INTRODUCTION

The chicken embryonic liver plays a crucial role in metabolism regulation, cell division, signal transduction, organogenesis, and other essential functions during embryo development and growth (Jianzhen et al., 2007). It is further responsible for maintaining energy homeostasis via the regulation of carbohydrate, protein, and lipid metabolic pathways (Berg et al., 2002; De Oliveira et al., 2008). In addition to glycogen storage, the liver is also responsible for glycogen mobilization to glucose, and the metabolism of protein, fat, and carbohydrates (Jianzhen et al., 2007). Embryos depend on yolk lipids for energy production, with more than 90% of the embryonic energy requirement provided for by yolk lipids (Noble and Cocchi, 1990; Speake et al., 1998). Moreover, some polyunsaturated fatty acids are crucial in brain, heart, and retinal development (Speake et al., 1998; Zhai et al., 2008). However, the potential for embryonic ketosis exists when the yolk nutrient profile is high in lipid and protein but low in carbohydrates (Burley and Vadehra, 1989). Acetyl coenzyme A, formed in fatty acid oxidation, enters the citric-acid cycle only if fat and carbohydrate degradation are appropriately balanced (Berg et al., 2002); therefore, ke-
Glucose is very important during the period in which the chick emerges from the egg because glucose requires less oxygen than does fat for the production of the same amount of energy (Christensen et al., 2001; Moran, 2007). To maintain blood glucose levels, fat and protein in the egg are metabolized to produce glucose and glycogen, the storage form of glucose in liver and muscle (Pearce, 1971). The avian embryonic liver is an important source of glucose through hepatic gluconeogenesis. During incubation, the gluconeogenic enzymes, pyruvate carboxylase, phosphopyruvate carboxylase, hexose-diphosphatase, and glucose-6-phosphatase increase to their maximum activities at approximately d 17 of incubation. Subsequent increases in the activities of other enzymes, including uridine diphosphoglucose α-glucosyl glucosyl transferase, α-glucan phosphorylase, glucose-1-phosphate uridyl transferase, and phosphoglucomutase, are necessary to allow hepatic concentrations of glycogen to reach its maximum on d 19 of incubation (E19; Ballard and Oliver, 1963; Pearce, 1971). At the end of the incubation period, hepatic glycogen is rapidly metabolized to glucose, which serves as an energy source for most active muscles during hatching (Freeman, 1965, 1969; Pearce, 1971; Moran, 2007). However, after pipping, lipids again become the major source of energy (Goodridge, 1968a,b; Pearce, 1971; Zhao et al., 2010).

Ohi et al. (2010) reported that the activity of 3-hydroxy acyl coenzyme A dehydrogenase, an enzyme involved in the β-oxidation of fatty acids in the liver, was positively related to the rate of water loss from embryonated eggs. Therefore, the possibility exists that water present in carbohydrate solutions injected in ovo may affect fatty acid oxidation in the livers of broiler embryos and hatchlings (McGruder et al., 2011). Uni et al. (2005) reported that the injection of a mixture of carbohydrates and a leucine metabolite improved the energy status of late-term chicken embryos. However, only liver glycogen concentration was examined in that study, and the effects of the injection of the carbohydrates on total protein, glucose, and fat concentrations in the liver remained undetermined.

Previous studies have shown that the BW of hatchlings was improved in response to the in ovo injection of carbohydrate (Tako et al., 2004; Uni et al., 2005; Zhai et al., 2011a,b). However, Zhai et al. (2011a,b) reported that high volumes (1.0 and 1.2 mL) of various carbohydrate solutions that were injected in ovo can be detrimental to hatchability of fertilized eggs (HF) and subsequent yolk nutrient utilization and body tissue deposition in broiler embryos and hatchlings. Furthermore, studies have shown that HF was negatively related to the injection volume of the carbohydrate solutions. To realize an HF of 90%, injection volume could not exceed 0.4 mL for fructose or sucrose solutions, and 0.7 mL for glucose, maltose, or dextrin solutions (Zhai et al., 2011b). Therefore, in the current study, it was hypothesized that the injection of carbohydrates at a low volume (0.4 mL) may improve embryo energy status (liver nutrient profile) and facilitate the hatching process without detrimentally affecting embryo development. Also, because the integrated changes in the energy profiles of the pipping muscle and liver before and during pipping are integral to the broiler embryo’s preparation for hatch (Moran, 2007; Pulikanti et al., 2010), our aim was to further understand the effects of the in ovo injection of carbohydrates on liver metabolic profile, concentration changes, and relationships among all 4 main nutrients in the liver.

**MATERIALS AND METHODS**

**Incubation**

The current experimental protocol was approved by the Institutional Animal Care and Use Committee of Mississippi State University. Ross × Ross 708 broiler hatching eggs were obtained from a commercial flock at 34 wk of age. All eggs were held for 3 d under standard storage conditions before setting. Each egg was weighed individually, and only those that were ± 10% of the mean weight (59 ± 5.9 g) of all eggs weighed were randomly set on each of 8 incubator tray levels in a Jamesway Model PS 500 (Jamesway Incubator Co., Inc., Cambridge, Ontario, Canada) single stage incubator. In total, approximately 1,728 eggs were set, with all treatment groups being represented on each tray level, and with 36 eggs being set in each of 6 treatment groups on each replicate tray level. On d 12 of incubation, eggs were candled and unfertilized eggs were removed from the incubator. Incubator dry and wet bulb temperatures were set at 37.5 ± 0.1 and 28.9 ± 0.1°C, respectively.

**Treatment Solutions and Injection**

All injected solutions were prepared on the day of injection using commercial vaccine diluent (Merial Select Inc., Gainesville, GA). Glucose, sucrose, maltose, and dextrin were dissolved in the diluent to achieve the following concentrations: 1) 6.25% glucose and 18.75% dextrin (G + D); 2) 6.25% sucrose and 18.75% dextrin (S + D); 3) 6.25% maltose and 18.75% dextrin (M + D); and 4) 25% dextrin. The diluent and the carbohydrate solutions were then autoclaved at 121°C for 15 min, and were subsequently allowed to cool to 37.5°C before injection. All treatment solution volumes were 0.4 mL. In addition, a noninjected control and a 0.4-mL volume diluent-injected control were included. Injections were performed on d 18 of incubation using a modified IntelliJect multi-egg injector (AviTech, LLC, Salisbury, MD) capable of simultaneously injecting 56
eggs at a time. The needles equipped on the injector provided an injection depth of approximately 2.49 cm from the top of the large end of the egg. The detailed injection procedure was described previously by Ker-alaparuth et al. (2010a,b) and Zhai et al. (2011a,b).

**Data Collection**

Individual egg weights at set (SEW) were recorded. On E19, which was 1 d after injection, 3 embryos per treatment replicate were sampled. Two out of the 3 embryos were used to collect liver samples (total of 96 livers). All of the 3 embryos were used to collect yolk sac samples (total of 144 yolk sacs). On the day of hatch (d 21 of incubation), all hatchlings were used to determine HF and the group BW of chicks in each treatment replicate. Three chicks per treatment replicate (total of 144 chicks) were subsequently wing-banded, weighed individually, and euthanized. Two out of the 3 chicks were used to collect liver samples (total of 96 livers). All of the 3 were used to collect yolk sac samples (total of 144 yolk sacs). Total chick BW (yolk included), yolk sac weight (YSW), and yolk-free BW (YFBW) proportional to SEW (BW/SEW × 100, YSW/SEW × 100, and YFBW/SEW × 100, respectively) were calculated to eliminate any effects of SEW variation on BW.

Within 1 min after the birds were euthanized, a 0.25-g liver sample was taken from the same lobe of the liver in all of the birds, and preserved in 10% perchloric acid for further biochemical analysis (Bennett et al., 2007). Liver glycogen and glucose concentrations were determined using the phenol-sulfuric acid method, as described by Bennett et al. (2007). Colorimetric protein determinations were made according to the procedures of Lowry et al. (1951), and colorimetric fat determinations were performed according to the methodology described by Van Handel (1985). Liver glycogen, glucose, protein, and fat concentrations were expressed as percentages of fresh sample weight. Total liver contents of the above parameters were calculated as concentration × liver weight. Also, liver weight proportional to BW was calculated to eliminate any effects due to variations in BW.

Yolk sacs, yolk-free carcasses, and portions of the liver samples that were not used for the aforementioned analyses, were collected for weight and moisture determination. Yolk sac and yolk-free carcass samples were dried in drying ovens set at 70°C until no further weight loss was observed. Later, samples were kept for 24 h at room temperature before their dry weights were determined. Tissue moisture content was calculated as a percentage of fresh tissue sample weight (Peebles et al., 1998, 1999).

**Statistical Analysis**

A randomized complete block design was used, with each tray level representing a block, and with all treatments being equally represented in each block. All data were analyzed using the procedures of SAS software (SAS Institute, 2008). Using the MIXED procedure, a 1-way ANOVA was used to test for all parameter differences among treatments, with treatment designated as a fixed effect and tray level as a random effect. Least squares means were compared in the event of significant global effects (Steel and Torrie, 1980). Using the GLM procedure, partial correlations between liver weight relative to SEW, YSW relative to SEW, and YFBW relative to SEW on E19 and on the day of hatch were analyzed. Also, partial correlations between liver protein, glycogen, glucose, and fat concentrations on E19 and on the day of hatch were analyzed. Global effects, differences among least squares means, and correlations were considered significant at $P \leq 0.05$.

**RESULTS**

Supplementation of carbohydrates increased embryo BW relative to SEW as compared with noninjected control and 0.4-mL diluent-injected control groups on E19, which was 1 d after injection. However, absolute BW, YSW, relative YSW to SEW, yolk moisture, YFBW,
relative YFBW to SEW, or yolk free body moisture were not affected by any injection treatments as compared with those of the controls (Table 1).

The current results showed that HF was not affected by any injection treatment on the day of hatch, which was 3 d after injection (Table 2). As compared with noninjected controls, chick BW and BW relative to SEW on the day of hatch were increased in the diluent-injected, S + D, and M + D groups, but not in the G + D and dextrin-only groups. As compared with the noninjected control groups, the injection of 0.4 mL of diluent did not affect yolk sac moisture; however, the injection of 0.4 mL of any of the carbohydrate solutions tested did increase yolk sac moisture. Absolute YSW, relative YSW to SEW, YFBW, relative YFBW to SEW, or yolk-free body moisture on the day of hatch were not affected by any of the treatments.

In the current study, the injection of any of the carbohydrate solutions or diluent at 0.4 mL did not affect absolute liver weight as a percentage of BW, or liver DM weight on E19 and on the day of hatch (Figure 1A, 1B, and 1D). Fresh liver weight increased from 0.61 g on E19 to 0.95 g on the day of hatch, and liver DM weight increased from 0.2 g on E19 to 0.32 g on the day of hatch (Figure 1A and 1D). Moreover, liver weight relative to BW increased from 1.43% on E19 to 2.18% on the day of hatch (Figure 1B). As compared with the noninjected control, liver moisture was not affected by any injection treatment on E19; however, it did increase in the diluent-injected and M + D groups on the day of hatch (Figure 1C).

Correlation analyses among relative liver weight, YSW, and YFBW to SEW showed that relative YSW was negatively correlated to relative YFBW on E19 and the day of hatch, and was also negatively correlated to relative liver weight on the day of hatch (Table 3).

Liver protein concentration (Figure 2A) was not affected by any injection treatments on E19 or the day of hatch. As compared with the noninjected control, diluent injection decreased glycogen concentration in the liver on E19. All carbohydrate solutions injected increased liver glycogen concentrations as compared with that of the diluent-injected control. Moreover, dextrin supplementation increased glycogen concentration in the liver compared with those of the noninjected control, diluent-injected control, S + D, and M + D groups. The glycogen concentrations in the G + D, S + D, and M + D groups were not significantly different from the noninjected control (Figure 2B). Similar to the effect of diluent on glycogen concentration in the liver, the injection of 0.4 mL of diluent also decreased glucose concentration when compared with that of the noninjected control on E19. Supplementation of S + D, M + D, and dextrin, but not G + D increased glucose concentration as compared with that of the diluent injection. The liver glucose concentrations in all carbohydrate solution-injected groups were not significantly different from those of the noninjected control (Figure 2C). Supplementation with carbohydrates decreased fat
concentration in the livers of G + D, S + D, and M + D, but not the dextrin groups on E19. Dextrin-injected groups had higher fat concentrations than did the G + D and M + D groups (Figure 2D). As compared with the noninjected control, a numerical ($P = 0.06$, Figure 2D) increase in fat concentration was observed in the diluent-injected group. From E19 to the day of hatch, liver glycogen concentrations decreased dramatically from an average of 3.2 to 0.6% (Figure 2B). Despite the differences in glycogen, glucose, and fat concentrations (Figure 2B, 2C, and 2D) observed on E19, these differences were lost by the day of hatch. Furthermore, a positive correlation (partial correlation coefficient = 0.29, $P = 0.005$) was found between liver glycogen and glucose concentrations on the day of hatch. To avoid redundancy, total liver content results were not reported because these results reflected the liver concentration data.

<table>
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<tr>
<th>Characteristic</th>
<th>Day 19 of incubation</th>
<th>Day of hatch</th>
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<tr>
<td></td>
<td>YSW/SEW</td>
<td>YFBW/SEW</td>
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<tr>
<td>LW/SEW</td>
<td>−0.12$^1$ (0.28)$^2$</td>
<td>0.21 (0.06)</td>
</tr>
<tr>
<td>YSW/SEW</td>
<td>−0.43 (&lt;0.0001)</td>
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$^1$Values represent 2 subsamples/replicate × 8 replicates × 6 treatments for all parameters. Values are partial correlation coefficients with $P$-values in parentheses.

Figure 1. Absolute liver weight (A), liver weight as a percentage of BW (B), liver moisture concentration (C), and liver DM (D) on d 19 of incubation (E19) and the day of hatch from noninjected control, 0.4-mL diluent-injected control, and 1) 6.25% glucose and 18.75% dextrin; 2) 6.25% sucrose and 18.75% dextrin; 3) 6.25% maltose and 18.75% dextrin; and 4) 25% dextrin-injected groups. All carbohydrates were dissolved in 0.4 mL of diluent. Eight replicates, 2 subsamples per replicate for all parameters. $^a,b$Means among treatments with no common letter differ significantly ($P \leq 0.05$).
A 0.4-mL solution volume of 25% dextrin was chosen for in ovo injection in the current study because the highest HF was observed for this particular solution volume and carbohydrate type in a previous study (Zhai et al., 2011b). In that same study, the other 3 carbohydrates used (25% glucose, sucrose, or maltose) were found to be safe at a 0.1-mL volume (HF was greater than 90%). In 3 of the 4 solutions that were injected at a 0.4-mL volume in the current study, 0.1 mL of a 25% dextrin solution was replaced with 0.1 mL of a 25% glucose, sucrose, or maltose solution. This resulted in a final solution volume of 0.4 mL containing glucose, sucrose, or maltose concentrations at 6.25%, in combination with dextrin at 18.75%.

The current results showed that HF was not affected by any injection treatment. However, as compared with the noninjected controls, embryo BW on E19 was increased in all carbohydrate solution-injected groups, and chick BW on the day of hatch was increased in the diluent-injected, S + D, and M + D groups, but not in the G + D and dextrin groups. It has been reported that the injection of a 1.0-mL mixture of carbohydrates increased embryo and chick BW without having any detrimental effect on HF (Tako et al., 2004; Uni and Ferket, 2004; Uni et al., 2005). However, Zhai et al. (2011a) recently reported that as compared with the noninjected controls, the injection of 1.2 mL of 0.3 g/mL of glucose, fructose, maltose, sucrose, or dextrin dissolved in 0.85% saline decreased HF, even though hatchling whole BW and BW relative to SEW were improved by the injection of these same carbohydrate solutions. A further study showed that the combination of the type and volume of carbohydrate used had differential effects on HF. Even though BW was positively related to injection volume, to achieve an HF of 90%, injection volume could not exceed 0.4 mL if fructose or sucrose solutions were injected, and could not exceed 0.7 mL if glucose, maltose, or dextrin solutions were injected (Zhai et al., 2011b).

In the current study, the supplementation of carbohydrate increased yolk sac moisture on the day of hatch but did not affect yolk free body moisture. This was
consistent with a previous study (Zhai et al., 2011b), which showed that yolk sac moisture, but not yolk-free body moisture, was positively correlated to the injection of various carbohydrate solutions. In the current study, the injection of a low volume (0.4 mL) of any of the carbohydrate solutions did not affect embryo and chick yolk sac nutrient utilization. Similarities among all of the treatments for absolute and relative YSW and YFBW were indicative of their lack of effect. These results were different from those that were found by Zhai et al. (2011a), in which the injection of a high volume (1.2 mL) of various carbohydrate solutions decreased yolk sac nutrient utilization. This was indicated by a higher residual YSW, a decrease in nutrient deposition in somatic tissues, and a lower YFBW on the day of hatch (Zhai et al., 2011a). This suggests that the use of carbohydrates requires an appropriate volume to improve energy status and growth without disrupting internal nutrient balance.

Zhai et al. (2011a) reported that injection volumes of 1.2 mL for sucrose, maltose, or dextrin dissolved in saline were associated with a lower liver weight at hatch. However, in the current study, the injection of 0.4 mL of any of the carbohydrate solutions or diluent did not affect liver weight. Furthermore, liver size increased by about 50% of its original size in just 2 d. The embryonic liver grows extensively during the last few days of incubation to accommodate increasing metabolic requirements for energy and nutrients by the embryo as it approaches hatch. Romanoff (1960) reported that the absolute liver weight of chicken embryos was 0.58 to 0.65 g by E19 and 0.82 to 1.5 g by the day of hatch. The rapid growth of the embryonic liver confirms that its functions are crucial for normal embryo development and growth. Both DM deposition and the concentration of solids in the embryonic liver increase much faster than that in all of the other major organs (Romanoff, 1960, 1967). Zhai et al. (2011a) also reported that a large decrease in liver weight relative to YFBW occurred in response to the injection of sucrose or dextrin solutions. Liver moisture was increased in the diluent-injected and M + D groups on the day of hatch. Furthermore, before pipping, the injection of external water did not increase the moisture content of the livers of the embryos. However, the higher moisture content in the livers of the hatchlings in the diluent-injected and M + D groups may have been because of an increase in their metabolic rates.

On the day of hatch, YSW relative to SEW was found to be negatively correlated to liver weight relative to SEW and YFBW relative to SEW. Yolk contains various nutrients, including protein, fat, carbohydrates, minerals, and vitamins (Burley and Vadehra, 1989), which are essential to the growth of embryos. The decrease in yolk nutrient absorption and mobilization would directly lead to a decrease in the availability of nutrients for embryo tissue growth, which may also include the liver. Another explanation for the negative correlation between liver weight and YSW might be that the rate of yolk sac nutrient mobilization is associated with liver size in chicks. The liver can produce glucose by the catabolism of its glycogen reserves and through the gluconeogenesis of protein and fat (Berg et al., 2002). Furthermore, although glucose and lipids are not able to stimulate amino acid deposition in muscle protein, their increased availability may help spare body protein (Vazquez et al., 1988). Although all of the carbohydrates injected in this study increased liver glycogen concentrations on E19 in comparison to diluent-injected controls, liver protein concentration was not affected by any injection treatments on E19 or the day of hatch. As compared with the noninjected control, the injection of diluent decreased glycogen and glucose concentrations in the liver on E19 and numerically (\( P = 0.06 \)) increased fat concentration. Carbohydrate supplementation alleviated or even reversed the effects of the diluent on liver glycogen and glucose concentrations. As compared with the diluent-injected group, use of all supplementary carbohydrates increased liver glycogen and glucose concentrations (except for the G + D group), and decreased fat concentration (except for the dextrin group) on E19.

In the last 2 d of incubation, liver glycogen concentrations dropped dramatically from an average of 3.2 to 0.6%. This would suggest that glycogen was preferentially utilized over fat and protein as an energy source by the chick embryo at the time of hatch (Freeman, 1965, 1969; Pearce, 1971; Moran, 2007). Borrebaek et al. (2007) reported that liver glycogen decreased from 2.8% on E19 to 0.8% on the day of hatch, but as compared with glycogen, liver protein concentration was relatively constant (13.6% on E19 and 14.2% on the day of hatch). The results of the current study were similar to (or confirmed) these results.

The positive correlation between liver glycogen and glucose concentrations on the day of hatch in the current study would be related to the fact that glycogen can be broken down to glucose quickly to meet the embryo’s high energy requirements. These current and past findings would also be related to the fact that the body prefers carbohydrates as an energy source over lipids and protein (Berg et al., 2002). It has been reported that the most active muscle groups exclusively use glucose from glycogen as an energy source during pipping, largely because glucose provides more energy than fat with use of same amount of oxygen (Freeman, 1969; Moran, 2007). This is crucial before external piping when the oxygen supply is limited (Christensen et al., 2001; Moran, 2007).

In conclusion, the use of 0.4-mL volume solutions of various carbohydrates dissolved in commercial diluent increased embryo and hatching BW. It is suggested that this resulted from changes in the liver nutrient profile. In addition, the supplementation of carbohydrates alleviated the decrease in glycogen and glucose concentrations in the embryonic liver, which were induced by the injection of commercial diluent. This occurred through the provision of a readily available source of...
energy and by helping to balance the embryo’s metabolism of carbohydrates, proteins, and lipids in the liver. Nevertheless, these changes in response to treatment were subsequently modified by the pattern of energy utilization by the embryo during the hatching process. However, to fully determine the effect of the treatments on their pattern of energy utilization, oxygen consumption and carbon dioxide production levels should also be determined in future studies. Furthermore, gluconeogenesis is critical to hatching. Therefore, the results of this research provide important information that is of interest to hatchery personnel, given that it concerns changes in the gluconeogenic nutrient status of the liver during the hatching process in response to the in ovo administration of commercial diluent and supplemental carbohydrates.

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REFERENCES


