transport of FABP6 in the blood to be cleared by the liver. It therefore was thought that FABP6 was the first FABP to have an extracellular function. FABP6 is a 127 residue-protein with an acetylated alanine at the NH2 terminus and an alanine at the COOH terminus, and has a single cysteine residue at position 69. The molecular weight is 14, 054 Da based on the amino acid sequence, and has no prosthetic groups attached to the native protein. Its isoelectric point is 8.68. The NH2 terminal-regions of the FABPs are thought to be associated with the interactions of intracellular membranes or other proteins [12].

Gantz et al, (1989) provided further evidence that FABP6 could not act as an enterooxyntin by finding that the primary translation product of porcine FABP6 mRNA does not contain signals for con-translational entry into the secretory pathway. The study suggested cytoplasmic targeting of the protein due to a methionine at the site of its proposed translocation site that would inhibit the removal of an amino acid terminal extension for proteolytic

processing. Therefore, this study concluded that if FABP6 were to be exported to stimulate distal small intestine cells, it would require a novel pathway. However, administration of FABP6 did not stimulate gastric acid secretion in the rat. Thus, it was concluded that FABP6 was not an enteric hormone. Indeed, comparative sequence analysis showed that that the primary structure of porcine gastropin was similar to rat liver fatty acid binding protein [15].

Sacchetini et al, (1991) characterized the protein they labeled Porcine Ileal Polypeptide (FABP6) by first looking at the regional specifications of the transcripts in the rat. They found that the mRNA of FABP6 is most abundant in the ileum compared to the FABP1 and FABP2 that were most abundant in the proximal jejunum (Fig. 2). No other tissues (liver, stomach, pancreas, brain, kidney, spleen, testis, skeletal muscle, heart or lung) produced any detectable signal. This study suggests that the protein plays a role in the intracellular transport of bile salts in the ileal epithelium. Therefore, they demonstrated the ability of FABP6 to bind palmitate and oleate, but also the bile acid chenodeoxycholate using NMR spectroscopy. In the presence of chenodeoxycholate, fatty acid binding is blocked[16, 17].

Figure 2. FABPs in the Small Intestine. FABP1 (Liver) is dominant in the duodenum and FABP2 in the liver are important for the uptake of Fas; while in the ileum, FABP6 is important in the uptake of bile acids [17],

Kanda et al, (1991) identified a gene that they thought was unique to the porcine gastrotropin since the sequences shared only a homology of 71% in total residues. Furthermore, this protein did not share the oxyntic activity of gastrotropin. Therefore they labeled the protein as I-15P (FABP6) that was found in the course of purifying rat intestinal FABP (FABP2). This protein was estimated to be 2% of cytosolic proteins of enterocytes. They determined that although structure of the protein was similar to that of the other FABP members, it had a limited binding affinity for long chain fatty acids such as palmitate and oleate as demonstrated in *in vitro* binding assays [18]. FABP6 instead has a high affinity to binding bile acids and possibly steroid hormones [4, 19-21].

In 1993, Kramer investigated bile acid reabsorption in the intestinal segments of the rabbit, looking at both the Apical Sodium Bile Acid Transporter (ASBT) and FABP6 using brush border membrane vesicles. The transport activity of bile acids [^{3}H]cholate and [^{3}H]taurocholate uptake was only in the distal segments of the small intestine, coinciding with the expression of ASBT and FABP6 transporters. To obtain the specificity of the proteins for bile acids, photoaffiniity labeling was performed, and it was

found that ASBT and FABP6 had a greater binding affinity for physiological bile acids rather than bile acid derivatives. An α-oriented hydroxyl group at the steroid nucleus of the bile acid is important for bile acid recognition. This was the first study that suggested the co-expression of ASBT and FABP6 in the terminal ileum to be essential components of Na^+/bile acid cotransporter system [22].

Gong et al., (1994) deduced that the FABP6 was immunologically distinct from the L-FABP and I-FABP and had only 25% similarity with rat I-FABP and L-FABP. Furthermore, the study identified the presence of FABP6 mRNA as limited to the ileum of the small intestine, with no presence in the liver or the rest of the gastrointestinal tract. Therefore, the authors suggested FABP6 has an importance in mediating the bile acid absorption in the ileum, since the ileum is the only site of active bile acid transport of mammals. FABP6 was suggested as serving as the cytoplasmic carrier that takes transports bile acids from the apical side of the brush border membrane to the basolateral [23, 24].

Based on crystallographic studies of the FABP6 structure, both FABP1 (Liver FABP) and FABP6 have two shortened beta-strands compared to the other FABP structures as seen in NMR results. The overall protein conformation is not influenced by this change, thereby maintaining the traditional β-clam structure. This variation of structure may help to explain the ability of FABP6 to bind the more bulky and rigid ligands—bile acids and steroids—since there is a less stable hydrogen bonding network compared to the FABPs from the heart or intestine that strictly bind fatty acids. In addition, a gap of 4-6 residues in the sequence alignment of FABP6 in comparison to FABP2 (intestinal FABP) appears to produce a second wider entry portal for bile acid ligands. In comparison to FABP6, FABP2 is suggested to have an additional 6-residue sequence that occurs at the turn between the beta-strand G and H in FABP2, thereby shortening the length of

the beta-strands in FABP6 [15]. Further investigation in to the ligand specificity, demonstrates that bile acids block the binding of fatty acid to FABP6; and that the binding of fatty acids to the FABP6 is relatively weak compared to other FABPs [8, 20].

Further interest in the protein includes its role in the production of steroid hormone metabolism. There have been reported cases of the presence of the FABP6 within the ovary and the endocrine cells of the adrenal glands. Amano et al. (1992), identified the presence of FABP6 was not limited to the epithelial cells of the ileum but was localized in the lutein cells of the ovary and subpopulations of cortical cells of the adrenal gland within rats [25]. This was further supported by Fujii et al, (1993) which showed that the FABP6 was abundantly localized in the ileum and steroid hormone-producing tissues of rat. This study measured 35% intensity of FABP6 mRNA in the ovary compared to that found in the ileum [26]. Tissue distribution was further determined by Iseki et al, (1993) by immunoblot to detect the presence of FABP6 in the distal regions of the small intestine, ovary and adrenal gland. In the ovaries, it is has been proposed that the expression of FABP6 is controlled by the ovarian cycle, being developmentally and hormonally controlled [27]. These studies stress the importance of identifying the function of the protein within each tissue and determine the ligands that specifically bind to the FABP6.

To investigate the biological function of FABP6, competitive binding between bile acids and steroid hormones progesterone and estrogen, and fatty acids was executed. A cloned FABP6 cDNA was inserted into a pSV-SPORT1 plasmid which was contransfected in COS-7 cells. To test the bile acid binding activity the COS-7 cellular lysates were incubated with 7,7 azo [^{3}H]Taurochlorate irradiatied with UV light, and the inhibition was observed based upon possible ligands of FABP6 when contransfected. 7,7 azo [^{3}H]-taurchlorate was significantly inhibited by coincubation with taurchlorate

(96%), tuarodeoxycholate (83.1), chenodeoycholate (74.6%), and cholate (50.5%)—clear inhibition by bile acids. Progesterone also inhibited 7,7-azo-[^{3}H]taurchlorate. Oleic acid and estradiol on the otherhand did not inhibit the binding of 7,7-azo-[^{3}H]taurcholate[24]. Therefore they suggested a role for FABP6 in the cytosolic transport of progesterone in the ovary.

Alves-Costa et al., (2008) reported the first FABP6 gene described for non-mammalian vertebrae in the embryos, larvae, and adult zebrafish. The FABP6/Fabp6 genes and cDNAs have been investigated in mammalian species such as human, rat, mouse, and pig. The zebrafish FABP6 protein had a 55.3% similarity to human FABP6. In addition, the localization of the Fabp6 transcripts and protein were present in the distal region of the intestine, liver, heart, ovary and kidney as detected by RT-PCR. Therefore it is possible that based on the presence of the FABP6 in the zebrafish, that this gene might be highly conserved across species—including avian. It may be interesting to note the conservation/difference of roles of FABP6 across species [10].

Evidence of Bile Acid Binding Proteins in Poultry

In 1988, a protein similar to a FABP was identified in the liver of the chicken with a low sequence similarity to the mammalian species and named chicken liver bile acid FABP (Lb-FABP) by Scapin et al which was later identified in the liver of axolotl, catfish, shark, frog, and iguana [28]. Similarly, two lipid binding proteins were identified as low molecular weight protein (14 kDa) from the liver and intestinal mucosa of the *Gallus domesticus* [29]. Measurement of relative binding activities identified a non-specific Liver Binding protein (ns-LTP) and Liver FABP (FABP1). The ns-LTP was capable of binding cholesterol, while FABP1 was not. FABP1 was observed to have significantly higher binding capacities of the fatty acids palmitate, linoleate, and oleate. Amino acid composition data was shown to have a 44% homology between FABP1 and chicken ns-LTP [30]. These 3 studies appear to be the initial evidence of bile acid binding proteins in

poultry, having similar characteristics to both the Lb-BABP (FABP10) and FABP6 gene that is now characterized in various species. Lb-BABP has not been identified in mammalian vertebrates.

The Lb-BABP in chickens is located on chromosome 23; while the ileal bile acid binding protein, FABP6, is found on chromosome 13. Both have the similar structure of the β–barrel and thought to have tissue specific functions in transporting bile acids in the cytosol, or protection from high levels of bile acids that can cause abnormal apoptosis or cytotoxicity.

It was not until 2007 that Guariento et al., (2007) first identified FABP6 in chickens. The chicken RNA database was searched for similarities in sequence to the human FABP6 and the sequence identified to produce recombinant proteins. Following spectral analysis by NMR the apo- and lipo-protein forms were shown to undergo a conformational rearrangement upon binding of a ligand. This protein is thought to be capable of binding two bile acids per protein molecule. The first binding event triggers a global structural rearrangement [31, 32].

Guariento et al. (2010), subsequently studied the structure of the chicken FABP6, looking at the binding model using glycholic acid and glycochenodeoxycholic acid (bile acids) as the ligands. The study identified the two binding sites for ligands in the FABP6 structure by isothermal titration calorimetry. The binding of the first ligand promotes a global structural rearrangement as demonstrated in NMR techniques. This structural change increases the stability of the protein. Further studies need to be conducted to demonstrate the binding affinity to other ligands such as steroids, to identify their binding capabilities and further elucidate the function of the FABP6 [31].

Although work has been conducted on the structure of the chicken liver bile acid binding protein and the chicken ileal lipid binding protein in their apo-protein and holo-protein isoforms, no work has been done to

characterize the distribution beyond the scope of the enterohepatic circulation.

Cholesterol Homeostasis

Cholesterol homeostasis is dependent upon the several mechanisms that control its synthesis, use in tissues, and excretion. Cholesterol is a 27-Carbon sterol molecule that is synthesized from acetate. Cholesterol's backbone is used as precursor for biologically active molecules, including all steroid hormones, bile acids, vitamin D, and oxysterols. It is a primary component of membranes and growing cells need to take up cholesterol according to growth and demands. It is primarily synthesized by the liver and is taken up by in tissues through lipoproteins. Cholesterol plays essential roles in cellular membrane physiology, dietary nutrient absorption, stress responses, salt and water absorption, salt and water balance, calcium metabolism, and reproductive physiology [33, 34]. The body's cholesterol homeostasis is essential in regulating all of these pathways. The cholesterol balance is dependent upon the dietary and biliary cholesterol absorption and the enterohepatic circulation. The tight regulation the varying pathways cholesterol is important to prevent buildup of cholesterol in the cell. Excess cholesterol is toxic to the cell. The imbalance of cholesterol modulating mechanisms leads to the formation and development of metabolic disorders such as gallstones and arteriosclerosis [35, 36]. Therefore, there are parallels in the processes that lead to the synthesis and the flux of the cholesterol byproducts is vital to understanding the role of FABP6.

Cholesterol is an isoprenoid that is synthesized de novo from acetyl coenzyme A and from dietary sources via fatty acid degradation. The first stage in the synthesis of cholesterol is the conversion of acetyl-coA to isopentyl pyrophosphate. 3-hydroxy-3-methylglutaryl CoA (HMG CoA) is formed from acetoacetyl CoA, which is reduced to mevalonate for cholesterol

synthesis being the first committed step in cholesterol formation via HMG-CoA reductase. Mevalonate is then converted in to 3-isopentyl pyrophosphate, in a process that requires ATP, and decarboxylation forms isopentyl pyrophosphate. Six molecules of isopentyl phosphate lead to the formation of squalene. Squalene then cyclizes to form lanosterol via a reaction that uses O^2 and NADPH. The removal of three methyl groups, reduction of a double bond, and migration of the other double bond convert lanosterol to cholesterol.

Cholesterol delivery to the cells is dependent on the conversion of acetate to cholesterol; and the exogenous delivery of cholesterol from the lipid lipoproteins (LDL, HDL, VLDL) that are internalized by their lipoprotein receptors; and the catabolism of bile acids[37]. The liver synthesizes up 50% of cholesterol for export to the blood stream, while most cells are capable to synthesize cholesterol from acetyl coenzyme A. Nutritionally, there is no dietary requirement of cholesterol since the cholesterol needed within the body can be met by *de* novo synthesis. However, significant amounts of cholesterol enter the intestinal lumen. Since animals are unable to degrade the steroid nucleus of cholesterol, this results in the modification of cholesterol by the addition of hydroxyl groups and shortening the conjugated side-chains to create bile acids with the enzyme cholesterol 7α-hydroxylase (CYP7A1) [34].

Cholesterol Homeostasis in Poultry

In contrast to mammals, the cholesterol pool is essential to regulate in the hen's ovulatory cycle in its ability for daily ovulation and the abundance of cholesterol in the egg yolk. The luteinizing hormone surge, follicular maturation, and ovulation are compressed into a 24-hour period. The ovary of the domestic hen consists of the stroma and a pool of undifferentiated follicles that is established during embryogenesis. These follicles eventually

develop into small follicles that contain white yolk (SWF), and when mature they are recruited into a pool of larger follicles and the yolks become yellow (SYF). The pool of five to seven large yellow follicles (Fn-F1) varies in size and degree of maturation and the follicles ovulate successively on a daily basis until a photorefractory period. Ovulation is due to a fluctuation of gonadotrophins and hormones, and ultimately to the response by the ovulating follicle's (F1) sensitivity to the luteinizing hormone (LH) and subsequent preovulatory LH surge[38, 39].

The liver and the ovary are the primary sites of cholesterol synthesis in the hen. Nutritional feeding of cholesterol affects the cholesterol pool and bile acid synthesis. In chickens, a 3% cholesterol supplemented diet causes a rise in mRNA expression levels of CYP7A1, indicating the increase of production of bile acids, and most likely FABP6. The cholesterol that is present in the chicken egg yolk is derived from the triglyceride rich lipoproteins derived from the liver transported in the plasma to the ovary. The plasma and the yolk cholesterol have little correlation [40].

Age of maturity may have an effect on the cholesterol pool at varying developmental stages. Mature birds (58 weeks of age) excrete more cholesterol via bile acids than at 8 and 26 weeks of age, suggesting a change in bile acid synthesis in maturity of white leghorn hens [41]. HMG-CoA reductase and CYP7A1 activity was monitored in males, and both normal and restricted ovulating hens developmental differences in bile acid production occur. There was a rise in HMG-CoA reductase in 26 weeks old birds compared to birds 18 weeks of age, likely in response to the demands for cholesterol in yolk synthesis of hens. For birds of 56 weeks of age, the activity of HMG-CoA was reduced probably due to the decreased demand of ovulation. The CYP7A1 activity decreased after maturation in all three groups [42].

It has been proposed that the demands of the developing oocytes in laying hens cause the augmentation of cholesterol synthesis and bile acid production during oocyte growth in poultry. Similar to the study by Mitchell et al., Sato et al., showed an age-dependent rise of CYP7A1 and HMGR (3-hydroxy-3-methylglutarate) when layers were measured at 16, 19, and 30 weeks of age, perhaps to support the enhanced cholesterol metabolism for egg production. In contrast, the male chicken liver expression of both HMGR and CYP7A1 are not influenced by maturation [43]. Therefore, there may be sex differences when looking at the cholesterol metabolism of poultry in studying FABP6.

FABP6 and Cholesterol

Changes in cholesterol levels leads to the proteolytic activation of sterol-responsive element binding proteins (SREBP) that act with sterol-responsive elements (SRE) located in the promoter of sterol genes, including SREBP1c, SREBP1a and SREBP [6, 44]. SREBPs are members of the helix-loop-helix family, synthesized as inactive precursors bound to the endoplasmic reticulum membrane and nucleus envelope. Low levels of cholesterol in a cell culture results in translocation of SREBP from the ER to the Golgi where proteolytic cleavage liberates the transcriptional active NH2-terminal domain to induce the transcription of sterol target genes [35]. The cholesterol balance is modulated by the activity of transcription by the SREBPs and in turn, the transcriptional activity of SREBP is dependent on the intracellular concentration of cholesterol[44]. The positive feedback loop of FABP6 in response to cholesterol is indirectly regulated through the Liver X Receptor (LXR) mediated induction of SREBP1c by oxysterols; and feedback loop by the Farnesoid X receptor (FXR) by bile acids (Figure 3). To study the importance of sterols on FABP6 expression, Zaghini et al. (2002), induced the FABP6 gene with a bile acid, CDCA; they then compared the addition of cholesterol and depletion (using HMG-CoA reductase inhibitor) within a Caco-2 cell culture. The FABP6 mRNA levels were decreased in response to

cholesterol depletion [35]. This study was the first to demonstrate that FABP6 as a sterol target gene by the identification of a sterol response element in the Human FABP6 promoter via the cloning of FABP6 promoter fragments into a CAT reporter vector. The response of CAT activity was monitored in the response to the addition and deprivation of sterols. Further characterization of the FABP6 promoter revealed that it is similar to the SREBP-binding site found in the promoters found in the LDL receptor and included a flanking Sp-1 binding site (GC box) that enhances SRE transcriptional activity. The presence of the SRE and GC box in the FABP6 promoter is highly conserved in the human, mouse, and rabbit sequences[35].

Paradoxical to the in vitro results, however, cholesterol enriched diets fed to mice induced an increase in levels of FABP6 mRNA levels in the ileum. This lack of congruence between the effects of levels of cholesterol on transcriptional activation of FABP6 may be explained in part by the effects of cholesterol metabolites on the LXR receptor *in vivo*. Activation of the LXR receptor by oxysterols or LXR agonists resulted in the up-regulation of SREBP1c in which in turn activated the transcription of FABP6. In addition, bile acids are present in vivo (but not *in vitro*) that are expected to up-regulate FABP6 through the mediation of the FXR receptor [35]. In the absence of bile acids, LXR agonists have reduced but still significant effects on stimulating FABP6 transcription and both direct and indirect effects are mediated by LXR/RXR (Retinoid X Receptor) heterodimers binding to FXRE, the bile acid response element (BARE), sequence in the promoter [44]. Thus, increases in dietary cholesterol increases bile acid and oxysterol production stimulate the activation of various transcription factors to up-regulate the transcription of FABP6 (Figure 3) [44, 45].

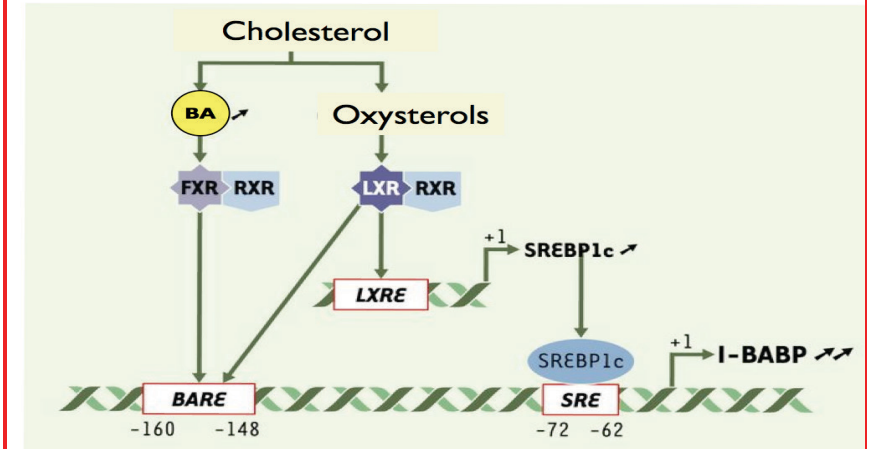

Figure 3. Regulation of FABP6 expression by sensing intracellular cholesterol and cholesterol derivatives. In the presence of cholesterol, oxysterols are a metabolite of cholesterol and the physiological ligand of LXR. LXR/RXR activation binds to the LXR response element on SREBP. SREBP then binds to the SRE on FABP6, activating transcription. Cholesterol is also oxidized in the liver into bile acids. These in turn are excreted from the liver into bile in the small intestine. Bile binds to the FXR/RXR heterodimer which binds to the Bile Acid Response Element (BARE) to up-regulate transcription of FABP6 [46]

Cytochrome P450 Enzymes

Cytochrome P450s (CYP) are heme-containing enzymes responsible for the hydroxylation of lipophylic substrates in species. They can be transcriptionally regulated by their own substrates or by other compounds. CYPs have relevant roles in the biosynthesis and metabolism of steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenetic amines, or retinoids. Thus, CYP-catalyzed reactions are integral in the regulation of cholesterol homeostasis. Transcriptional regulation is by the super family of nuclear receptors. These compounds are capable to enter through direct diffusion or facilitated transport into the cell. The nuclear receptors that are responsible in controlling the action of the CYP reactions are the LXR that respond to the dietary cholesterol and oxysterols; and FXR that responds to the presence of bile acids and steroid hormones[47-49]

Bile Acids

Bile Acid Biosynthesis

Bile acids are amphipathic molecules that are synthesized from cholesterol in hepatocytes by a catabolic process to promote the secretion of the hydrophobic and insoluble cholesterol molecule into bile acids that can be excreted in the feces. They are also the primary component of bile that participates in the absorption of dietary lipids, cholesterol and fat-soluble vitamins in the intestine[31, 37, 50-52]. Bile acids act as detergents that form micelles with cholesterol and dietary fats. Approximately 95% of bile acids are taken up in the enterocytes during enterohepatic circulation to return to the liver. The body efficiently reuses bile acids and limits the need to synthesize excess bile acids de novo [37]. The primary secretion and absorption of bile acids is generated by osmotic gradients.

There are at least 14 different liver enzymes that add polarity to the cholesterol ring structure and side chain to create the ideal micellular solubilization agents [33, 37]. Bile acids promote the digestion and absorption of fat in the small intestine by emulsifying fat and aiding in the hydrolysis of fat by pancreatic lipase and forming the micelles of fatty acid-monoacylglycerol-bile salt [53]. Synthesis of bile acids from cholesterol is dependent on the action of the CYP P450 hydroxylases. The pathways leading to the synthesis and excretion of bile acids need to be tightly regulated because of their intrinsic toxicity. The two nuclear hormone receptors LXR and FXR modulate the flux of target genes involved in bile acid synthesis in response to cholesterol to up regulate or down regulate cholesterol metabolic pathways [54].

Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized from cholesterol in the liver where they are conjugated with glycine or taurine, and then secreted into the bile. Bile acids are released into

the duodenum to aid in the absorption of dietary fat and lipid-soluble vitamins where they form micelles. The bile acids are then reabsorbed in the distal region of the intestine by enterocytes and return through the portal blood to the liver to be secreted again into bile. This loop describes the enterohepatic circulation that demonstrates the importance of bile acids to maintain the homeostasis of body cholesterol, being responsible for 50% of cholesterol catabolism [55, 56]. Bile acids not reabsorbed by the small intestine are the main physiological way of cholesterol elimination [44, 50]. In turkey and chickens, the most predominant bile acids in the bile are cholyltaurine and chenodeocxycholyltaurine and to a lesser extent allocholyltaurine [57].

Enterohepatic Circulation

Bile acids synthesized by cholesterol in the liver are secreted into the small intestine (Fig. 4). Bile acids are essential in the solubilization and absorption of intestinal lipids. The uptake of unconjugated bile acids in the intestinal lumen is dependent upon the passive absorption along the length of the entire intestine; and the transport-mediated absorption of the conjugated bile acids in the ileum [58]. The process of conjugated bile acid reabsorption involves the apical sodium-dependent bile acid transporter (ASBT) uptake of the bile acids from the enterocyte brush border membrane at the ileum. Bile acids then bind to the FABP6 proteins in the ileocytes with high affinity and specificity, after which, the bile acids are secreted into the portal blood via Na+-independent organic anion exchange system in the basolateral membrane of ileocyte via the organic solute transporters α and β (OSTα and OSTβ) and the multidrug resistance associated protein 3 (Mrp 3).

It is thought the ileal lipid binding protein (FABP6) is the rate-limiting step for enterohepatic bile acid circulation since it is an essential component for efficient bile acid desorption from the apical plasma membrane and bile acid intracellular trafficking [6, 22, 51, 56, 59].

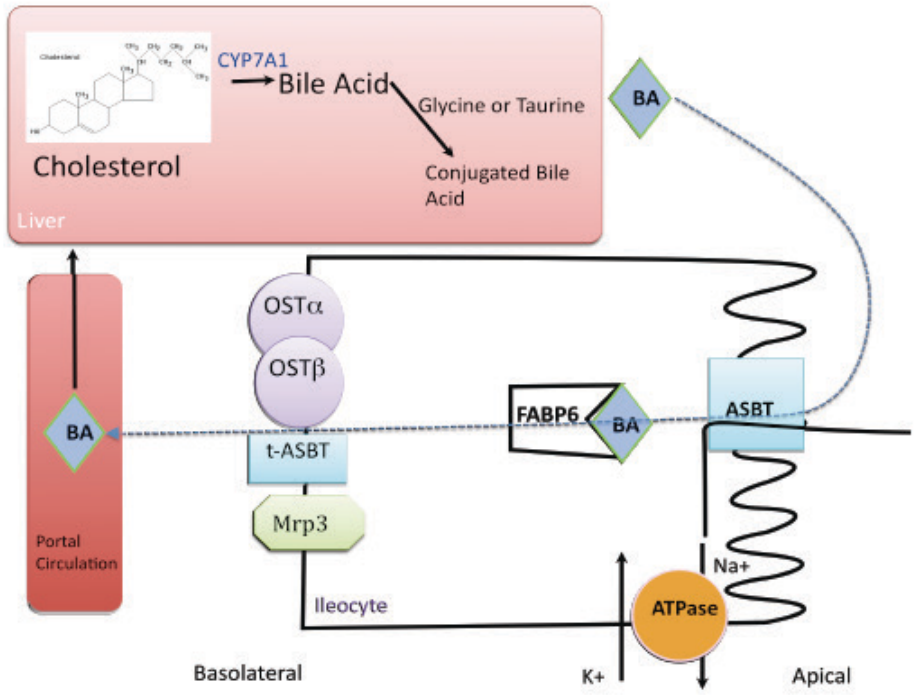

Figure 4. Enterohepatic circulation. Cholesterol creates bile acids in the liver; the bile is then transported into the ileocyte via ASBT. The FABP6 transports the bile acids from the apical to basolateral membrane. The bile acid efflux of the bile acid is then regulated by the transporters OSTα-β, tASBT, or Mrp3 back into portal circulation to the liver

The enterohepatic circulation is under the control of nuclear hormone receptors. Oxysterols are the metabolites of cholesterol and the physiological ligand of LXR. LXR binds to its respective response element LXRE in the promoter of CYP7A1 which is the initial enzyme and rate-limiting step in bile acid synthesis. The presence of cholesterol accelerates the conversion of cholesterol to bile acids[48, 52-54, 60]. Bile acids are the physiological ligand of FXR that binds to its response element FXRE. The activation of LXR and FXR in the ileum results and the upregulation of FABP6 expression, promoting transport of bile acids back to the liver. The presence of bile acids in the liver causes FXR binding to its response element in the CYP7A1 gene in to inhibit the conversion of cholesterol and repress other enterohepatic genes[22, 23, 45, 52]

Apical and Basolateral Ileocyte Bile Acid Transport

The apical sodium bile acid transporter (ASBT or SLC10A2) is localized to the apical surface of ileal enterocytes, cholangiocytes, and the renal proximal tubular cells. In the intestine ASBT is responsible for the uptake of bile acids from the intestinal lumen. ASBT is abundantly expressed in the distal ileum at the epithelial apical membrane of mammals. Similarly, the solute carrier in hepatocytes (SLC10A1) is expressed on the basolateral membrane to mediate the uptake of bile acids from portal blood. Both of these proteins are dependent on the flux of Na^+ [58, 61].

Similar to the FABP6 gene, ASBT is also regulated through the actions of bile acids using the nuclear hormone receptor FXR to induce SHP expression and subsequently the LRH-1 to antagonize ASBT in mice and rabbits; however there is no LRH-1 in rats, and no bile acid repression of rat ASBT [62, 63].

An alternatively spliced version of ASBT is located on the basolateral membrane of the ileocyte. This truncated ASBT (tASBT) along with the organic solute transporter α and β (OSTα-β) and multidrug resistance protein 3 (Mrp3) effect the efflux of bile acids into the portal circulation [64-66].

FABP6 in the Enterohepatic Circulation

FABP6 has shown to be the only physiologically relevant bile acid carrier found in the cytosol of enterocytes and expressed primarily in the ileum, corresponding to the site of uptake of bile acids [17, 67]. Unlike mammals, both the chicken pancreatic and bile ducts enter the duodenum at its distal end (in mammals entry is in the middle), forcing the secretion of bile toward the duodenum, opposite to the flow of digesta. In laying fowl and pigeons, bile salts are rapidly absorbed at similar rates in the jejunum and ileum [68]. The fluctuation of bile acid reabsorption in the small intestine should therefore cause a differential pattern of FABP6 expression and corresponding genes associated with bile acid metabolism in poultry

than in mammals, with an increased presence of FABP6 in the jejunum and ileum, rather than solely in the distal portion of the small intestine. To date the distribution of intestinal FABP6 in poultry has not been assessed.

Steroid Hormones

Steroidogenesis is dependent on the presence of cholesterol. Cholesterol sources are from de *novo* from acetate; plasma low-density lipoproteins and high-density lipoproteins; cholesterol esters stored in the form of lipid droplets; and cholesterol interiorized from the plasma membranes [69]. Cholesterol uptake and hormone synthesis by the cells is dependent upon the tropic hormones—luteinizing hormone (LH), Follicle Stimulating hormone (FSH) or adenocorticotropic hormone (ACTH). The acute stimulation involves the rapid mobilization of cholesterol and the increased delivery of cholesterol by the Steroid Acute Regulatory Protein (StAR) on the outer mitochondrial membrane to the Cytochrome P450 side chain cleavage protein (P450scc) in the in the mitochondria that initiates the conversion of cholesterol to pregnenolone. Further oxidation by CYP450s and the movement between the endoplasmic reticulum and mitochondria to synthesizes the tissue's respective steroid hormone (Figure 5) [69, 70]. The mechanism facilitating the movement in the cytosol of the steroid hormones has yet to be defined [71]. Evidence of LXR, FXR, and SREBP in the steroidogenic tissues and regulation of the steroidogenic pathway suggest that the mechanisms governing the control of bile acid biosynthesis may similarly modulate steroidogenesis [35, 72-74].

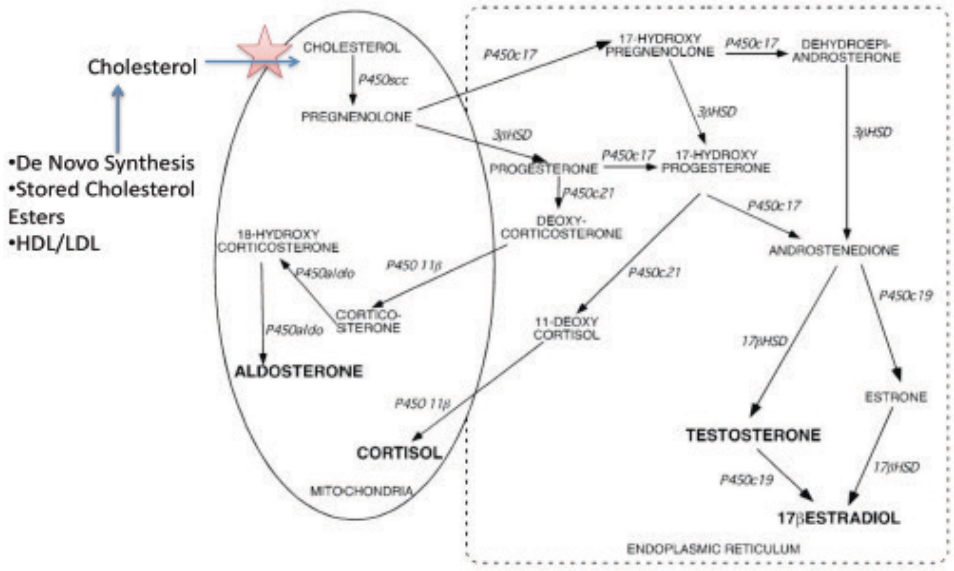

Figure 5. Cholesterol metabolism into steroid hormones. Steroidogensis involves the CYP450 enzymes and the movement between the mitochondria and endoplasmic reticulum[75].

FABP6 in Steroid Producing Tissues

Iseki et al., (1993) detected FABP6 predominantly in the enterocytes of the ileum however significant levels were also noted in the corpora lutea of ovaries and adrenal gland of the rat. No signal was detected in the granulosa or theca cells of ovarian follicles in various growing phases. The adrenal gland also had a very intense immunoreactivity and RNA signal located to the cortico-medullary boundary and in the medulla. Electron microscopy results revealed that the FABP6 expressing cells belonging to steroid-endocrine cells [14]. In neonatal or sexually immature rats, FABP6 was undetectable in the ovary. However, treatment of juvenile rats with exogenous gonadotrophins induced transient increases in FABP6 in the granulosa cells during the process of luteinization, whereas, corpora lutea had the highest levels of expression [76]. Thus, the expression of FABP6 would appear to be developmentally and hormonally controlled in a tissue specific fashion and is associated with progesterone. Ovarian expression of FABP6 is not limited to mammals since FABP6 is also present in the ovary of zebrafish [10]. No studies have been published on poultry where unlike

mammals, progesterone is the positive feedback signal for ovulation within the follicular hierarchy and an analog of the corpus luteum does not exist.

In relation to the hen that has a different steroid hormone production profile in the ovary, the presence of FABP6 has yet to be determined. Since the FABP6 was localized to a site of high progesterone production and not in relation to estrogen or other androgens. It can be proposed that progesterone or an intermediate in the progesterone synthesis requires the binding of FABP6 for transport in the granulosa of the follicles, as based on the progesterone content being greatest in the granulosa of the first three follicles in the ovary, with a peak 4 hours prior to ovulation, in comparison to the thecal tissue. Otherwise, FABP6 may be evident in the interstitial cells of the theca interna in small ovulating follicles. Likely, FABP6 will have variable expression in the varying stages of the follicular hierarchy [77].

Interestingly, a study conducted by Smith et al. (2009) demonstrated all elements of the bile acid synthesis pathway including the nuclear hormone receptors, LXR, FXR, RXR and the synthesis of bile acids in the human ovary in cell culture. This study found that treatment to hCG caused a decrease of bile acid content in the serum. This study is the first demonstration of bile acids in the ovary. Furthermore, a gene chip array was conducted and the enzymes responsible for the bile acid pathways CYP7A1, CYP27A1, CYPB1 and CYP8B1 are present in the human ovary that were verified by immunoblots. This study also exposed cumulus granulosa cells to varying concentrations of cholesterol, and measured a dose dependent increase of bile acids in the cholesterol-induced media [78]. Perhaps the increased presence of bile acids prior to the hCG stimulation is in a regulation within the ovary to control cholesterol homeostasis via degradation of cholesterol in the biosynthesis of bile acids. If this proves true, FABP6 would play an integral part in regulating the flux of bile acids and/or steroid hormones within steroid hormone producing tissues.

Conclusion

Due to the morphological differences of poultry in both the small intestine and reproductive tissues, there should be physiological differences in the presence of the FABP6 gene and its corresponding nuclear hormone receptors than what is observed in mammals. This would mean a presence of FABP6 in more proximal regions of the small intestine—the jejunum and ileum should both express FABP6, as these are the sites of reabsorption of bile acids. If FABP6 does play a role in steroid hormone metabolism, this would mean its presence should be observed in the adrenal glands, testes and ovary; otherwise the possibility that bile acids are present in these tissues would indicate the role of FABP6 in cholesterol homeostasis via the transport of bile acids throughout the body. In layers, there should be a differential expression in folliculogenesis, corresponding to the varying stages of development, likely with higher expression of the FABP6 gene in the rapid growth stages of development rather than in smaller follicles. The purpose of this study is to characterize the presence of FABP6 in poultry and to gain further understanding of cholesterol homeostasis via parallels between bile acid metabolism and steroid hormone synthesis.

Materials and Methods

Animals

Tissues were generously donated post-mortem from Ross 508 broilers (13 weeks of age) and white leghorn layers (49 weeks of age) housed in local Macdonald Campus Farm were used for the experiments under standard conditions. I was not involved in the handling of live animals for these experiments. The turkey embryo tissues were obtained from Couvoir Unik Inc. (Mont-Saint-Gregoire, QC).

Tissue Collection:

Tissues were collected from male broilers (total n=5) at 13 weeks of age and female layers (n=20) at 49 weeks of age. Embryonic turkey tissues were also collected (n=3) at 18th embryonic day (ED) of development; (n=4) on the 24ED; and (n=6) 28ED. The abdominal cavity was cut open and sections of the small intestine (duodenum, jejunum, and ileum), testes, and ovaries were collected. Follicles of the hen were separated based on follicular maturation into F1 follicle, small yellow follicles, and small white follicles and thecal and granulosa cells layers were separated from the yolk mass. All tissues were initially stored in RNALater (Ambion, Cat. AM7021M) followed by storage at -80°C until further processed.

RNA Isolation:

RNA was extracted from the tissues using TRIzol Reagent. About 0.05-0.1 g of tissue were placed in 2mL screw-cap tubes with 0.8g of zirconia-silica ceramic beads and 1mL of Trizol reagent. The tubes were then placed in the MagNA Cell Lyser (Roche) at 7000 for 40 second intervals for two minutes. Briefly the homogenate was centrifuged at 12,000 x g for 1 minute. The supernatant was then transferred to 1.5mL eppendorf tube and

centrifuged at 12,000 x g for 10 minutes to remove insoluble material from the homogenate. The supernatant was then transferred to a fresh tube and 0.2mL of chloroform was added. The solution was shaken by hand for 15 seconds and incubated for 2-3 minutes, followed by centrifugation of the mixture for at 12,000 x g for 10 minutes. The clear aqueous phase was then transferred to a new tube, and 0.50mL of isopropyl alcohol was added, mixed, and incubated for 10 minutes. After centrifugation at 12, 000 x g for 10 minutes. The alcohol was discarded and the resulting RNA precipitate was briefly dried. The RNA pellet, was washed with 1mL of 75% ethanol, vortexed and centrifuged for 10 minutes at 12, 000 x g. The alcohol supernatant was then removed, and the pellet was air-dried for 5 minutes. The pellet was re-dissolved in 50μL of ddH_2O and placed in a hot water bath of 55°C for 10 minutes and stored at -80°C. RNA concentration and purity was determined using a Nanodrop-1000 spectrophotometer (Thermo Fisher ND 1000). RNA was considered pure with a of a 260/280 value of ~2.0; and a 260/230 value of 1.8-2.2. Lower ratios are indicative of contaminants, and were not used in this study. Total RNA concentration was adjusted to 300 ng/μl prior to storage.

cDNA Synthesis:

Total RNA obtained from animals was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Part 18080) for males in the study ; while the High Capacity RNA-to-cDNA kit (Applied Biosystems Part 387406) for females and embryo groups for first-strand cDNA synthesis. All samples were normalized to 2000ng and manufacturer protocols were followed to specifications. The cDNA was then stored at -20°C for further processing.

Polymerase Chain Reaction:

Primers for the amplification (Table 1) were designed based on the predicted mRNA sequences from the NCBI GenBank for the Chicken (*Gallus gallus)* FABP6 (XM_414486.2), ASBT(XM_425589.2), FXR(NM_204113), LXR(NM_204542.2); and Turkey (*Meleagris gallopavo)* FABP6 (XM_003210281.1) and ASBT (XM_003203223.1). All primers were checked using BLAST to ensure specific binding to the target sequence. Sequences obtained from the current study were used to compare to the predicted sequence reported by the genomic sequencing projects on *Gallus gallus* and *Meleagris gallopavo.* BLASTn and BLASTp suite were used to analyze nucleotide and amino acid sequence identities, respectively.

To a sterile 0.20-ml micro centrifuge tube, the final concentrations of a PCR buffer minus Mg (1X), were dNTP mixture (0.2mM), MgCl2 (0.5μM), forward primer (0.5μM), reverse primer (0.5μM), Taq DNA polymerase (0.5unit), cDNA (1μl) and distilled water to 25μl. Tubes were placed in a thermal cycler at 94°C for 3 minutes to denature the template, and 34 cycles of denaturing at 94°C for 45 seconds, annealing at 60°C for 30 seconds, and extending at 72°C for 1 minute and 30 seconds, followed by incubation at 72°C for 10 minutes and maintenance of the reaction at 4°C were conducted. The samples were then stored at -20°C for further use. The amplicons were separated by 2% agarose gel electrophoresis followed by with ethidium bromide staining and image analysis

Table 1. Primers for Standard PCR

Gene of Interest	Forward Primer (5'→3')	Reverse Primer (5'→3')	Length (bp)
Chicken FABP6	TCTCCTGTGGGATGTTTGAG	CCAGAAGACTGGCTTTGATG	615
Chicken FABP6 Q-PCR	TGATTTCCCTGGACTCAGC	CCCACCTTCCATTTTGACTG	130
Chicken FXR	GCAAGTCAAAAAGCTACGG	ATCTCAGCTGAACGGAGGAA	390
Chicken LXR	ACGCTGCAGACTTCATTTCCC	GCTTGGCAAAGTCCACAATC	172
Chicken ASBT	GTGATGATCATGGGATGCTG	TGCAAGAAGTGTGGAGCAAG	102
Turkey FABP6	CATTCGCAGGCAAATATGAA	GCAATCTTTTTGCTGGTTCTC	378
Turkey ASBT	CCAAGTTGCTGGACAACTCC	CCATTGCAAGAAGTGGAG	449

PCR Cloning:

Using the Qiagen PCR Cloning kit (Catalog 231122) the standard protocol was followed to amplify the chicken FABP6 Q-PCR amplicon to . Briefly, the purified PCR product was combined with pDrive Cloning Vector, Ligation Master competent cells and incubated for 5 minutes and heat shocked at 42°C, followed by a brief incubation on ice for two minutes. The transformation mixture was then added to 250μl the of SOC medium, and the transformation mixture was spread one LB agar plates containing 50μM IPTG, 80μg/ml X-gal, and ampicillin (100 μg/ml). Using blue and white screening, white colonies were selected from the plate and placed in LB medium for further growth at 37°C overnight.

Following harvest, QIAPrep spin protocol using a microcentrifuge based on manufacturer's specifications was followed for plasmid DNA purification. The plasmid DNA was then sent for sequencing

Sequencing:

To validate primers, following PCR, at least two samples per primer set were sent to be sequenced at Genome Quebec to verify the gene of interest using Sanger Sequencing methodology. All samples were sent for direct sequencing except for the samples with the Chicken FABP6 Q-PCR primers. The sequence of the FABP6 Q-PCR plasmid was sent to the Genome center and analyzed with the M13 primers, and the chicken FABP6 subset was sequenced. The product sequence was read on 4Peaks analysis software.

Relative mRNA Quantitation by Quantitative Real- Time PCR:

The ABI Prism 7500 Sequence Detection System was used for Relative Quantitative PCR. SYBR Green PCR Master Mix (Applied Biosystems, Part 3454344) was used to conduct real-time PCR with volumes for the gene of interest (FABP6 and ASBT) and the 18S Internal Standard (Ambion, Part.

AM1716). All samples used the endogenous control 18S Internal Standard to normalize the differences in the amount of total RNA in each reaction. In both the gene of interest and 18S reaction were composed of 10μl 2X SYBR Green Master Mix, 1μl cDNA (1/1000 dilution for 18S), 0.1μl sense and anti-sense primers or 0.1μl of the 18S primer pair, and water to 20μl . Amplifications were conducted in duplicate. The thermo cycler conditions were performed with a two-step amplification of 95°C for 15 minutes; 39 cycles at 95°C for 15 seconds and 62.5°C for 1 minute. Specificity of the amplifications was determined by melting curve analysis ((63°C for 15 seconds to 95 °C in 0.1°C per second increments) and by gel electrophoresis. Primer sequences and expected size of the amplicons are listed in Table 2.

Table 2. Quantitative PCR primers

Gene of Interest	Forward Primer (5'→3')	Reverse Primer (5'→3')	Length (bp)
Chicken FABP6 Q-PCR	TGATTTCCCTGGACTCAGC	CCCACCTTCCATTTTGACTG	130
ASBT chicken	GTGATGATCATGGGATGCTG	TGCAAGAAGTGTGGAGCAAG	102
FABP6 turkey	ACAAGATCGAACTGGGAAGG	TCTCCATGTCTGCTTCCTTG	140
ASBT turkey	GTGATGATCATGGGATGCTG	CCATTGCAAGAAGTGTGGAG	117

Relative mRNA quantification was calculated by using the comparative Ct Method (ΔΔCt). Initially, a ΔΔCt validation experiment was conducted using dilution series of cDNA containing the gene of interest and the 18S control, and the slopes of the dilution series were compared to ensure that there was no change between the primer efficiencies. The ΔΔCt requires normalization of the endogenous control and the normalization to the reference sample that are used to calculate the fold changes (Refer to calculations below). The calculation refers to the fold changes of the amplicon between cycles of the sample tissue in comparison to the reference tissue sample. For example, the reference sample used for a majority of this study was the duodenum in the tissues of the broilers, layers, and embryo, all mRNA abundance is in relation to duodenal mRNA content. The F1 follicular

layers were separated into theca and granulosa. The theca was used as the reference tissue, therefore mRNA abundance was expressed as a function of the theca content.

Equations:

Normalize to Endogenous Control

Ct Target gene – Ct Endogenous control = ΔCt

Normalize to the Reference tissue

ΔCt Sample - ΔCt Reference = $\Delta\Delta$Ct

Calculation of Relative Fold Change

Fold Change = $2^{-(\Delta\Delta Ct)}$

Protein Extraction:

Protein extraction of samples was conducted by placing 0.05g of tissue in a 2.0 mL screw-cap tube with 0.4g of 1.0mm diameter zirconia-silica ceramic beads (BIOSPEC Products Cat. 11079110z). A 100µL buffer of 10mM Tris-HCl, 1mM EDTA, and 0.5% Tween 20 is added to each tube. The samples were then homogenized in the MagNA Lyser (Roche) at 6500 for 45 seconds. The homogenate was then centrifuged at 9000 x g and the supernatant was then transferred to a 0.6mL eppendorf tube, and an equal amount (100µL) of buffer composed of 10mM Tris-HCl, 1mM EDTA, 0.5% Tween 20, and 0.1mM PMSF (phenylmethylsulfonyl fluoride) is added to the tube. The samples are then centrifuged for 15 minutes at 12,000 x g and the resulting supernatant transferred to new tube. The protein concentration was then measured via Bradford Assay using Bio-Rad Protein Assay and Bovine Serum Albumin as a standard. Samples were stored at -80°C until further analysis.

Antibodies.

The primary antibody used to probe the membrane for immunoblotting and immunohistochemistry was a rabbit antisera against recombinant murine FABP6 to detect the immunoreactive 14.3 kDa protein. The primary antibody was generously donated by LB. Agellon of McGill University [7]. Murine FABP6 has 69% identity (amino acid) to the chicken FABP6 protein. The secondary antibody was a horseradish peroxidase (HRP) conjugated goat anti-rabbit.

Immunoblot:

Proteins (40 μg homogenate) were separated by electrophoretically on 13.5 % SDS-PAGE and then transferred onto PVDF membrane The membrane was incubated for 1 hour in a blocking solution of a 5% fat free milk solution with 0.01% PBS-Tween at room temperature. The membrane was then washed with 0.01% PBS-Tween solution for 15 minutes followed by three additional washes of 5 minutes. The membrane was then incubated with primary antibody (rabbit anti-rat FABP6) at a dilution of 1/5000 in the PBS-Tween solution for 1 hour. After the membrane was washed again, it was incubated with secondary antibody (HRP-conjugated anti-rabbit IgG) at a dilution of 1/14000 for 1 hour. Peroxidase activity was visualized using SuperSignal West Pico Chemiluminescent Substrate and exposure on radiographic films . ImageJ was used to analyze optical density of bands.

Immunohistochemistry:

Segments of fresh tissue (1 cm) from the duodenum, ileum, and whole ovarian follicles were fixed in 10% Formalin prior to paraffin embedding. Samples were washed in PBS three times (15 minutes) in 0.1 M phosphate buffered saline (pH 7.4). Samples were dehydrated 4 step dilutions of ethanol until they reached 100% in one hour intervals (70%, 80%, 90%, 100%(x3)). Then, the sections were embedded in paraffin wax overnight and

stored. A microtome was used to cut 4μm cross sections which were placed into a warm water bath and mounted onto a ULTIDENTBRAND Positive-Charged Slides to eliminate tissue loss. Slides were then dried and processed for immunohistochemistry.

Slides were placed in three washes of Citrisolv (Fisherbrand, Product 22-143-95) of 15 minutes each. The slides were then serially washed in dilutions of ethanol (100%x2, 95% x2 , 70% x2) in 5 minute intervals, and briefly washed with ddH2O, followed by 10 minutes in tap water. Slides were then washed with 0.03% Brij35 for 10 minutes. An antigen retrieval step was then implemented by placing slides in a solution with a final concentration of 0.0018M citric acid and 0.0082M tri-sodium-citrate-dihydrate and microwaved for 6 minutes. After the slides returned to room temperature, they were briefly washed in ddH20, and washed with the 0.03% Brij35 in PBS for 5 minutes. The endogenous peroxidase block consisted 0.3% of Hydrogen Peroxide in methanol for 30 minutes followed by a wash in the PBS-Brij35 solution.

Samples were then blocked with 5% BSA serum in the PBS-Brij35 for 1 hour. The samples were then incubated overnight with the primary antibody at a 1:200 dilution in the blocking solution, washed with Brij35 three times for 5 minutes, then incubated with a 1:500 dilution of secondary HRP-conjugated anti-rabbit antibody in the blocking solution for 1 hour. The Liquid DAB+ Substrate chromagen system of 1mL liquid buffer + 30ul of Chromagen (Dako, K3467) was immediately added to the sections and color development was monitored under microscope for 15 minutes and the reaction stopped in ddH2O. Hematoxylin was applied for 2 minutes to counterstain the tissue samples on the slide. Following incubation in tap water for 10 minutes, the samples were rehydrated in PBS (1 min), Ethanol (95% x2, 100%x2 in 5 minute increments) and Citrisolv (x2, 5 minutes) . Slides were mounted with VectaMount permanent mounting medium and let

dry prior to visualization using a Nikon DXM 1200c microscope and NIS-Element BR v. 2.3 software.

Statistical Analysis

Data was analyzed as a Repeated-Measures ANOVA using GraphPad Prism Software. Differences among treatment means were tested using the Bonferroni post-test. Pearson correlation was used to measure the association between FABP6 and ASBT expression. Statistical significance was declared at $P<0.05$.

Results

Validation of Primers

A primer pair was designed to amplify a region of RNA from the 5' to 3' UTR of FABP6 in Leghorn chickens which encoded the 128 amino acid open reading frame (ORF). Following sequence analysis this 615 bp fragment was identical to the predicted gene sequence of the chicken genome project. Thus, there were no apparent sequence deviations between Leghorns and *Gallus gallus.* Likewise, the 130 bp chicken Q-PCR amplified product between exons 4 and 5. There were no sequence deviations from the *Gallus gallus* sequence (Figure 6).

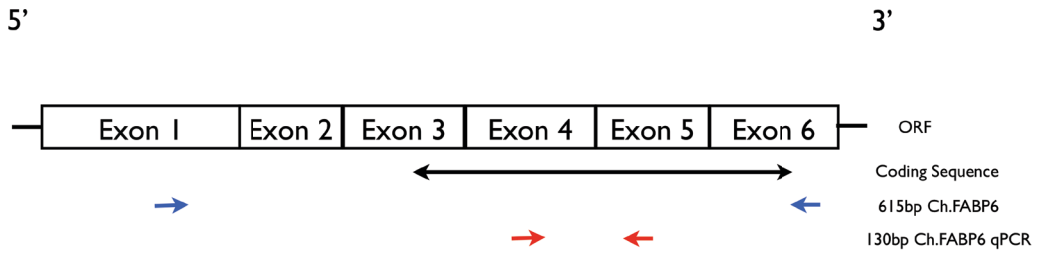

Figure 6 Location of primers on the chicken FABP6 transcript. *Gallus gallus.* FABP6 open reading frame has 6 exons. The coding sequence (black arrow) spans exon 3 to exon 4. Primers used for standard PCR are from 5'UTR in exon 1 to 3'UTR in exon 6. Chicken FABP6 Q-PCR primers amplify sequence between exon 4 and exon 5.

Similarly, in the turkey, a 373bp amplicon spanned a majority of the coding sequence from exon 1 to exon 4. Unlike the sequence of the chicken provided on the NCBI website, the 5'-UTR was relatively short, and no 3'-UTR has been defined. The Q-PCR amplified product was also validated by sequence analysis and extends from exon 2 to exon 3 (Figure 7).

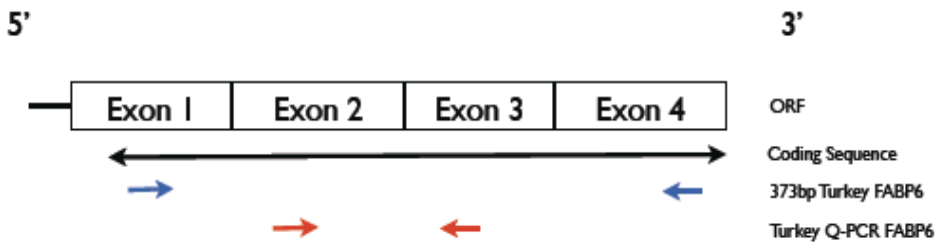

Figure 7 FABP6 primers in Turkey. Coding Sequence includes exons 1-4. Standard PCR primers are within the coding sequence from exon 1-4. Q-PCR primers are in between exon 2 and 3.

The primers used for ASBT in both the chicken and turkey were validated by sequencing the amplicons from both standard PCR and Q-PCR and there were no deviations from the gene sequence of the chicken genome project. ASBT is composed of 6 exons, the primer in the chicken spans from exon 1 to exon 2. Turkey ASBT primers for Q-PCR extend from exon 1 to exon 2, and the 449 bp Turkey ASBT primers capture a majority of the ORF. However turkey ASBT could not be validated using tissues obtained from the embryo since there was no amplification at this stage in development.

FXR and LXR amplicons were detected using standard PCR in the chicken. FXR primers amplified a 390 bp product between exons 4-7; and LXR primers amplified a 172 bp product between exons 3-4. However, further analysis of LXR and FXR beyond ORF comparisons in *Gallus gallus* and *Meleagris gallopavo* were beyond the scope of this initial characterization. Preliminary results show the presence of both these receptors in segments of the intestine, kidney, testis and follicular hierarchy.

Sequence Alignment

Based on the predicted ORFs of FABP6 in *Gallus gallus* and *Mus musculus*, the nucleotide and amino acid sequence identity between these two species is 69% and 66 %, respectively(Figure 8,9). Based on the chemical properties of amino acids, similarity of the proteins was 83 %.

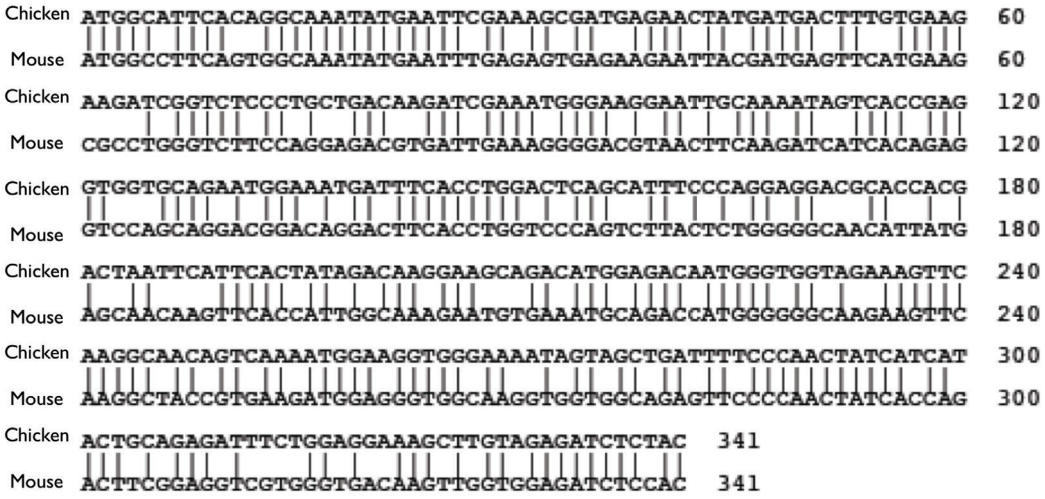

Figure 8. Chicken and Mouse FABP6 mRNA Sequence Alignment of coding sequence between *Gallus gallus* (XM_414486.2) and *Mus musculus (NM_008375.1)* has 69% sequence identity. Bars connecting mouse to chicken bp indicate nucleotide sequence identity

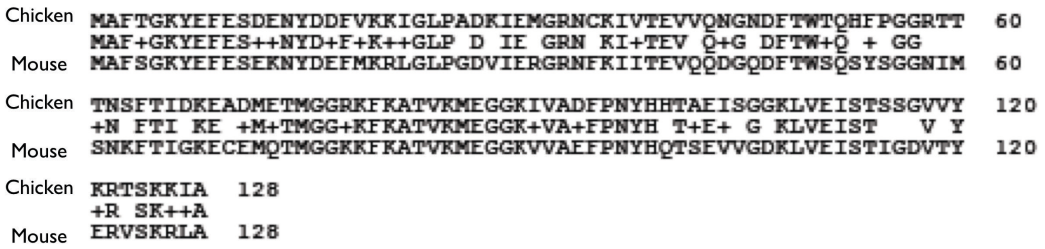

Figure 9. Chicken and Mouse FABP6 Protein Amino Acid Sequence Alignment. Comparision of amino acids between *Gallus gallus* (XP_414486.1) and *Mus musculus* (NP_032401.1) with 66% identity and 83% amino acid identity. . Middle sequence shows sequence identity. If middle sequence has a + this is indicative of a similar amino acid, if left blank, amino acids are different.

Similarly, sequencing of the ORF amplified from turkeys showed that FABP6 was highly conserved in aves. The chicken and turkey FABP6 had 96% and 98 % nucleotide and amino acid sequence identity , respectively (Figure 10, 11). The majority of differences in nucleotide sequence resulted in the use of alternative codons, whereas, variations at position 4 and 31 resulted in substitutions of threonine and leucine in chickens to cysteine and methionine in turkeys, respectively.

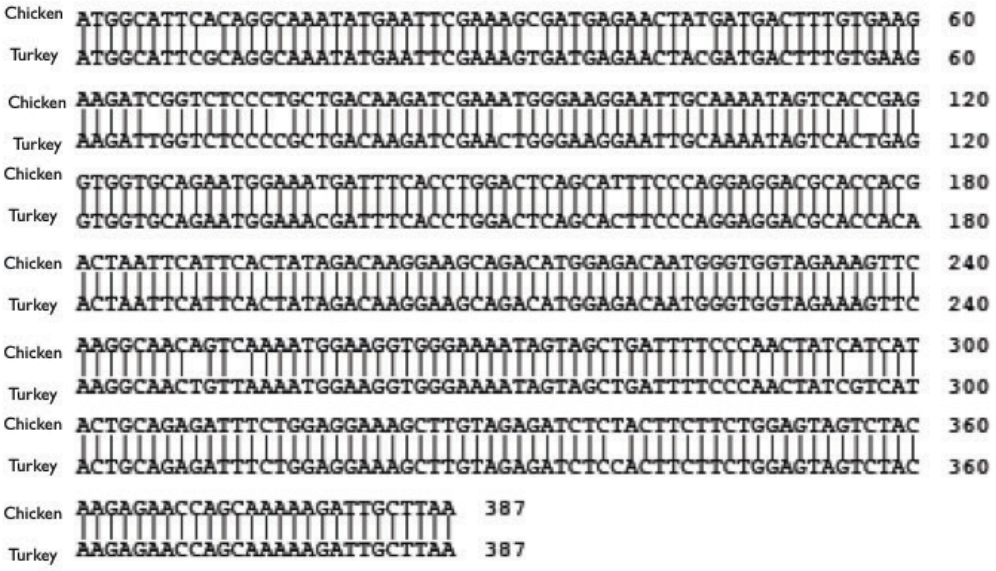

Figure 10. Chicken and Turkey FABP6 mRNA Sequence Alignment. A 96% nucleotide sequence identity between the *Gallus Gallus* (XM_414486.2) and Turkey (*Meleagris gallopavo)* (XM_003210281.1) FABP6. Bars connecting mouse to chicken bp indicate nucleotide sequence identity

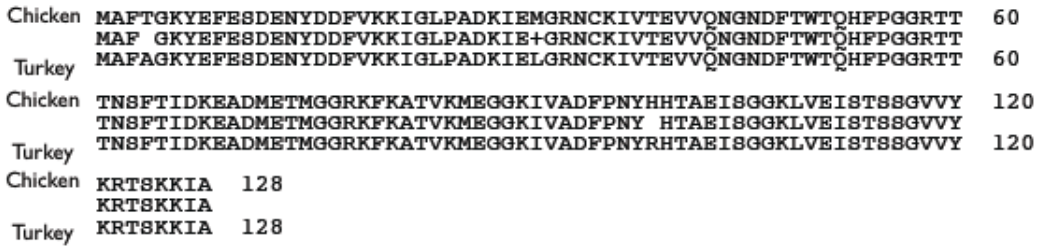

Figure 11. Chicken and Turkey FABP6 Amino Acid Sequence Alignment. Chicken (XP_414486.1) and Turkey (XP_003210329.1). If middle sequence has a + this is indicative of a similar amino acid, if left blank, amino acids are different.

The ORF amplified from chicken and turkeys for ASBT showed that the gene is highly conserved in the poultry. There is a 97% and 99% sequence identity for nucleotide and amino acids, respectively. Differences in acids were primarily alternative codons. Amino acid variations at position 25 and 104 in chickens of serine and histidine to lysine and leucine, respectively (Figure 12 and 13).

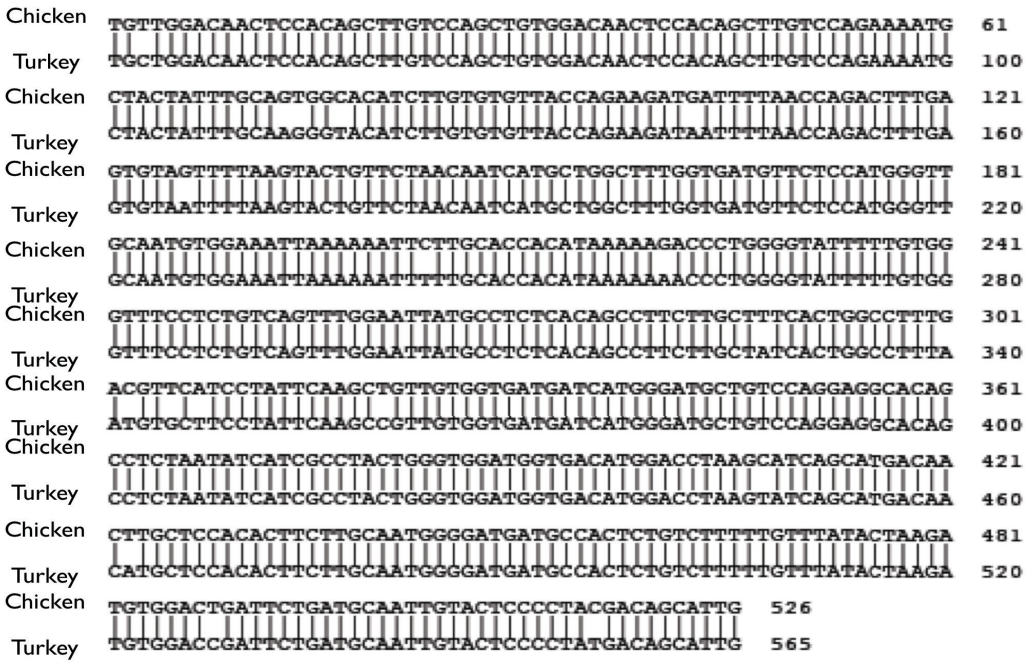

Figure 12. Chicken and Turkey ASBT mRNA sequence alignment. *Gallus gallus* (XM_425589.2 and *Meleagris gallopovo* (XM_003203223.1) share 97% sequence identities. Bars connecting mouse to chicken bp indicate nucleotide sequence identity

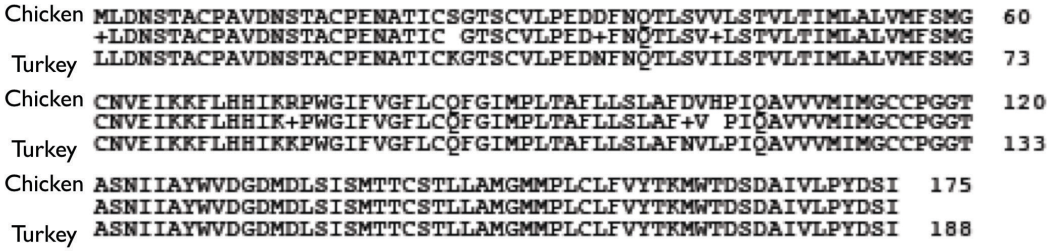

Figure 13. Chicken and Turkey ASBT amino acid sequence alignment. *Gallus gallus* and *Meleagris galloprovo* have 96% amino acid sequence identity with 99% amino acid similarity. Middle sequence shows sequence identity. If middle sequence has a + this is indicative of a codon variant, if left blank, amino acids are different.

Comparisons of the ORF of LXR in chicken (NM_204542.2) and turkeys (XM_003206414.1) demonstrate a 96% nucleotide sequence identity in 3153bp (data not shown). There was a 100% amino acid sequence identity (Figure 14) between Chicken (NP_989873.1)) and Turkey (XP_003206462.1). See figure 14.

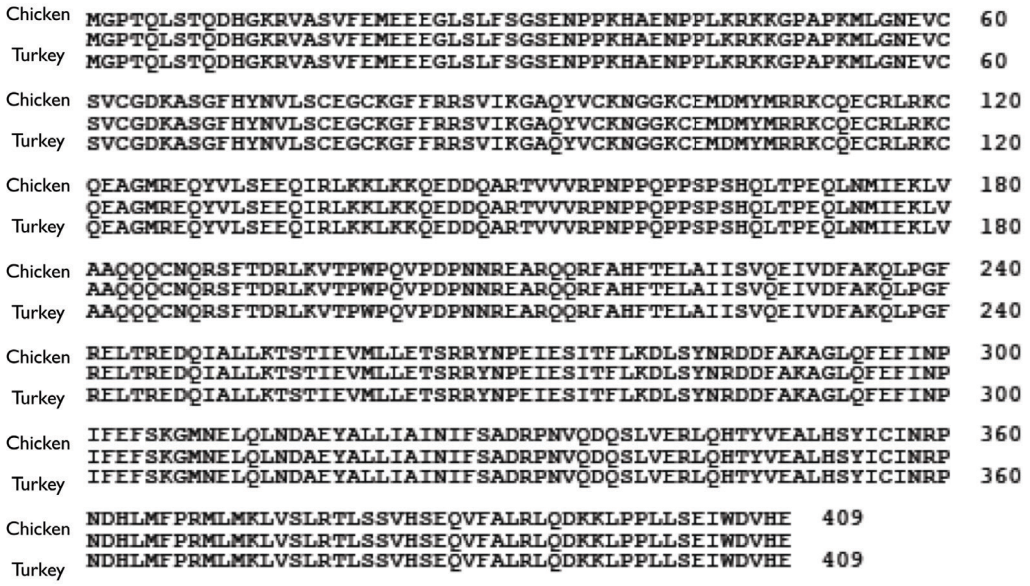

Figure 14. Comparison between Chicken and turkey LXR amino acid sequence. There is 100% sequence identity between the Chicken (NP_989873.1) and Turkey (XP_003206562.1). Middle sequence is the sequence identities.

The FXR sequence had a 96 % nucleotide sequence identity between chicken (NM_204113.1) and turkeys (XM_003202169) ORF (Data not shown). The amino acid sequence identity between chicken (NP_989444.1) and turkey (XP_003202217.1) was 99% and variations were noted at 3 positions: position 10 proline was replaced by serine, and at positions 13 and 48 threonine was replaced with alanine (Figure 15).

Figure 15. Comparison between Chicken and turkey FXR amino acid sequence. Chicken (NM_204113.1) and Turkey (XM_003202169) have 99% amino acid identities. If middle sequence has a '+' is indicative of codon variant, if left blank, amino acids are different.

Broilers:

mRNA

To determine the expression of FABP6 at the transcript level a quantitative PCR test was developed to amplify a 130 bp fragment spanning exons 3-4 in FABP6 . Specificity of the reaction was confirmed by melting curve analysis, gel electrophoresis and sequencing of the amplicon. All tissue mRNA abundance is measured in relation to the duodenal content, which was used as a reference tissue for this study. Tissue levels of transcript were observed to increase from low levels in the duodenum to the jejunum (about 4 fold higher than the duodenum) and to reach significantly higher levels (about 600 fold higher than the duodenum) in the ileum (Figure 16). In these tissues a similar fold increase in levels of ASBT mRNA were observed from the duodenum to the ileum (Figure 17). Levels of FABP6 mRNA were also higher compared to the duodenum, albeit non-significantly, in kidney, liver and testicular tissue. The expression of ASBT in those tissues were also not statistically significant. There was a high correlation between ASBT and FABP6 in respect to the tissue distribution resulted in an R^2 value of 0.987, ($p<0.0001$).

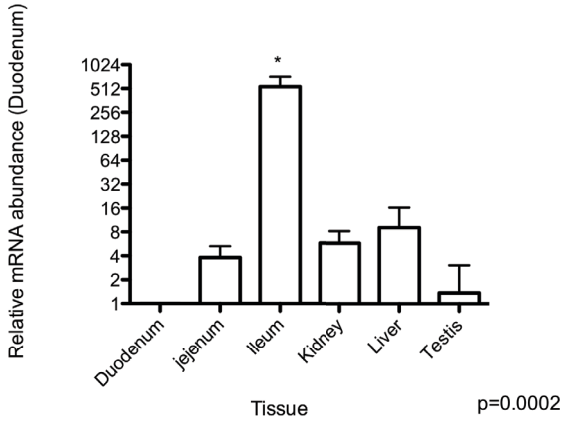

Figure 16. Fold changes in levels of FABP6 mRNA in broilers expressed as a function of duodenal content (n=5) in a log 2 scale. Means with an asterisk are significantly different ($p<0.05$).

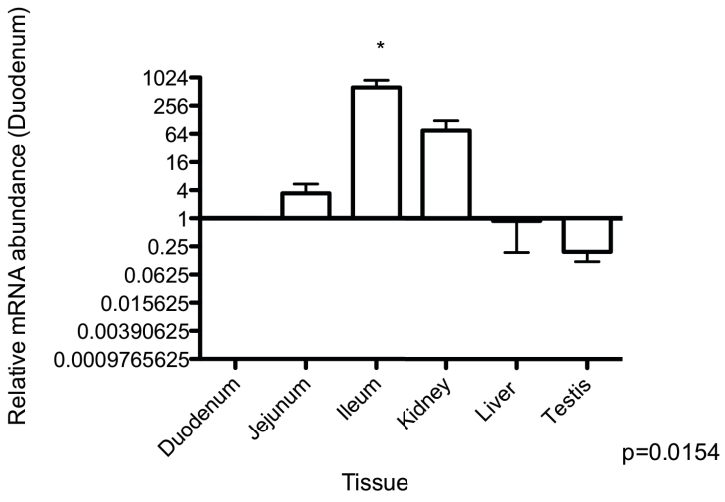

Figure 17. Fold changes in levels of ASBT mRNA in broilers expressed as a function of duodenal content (n=5) in a log 2 scale. Means with an asterisk are significantly different ($p<0.05$).

Protein

To identify the FABP6 protein in tissues of the broiler a murine antibody that cross-reacted with avian FABP6 was used., The western blot analysis show that the protein was abundantly expressed in the mouse ileum with a singular band at 14 kDa. Interestingly, in chicken tissues there appears to be two bands, a 14 kDa and 23 kDa band (Figure 18 A). Based on the 14 kDa band (Figure 18 B) levels in the ileum (4.5); jejunum (0.7); the kidney (2); liver (7.3); and testis (1.1) fold different in comparison to the duodenum. When the two bands are analyzed together (Figure 18C), the expression pattern of FABP6 follows closely with the mRNA. In the intestine the ileum has the highest abundance of FABP6 protein (17); liver (7.3); kidney (2.5). In the testis, the combination of the 14 kDa and 23 kDa band is 15 times higher relative to the duodenum. This does not correlate well to the mRNA, in which there was only a 1.37 fold change (137%).

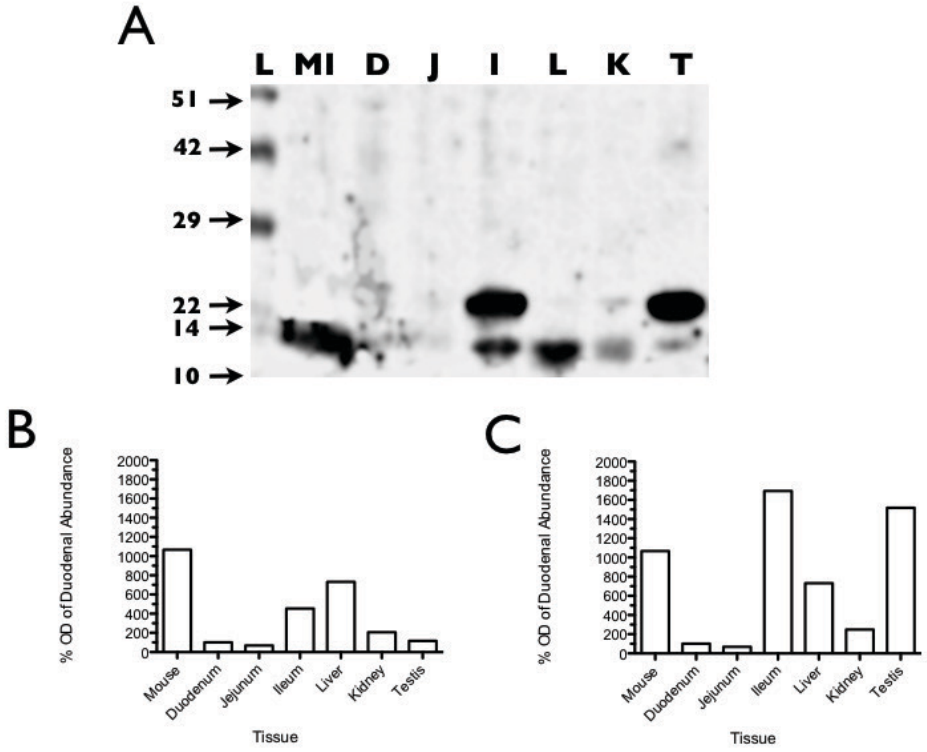

Figure 18. Immunoblot of FABP6 in Broilers A) Immunoblot of broiler tissues: (MI) Mouse Ileum as positive control, (D) Duodenum, (J) Jejunum, (I) Ileum, (L) Liver, (K) Kidney, (T) Testis. Graphs B and C are calculations of the optical density as a percentage relative to the duodenal segment. B) OD of 14 kDa band C) OD of all bands in lane

Layers

mRNA

The laying hens had a similar distribution of FABP6 in the small intestine as the male broilers. The duodenum was used as a reference tissue, meaning all data is presented as a function of the duodenal tissues. Levels of FABP6 increased by 1.5 fold from the duodenum to jejunum and levels in the ileum were about 800 fold ($p<0.05$) higher than in the duodenum. To determine the FABP6 gene expression in follicular development of the ovary in the hen, tissues collected from the ovarian follicles were separated based on size in the hierarchy (Figure 19). To define the follicles examined in this study, the small white follicles lack yolk and have a small diameter of 1mm-3mm. The small yellow follicles contain oocytes that take up yolk (5-6mm). The F1 follicle is >30mm diameter and is the next follicle to ovulate. There was no significant difference in FABP6 abundance in relation to size in the hierarchy. However, the small yellow follicle had the greatest fold change (15.9) relative to the duodenum($p>0.05$) ; while lower levels of FABP6 mRNA were identified in the stroma (1.54); F1 follicle (3.5); and small white follicle (2.1).

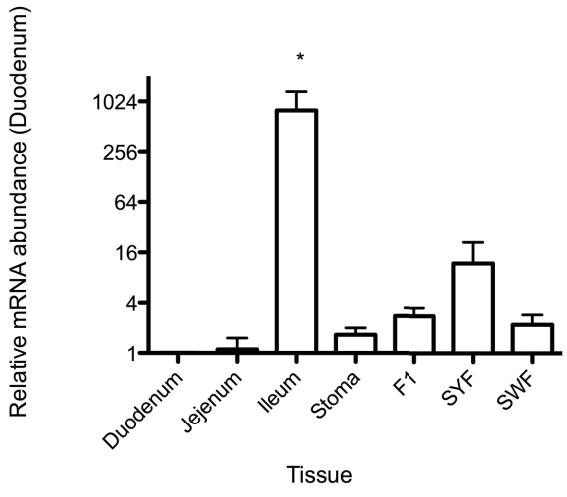

Figure 19. FABP6 in the female Intestine and Ovarian Follicles. Fold changes in levels of FABP6 mRNA in laying hen tissues are expressed as a function of duodenal content (n=5) in a log 2 scale. Means with an asterisk are significantly different ($p<0.05$).

Corresponding to the FABP6 abundance in the intestine and ovarian follicles, levels of ASBT mRNA in layers (Figure 20) was found to have greatest amounts of ASBT in the ileum (300 times the duodenum, P<.05)and small yellow follicles (11.9 times the duodenum, p>0.05). High correlation between FABP6 and ASBT with respect to tissue distribution resulted in an R^2 value of 1 (p<0.0001).

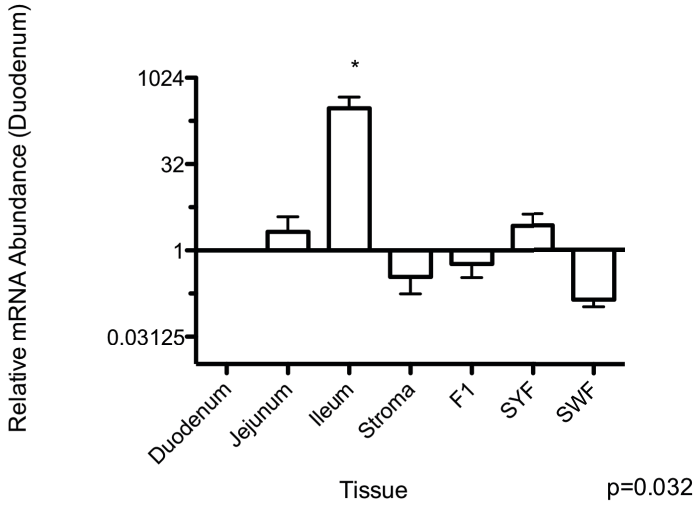

Figure 20. ASBT Female Intestine and Ovarian Follicles Relative Fold Change to Duodenum/18S. on a log 2 scale (n=5) Means are significantly different (p<0.05) Fold changes in levels of ASBT mRNA in laying hen tissues are expressed as a function of duodenal content. (n=5) Means with an asterisk are significantly different (p<0.05) .

The tissues of the theca and granulosa were separated in the F1 follicle, and their mRNA abundance of FABP6 and ASBT were compared. The granulosa cell laver was observed to have 8.4 times higher levels of FABP6 than the thecal cell layer (p>0.05) (Figure 21 A). Conversely, the granulosa cells were observed to have 0.63 times the ASBT mRNA abundance of the theca cells (p>0.05) (Figure 21 B). This lack of concordance between FABP6 and ASBT in ovarian steroidogenic tissue was confirmed by immunohistochemistry, in which the layers were viewed together.

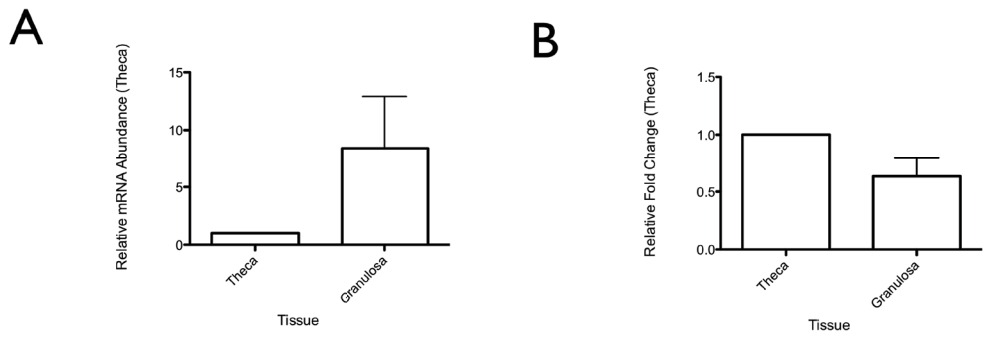

Figure 21. FABP6 and ASBT in the F1 Follicle Tissues (Granulosa and Theca) as a function of theca FABP6 mRNA content (n=6) in both A) FABP6 and B) ASBT. In A) Granulosa has 8.4 times greater FABP6 mRNA than theca (p>0.05) B) Thecal cells have 0.63 times ASBT mRNA compared to granulosa (p>0.05)

Protein

FABP6 was analyzed in the layer's intestinal segments and follicular hierarchy using 40μg of protein (Figure 22 A). Mouse ileal tissue was used a as a control, since a murine FABP6 antibody was used; and muscle tissue was used as a negative control, since there is no evidence that FABP6 is expressed in muscle tissue. A single band at 14 kDa is observed in the mouse ileum to verify the specificity of the antibody; while in the chicken three bands were observed. The 14 kDa and 23 kDa band that was present in the male immunoblot is also shown in the female immunoblot. There also appears to be a 29 kDa band in the intestinal segments and the ovarian stroma; and a 40 kDa band in the F1 and F2 follicle.

Based on the 14 kDa band (Figure 22 B) FABP6 has a similar expression to that of the male small intestine. There is slightly less FABP6 in the jejunum than in the duodenum, and a greater amount of FABP6 in the ileum (131%). In the follicular hierarchy the 14 kDa band can only be visualized in the small yellow follicle, 119% of the optical density in the duodenum. There is no FABP6 protein band in the small white follicle or the muscle.

The collective analysis of all bands in the lane reveal that the ileum has 210% of the FABP6 abundance in comparison to the duodenum; some

evidence of FABP6 in the stroma (37%), F1(14%), F2 (18%), and SYF (155%). (Refer to Figure 22 C)

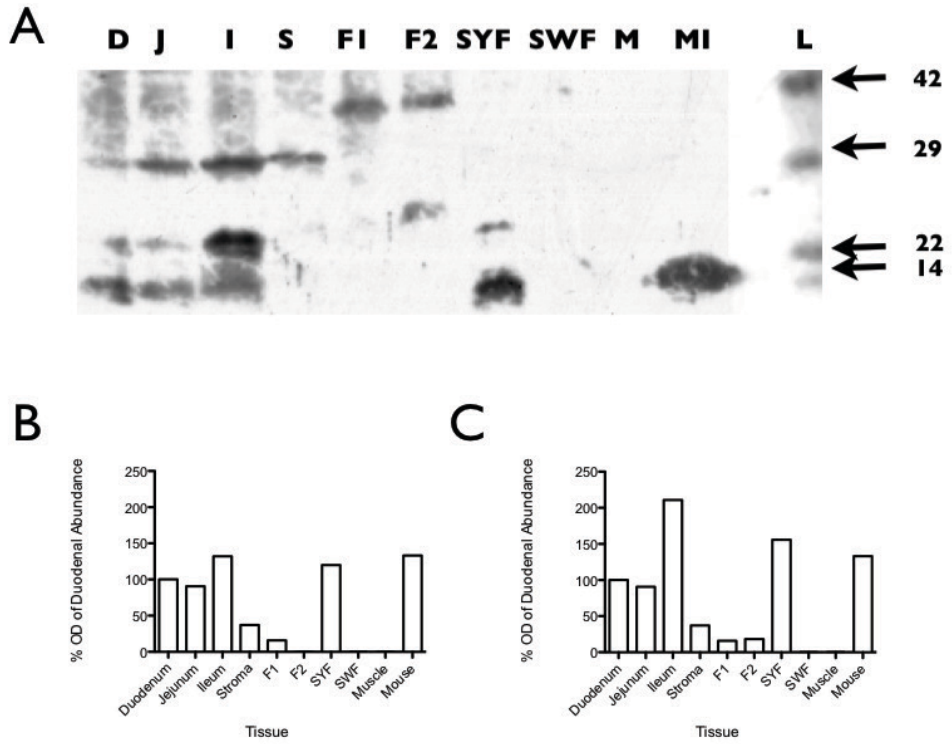

Figure 22. FABP6 Immunoblot Analyses. A) Immunoblot of FABP6 in the (D) duodenum, (J) Jejunum, (I) Ileum, (S) Stroma, (F1) F1 whole follicle (F2) F2 whole follicle, (SYF) Small Yellow Follicle, (SWF) Small White Follicle, (M) Muscle, and (MI) Mouse Ileum. Graphs B and C, measure optical density relative to the duodenum. B) OD 14 kDa band, C) OD of all bands in lanes

Immunohistochemistry detection was used to localize the FABP6 protein in the tissues of the laying hen's tissues of the duodenum, ileum, and follicles. The respective negative controls with no primary antibody were used to demonstrate the specificity of the primary antibody.

In the duodenum, there is some staining of FABP6 in the epithelium of the villus of the immunostained FABP6 tissue, with no staining in the control (Figure 23). In the ileum, there is ubiquitous staining in the epithelium in comparison to the duodenum (Figure 24).

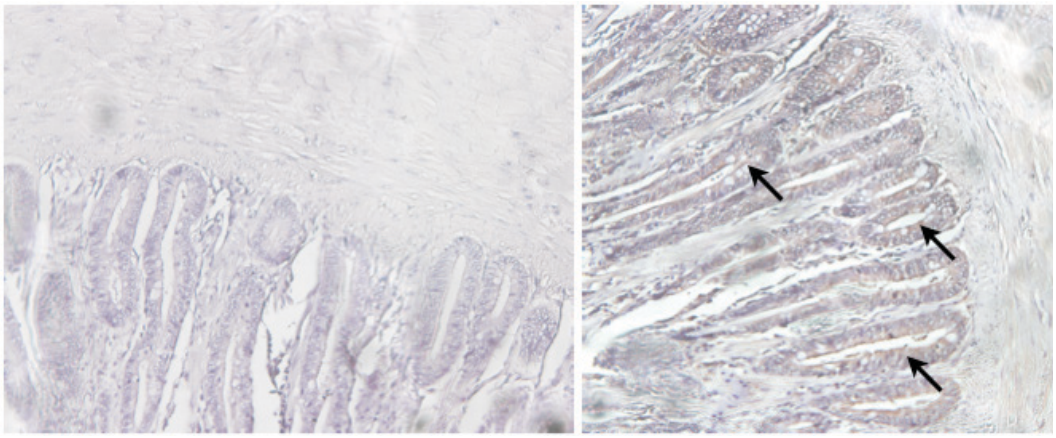

Figure 23. Immunoreactivity of the Duodenum in control (Left) and FABP6 (Right). In the duodenum there is a limited abundance of FABP6 immunoreactive staining with the Rabbit-antimouse antibody (1:200) dilution and HRP-conjugated anti-rabbit IgG (1:500). The control tissue has no primary antibody. In the FABP6 stained duodenum, arrows indicate sites of immunoreactivity with brown staining in the epithelium. Visualized using the Nikon DXM 1200C at 200X magnification

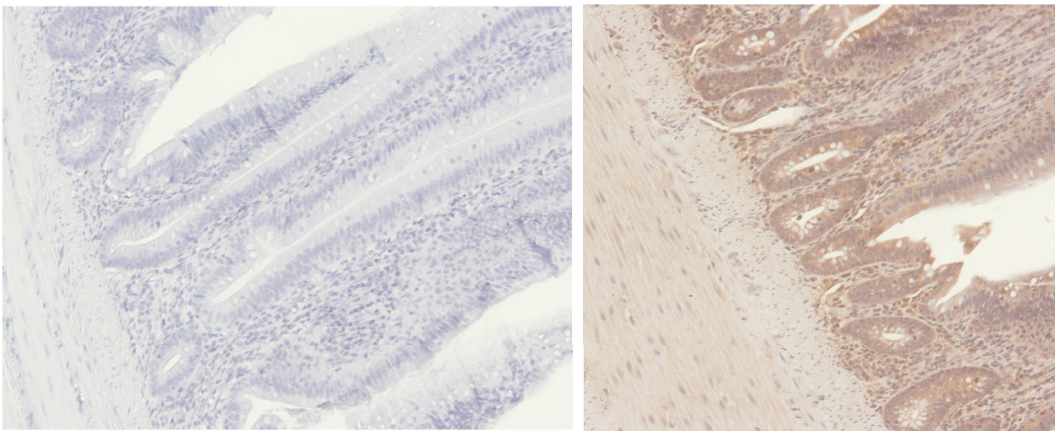

Figure 24. Immunoreactivity of the Ileum in control (Left) and FABP6 stained (Right) In the ileum, ubiquitous staining of the epithelium in the FABP6 tissue stained with rabbit antimouse antibody (1:200) dilution and HRP-conjugated anti-rabbit IgG (1:500). Control tissue has no primary antibody applied, therefore no reaction to DAB. Visualized using the Nikon DXM 1200C at 200X magnification

The immunostaining of follicles are presented in Figure 24. It was important in this study to localize the FABP6 in the tissues of the follicular hierarchy, since at varying stages of follicle development there are different steroidogenic roles and growth.

Immunoreactivity in the largest follicle, F1, is shown in Figure 24A. Based on this image, staining with the FABP6 primary antibody was restricted to both the granulosa and thecal tissues with no presence in the yolk. Figure 24B is a small yellow follicle, with very intense immunohistochemical staining in the

granulosa. Albeit, FABP6 was not restricted to the granulosa cells, but is also seen across the theca layer. It does appear that there is some also some staining in the yolk of the small yellow follicle; while no staining appeared in the larger follicle. In the small white follicles (Figure 24C), there unexpectedly appears to be some staining of the granulosa cells and the theca externa; with no staining in the theca interna cells.

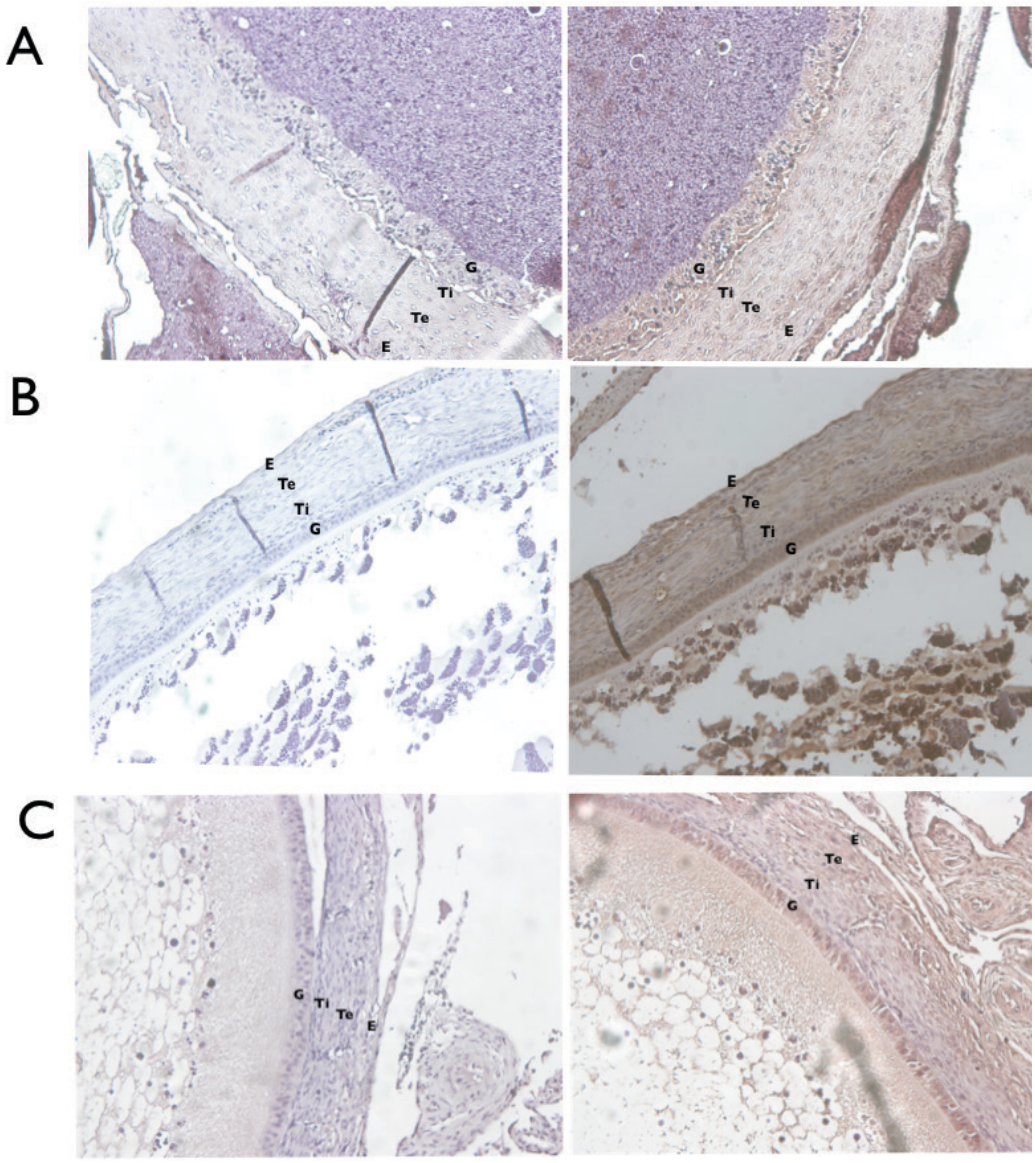

Figure 25. Immunoreactivity of Follicles. For all groups of images A-C images on the left panel are control tissues (no FABP6 antibody) to validate specificity of the primary antibody. Images on the Right are tissues that were incubated with the FABP6 Rabbit-Antimouse (1:200) and HRP-conjugated anti-rabbit IgG antibodies (1:500). Visualized using the Nikon DXM 1200C with 200X Magnification. Tissue Acronyms: Granulosa (G), Theca interna (Ti), Theca Externa (Te), Epithelium (E). Image groups: A) F1 Follicle of the follicular hierarchy B) Small Yellow Follicle C) Small White Follicle. There is no immunoreactive staining in tissues A-C. A) In the F1 follicle there is staining in the granulosa and thecal layers B) SYF follicles there is intense staining in the granulosa, and lower staining in the thecal layers and yolk. C) Light staining in the SWF, primarily in the granulosa and theca externa.

Turkey Embryo

The turkey embryo was studied to determine if FABP6 expression is present prior to hatch in an aim to support work in mammals and zebrafish that have shown FABP6 as being developmentally controlled.

At day 18ED, FABP6 mRNA was not detectable by Q-PCR (Ct>34) for all tissues. By 24ED, FABP6 mRNA was detectable in the tissues collected (intestinal segments, liver, gonad) but not in the muscle (Ct >34) (Figure 26). The distal segments of the small intestine exhibited a greater abundance of FABP6, than the duodenum—jejunum (3.05 fold greater) and ileum (3.49 fold greater) ($p>0.05$). Similar to the adult animals the gonad also expressed FABP6 a 1.27 fold the expression of the duodenum. At 28ED, there is a pronounced difference of FABP6 expression across the small intestine with a significant abundance ($p<0.05$) in the ileum (6966 fold greater than duodenum). FABP6 was also detected in abundance in reference to the duodenum: in the jejunum (4026 fold), liver (57.31 fold), kidney (2 fold), and gonad (50.4 fold).

Similar to the FABP6 expression profile, ASBT mRNA abundance was not detectable using Q-PCR at 18ED or 24ED; however at 28ED, ASBT is almost exclusively expressed ileum (5404 fold greater abundance that duodenum), with some expression in the jejunum (42.67 fold greater than the duodenum), with all other tissues being undetected (Ct >34) (Figure 28). FABP6 mRNA and ASBT mRNA likely are developmentally controlled, with rises in abundance close to the time of hatch.

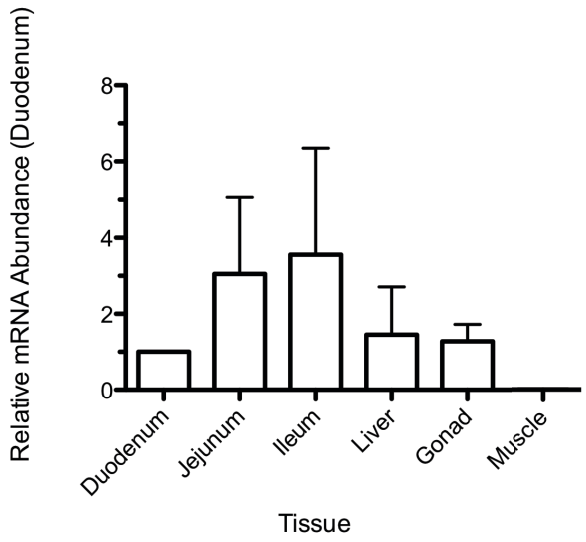

Figure 26. FABP6 mRNA in 24ED turkey embryo tissues measured as a function of the duodenal content (n=4). Means with are not significantly different (p>0.05).

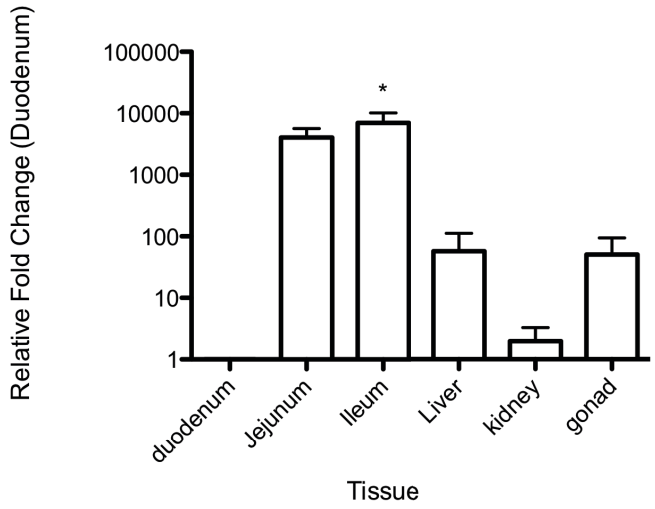

Figure 27. FABP6 mRNA in 28ED turkey embryo tissues measured as a function of the duodenum (n=6) on a log 10 scale. FABP6 was not detected in the muscle (Ct>34). Means are considered significantly different (p<0.01). Means with an asterisk are significantly different (p<0.05)

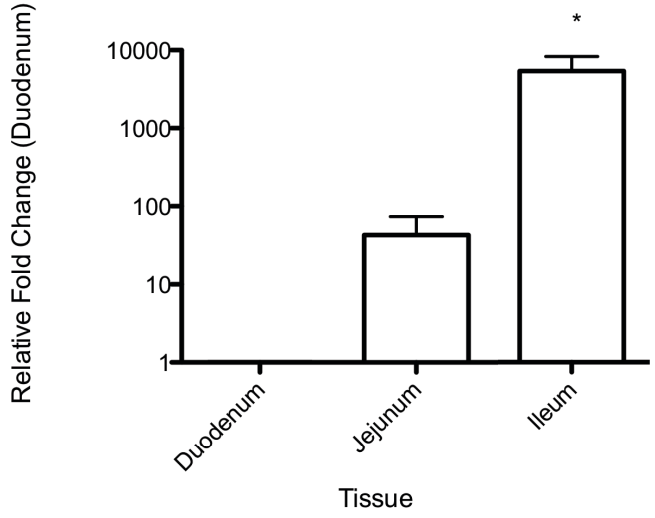

Figure 28. ASBT mRNA in 28ED turkey embryo tissues measured as a function of the duodenum (n=3) on a log 10 scale. ASBT was not detected in the muscle; liver, kidney, or gonad (Ct>34). Means are considered significantly different ($p<0.05$). Means with an asterisk are significantly different ($p<0.05$)

Discussion:

Studies focused on the roles of FABPs in non-mammalian vertebrates are limited. Most studies of FABP6 in the chicken have focussed on examining the molecular determinants of bile acid binding to recombinant FABP6 protein [31]. Localization of this protein to tissues and cell types have not been thoroughly investigated and indeed FABP6 has only been identified in the intestine of chicken and even then at a very superficial level. For example, FABP6 was cloned from ileal tissue in chickens [79]. However, specific distributions within tissues or changes in expression with differing physiological states have not been studied. The aim of the present study was to characterize the FABP6 gene at a physiological level by localizing of FABP6 in the tissues of poultry that are known to have abundance in mammals and zebrafish in an aim to make a comparative analysis and to give insight into possible functions of the FABP6. For this reason, this study included the use of males, females, and embryonic birds.

As a first step in characterization, FABP6 mRNA in both chickens and turkeys was sequenced. The sequence of the UTRs and the ORF fully matched the mRNAs predicted by the NCBI databases associated with the genomic sequencing projects of *Gallus gallus* and *Meleagris gallopavo.* Chicken and turkey FABP6 were highly conserved and sequence identity was 96 % and 98 % at the nucleotide and amino acid levels, respectively. The predicted 128 amino acid protein differed by 2 residues at positions 4 (threonine to cysteine) and 31 (methionine to leucine) in chickens and turkeys, respectively. In comparison to other species, chicken FABP6 had lower amino acid sequence identity (mouse 66% human 62%, rat 59% and zebrafish 55%). Nevertheless, FABP6 is considered to be highly conserved especially in tertiary structure across species and the gene likely arose from a common ancestor of the avian, fish, and mammalian species [10, 79].

An antibody generated against recombinant murine FABP6 [7] was used to analyze the immunoreactive staining in this study. Although mouse FABP6 protein has a 66 % identity to the chicken protein, the similarity (substitution of amino acid with another of similar physic-chemical properties) is 83 %. Given the high degree of similarity and the high degree of structural conservation, it was considered likely that the murine antibody would recognize similar epitopes in the chicken FABP6 protein. Indeed, a 14.3 kDa band was stained in protein homogenates from various tissues of the chicken which co-migrated with a band in the control murine ileal isolate in western blot. However, only a single band appeared in mouse isolates, whereas in the chicken, additional bands of approximately 23 kDa, 29 kDa, and 40 kDa were present in some chicken samples. Increasing the stringency of the hybridization and/or washing conditions did not prevent cross-hybridization with these additional bands. These additional bands may represent hybridization artefacts, although it is notable that in the muscle, which does not express FABP6 in mammalian species, no bands appear. It is also possible that these bands may represent splice variants of the FABP6 transcript, or be the result of post-translational modifications or dimerization. In humans, there is an isoform of FABP6 (FABP6 isoform 2, NM_001445.2) that contains seven exons. Both isoforms share a part of exon 4 and exons 5 to 7, however FABP6 isoform 2 has three unique exons at the 5' end of the gene. Thus, the open reading frame encodes an additional 49 residues following the initiator ATG that is absent in FABP6 and has a molecular weight of 19.8 kDa. In humans, FABP6 isoform 2 appears to be physiologically relevant acting as a buffer to prevent bile acid induced apoptosis in certain cells and is under the transcriptional regulation of NF-kB, unlike the FABP6 isoform 1 counterpart [80, 81]. Furthermore, it apparent that the FABP6 over-expressed lysate product (NBL1-10422, Novus Biologicals) has a similar expression profile of FABP6 with bands at molecular weights 14, 23, and 40, and 47 kDa to this study. Interestingly, the

bands in our current study at 14 kDa and 23 kDa appear to be identical to those bands in the silver-stained SDS-PAGE for ns-LTP conducted by Sams et al 1991. However, western blot analysis in the Sams et al., study show a distinct 14 kDa band that reacted with both purified ns-LTP and FABP1 with a goat-anti chicken ns-LTP[30]. Taken together, these results suggest that the additional bands observed in the current study may be related to the FABP6 protein. However, further experiments are required to resolve this issue.

ASBT in the turkey and chicken share 97% and 99% nucleotide and amino acid sequence identity, being highly conserved in *Aves*. ASBT orthologs have previously been reported in the rat, mouse, hamster, and rabbit [82]. Chicken amino acid sequence analysis previously conducted on ASBT indicate that FABP6 is conserved across numerous species, with mammalian, reptilian, and ascidiacea orthologs [81, 83, 84]. LXR and FXR have high nucleotide and amino acid identity between chickens and turkeys. Other studies indicate that LXR is also orthologous between humans and rats, sharing 86% identity [48]. Similarly, FXR is highly conserved between the chicken and turkey with orthologs in the Syrian hamster, mouse, rat, and human [85, 86]. The remarkable similarity between the sequences of these genes in various species suggests that they are involved in an evolutionarily conserved metabolic pathway, such as bile acid and steroid metabolism.

Tissue-Specific Distribution

The present study identified FABP6 mRNA in all three segments of the small intestine, liver, kidney, testis and ovarian follicles but not in the negative control of the muscle. No other studies have characterized FABP6 expression in the testis in mammalian and non-mammalian species. In concordance with our hypothesis we detected both mRNA and protein in steroidogenic tissues suggesting a potential link between steroid hormone biosynthesis and FABP6 function.

Intestine

In the chicken and turkey FABP6 is localized along the length of small intestine, with increasing expression in the ileum. This is similar to studies of FABP6 which reported the greatest abundance in the distal portion of the small intestine in other species. With the exception of Amano et al., most studies did not have measureable expression of FABP6 in the proximal regions [10, 17, 24]. In contrast our data indicate that the duodenum expressed low levels of FABP6 at both the transcript and protein levels as shown by mRNA abundance and immunoblot respectively. Moreover, immunohistochemical staining of the duodenum indicated immunoreactivity in the epithelium that may suggest the possible uptake of small amounts of bile acids in earlier regions of the chicken small intestine. However, the majority of bile acid uptake is in the ileum where levels of transcript, protein and immunohistochemical staining are most intense. This may be due to the observation that the major sites of active bile salt transport in poultry are not the major sites of lipid transport in mammals. In contrast to mammals, the contents of the small intestine are refluxed into the gizzard and lipid absorption occurs primarily in the duodenum in poultry. The active uptake of bile acids have similar rates of absorption in the jejunum and ileum in the chicken; and primarily in the duodenum turkey [68, 87, 88]. Furthermore, decreased levels of ileal FABP6 as assessed by DNA microRNA are associated with increased susceptibility to malabsorption syndrome (MAS) in broilers [89]. Therefore, it would seem likely that FABP6 abundance would correspond to the active uptake of bile acids along the entire length of the poultry intestine

In order to support active uptake of bile acid from the ingesta into the intestinal cell, the apical sodium bile acid transporter (ASBT) is required. This protein catalyzes the active transport of bile acids across the brush border cell membrane where FABP6 mediates its intracellular transport. Thus, FABP6 and ASBT are coexpressed in intestinal cells involved in bile acid transport [22]. In the current study, the levels of ASBT in tissues from

the proximal to distal small intestine increased in tandem with increases in levels of FABP6 and were highly correlated (R^2=0.98).

Kidney

FABP6 was detected in the kidney of the chicken broiler in this study at both the mRNA and protein levels. This observation may be physiologically relevant since bile acids are filtered by the glomerulus and reabsorbed in the proximal tubules [90] and both ASBT mRNA and protein are expressed at the apical membrane of the renal proximal convoluted tubule [91]. In agreement, the highest levels of ASBT are reported in the ileum and kidney, with less abundance in other tissues of mice[92]. Therefore the expression of both FABP6 and ASBT in kidney of the chicken suggest that these genes play an integral role in bile acid metabolism in this tissue. In the zebrafish, substantial levels of FABP6 have been identified in kidney and/or the adrenal homolog [10].

Liver

In this study, FABP6 was detected in the liver of poultry. The liver of zebrafish also had FABP6 but FABP6 has not been detected in the liver of the rat, mouse, or human [10, 20, 24, 26]. The role of FABP6 may have diverged during the evolution of mammalian and non-mammalian species. In concordance, the fatty acid binding proteins FABP9, FABP12, and CRABP2 all have no apparent avian orthologs [79].

The Na+/Taurocholate cotransporting polypeptide (NTCP) otherwise known as SLC10A1 is thought to be the homolog of ASBT in hepatocytes [82]. For this reason, ASBT was not predominant in the liver. FABP6 on the other hand could potentially function in a similar pattern in hepatocytes as it does in the ileum—intracellular transport of bile salts that are actively removed

from the blood by NTCP substituting for ASBT at the apical membrane of the hepatocyte.

The avian liver also expressed FABP1 (Liver FABP) and FABP10 (Chicken Liver Bile Acid Binding Protein). FABP10 is thought to take on the role of FABP6 in the liver functioning to bind to bile acids[93]. Presently it appears that potentially both bile acid transporting FABPs (FABP6 and FABP10) may be expressed simultaneously and have overlapping function. The distinction between the roles of these protein in the liver is unclear.

Testis

FABP9 was the only FABP that had been identified in the testis thus far. Its roles have been implicated in determining germ cell fate and the preservation of sperm quality[94].

FABP6 has not been detected in the testes of any species other than the chicken in the current study. FABP6 was predicted to be in the testes to support steroidogenic activity. The avian testes differs from the mammalian testes being situated in the central body cavity ventral and toward the cephalic border of the kidney. In contrast to mammals, spermatozoa in the testes are produced at a higher proliferation rate, requiring 14 days for the spermatogenic process in comparison to 35 days and 64 days in mice and humans, respectively. Furthermore, in birds steroidogenesis is not limited to the leydig cells; sertoli cells actively express 3β-HSD, an enzyme involved in steroidogenesis in contrast to rodent sertoli cells [95, 96]. However, a putative role in testicular steroidogenesis requires further investigation since although FABP6 is known to bind progesterone [24] , interactions with other steroid metabolites in not known.

Ovarian Follicles

FABP6 was expressed in the ovarian follicles of the laying hens.. Small yellow follicles had the greatest expression of FABP6 and ASBT in the

follicular hierarchy. The expression of FABP6 was predicted to be localized to the granulosa of the large ovulating follicle because progesterone bound to FABP6 more effectively than estrodiol [24]. Furthermore in the ovary of rats FABP6 was found exclusively in the corpora lutea—the site of progesterone biosynthesis in mammals. In addition, FABP6 was not expressed in the granulosa or theca cells within rats when examined by immuno-electron microscopy [14]. Unlike mammals, FABP6 was identified in chickens in the granulosa, theca, and stroma layers of follicles of all sizes. Although it appears that there is a greater mRNA and intense immunoreactive staining of FABP6 in the F1 follicle's granulosa; FABP6 is not restricted to the progesterone producing cells of the ovary. It does appear that the ASBT and FABP6 in the ovarian expression is uncoupled, since the ASBT has a greater abundance in the theca. But based on our overall tissue analysis of mRNA, there is a relatively low abundance of ASBT in most of the tissues other than the small intestine and small yellow follicles.

The observation that FABP6 being more highly expressed in the small yellow follicles is important to note, since the granulosa layer at this stage of follicular development is incapable of producing progesterone due to a low level of steroid acute regulatory (StAR) protein and P450scc [97, 98]. Individual whole follicles less than 10mm in diameter are unable to produce progesterone, but can produce DHEA, androstendione, and estrodiol [98]. Estrodiol is synthesized in the theca externa, while progesterone is converted to the testosterone in the theca interna [99, 100]. For this reason, in the SWF immunoreactivity is primarily stained at the site with the greatest steroidogenesis the theca externa, with less intensity in the theca interna.

Small ovarian follicles produce estrogens and androgens to stimulate the calcium deposition during egg shell formation. The smaller follicles convert pregnenolone to androstendione via the Δ^5 pathway in the conversion from pregnenolone to 17α-hydroxypregnenolone to androstendione in the theca. After the follicles are recruited into the yolk-

filled hierarchy, the pregnenolone is converted into androstendione via the Δ^5 pathway (Pregnenolone→dehydroepiandrosterone→Androstensterone) [101]. Once the follicles begin to sequester yolk there is a decline in estrogen production and an increase in androgens [102, 103]. This is due to the low levels of StAR protein and P450scc in the granulosa of white follicles [97]. In accordance to this change in hormone production, there is a gradual shift of 3β-HSD from the theca layer of small follicles to the granulosa of preovulatory and postovulatory follicles with follicular maturation [104]. Our results are similar to this steroid hormone synthesis pattern since FABP6 is more predominant in yolk filled follicles (SYF) and the F1 follicle in comparison to the SWF. Stromal tissues had limited FABP6 abundance. However, stroma cells do respond to LH, producing progesterone androstendione, and estrogen and thus contribute to steroid hormone metabolism and to total steroidogenic output.

LXRα gene expression that were measured at different stages in the chicken follicle development by Seol et al., demonstrating that chicken follicles express higher LXRα in the rapid growth stages of follicle development (F5 to F1) compared to the SWF. However levels of LXRα were greater in the F3 and F2 compared to SYF[105]. Corresponding to that study, FABP6 was greater in the SYF in comparison to SWF; however, levels of FABP6 protein was greater in the SYF than both the F1 and F2 follicles. Thus, it can be postulated that FABP6 abundance is under the regulation of LXRα in the ovary.

ASBT's presence in the ovary primarily at the small yellow follicle phase could be indicative of its role in steroid metabolism; or FABP6 and ASBT may interact to have a role in the growth of the follicular hierarchy by functioning in the recruitment of lipids and cholesterol for growth and yolk development. The ovary of the hen, in addition to the liver, is a primary site of cholesterol biosynthesis[105]. Furthermore, at least three components of bile acid metabolism are localized in the ovary (LXRα, FABP6, and ASBT).

Ten days prior to ovulation yolk is rapidly deposited from precursors synthesized by the liver stimulated by estrogens. About 10% of the yolk are plasma proteins that are actively uptaken from the plasma. Specific binding proteins for riboflavin, thiamine, vitamin A, calciferol are found in the yolk, including retinol binding protein. Therefore FABP6 may be important in the regulation of cholesterol metabolism which would be expected to vary throughout the egg laying cycle. Parameters associated with the timing of ovulation and oviposition were not measured in the current study but major effects of daily hormonal rhythms should have substantial effects on the expression patterns of FABP6 as the ovarian hierarchy progresses throughout the ovulatory cycle. For example, FSH and LH-induced steroidogenesis are important in the transition from pre-hierarchical follicles to the preovulatory follicles in stimulating the expression of both StAR protein and P450scc, and ultimately progesterone from 3β-HSD activity in the growing hierarchal follicles[97, 98, 106].

Embryos

At the 18, 24 and 28 embryonic days of the turkey, FABP6 and ASBT were measured to gain insight into their expression prior to hatch. FABP6 and ASBT were undetected at 18ED. At 24 ED, FABP6 was expressed , having a similar distribution pattern in tissues to adults—it was present in the all segments of the small intestine, liver, and gonad. In the current study ASBT is not observed in the embryo at this time point, implying that ASBT is developmentally controlled in poultry. By 28ED, both FABP6 was detected in the duodenum, jejunum, ileum, liver, kidney, and gonad—with greatest abundance in the jejunum and ileum. ASBT on the other hand, was only detected in the intestinal segments of the jejunum and ileum. Previous studies on FABP6 and ASBT have reported a developmentally related increase in FABP6 and ASBT associated the weaning period in mammals [107]. In precocial birds, the yolk represents a major nutrient supply for the

developing embryo prior to hatch. Yolk is transported to the small intestine by the yolk stalk and through antiperistaltic contractions of the small intestine and is digested by pancreatic enzymes at the brush border prior to absorption. Within the first 48 hours post-hatch, the chick needs to transition from digestion of energy from the lipids in the yolk to exogenous complex carbohydrates [108, 109]. Corresponding to changes of villi growth for enhanced nutrient absorption it can be predicted that the expression of ASBT and FABP6 would also increase in abundance in the small intestine as the bird develops [110]

In mammals, the FABP6 gene was not expressed in the fetal ileum during the suckling period of rats, but rose in abundance during the weaning period [16]. Therefore, FABP6 may be developmentally controlled in the rat and may respond to the rise of free corticosterone during the weaning period or in response to the change of diet. In contrast, turkey FABP6 transcript was expressed by 24ED throughout the small intestine with increasing levels from the duodenum to the ileum similar to the pattern observed in larval zebrafish [10]. This increase in the turkey embryo was not accompanied by the concurrent expression of ASBT. However, at 28ED there is significant evidence of ASBT expression. Therefore, further studies should be undertaken to examine the stage when the intestine is capable of ASBT expression, and whether this is related to digestive maturity similar to the developmental pattern observed in mammals.

In poultry, the small intestine enters the final stages of differentiation 3 to 4 days before hatching[111, 112]. Turkeys have an incubation period of 28ED—for this reason the rise in abundance of FABP6 and ASBT mRNA in the distal segments of the small intestine may be exhibited at this stage of development in comparison to the 24ED time point. The changes of FABP6 and ASBT abundance in the small intestine could be a result of a corresponding increase in bile acid concentrations in the plasma and liver. Sato et al., (2008), examined chicken embryos—that have an incubation

period of 24ED—at 14ED, 18ED, and hatch measuring bile acid and genes associated to bile acid synthesis (CYP7A1, SREBP, LXR, and FXR) in the liver. There is a marked increase of bile acids and CYP7A1 activity from 14ED to the newly hatched chicks[53]. Interestingly, FABP6 was also expressed in the liver at 24ED and 28ED. FABP6 expression could contribute to the transport of bile acids in both sites of bile acid metabolism during development.

It would be interesting to note the simultaneous changes of FABP10 in the liver, as it is also a known bile acid transporter in chickens. It has been previously shown that the expression of FABP1 and FABP10 were co-ordinately increased in the livers of J. Quail during the hatch stage and are differentially expressed in response to the diurnal cycle and feeding conditions [113]. How FABP6 may be interacting with FABP1 and FABP10 in the liver is unclear.

Further study should be done to elucidate the bile acid absorption and correlation FABP6, ASBT, FABP10 and nuclear hormone receptors in the both the liver and small intestine of embryos and post-hatch birds and to fully understand the enterohepatic circulation during embryonic development and chick growth.

Conclusion

FABP6 is a highly conserved gene across species linked to regulating cholesterol metabolism in tissues involved in bile acid metabolism and steroid hormone biosynthesis. This study characterizes the FABP6 gene expression in poultry involved in the enterohepatic circulation (intestine, liver, kidney) and steroidogenesis (testis and ovarian follicles).

The expressional pattern of FABP6 in the poultry small intestine is comparable to mammals and zebrafish, with an increasing abundance towards the ileum. In all of the three study groups of poultry conducted, there was the detection of FABP6 and ASBT proximal to the ileum. In contrast, FABP6 and ASBT potentially respond to the active uptake of bile acids in the segments proximal to the ileum due to the repetitive reflux of bile back to the gizzard from the entry of the bile duct at the distal end of the duodenum—a physiological mechanism not exhibited by mammals. FABP6 and ASBT were also localized to the kidney of poultry, which is capable of bile acid reabsorption through the renal proximal tubules. Furthermore, FABP6 was detected in the liver of poultry possibly as an adjunct to FABP10 in binding of bile acids on their return from the portal circulation.

Collectively, the findings in this study and reference to previous work on FABP6 and bile acid uptake suggest FABP6 has an essential role of tightly regulating intracellular bile acid transport.

FABP6 is clearly involved in bile acid metabolism; however, its presence in the ovarian follicles, testis, and gonad (in the embryo) extend the notion that FABP6's function is not limited in regulating bile acids. Steroid hormones and bile acids are derivatives of cholesterol sharing similar biosynthetic pathways and physicochemical properties and are likely both ligands of FABP6. Further work must be conducted to determine the exact mechanisms and identifying the specific steroid hormones capable of binding

to FABP6 and ASBT. This study shows that FABP6 in the hen is not limited to the progesterone producing tissues of the F1 follicle, having a greater abundance in the small yellow follicles that are essential in the production of androgens using the Δ^4 pathway. Likely FABP6 is important in the intracellular trafficking of steroid hormone metabolites. FABP6 appears to have differential expression in folliculogenesis of the hen.

In contrast studies to mammals, FABP6 was identified in the tissues of the turkey embryo. ASBT however, was not detected until a later stage of development. It would be interesting to conduct further developmental studies on the expression of FABP6 since poultry are precocial creatures that internalize the yolk by hatch during the transition from feed dependence--analogous to the weaning period of mammals.

In characterizing FABP6 in poultry further analysis of the functional roles of FABP6 should be conducted. Ideally the research conducted in this field will further examine the similarities of bile acids and steroid hormones to gain a better understanding of cholesterol homeostasis and reproductive efficiency.

References

1. Ockner, R.K. and J.A. Manning, *Fatty acid-binding protein in small intestine. Identification, isolation, and evidence for its role in cellular fatty acid transport.* J Clin Invest, 1974. **54**(2): p. 326-38.
2. Storch, J. and A.E. Thumser, *The fatty acid transport function of fatty acid-binding proteins.* Biochim Biophys Acta, 2000. **1486**(1): p. 28-44.
3. Yamamoto, T., et al., *Classification of FABP isoforms and tissues based on quantitative evaluation of transcript levels of these isoforms in various rat tissues.* Biotechnology Letters, 2009. **31**(11): p. 1695-1701.
4. Hertzel, A.V. and D.A. Bernlohr, *The mammalian fatty acid-binding protein multigene family: Molecular and genetic insights into function.* Trends in Endocrinology and Metabolism, 2000. **11**(5): p. 175-180.
5. Levin, L.B., et al., *Insight into the interaction sites between fatty acid binding proteins and their ligands.* Journal of Molecular Modeling, 2010. **16**(5): p. 929-938.
6. Landrier, J.F., et al., *Regulation of the ileal bile acid-binding protein gene: an approach to determine its physiological function(s).* Mol Cell Biochem, 2002. **239**(1-2): p. 149-55.
7. Labonte, E.D., et al., *The relative ligand binding preference of the murine ileal lipid binding protein.* Protein Expr Purif, 2003. **28**(1): p. 25-33.
8. Lucke, C., et al., *Flexibility is a likely determinant of binding specificity in the case of ileal lipid binding protein.* Structure, 1996. **4**(7): p. 785-800.
9. Haunerland, N. and F. Spener, *Properties and physiological significance of fatty acid binding proteins.* Advances in Molecular and Cell Biology, 2003. **33**: p. 99-123.
10. Alves-Costa, F.A., et al., *Spatio-temporal distribution of fatty acid-binding protein 6 (fabp6) gene transcripts in the developing and adult zebrafish (Danio rerio).* Febs Journal, 2008. **275**(13): p. 3325-3334.
11. Wider, M.D., A.I. Vinik, and A. Heldsinger, *Isolation and partial characterization of an entero-oxyntin from porcine ileum.* Endocrinology, 1984. **115**(4): p. 1484-91.
12. Walz, D.A., et al., *The complete amino acid sequence of porcine gastrotropin, an ileal protein which stimulates gastric acid and pepsinogen secretion.* J Biol Chem, 1988. **263**(28): p. 14189-95.
13. Borgstrom, A., et al., *Immunohistochemical localization of a specific ileal peptide in the pig.* Histochemistry, 1986. **86**(1): p. 101-5.
14. Iseki, S., et al., *Expression and localization of intestinal 15 kDa protein in the rat.* Mol Cell Biochem, 1993. **123**(1-2): p. 113-20.
15. Gantz, I., et al., *Gastrotropin: not an enterooxyntin but a member of a family of cytoplasmic hydrophobic ligand binding proteins.* J Biol Chem, 1989. **264**(34): p. 20248-54.

16. Sacchettini, J.C., et al., *Developmental and structural studies of an intracellular lipid binding protein expressed in the ileal epithelium.* J Biol Chem, 1990. **265**(31): p. 19199-207.
17. Agellon, L.B., M.J. Toth, and A.B. Thomson, *Intracellular lipid binding proteins of the small intestine.* Mol Cell Biochem, 2002. **239**(1-2): p. 79-82.
18. Kanda, T., et al., *Primary structure of a 15-kDa protein from rat intestinal epithelium. Sequence similarity to fatty-acid-binding proteins.* Eur J Biochem, 1991. **197**(3): p. 759-68.
19. Bonne, A., et al., *Sequencing and chromosomal localization of Fabp6 and an intronless Fabp6 segment in the rat.* Molecular Biology Reports, 2003. **30**(3): p. 173-176.
20. Fujita, M., et al., *Molecular cloning, expression, and characterization of a human intestinal 15-kDa protein.* Eur J Biochem, 1995. **233**(2): p. 406-13.
21. Agellon, L.B., et al., *Adaptations to the loss of intestinal fatty acid binding protein in mice.* Mol Cell Biochem, 2006. **284**(1-2): p. 159-66.
22. Kramer, W., et al., *Intestinal bile acid absorption. Na(+)-dependent bile acid transport activity in rabbit small intestine correlates with the coexpression of an integral 93-kDa and a peripheral 14-kDa bile acid-binding membrane protein along the duodenum-ileum axis.* J Biol Chem, 1993. **268**(24): p. 18035-46.
23. Stengelin, S., et al., *The rabbit ileal lipid-binding protein. Gene cloning and functional expression of the recombinant protein.* Eur J Biochem, 1996. **239**(3): p. 887-96.
24. Gong, Y.Z., et al., *Molecular cloning, tissue distribution, and expression of a 14-kDa bile acid-binding protein from rat ileal cytosol.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4741-5.
25. Amano, O., et al., *Immunocytochemical localization of rat intestinal 15 kDa protein, a member of cytoplasmic fatty acid-binding proteins.* Anat Rec, 1992. **234**(2): p. 215-22.
26. Fujii, H., et al., *Cloning of a cDNA encoding rat intestinal 15 kDa protein and its tissue distribution.* Biochem Biophys Res Commun, 1993. **190**(1): p. 175-80.
27. Ono, T., *Studies of the FABP family: A retrospective.* Molecular and Cellular Biochemistry, 2005. **277**(1-2): p. 1-6.
28. Vasile, F., et al., *Solution structure of chicken liver basic fatty acid binding protein.* J Biomol NMR, 2003. **25**(2): p. 157-60.
29. Sewell, J.E., et al., *Isolation and characterization of two fatty acid binding proteins from intestine of Gallus domesticus.* Comp Biochem Physiol B, 1989. **92**(4): p. 623-9.
30. Sams, G.H., B.M. Hargis, and P.S. Hargis, *Identification of two lipid binding proteins from liver of Gallus domesticus.* Comp Biochem Physiol B, 1991. **99**(1): p. 213-9.

31. Guariento, M., et al., *Chicken ileal bile-acid-binding protein: a promising target of investigation to understand binding co-operativity across the protein family.* Biochem J, 2010. **425**(2): p. 413-24.
32. Guariento, M., et al., *Identification and functional characterization of the bile acid transport proteins in non-mammalian ileum and mammalian liver.* Proteins-Structure Function and Bioinformatics, 2008. **70**(2): p. 462-472.
33. Tabas, I., *Cholesterol in health and disease.* J Clin Invest, 2002. **110**(5): p. 583-90.
34. Repa, J.J. and D.J. Mangelsdorf, *The role of orphan nuclear receptors in the regulation of cholesterol homeostasis.* Annu Rev Cell Dev Biol, 2000. **16**: p. 459-81.
35. Zaghini, I., et al., *Sterol regulatory element-binding protein-1c is responsible for cholesterol regulation of ileal bile acid-binding protein gene in vivo. Possible involvement of liver-X-receptor.* J Biol Chem, 2002. **277**(2): p. 1324-31.
36. Matsuyama, H., et al., *Modulation of regulatory factors involved in cholesterol metabolism in response to feeding of pravastatin- or cholesterol-supplemented diet in chic.* Biochemica et Biophysica Acta - Molecular and Cell Biology of Lipids, 2005. **1734**(2): p. 136-142.
37. Russell, D.W. and K.D. Setchell, *Bile acid biosynthesis.* Biochemistry, 1992. **31**(20): p. 4737-49.
38. Etches, R.J., et al., *Follicular growth and maturation in the domestic hen (Gallus domesticus).* J Reprod Fertil, 1983. **67**(2): p. 351-8.
39. Rangel, P.L., P.J. Sharp, and C.G. Gutierrez, *Testosterone antagonist (flutamide) blocks ovulation and preovulatory surges of progesterone, luteinizing hormone and oestradiol in laying hens.* Reproduction, 2006. **131**(6): p. 1109-14.
40. Griffin, H.D., *Manipulation of Egg-Yolk Cholesterol - a Physiologists View.* Worlds Poultry Science Journal, 1992. **48**(2): p. 101-112.
41. Burczak, J.D., J.L. Mcnaughton, and T.F. Kellogg, *Cholesterol-Metabolism in Poultry, Gallus-Domesticus - Fecal Neutral Sterol and Bile-Acid Excretion.* Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 1980. **66**(3): p. 385-389.
42. Mitchell, A.D., et al., *Hepatic HMG CoA reductase and cholesterol 7 alpha-hydroxylase activities in normal and hyperlipidemic-restricted ovulator atherosclerosis-prone chickens before and after the commencement of egg laying.* Atherosclerosis, 1979. **32**(1): p. 11-21.
43. Sato, K., et al., *Changes in mRNA expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol 7 alpha-hydroxylase in chickens.* Biochim Biophys Acta, 2003. **1630**(2-3): p. 96-102.
44. Landrier, J.F., et al., *FXRE can function as an LXRE in the promoter of human ileal bile acid-binding protein (I-BABP) gene.* FEBS Lett, 2003. **553**(3): p. 299-303.

45. Chiang, J.Y.L., R. Kimmel, and D. Stroup, *Regulation of cholesterol 7a-hydroxylase gene (CYP7A1) transcription by the liver orphan receptor (LXRa)* GENE, 2001: p. 257-265.
46. Besnard, P., et al., *[Is the ileal bile acid-binding protein (I-BABP) gene involved in cholesterol homeostasis?].* Med Sci (Paris), 2004. **20**(1): p. 73-7.
47. Handschin, C., et al., *Cholesterol and bile acids regulate xenosensor signaling in drug-mediated induction of cytochromes P450.* J Biol Chem, 2002. **277**(33): p. 29561-7.
48. Demeure, O., et al., *Liver X receptor alpha regulates fatty acid synthase expression in chicken.* Poult Sci, 2009. **88**(12): p. 2628-35.
49. Gnerre, C., et al., *LXR deficiency and cholesterol feeding affect the expression and phenobarbital-mediated induction of cytochromes P450 in mouse liver.* J Lipid Res, 2005. **46**(8): p. 1633-42.
50. Alrefai, W.A. and R.K. Gill, *Bile acid transporters: structure, function, regulation and pathophysiological implications.* Pharm Res, 2007. **24**(10): p. 1803-23.
51. Agellon, L.B. and E.C. Torchia, *Intracellular transport of bile acids.* Biochim Biophys Acta, 2000. **1486**(1): p. 198-209.
52. Zhang, Y. and D.J. Mangelsdorf, *LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing.* Mol Interv, 2002. **2**(2): p. 78-87.
53. Sato, M., K. Sato, and M. Furuse, *Change in hepatic and plasma bile acid contents and its regulatory gene expression in the chicken embryo.* Comp Biochem Physiol B Biochem Mol Biol, 2008. **150**(3): p. 344-7.
54. Goodwin, B., et al., *Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha.* Mol Endocrinol, 2003. **17**(3): p. 386-94.
55. Capaldi, S., et al., *The X-ray structure of zebrafish (Danio rerio) ileal bile acid-binding protein reveals the presence of binding sites on the surface of the protein molecule.* J Mol Biol, 2009. **385**(1): p. 99-116.
56. Grober, J., et al., *Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene - Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer.* Journal of Biological Chemistry, 1999. **274**(42): p. 29749-29754.
57. Elkin, R.G., K.V. Wood, and L.R. Hagey, *Biliary bile acid profiles of domestic fowl as determined by high performance liquid chromatography and fast atom bombardment mass spectrometry.* Comp Biochem Physiol B, 1990. **96**(1): p. 157-61.
58. Saeki, T., et al., *Characterization, cDNA cloning, and functional expression of mouse ileal sodium-dependent bile acid transporter.* Journal of Biochemistry, 1999. **125**(4): p. 846-851.
59. Barley, N.F., et al., *Human ileal bile acid-binding protein promoter and the effects of CDX2.* Biochim Biophys Acta, 2003. **1630**(2-3): p. 138-43.

60. Krasowski, M.D., et al., *Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR.* Molecular and Cellular Endocrinology, 2010.
61. Ho, R.H., et al., *Functional Characterization of Genetic Variants in the Apical Sodium-Dependent Bile Acid Transporter (ASBT; SLC10A2).* J Gastroenterol Hepatol, 2011.
62. Chen, X., et al., *Transactivation of rat apical sodium-dependent bile acid transporter and increased bile acid transport by 1alpha,25-dihydroxyvitamin D3 via the vitamin D receptor.* Mol Pharmacol, 2006. **69**(6): p. 1913-23.
63. Shneider, B.L., K.D. Setchell, and M.W. Crossman, *Fetal and neonatal expression of the apical sodium-dependent bile acid transporter in the rat ileum and kidney.* Pediatr Res, 1997. **42**(2): p. 189-94.
64. Kanchanapoo, J., et al., *Weanling but not adult rabbit distal colon exhibits net bile acid absorption putative roles for Na-dependent bile acid transporter (ASBT) and lipid binding protein (LBP).* Gastroenterology, 2004. **126**(4): p. A298-A298.
65. Rao, A., et al., *The organic solute transporter alpha-beta, Ostalpha-Ostbeta, is essential for intestinal bile acid transport and homeostasis.* Proc Natl Acad Sci U S A, 2008. **105**(10): p. 3891-6.
66. Soroka, C.J., N. Ballatori, and J.L. Boyer, *Organic solute transporter, OSTalpha-OSTbeta: its role in bile acid transport and cholestasis.* Semin Liver Dis, 2010. **30**(2): p. 178-85.
67. Borgstroem, B., G. Lundh, and A. Hofmann, *The Site of Absorption of Conjugated Bile Salts in Man.* Gastroenterology, 1963. **45**: p. 229-38.
68. Hurwitz, S., et al., *Absorption and secretion of fatty acids and bile acids in the intestine of the laying fowl.* J Nutr, 1973. **103**(4): p. 543-7.
69. Hu, J., et al., *Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones.* Nutr Metab (Lond), 2010. **7**: p. 47.
70. Stocco, D.M., *StAR protein and the regulation of steroid hormone biosynthesis.* Annu Rev Physiol, 2001. **63**: p. 193-213.
71. Payne, A.H. and D.B. Hales, *Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.* Endocrine Reviews, 2004. **25**(6): p. 947-70.
72. Catalano, S., et al., *Farnesoid X receptor, through the binding with steroidogenic factor 1-responsive element, inhibits aromatase expression in tumor Leydig cells.* J Biol Chem, 2010. **285**(8): p. 5581-93.
73. Nilsson, M., et al., *Liver X receptors regulate adrenal steroidogenesis and hypothalamic-pituitary-adrenal feedback.* Mol Endocrinol, 2007. **21**(1): p. 126-37.
74. Cummins, C.L., et al., *Liver X receptors regulate adrenal cholesterol balance.* J Clin Invest, 2006. **116**(7): p. 1902-12.
75. Sewer, M.B. and D. Li, *Regulation of steroid hormone biosynthesis by the cytoskeleton.* Lipids, 2008. **43**(12): p. 1109-15.

76. Sato, E., et al., *Tissue-specific regulation of the expression of rat intestinal bile acid-binding protein.* FEBS Lett, 1995. **374**(2): p. 184-6.
77. Etches, R.J. and C.E. Duke, *Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (Gallus domesticus).* J Endocrinol, 1984. **103**(1): p. 71-6.
78. Smith, L.P., et al., *The bile acid synthesis pathway is present and functional in the human ovary.* PLoS One, 2009. **4**(10): p. e7333.
79. Hughes, A.L. and H. Piontkivska, *Evolutionary diversification of the avian fatty acid-binding proteins.* Gene, 2011. **490**(1-2): p. 1-5.
80. Fang, C., J. Dean, and J.W. Smith, *A novel variant of ileal bile acid binding protein is up-regulated through nuclear factor-kappaB activation in colorectal adenocarcinoma.* Cancer Res, 2007. **67**(19): p. 9039-46.
81. Flicek, P., et al., *Ensembl 2011.* Nucleic Acids Research, 2011. **39**: p. D800-D806.
82. Hagenbuch, B. and P. Dawson, *The sodium bile salt cotransport family SLC10.* Pflugers Arch, 2004. **447**(5): p. 566-70.
83. Saeki, T., et al., *Mutational analysis of uncharged polar residues and proline in the distal one-third (Thr130-Pro142) of the highly conserved region of mouse Slc10a2.* Biosci Biotechnol Biochem, 2009. **73**(7): p. 1535-40.
84. Hussainzada, N., A. Banerjee, and P.W. Swaan, *Transmembrane domain VII of the human apical sodium-dependent bile acid transporter ASBT (SLC10A2) lines the substrate translocation pathway.* Mol Pharmacol, 2006. **70**(5): p. 1565-74.
85. Lefebvre, P., et al., *Role of Bile Acids and Bile Acid Receptors in Metabolic Regulation.* Physiological Reviews, 2011. **89**(1): p. 147-191.
86. Otte, K., et al., *Identification of farnesoid X receptor beta as a novel mammalian nuclear receptor sensing lanosterol.* Mol Cell Biol, 2003. **23**(3): p. 864-72.
87. Krogdahl, A., *Digestion and absorption of lipids in poultry.* J Nutr, 1985. **115**(5): p. 675-85.
88. Sklan, D., *Site of Digestion and Absorption of Lipids and Bile-Acids in the Rat and Turkey.* Comparative Biochemistry and Physiology a-Physiology, 1980. **65**(1): p. 91-95.
89. van Hemert, S., et al., *Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome.* Poult Sci, 2004. **83**(10): p. 1675-82.
90. Kwakye, J.B., et al., *Identification of Bile Acid-Coa - Amino-Acid N-Acyltransferase in Rat-Kidney.* Biochemical Journal, 1991. **280**: p. 821-824.
91. Christie, D.M., et al., *Comparative analysis of the ontogeny of a sodium-dependent bile acid transporter in rat kidney and ileum.* Am J Physiol, 1996. **271**(2 Pt 1): p. G377-85.

92. Cheng, X. and C.D. Klaassen, *Tissue distribution, ontogeny, and hormonal regulation of xenobiotic transporters in mouse kidneys.* Drug Metab Dispos, 2009. **37**(11): p. 2178-85.
93. Guariento, M., et al., *Identification and functional characterization of the bile acid transport proteins in non-mammalian ileum and mammalian liver.* Proteins, 2008. **70**(2): p. 462-72.
94. Selvaraj, V., et al., *Mice lacking FABP9/PERF15 develop sperm head abnormalities but are fertile.* Developmental Biology, 2010. **348**(2): p. 177-189.
95. Guibert, E., et al., *Characterization of chicken Sertoli cells in vitro.* Poult Sci, 2011. **90**(6): p. 1276-86.
96. Purohit, V.D., P.K. Basrur, and M.K. Bhatnagar, *Histochemical localization of 3 beta-hydroxysteroid dehydrogenase in the testes of chicken-pheasant hybrids.* Histochemical Journal, 1977. **9**(3): p. 293-9.
97. Sechman, A., K. Pawlowska, and A. Hrabia, *Effect of 3,3',5-triiodothyronine and 3,5-diiodothyronine on progesterone production, cAMP synthesis, and mRNA expression of STAR, CYP11A1, and HSD3B genes in granulosa layer of chicken preovulatory follicles.* Domest Anim Endocrinol, 2011. **41**(3): p. 137-49.
98. Kowalski, K.I., J.L. Tilly, and A.L. Johnson, *Cytochrome P450 side-chain cleavage (P450scc) in the hen ovary. I. Regulation of P450scc messenger RNA levels and steroidogenesis in theca cells of developing follicles.* Biol Reprod, 1991. **45**(6): p. 955-66.
99. Nitta, H., Y. Osawa, and J.M. Bahr, *Immunolocalization of steroidogenic cells in small follicles of the chicken ovary: anatomical arrangement and location of steroidogenic cells change during follicular development.* Domest Anim Endocrinol, 1991. **8**(4): p. 587-94.
100. Nitta, H., Y. Osawa, and J.M. Bahr, *Multiple steroidogenic cell populations in the thecal layer of preovulatory follicles of the chicken ovary.* Endocrinology, 1991. **129**(4): p. 2033-40.
101. Proszkowiec, M. and J. Rzasa, *Variation in the ovarian and plasma progesterone and estradiol levels of the domestic hen during a pause in laying.* Folia Biologica-Krakow, 2001. **49**(3-4): p. 285-289.
102. Etches, R.J. and J.N. Petitte, *Reptilian and avian follicular hierarchies: models for the study of ovarian development.* J Exp Zool Suppl, 1990. **4**: p. 112-22.
103. Robinson, F.E. and R.J. Etches, *Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus domesticus).* Biol Reprod, 1986. **35**(5): p. 1096-105.
104. Nitta, H., J.I. Mason, and J.M. Bahr, *Localization of 3-Beta-Hydroxysteroid Dehydrogenase in the Chicken Ovarian Follicle Shifts from the Theca Layer to Granulosa Layer with Follicular Maturation.* Biology of Reproduction, 1993. **48**(1): p. 110-116.

105. Seol, H.S., et al., *Modulation of sterol regulatory element binding protein-2 in response to rapid follicle development in chickens.* Comp Biochem Physiol B Biochem Mol Biol, 2007. **147**(4): p. 698-703.
106. Johnson, A.L., E.V. Solovieva, and J.T. Bridgham, *Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development.* Biol Reprod, 2002. **67**(4): p. 1313-20.
107. Hwang, S.T. and S.J. Henning, *Hormonal regulation of expression of ileal bile acid binding protein in suckling rats.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(6): p. R1555-63.
108. Uni, Z., et al., *Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo.* Poult Sci, 2003. **82**(11): p. 1747-54.
109. Yegani, M. and D.R. Korver, *Factors affecting intestinal health in poultry.* Poult Sci, 2008. **87**(10): p. 2052-63.
110. Kawalilak, L.T., A.M. Ulmer Franco, and G.M. Fasenko, *Impaired intestinal villi growth in broiler chicks with unhealed navels.* Poult Sci, 2010. **89**(1): p. 82-7.
111. Runde, H. and H. Luppa, *[Histochemical study on the differentiation of the duodenal epithelium in chick embryos].* Acta Histochem, 1973. **45**(1): p. 75-90.
112. Esteban, S., et al., *Gastrointestinal emptying in the final days of incubation of the chick embryo.* Br Poult Sci, 1991. **32**(2): p. 279-84.
113. Murai, A., et al., *Characterization of critical factors influencing gene expression of two types of fatty acid-binding proteins (L-FABP and Lb-FABP) in the liver of birds.* Comp Biochem Physiol A Mol Integr Physiol, 2009. **154**(2): p. 216-23.