# EXPRESSION OF LIVER FATTY ACID BINDING PROTEIN DURING EMBRYIONIC AND POST-HATCH DEVELOPEMENT OF *GALLUS DOMESTICUS*

A Senior Thesis

By

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# ABSTRACT

**EXPRESSION OF LIVER FATTY ACID BINDING PROTEIN DURING EMBRYONIC AND POST-HATCH DEVELOPMENT OF *GALLUS DOMESTICUS.***
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To date, developmental studies investigating liver fatty acid binding protein (L-FABP), an intracellular protein that binds fatty acids, have been performed predominantly in mammals. The purpose of the current study was to evaluate avian L-FABP expression during embryogenesis and early post-hatch. L-FABP expression was measured at days 10, 14, and 17 of embryogenesis and days 1, 4, 7, and 11 post-hatch using western blot analysis with goat anti-chicken L-FABP as the primary antibody. The L-FABP protein bands were visualized using chemiluminescence and quantified using densitometry. Levels were low during early embryogenesis. The greatest increase occurred between days 10 and 14. After day 14, L-FABP expression increased at a steady rate. Results from the current study suggest that, like the rat, chickens do not reach adult levels of L-FABP expression by the time of hatch or birth. Results also suggest that the change in diet from a high fat diet provided by the yolk to a high carbohydrate feed ration does not affect the steady increase in L-FABP expression observed in the developing chicks. Future studies may focus on how dietary mactronutrients as well as developmental energy demands affect L-FABP expression in the chick.

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# LITERATURE REVIEW

Lipids are hydrophobic compounds that are insoluble in water and soluble in nonpolar solvents. They are important biologically for supplying metabolic energy, giving structure to cell membranes, insulating and protecting vital organs, carrying lipid soluble nutrients such as the fat soluble vitamins A, D, E and K, and serving as electrical insulators for sending nerve impulses. Lipids can be classified as: A) simple or neutral lipids such as fats (solid at room temperature), oils (liquid at room temperature), or waxes; B) complex lipids such as phospholipids; or C) precursor and derived lipids including fatty acids, glycerol, and steroids. Triglycerides, the most common form of lipids in foods, are a type of neutral lipid containing three ("tri") fatty acids esterified onto a glycerol backbone ("glyceride") (Figure 1). Fatty acids serve as building blocks for most lipids. Because of the hydrophobic nature of fatty acids, they are not effectively transported in aqueous environments such as the blood and therefore require binding to hydrophilic proteins. Additionally, at high levels, free (nonesterified) fatty acids can have a deleterious, detergent-like effect on the cell. Albumin, a 66 kDa protein, is responsible for the transport of free fatty acids throughout the blood. In order for the fatty acids to deliver the nutrients they offer, they must be taken up by the cell. Currently, little is known about

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Literature cited follows the style of Poultry Science.

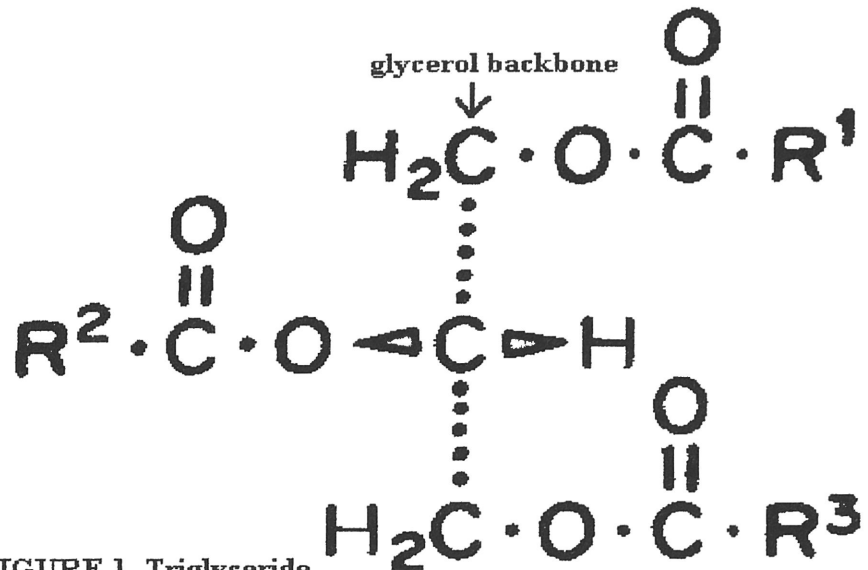


**FIGURE 1. Triglyceride**
**$R^1$, $R^2$, and $R^3$ represent fatty acids**

how the transfer from the blood (extracellular environment) to the cell (intracellular environment) occurs. However, Paulussen and Veerkamp (1990) reviewed several proposed mechanisms for the cellular uptake of fatty acids and concluded that, in general, fatty acids are taken up by cells, and once inside the cell, they are associated with intracellular lipid binding proteins.

Sterol carrier protein-2 (SCP-2) and fatty acid binding protein (FABP) are two classes of lipid binding proteins responsible for the intracellular binding of nonesterified fatty acids. Sterol carrier protein-2, also referred to as non-specific lipid transport protein, was named for its very broad ligand specificity. Sterol carrier protein-2 is a 13.2 kDa protein located in the cytosol, mitochondria, peroxisomes and near the endoplasmic reticulum (Gosset *et al.*, 1996). Fatty acid binding proteins belong to a family of seventeen members, each named for the primary tissue location in which they were first identified (Gosset *et al.*, 1996). They are 14-16 kDa, clam-shaped proteins found in a number of

tissues known to actively metabolize lipids including the small intestine, liver, adipose, heart, brain, muscle, kidney, and arteriole wall (Matarese *et al.*, 1989). Fatty acid binding proteins are highly conserved among species and are believed to have been present prior to the emergence of vertebrates (Chan *et al.*, 1985). Liver-FABP (L-FABP), the most abundant of these proteins, are expressed in both the liver and small intestinal epithelium (Bass and Manning, 1986). Liver-FABP has been shown to form 2-5% of the cytosolic proteins in these tissues (Kaikaus *et al.*, 1993). Liver-FABP binds a large array of compounds including fatty acids, fatty acyl-CoA (activated FA), prostaglandins, bile acids, and carcinogens (Gossett *et al.*, 1996). There is *in vitro* evidence to support a role for L-FABP as a regulator of lipid metabolism. Specifically, there are studies which have shown L-FABP to affect the activity of enzymes involved in lipid synthetic (intracellular storing) and oxidative (energy producing) biochemical pathways (Gossett *et al.*, 1996). For example, L-FABP stimulates the synthesis of long chain fatty acyl CoA by enhancing the enzyme, long chain fatty acyl CoA synthetase, in the mitochondria (Rasmussen *et al.*, 1994). Long chain fatty acyl CoA may be directed toward esterification or elongation and desaturation. Alternatively, L-FABP has been shown to influence beta-oxidation, by stimulating the outer mitochondrial membrane enzyme, carnitine palmitoyl-transferase.

This enzyme is responsible for converting long chain acyl CoA to acylcarnitine, a molecule capable of penetrating the mitochondria for participation in beta-oxidation (Bhuiyan and Pande, 1994).

Liver-FABP has been isolated from a variety of vertebrate classes including mammals, aves, and osteichthyes as well as invertebrates. Specifically, L-FABP has been purified from the rat, mouse, human, cow and pig (Matarese *et al.*, 1989), catfish (Di Pietro and Santome, 1996), the domestic chicken or *Gallus DOMESTICUS* (Sewell *et al.*, 1989), insects (Price *et al.*, 1992) and worms (Smith et al, 1992).

One of the interests of our laboratory is chicken L-FABP, specifically with respect to the influence of nutrients and development on L-FABP expression. As previously mentioned, chicken L-FABP was first isolated and characterized by Sewell and co-workers in 1989. These researchers purified chicken L-FABP from the cytosol of liver cells using a combination of gel filtration and anion exchange chromatography, two commonly utilized techniques for the purification of proteins. They also determined the amino acid composition of chicken L-FABP and learned that it contains 148 amino acid residues. Interestingly, when Sewell *et al.* (1989) compared seven of the most common residues of chicken L-FABP with those of rat L-FABP reported by Glatz *et al.* (1985), four of the amino acid residues were the same. This data suggests that chicken L-FABP and rat L-FABP are similar but not identical. Sams and co-workers (1991) later showed a 0.87 coefficient of correlation for amino acid composition homology of chicken L-FABP to rat L-FABP indicating that the overall amino acid composition of chicken L-FABP is very similar to rat L-FABP. It is important to note that this high correlation was found in regard to amino acid composition and not amino acid sequence. The correlation investigating rat L-FABP and chicken L-FABP sequence homology remains to be

determined. However, unpublished data from our laboratory has shown that antibodies against rat L-FABP do not recognize chicken liver cytosolic extracts containing L-FABP (McNeill, unpublished data). This lack of antibody recognition would suggest that differences exist in L-FABP structure and/or sequence of the two proteins.

In 1990, Scapin and coworkers elucidated the three dimensional structure of chicken L-FABP using X-ray crystallography. These authors showed the protein to be structured as a compact 10-stranded-β-barrel unit with one fatty acid bound to it. This structural characterization also revealed two short-α-helices on the $NH_2$-terminus portion of the molecule. They concluded that the structure of chicken L-FABP appears to be similar to the *Escherichia coli* derived rat intestinal FABP (Scapin *et al.*, 1990).

Sams and co-workers (1991) further characterized chicken L-FABP from a functional standpoint. They investigated the relative fatty acid binding activity of chicken L-FABP. The researchers investigated three fatty acids: 1) a saturated fatty acid, palmitic acid, containing 16 carbons and no double bonds, 2) an unsaturated fatty acid, oleic acid, containing 18 carbons and 1 double bond, and 3) linoleic acid, a polyunsaturated fatty acid containing 18 carbons and two double bonds. They illustrated L-FABP has an increased affinity for the unsaturated and polyunsaturated fatty acids as compared to the saturated fatty acids. Results from these studies support mammalian literature which shows L-FABP has a high affinity for unsaturated fatty acids, and this affinity appears to increase as chain length and degree of unsaturation of the fatty acid increase. In fact, up to 85% of the fatty acids endogenously bound to rat L-FABP are unsaturated. A high percentage (50-

75%) of these unsaturated fatty acids are polyunsaturated fatty acids (Cistola *et al.*, 1989; Ockner *et al.*, 1982). These authors also showed an increased affinity of unsaturated fatty acids for chicken L-FABP.

Although chicken L-FABP has been isolated/purified and some functional characteristics have been elucidated, little is known about L-FABP expression during embryogenesis. Developmental studies of fetal human L-FABP have shown that L-FABP is detectable after seven weeks of gestation, and throughout the rest of the gestational period, it remains at a level which is higher than the adult liver level (Paulussen and Veerkamp, 1990). Interestingly, L-FABP content is already at adult levels during the late fetal stage of guinea pigs and monkeys (Paulussen and Veerkamp, 1990). Conversely, postnatal FABP content in male rat livers reach adult values between day 40 and day 70 (Paulussen, 1989). In prenatal rats, L-FABP expression shows a rise from less than 0.1 pmol/ug at 18 days of gestation to about 0.3 pmol/ug protein at term or 22 days of gestation. Until now, all of the developmental studies have been performed in mammals. Therefore, the purpose of the current study was to evaluate avian L-FABP expression during embryogenesis and early post hatch.

# MATERIALS AND METHODS

This research was conducted in a series of three trials. Each trial utilized the same methodology as described below.

### *Incubation*

DeKalb Single Comb White Leghorn fertilized eggs were ordered from Feathercrest Farms[1]. For each trial, the eggs were incubated in a Petersime model S-11 setter with the large end of the egg pointing up so the head of the chick would develop at the large end of the egg near the air cell. A dry bulb temperature of 99.5°F and a wet bulb temperature of 87°F were maintained so relative humidity was approximately 58-60% which is essential for the proper development of the chick embryo. Eggs were rotated at 40-45 degree angles and one turn was completed every 1.5 hours by a mechanical turner. The turner in the incubator prevented the embryos from sticking to the side of the shell. At day 18 of incubation, the eggs were moved to a Petersime model S-1 hatcher. After hatch, the chicks were moved to a Petersime six level chick brooder unit. The chicks were placed on a carbohydrate rich corn-soybean based chick starter ration the third day after hatch. Prior to diet initiation, the chick uses nutrients from the egg yolk that is naturally encapsulated within the abdomen just before hatch.

### *Tissue Sampling*

Livers were excised on days 8 (second and third trials only), 10, 12, 14 and 17 of

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[1] Bremen, AL 35033

incubation. Samples were also taken on days 1, 3, 7 and 11 post-hatch (also considered days 21, 24, 28, and 32 after the initiation of incubation). All chicks and embryos were humanely handled and properly euthanized before sampling. Embryos were quickly decapitated after the shells were cracked. Chicks were euthanized by either cervical dislocation or $CO_2$ gas. Due to requirements of the FABP assay, at least one gram of liver was needed for each sampling day. For samples taken on day 8 of incubation, the embryos were placed in a dish of .1M Phosphate Buffered Saline (PBS) with a pH of 7.4 at 4°C. Livers were then floated out by "teasing" the liver away from the body. At this stage of development, any attempt to excise the liver with tweezers will cause it to burst, therefore it must be gently pried away from other organs. Trial one did not have a sampling on day 8 because this floatation method was not perfected until after trial one was completed. Based on average liver weight, approximately 200 livers (~5 mg each) were collected at day 8, and approximately 40 livers (~29 mg each) were collected at day 10. The number of livers collected for each sampling day continued to decrease as the livers increased in weight; however, to maintain adequate representation within days, each pool contained a minimum of seven livers.

*Cytosolic Protein Determination*

For each sampling, livers were collected in conical tubes with PBS and stored at -75°C until further analysis. Prior to analysis, each pool was thawed and homogenized for 3 minutes in 2.5 volumes PBS with a Precise® Virtis[2] at speed 30 on the virtis dial. The pools were then centrifuged at 800 x g for 15 minutes with a Sorvall RT6000B table top centrifuge. The supernatant was collected and centrifuged at 105,000 x g for 1.25 hours with a Sorvall T1270 rotor[3]. The pure cytosol (supernatant) was placed in eppendorf tubes and stored at 4°C. A bicinchoninic acid (BCA) protein assay[4] was used to determine total cytosolic protein concentrations of the samples. Cytosolic proteins were diluted with Sample Application Buffer (SAB) to a final concentration of either 2.5 or 5μg/μl.

***SDS Page and Western Blot Analysis (Chemiluminescence)***

Twenty-five μg of total cytosolic protein for each sample was separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using gels containing 17% polyacrylamide in the separating layer. The electrophoresed protein was then transferred to a polyvinylidene fluoride (PVDF) membrane. Each PVDF membrane was blocked overnight in approximately 400 mls of 5% Carnation Nonfat Dried Milk inTris Buffered Saline with Tween 20 (TBST) at 4°C. After blocking the non-specific binding sites, the membranes were incubated in a solution containing the primary antibody, goat

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[2] Precise Products Corp, Racine, Wisconsin 53401

[3] DuPont Medical Products, Newtown, CT 06470

[4] Pierce Chemical Co., Rockford Il. 06470-5509

anti-chicken L-FABP IgG, in 5% Carnation Nonfat Dried Milk in TBST at a concentration of 1:500. The membranes were subsequently washed 3 times for 5 minutes each in TBST. After the last wash, 90 mls of secondary antibody, consisting of a 1:90,000 concentration of rabbit anti-goat IgG in 5% Carnation Nonfat Dried Milk in TBST was added to the membrane and incubated for 1.5 hours. Secondary antibody was washed from the membrane using three 5 minute washes in TBST followed by two 5 minute washes in TBS.

Liver-FABP bands were visualized by incubating the membranes in 30 mls of the Pierce Super Signal Substrate Western Blotting solutions (kit #34080) for 5 minutes. Next, the membranes were exposed to Kodak Biomax MR scientific imaging film[5] for 35 seconds in an Amersham Life Science Hypercassette™[6]. A Konica QX-70 Medical Film Processor was used to develop the film. The amount of L-FABP was then quantified using densitometry and the results were given in the unit OD x $mm^2$.

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[5] Eastman Kodak, Rochester, NY 14650

[6] Amersham Life Science Inc., Arlington Heights, IL 60005

# RESULTS AND DISCUSSION

## *Liver Weights*

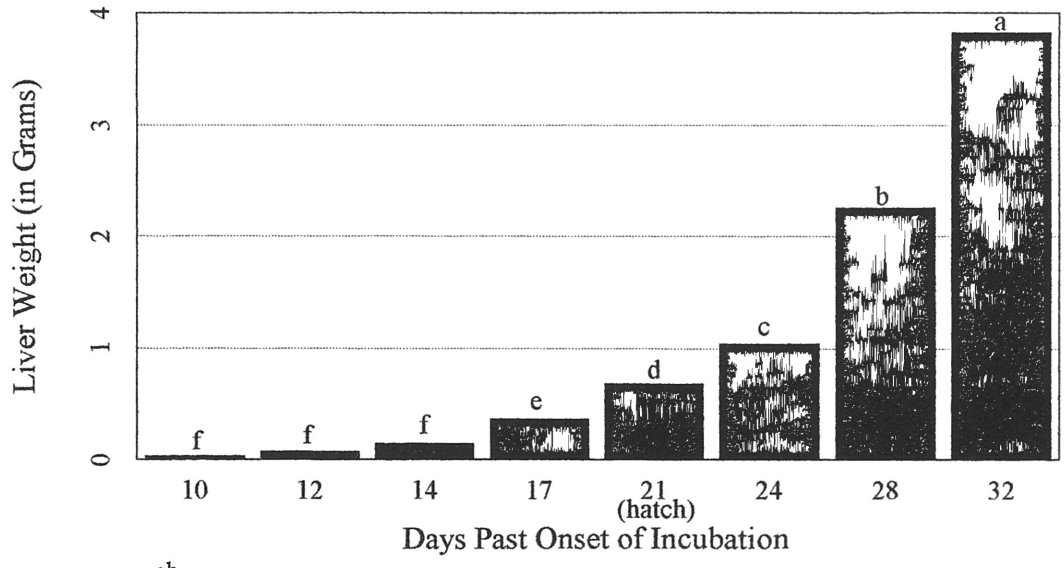
As expected, liver weights increased significantly ($P < .001$) during the transition between incubation and early post-hatch life. During incubation, significant changes in liver weight were observed only between the early and late stages of embryonic development. Specifically, liver weights were not significantly different in the early embryonic period (day 10-day 14) but increased significantly during the late embryonic period immediately prior to hatch (day 14 -day 17) (Table 1 and Graph 1). This increase is consistent with the period of rapid growth and rapid uptake of lipid from the yolk which starts around day 14 and continues through early post-hatch (Noble, 1987). Between day 17 and day 21, marking the transfer from incubation to hatch, another significant increase in liver weight was noted (Table 1 and Graph 1). Noble (1987) indicated that by day 19 of incubation, liver lipid accumulation accounts for approximately 5% of the lipid taken up from the yolk. Each day of investigation post-hatch continued to follow the pattern of significant increase in liver weight, as would be expected during this period of rapid growth and development.

TABLE 1. Liver Weights During Embryogenesis and Early Post-Hatch
(All Trials Combined)

| Days Past Onset of Incubation | Weight in Grams |
|---|---|
| 10 | $.027 \pm .001^{f}$ |
| 12 | $.069 \pm .003^{f}$ |
| 14 | $.138 \pm .006^{f}$ |
| 17 | $.357 \pm .011^{e}$ |
| 21 (hatch) | $.677 \pm .045^{d}$ |
| 24 | $1.04 \pm .033^{c}$ |
| 28 | $2.25 \pm .090^{b}$ |
| 32 | $3.82 \pm .086^{a}$ |

[ab]Means ± SEM differ significantly if superscripts differ (P<.001).

GRAPH 1. Liver Weights During Embryogenesis and Early Post-Hatch
Data from All Three Trials




[ab]Means ± SEM differ significantly if superscripts differ (P<.001)

*L-FABP Expression*

The limited supply of hepatic material for each day of incubation/post-hatch life allowed for a single liver pool to represent each sampling in the evaluation of L-FABP expression during each trial. This limitation prohibited the statistical evaluation of an interaction between trials and days; therefore, the trials are presented independently and the results observed are subjective (Tables 2-4 and Graphs 2-4).

Western blot analysis revealed the levels of L-FABP expression throughout embryogenesis and early post hatch. Day 8 of embryogenesis represented the earliest time point in which L-FABP expression was measured (Tables 2-4 and Graphs 2-4). At this stage of chick development, L-FABP expression was barely detectable in the second trial and was not detected by chemiluminescence in the third trial. However, L-FABP expression at day 8 was higher than levels found at day 10 of the same trial. Liver-FABP expression was detected in all three trials at day 10; however, expression remained low as compared to day 8 in Trial 2. For all three trials, the most dramatic increase (339% to 1808%, depending on the trial) in chicken L-FABP expression occurred between days 10 and 14 of incubation. During the first 14 days of embryogenesis, an embryo uses proteins and, to a lessor degree, carbohydrates from the albumin of the egg rather than lipid from the yolk (Noble, 1987). In fact, of the estimated 5 grams of total lipid in the egg yolk, only (approximately) 350 milligrams are absorbed during the first two weeks. Because the liver has a low amount of fat to transport, low levels of L-FABP expression were expected prior to day 14. However, the dramatic increase in L-FABP expression between days 10 and 14

was not expected because the embryo does not start absorbing lipids from the yolk until approximately day 14 (Noble, 1987). Surprisingly, samples taken after day 14 show a modest yet steady increase in L-FABP expression in all three trials. This includes samples taken after hatch on day 21 and after the birds were placed on the high carbohydrate corn-soy based feeding regime. The birds in this study were placed on a broiler starter diet instead of a typical layer starter diet due to cost constraints and availability. The broiler starter diet had approximately 3200 kcal/kg of feed whereas a typical layer starter ration may have around 2850 kcal/kg of feed. Layer chicks in any future trials, similar to the three trials in this study, should be placed on a layer starter diet if possible because layers require less calories than broilers for growth and maintenance.

The data from this study suggests that, like the rat, chickens do not reach adult levels of L-FABP expression by the time of hatch or birth. Comparable adult levels could not be quantified because chemiluminescence is too sensitive for samples from adult layers. The sensitivity of chemiluminescence was needed to pick up the levels of L-FABP expression in the embryos. Therefore, the adult levels of L-FABP expression are higher than levels in the embryo, but how much higher is not known. This study also suggests that the change from a high fat diet to a high carbohydrate diet does not affect the steady increase in L-FABP expression observed in the developing chick. Recall that fat and carbohydrates in the diet have been found to affect L-FABP expression (Noble, 1987). Future studies might involve calculating the number of calories from fat and carbohydrates that the birds receive from the yolk and from the chicken ration.

Liver-FABP expression in the chick may be influenced by energy demands. Two factors that cause increased energy demands include rapid growth and control of body temperature. It is interesting to note that around day 14 of embryogenesis, the chick enters a period of rapid embryonic growth and intense lipid metabolism (Noble, 1987). This occurs at the same time that L-FABP expression began its steady increase in the observations of the current study. Sometime during late embryogenesis, the chick begins to control energy metabolism for heat production. In fact, studies have shown at approximately day 18 of incubation, the chick embryo can maintain oxygen consumption in response to shifts in incubation temperature (Tazawa *et al.* 1989). After pipping, or initiation of hatch, a shift toward independent thermoregulation has been observed (French, 1997). This ability to efficiently metabolize energy to maintain heat production in response to cold stress continues to improve after hatch (French, 1997). Perhaps future studies will find whether a link exists between energy demands, such as these, and L-FABP expression.

In conclusion, the steady increase in L-FABP expression observed in layer chicks is not affected by the change in diet from a high fat diet provided by the yolk to a high carbohydrate diet provided by a starter ration. Adult levels of L-FABP expression are reached sometime after day 11 post-hatch; however, the amount of time after hatch was not determined in this study. When compared to developmental studies in mammals, L-FABP expression in the chick follows a pattern more similar to the rat than other mammals. This study can be expanded to elucidate answers to current questions regarding the role of L-FABP in liver lipid metabolism.

TABLE 2. L-FABP Expression (OD x $mm^2$) During Embryogenesis and Early Post-Hatch (Trial 1)

| Days Past Onset of Incubation | Expression (OD x $mm^2$) |
|---|---|
| 10 | .1106 |
| 12 | n/m[1] |
| 14 | .4853 |
| 17 | .6454 |
| 21 (hatch) | .6744 |
| 24 | 1.239 |
| 28 | 1.970 |
| 32 | 2.891 |

[1] n/m, Not measured

GRAPH 2. L-FABP Expression During Embryogenesis and Early Post-hatch Life (Trial 1)

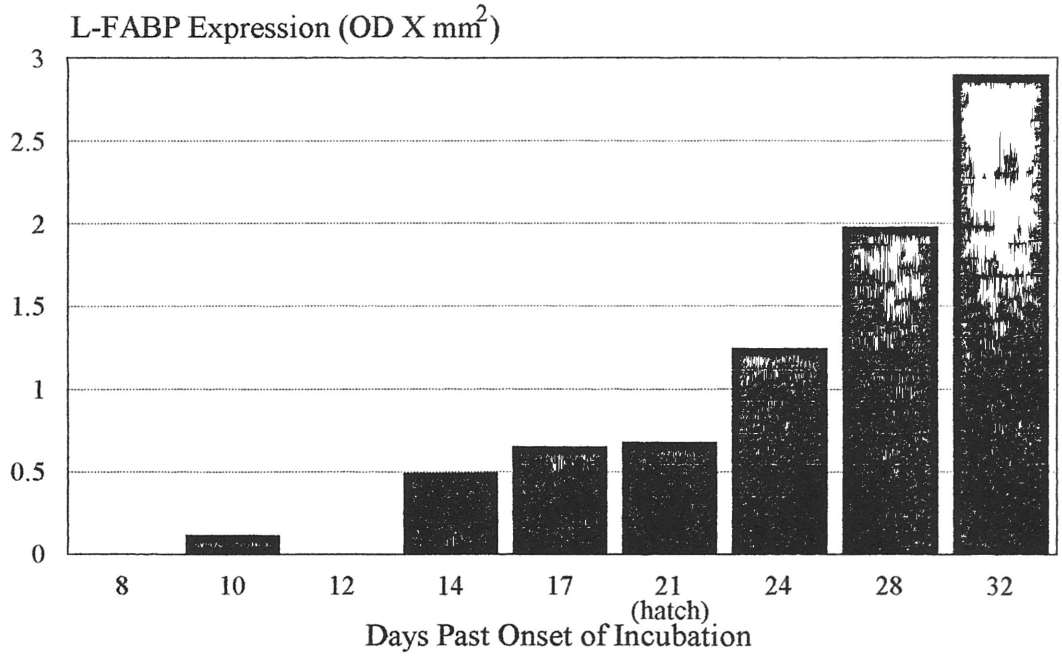


TABLE 3. L-FABP Expression (OD x $mm^2$) During Embryogenesis and Early Post-Hatch (Trial 2)

| Days Past Onset of Incubation | Expression (OD x $mm^2$) |
|---|---|
| 8 | .1806 |
| 10 | .0539 |
| 12 | n/m[1] |
| 14 | .5322 |
| 17 | .5530 |
| 21 | 1.709 |
| 24 | 2.238 |
| 28 | 2.931 |
| 32 | 2.838 |

[1]n/m, Not measured

GRAPH 3. L-FABP Expression During Embryogenesis and Early Post-Hatch (Trial 2)

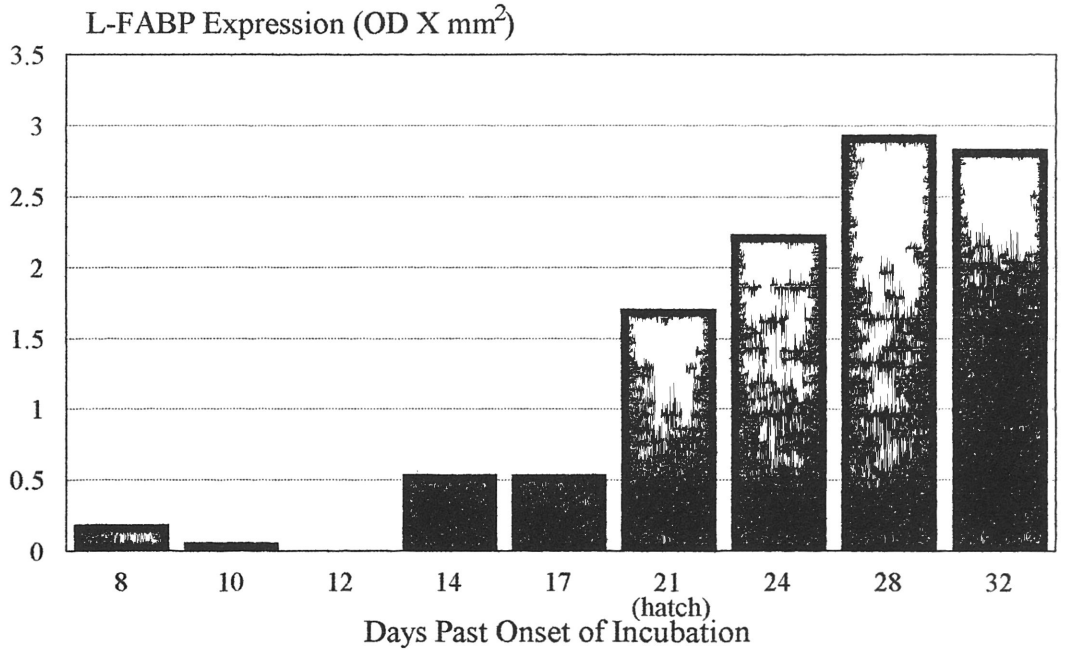
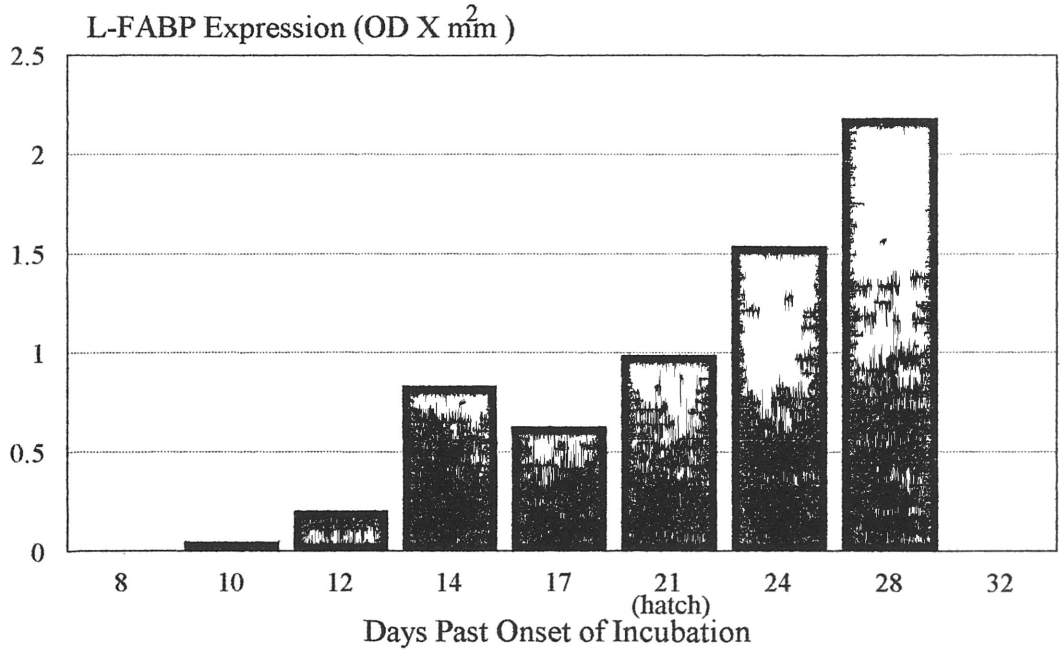


TABLE 4. L-FABP Expression (OD x $mm^2$) During Embryogenesis and Early Post-Hatch (Trial 3)

| Days Past Onset of Incubation | Expression (OD x $mm^2$) |
|---|---|
| 8 | n/d[1] |
| 10 | .0434 |
| 12 | .1994 |
| 14 | .8279 |
| 17 | .6240 |
| 21 | .9839 |
| 24 | 1.537 |
| 28 | 2.181 |
| 32 | n/$m^2$ |

[1] n/d, Not detectable
[2] n/m, Not measured

GRAPH 4. L-FABP Expression During Embryogenesis and Early Post-Hatch (Trial 3)



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