ABSTRACT: The objective of this study was to test the hypothesis that in ovo injection of carbohydrates into pigeon (*Columba livia*) amnion may improve the small intestine development. At d 14.5 of incubation, 80 fertile eggs were injected with 200 μL of carbohydrate solution, and 80 control eggs were not injected. The carbohydrate solution (wt/vol) contained 2.5% maltose + 2.5% sucrose, all dissolved in 0.75% saline. Twelve eggs from each treatment were randomly sampled at d 16 of incubation and the day of hatch, embryos or young pigeons were euthanized, and the jejunum samples were collected. Jejunal villus surface area, activity of the brush border enzymes, sucrase, maltase, aminopeptidase-N, and alkaline phosphatase, and mRNA expression of the digestion-absorption related genes *oligopeptide transporter 1*, *sodium glucose transporter 1*, *glucose transporter 2*, *aminopeptidase-N*, and *sucrase-isomaltase* were examined. Results showed that in ovo injection of carbohydrate solution caused a villus surface area increase of 38% on d 16 of incubation and 23% on day of hatch relative to controls (*P* < 0.05). The in ovo injected pigeons exhibited greater (*P* < 0.05) activities of jejunal sucrase, maltase, and alkaline phosphatase from d 16 of incubation to day of hatch compared with the controls. At day of hatch, aminopeptidase-N activity in embryos injected in ovo was approximately 27% greater (*P* < 0.05) than control embryos. Enhanced expressions of the jejunal *sodium glucose transporter 1*, *glucose transporter 2*, and *aminopeptidase-N* mRNA were found at d 16 of incubation in embryos that received carbohydrate solution into the amniotic fluid in comparison with the control group (*P* < 0.05). These results indicate that the in ovo injected pigeon may hatch with more mature enterocytes and greater intestinal digestive and absorptive capacity than the conventional hatchling. Therefore, the in ovo injected pigeons may become more precocial at hatch and easier to hand-rear during the immediate posthatch period.

**Key words:** amnion, carbohydrate, in ovo injection, pigeon, small intestine
In ovo injection of carbohydrate solution

Tsat et al., 1994). Therefore, to hand-feed young pigeons from the immediate posthatch period, it is desirable to enhance intestinal function and maturation before hatching. In ovo injection (Uni and Ferket, 2003) is a method of supplementing exogenous nutrients into the amnion of the late-term avian embryo. Therefore, in ovo injection may serve as a tool to improve the gut development prehatch in oviparous species, as the avian embryo orally consumes the amniotic fluid (together with the supplemental nutrients) before hatching. Studies have shown that in ovo injection increased villus dimensions and mRNA expression and activity of brush border digestive enzymes and transporters in precocial birds (Tako et al., 2004; Foye et al., 2007, 2009; Al-Daraji et al., 2012; Shafey et al., 2012). However, effects of in ovo injection on intestinal development studies have not been determined in young pigeons.

Previous studies in our laboratory demonstrated that in ovo injection of carbohydrates (2.5% maltose + 2.5% sucrose in 0.75% saline) on d 14.5 of incubation can improve the hatchability, BW, body glycogen reserves, and yolk sac nutrient use in domestic pigeons at hatch (X. Y. Dong et al., 2013). We hypothesize that in ovo injection of carbohydrates would improve enteric development in pigeons before hatching. Therefore, the aim of the current study was to test this hypothesis by evaluating the effects of in ovo injection of carbohydrates on the morphology, gene expression, and activity of brush border enzymes and transporters in the pigeon small intestine.

MATERIALS AND METHODS

All procedures were approved by the Animal Care and Welfare Committee of Animal Science College, Zhejiang University (Hangzhou, China).

Birds and Incubation

Fertilized pigeon (Columba livia) eggs were obtained from a commercial pigeon farm (Wenzhou, China). All the eggs used were collected from a 40-wk-old breeder flock and were laid within a 24-h period. The eggs were incubated (18-d incubation period) under optimal conditions (38.1°C and 55% relative humidity) in an incubator (Model CFZ; Keyu Incubation Equipment Co. Ltd., Dezhou, China). On d 6 of incubation, eggs were candled, and unfertilized eggs were removed from the incubator.

In Ovo Injection Procedure

At d 12 of incubation, 160 fertile eggs with viable embryos were identified by number and weighed. The eggs were then randomly allocated to 2 treatment groups of 80 eggs (4 trays in each treatment group) with similar weight (18.1 ± 2.5 g). Both treatment groups were randomly represented in each of 4 tray levels in the incubator. At d 14.5 of incubation, the amnion in the in ovo injection group was identified by candling. A hole was punched using a 21-gauge needle and 200 μL of carbohydrate solution was injected manually into the amnion using a 21-gauge needle as described by Uni and Ferket (2003). The carbohydrate solution (wt/vol) contained 2.5% maltose + 2.5% sucrose dissolved in 0.75% saline. The carbohydrate solution was autoclaved at 121°C for 15 min and then allowed to reach room temperature before injection. Preliminary experiments conducted in our laboratory demonstrated that sham injection (shell perforated but without solution injection) or injection of 200 μL 0.75% saline did not affect hatchability, embryo and chick BW, or intestinal development (data not shown). Therefore, the other group was not injected and served as the control group. Immediately after the injection, the holes in the eggs were disinfected with ethyl-alcohol laden swabs, sealed with cellophane tape, and transferred to hatching baskets. All eggs were held outside the incubator for fewer than 30 s to complete the in ovo injection procedure, including the noninjected control eggs.

Sample Collection

Twelve eggs from each treatment group (3 from each of 4 tray levels) were randomly sampled at d 16 of incubation and the day of hatch (within 2 h after hatch). Because the young pigeons are difficult to hand-rear in the first few days during the immediately posthatch period, the remaining birds in each treatment were transferred to the commercial pigeon farm and fed by adult pigeons. The adult pigeons were reared in an aviary with free access to food and water. They were fed a mixed-grain diet of cereals and pulses (16.89% CP and 11.47 MJ ME/kg) supplemented with vitamins and minerals. This diet was formulated according to local feeding standard for pigeons (DB34/T 541-2005; Quality and Technology Supervision Bureau, Anhui, China) using a diet formulation software (Brill Formulation version 7; Brill Corporation, Norcross, GA).

Unhatched eggs were opened at the blunt end using surgical scissors and the embryo was extracted. Embryos or young pigeons were killed by cervical disloca-
tion. The BW (with yolk sac) of all birds was recorded. The entire small intestine content and adherent material were removed carefully under ice-cold saline, and the weights were recorded. The jejunum was removed according to the method described by Uni et al. (2003) and 1-cm long segments were taken and placed in 2 separate tubes, with the one fixed in 4% neutral-buffered formalin solution for histology and the other frozen in liquid N, and then stored at –80°C for measurement of enzyme activity and mRNA abundance.

Morphological Examination

Jejunal samples were dehydrated, cleared, and embedded in paraffin. Serial sections (5 μm) were placed on glass slides and stained with hematoxylin and eosin. Sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and examined by light microscopy (Nikon Corp., Tokyo, Japan). Villus area was calculated from the villus height (from the tip of the villi to the villus crypt junction) and width at half height (Uni et al., 1998). Values presented are means from 10 adjacent villi and only vertically oriented villi were measured.

Intestinal Enzyme Activity

Enzyme activities were assayed in homogenized jejunal tissue. Samples were thawed at 4°C and homogenized in 10 times of the volume of cold normal saline. The homogenates were then centrifuged at 20,000 × g for 20 min at 4°C and the supernatant was collected for enzyme assays. Sucrase [Enzyme Commission (EC) 3.2.1.48] and maltase (EC 3.2.1.20) activity were assayed colorimetrically using sucrose and maltose as substrates, respectively (Dahlqvist, 1964; Palo et al., 1995). Activity was expressed as micromoles of glucose released per minute per gram of jejunal wet tissue. Aminopeptidase-N activity (EC 3.4.11.2) was determined by hydrolysis of L-leucine-p-nitroanilide for 15 min at 37°C, and p-nitroanilide was determined spectrophotometrically at 405 nm as described by Caviedes-Vidal and Karasov (2001). Activity was expressed as micromoles of p-nitroanilide released per minute per gram of jejunal wet tissue. Alkaline phosphatase (EC 3.1.3.1) activity was determined by measuring the hydrolysis of p-nitrophenol at 37°C according to Palo et al. (1995) and the unit of activity was expressed as per minute per gram of jejunal wet tissue.

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from pigeon jejunal tissues (approximately 50 mg) using a reagent according to the manufacturer’s instructions (TRIzol; Invitrogen, Carlsbad, CA). The quality of total RNA was checked by both native RNA electrophoresis on 1.0% agarose gel and the UV absorbance ratio at 260 and 280 nm. The cDNA was synthesized from 2 μg of total RNA by a reverse transcriptase (M-MLV; Takara, Dalian, China) at 42°C for 60 min with oligo dT-adaptor primer using the protocol of the manufacturer.

Real-Time PCR

The abundance of mRNA was determined (StepOne Plus Real-Time PCR system, ABI 7500; Applied Biosystems, Foster City, CA). Gene-specific primers (Table 1) for oligopeptide transporter 1, sodium glucose transporter 1, glucose transporter 2, aminopeptidase-N, sucrase-isomaltase, and endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were based on our previous work (Dong et al., 2012b).

Table 1. Primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PepT1</td>
<td>GGCACCCAGTCTGTAGTCTGGTGACT</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>GCACAAAGAGGCAAGGACATACCA</td>
<td></td>
</tr>
<tr>
<td>SGLT1</td>
<td>GCCATTGCTGGTGAGGAGGCGATT</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>CACTTTGAGCTGACCTGACCCACA</td>
<td></td>
</tr>
<tr>
<td>GLUT2</td>
<td>CCTCTGAGCTGTCGGCCATGTAT</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>GGGTATGAGGACATTCCCGGCAAGAGA</td>
<td></td>
</tr>
<tr>
<td>APN</td>
<td>GCTCCCTTACACAGGAGACATACC</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>GTCTGTCCGAGTCTGCTGAAAGA</td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>GGTTCGCCGACTCCAAGACTCACC</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>GTCTGTCCGAGTCTGCTGAAAGA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCAGGAACACATCCCGAAGAGC</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>CCCCAGGAAGTGACCAGTATGAG</td>
<td></td>
</tr>
</tbody>
</table>

Gene-specific primers for oligopeptide transporter (PepT1), sodium glucose transporter (SGLT1), glucose transporter (GLUT2), aminopeptidase-N (APN), sucrase-isomaltase (SI), and endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were based on our previous work (Dong et al., 2012b).
In ovo injection of carbohydrate solution

average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The calibrator for each studied gene was the average $\Delta C_t$ value of jejunum in the control group at d 16 of incubation.

**Statistical Analysis**

Data were statistically analyzed by SPSS (SPSS Inc., Chicago, IL). A generalized randomized block design was used, with each tray level representative of a block and with all treatments being equally represented on each of the 4 tray levels. For each sampling time, data were analyzed using GLM procedure, with treatment designated as a fixed effect and tray level as a random effect. All data are presented as means ± SE. Differences at $P < 0.05$ were regarded as statistically significant.

**RESULTS AND DISCUSSION**

Carbohydrates were chosen as the nutrient for in ovo injection in our study because they are critical during the later term of avian embryo development and very little carbohydrate remains in the amniotic fluid and the yolk sac (Noble and Oggunyemi, 1989; Christensen et al., 1993; ten Busch et al., 1997; Noy and Sklan, 1998). In addition, previous studies have demonstrated that supplemental carbohydrate in the intestine increases the capacity of the intestine to digest and absorb nutrients from diets (Pluske et al., 1996; Petersen et al., 2002). In the current study, in ovo injection of carbohydrate solution to the late-term pigeon embryo resulted in a 2.9% increase of BW at d 16 of incubation and 5.7% increase at day of hatch compared with the controls ($14.25 \pm 0.11$ g vs. $14.67 \pm 0.17$ g and $15.56 \pm 0.14$ g vs. $16.45 \pm 0.18$ g, respectively; $P < 0.05$).

At the same time, in ovo injection of carbohydrate solution caused a villus surface area increase of 38% on d 16 of incubation and 23% on day of hatch relative to controls ($P < 0.05$; Fig. 1). The results are consistent with those reported in a study conducted by Tako et al. (2004) who showed that chickens that were in ovo with carbohydrates exhibited increased villus surface area compared with the control group 48 h after an in ovo injection procedure. The trophic effect of in ovo injection on villus surface area might be attributed to the high concentrations of blood insulin caused by ingesting carbohydrates. Studies have demonstrated that insulin is a potent stimulator of intestinal epithelial proliferation (Chao and Donovan, 1996). Yamauchi et al. (1996) stated that the increased absorptive capacity was due to the expansion of villus surface area that occurs with villus growth. In addition, Sklan (2001) showed that the villus surface area was correlated with growth in the chicken. Therefore, it is reasonable to speculate that the increase in intestinal villus area in the in ovo injection treatment contributed to elevated nutrient digestion and absorption and, hence, enhanced BW.

The intestinal brush border enzymes play an important role in the final degradation of nutrients (e.g., carbohydrate and protein) before absorption. In the current study, jejunal disaccharidase (sucrase and maltase) activities were detectable in all groups at d 16 of incubation in pigeon embryo (Fig. 2A and 2B) although the amniotic fluid contained very low concentrations of carbohydrates (ten Busch et al., 1997). Studies have demonstrated that the activity of disaccharidase was increased by greater amounts of substrate in the intestine (Siddons, 1972; Sell et al., 1989). Our results showed that after in ovo injection, jejunal sucrase and maltase activities per gram of tissue increased ($P < 0.05$) by 39 and 43%, respectively, at d 16 of incubation and 26 and 52%, respectively, at the day of hatch compared with controls. Similarly, Tako et al. (2004) reported that the activity of chicken jejunal sucrase-isomaltase was increased 48 h after in ovo injection of carbohydrate. These results can be explained by the direct effect of carbohydrate substrates on brush border disaccharidase activity. Young pigeons are fed by their parents with only pigeon milk containing mainly proteins and lipids consumed in the first few days after hatch (Vandeputte-Poma, 1980). However, increased amounts of carbohydrate should be considered when nutritionists formulate diets for hand-rearing young pigeons immediately after hatch, as pigeons have increased potential for carbohydrate digestion and assimilation during the posthatch development (Dong et al., 2012b). Yang and Vohra (1987) showed that a diet containing 21.5% glucose supported the optimum growth of squabs (young pigeons) for the first 7 d. Therefore, the increased disaccharidase activity after in ovo injection in the current study may help the young pigeons adapt to a carbohydrate-containing diet that is formulated for hand-rearing young pigeons from the immediately posthatch period.
Brush border aminopeptidase-N plays a central role in protein digestion. No differences were observed for jejunal aminopeptidase-N activity per gram of tissue between in ovo injection and control treatments at d 16 of incubation (Fig. 3A). At day of hatch, aminopeptidase-N activity per gram of tissue of pigeons given the in ovo injection was approximately 27% greater (P < 0.05) than that of the control. The increased aminopeptidase-N activity caused by in ovo injection of carbohydrate solution at hatch is intriguing, as no specific substrate was provided for aminopeptidase-N during the in ovo injection procedure. During the period of embryonic transition towards an independent chick, yolk sac, rich in fat and protein, is the major nutrient source. There are 2 routes for yolk use. One is by direct absorption through the yolk sac membrane (Murakami et al., 1992), and the other is via the yolk stalk into the small intestine (Esteban et al., 1991). Our previous work showed that the shift from the route through the yolk sac membrane to the route through the intestine occurs between d 16 of incubation to the day of hatch in pigeons (Dong et al., 2012b). Evidently, the protein substrate from the yolk sac present in the intestine can elevate the intestinal aminopeptidase-N activity during this period. In addition, in ovo injection of carbohydrate increased yolk sac nutrient use as reflected by decreased residual yolk sac weight compared with conventionally raised pigeons at day of hatch (X. Y. Dong et al., 2013). Therefore, the increased aminopeptidase-N activity of in ovo injected pigeons at day of hatch may partly be associated with the increased yolk sac nutrient use.

Providing carbohydrates to late-term pigeon embryos increased the jejunal alkaline phosphatase activity per gram of tissue at d 16 of incubation and the day of hatch by 44 and 35%, respectively (P < 0.05; Fig. 3B). The alkaline phosphatase is a glycoprotein (Engstrom, 1961). Glucose is a substrate for the biosynthesis of macromolecules such as glycoproteins, proteoglycans, and glycolipids. Therefore, the presence of available glucose in the intestinal lumen of the chick embryo might be a trigger for enhancement in the activity of alkaline phosphatase. At day of hatch, changes of alkaline phosphatase activity between the in ovo treatment and the control paralleled those of villus surface area and brush border enzyme activity (sucrase, maltase, and aminopeptidase-N). It has been established that alkaline phosphatase is expressed in mature mucosal enterocytes and has been used as an enterocyte maturation marker (Weiser, 1973; Traber et al., 1991). Our results indicate that the intestinal enterocytes of in ovo injected pigeons may be more mature than enterocytes in conventionally raised pigeons at day of hatch.
The various nutrients in the diet are digested and absorbed through the brush border membrane proteins produced by the specifically expressed intestinal genes (Ferraris, 2001). Changes of digestive capacity by in ovo injection can be evaluated by studying the changes in the expression of the digestive and absorptive related gene mRNA (de Oliveira et al., 2009). Sucrase and maltase appear to be complexed as sucrase-isomaltase within the brush border membrane (Hunziker et al., 1986). No differences were observed for jejunal sucrase-isomaltase mRNA expression between in ovo injected and control treatments from d 16 of incubation to day of hatch (Fig. 4A). This result is not consistent with previous reports that showed that dietary sucrose enhanced sucrase-isomaltase mRNA abundance in rat small intestines (Broyart et al., 1990; Yasutake et al., 1995). However, in ovo injection had a marked effect on the activity of sucrase and maltase during the same period. It is suggested that in ovo injection of carbohydrates to the amnion may have a posttranscriptional regulation for jejunal sucrase-isomaltase gene in the pigeon. The processes of intestinal absorption of carbohydrates are mediated by transporters within the brush border membranes (Semenza, 1986; Thorens, 1996). The sodium glucose transporter 1 mediates the Na\(^+\)-dependent uptake of glucose and galactose across the apical membrane (Thorens, 1996) whereas glucose transporter 2 mediates the facilitated transport of these monosaccharides across the basolateral membrane (Uldry and Thorens, 2004). Enhanced expression of the jejunal sodium glucose transporter 1 and glucose transporter 2 mRNA was found at d 16 of incubation in embryos that received carbohydrate solution into the amniotic fluid in comparison with the control group (\(P < 0.05\); Fig. 4B and 4C). Similarly, Miyamoto et al. (1993) showed that a high-glucose diet stimulated glucose transport activity and increased the abundance of sodium glucose transporter 1 and glucose transporter 2 mRNA in rat jejunum. Upregulation of these monosaccharide transporter expressions before hatching is an apparent attempt to completely absorb incoming carbohydrates. At day of hatch, no difference was observed for monosaccharide transporter mRNA expression between the in ovo injection and control group. These results indicate that in ovo injection of carbohydrates can, in a short period of time, enhance the expression of the genes encoding monosaccharide transporters in the pigeon intestinal absorptive cells. These results are also compatible with the work of Kishi et al. (1999) who showed that mRNA abundance of sodium glucose transporter 1 and glucose transporter 2 were coordinately increased in rat jejunum within 12 h after force feeding a fructose or sucrose diet.

At d 16 of incubation, an increase (\(P < 0.05\)) was observed for jejunal aminopeptidase-N mRNA expression in the in ovo injected group compared with the control whereas no differences were observed on the day of hatch (Fig. 5A). Although mRNA expression is not necessarily correlated with protein activity (Ferraris and Diamond, 1997), increases in aminopeptidase-N activity of in ovo injected pigeons on the day of hatch were accompanied by an increase in mRNA expression on d 16 of incubation in this study. Therefore, for pigeon intestinal aminopeptidase-N, we speculate that changes in expression of mRNA would be expected to precede changes in activity. At the brush border membrane, oligopeptide transporter 1 transports di- and tripeptides from the lumen of the small intestine into the enterocyte (Leibach and Ganapathy, 1996; Chen et al., 2002). No differences were found in

![Figure 4. The mRNA expression of sucrase-isomaltase (A), sodium glucose transporter 1 (B), and glucose transporter 2 (C) on d 16 of incubation and the day of hatch (within 2 h after hatch) in the pigeon jejunum. Relative gene expression ± SE (\(n = 12\)) was calculated using the 2\(^{-}\Delta\Delta Ct\) method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt (ΔCt=Ct gene of interest - Ct endogenous reference gene) value of jejunum in the control group at d 16 of incubation as the calibrator. a,b Different letters within the same time points indicate differences between the 2 treatments (\(P < 0.05\)). The in ovo treatment involved injection with a carbohydrate solution (wt/vol) containing 2.5% maltose + 2.5% sucrose dissolved in 0.75% saline, and the control group was not injected.](image-url)
mRNA expression of jejunal oligopeptide transporter 1 on any of the days examined (Fig. 5B). Thus, the inclusion of carbohydrate in the in ovo injected solution did not enhance additional pigeon jejunal oligopeptide transporter 1 gene expressions although the jejunum lumen of the in ovo injected pigeon had more di- and tripeptides as reflected by greater aminopeptidase-N activity than those of the control on the day of hatch. Therefore, it seems that the basal expression of oligopeptide transporter 1 is sufficient to absorb these di- and tripeptides present in the lumen at the day of hatch.

In conclusion, in ovo injection of carbohydrate on d 14.5 of incubation enhanced pigeon intestinal development by increasing the size of the villi and the activity of brush border enzymes. In addition, the in ovo administration of carbohydrate showed an upregulation in digestion/absorption-related intestinal genes mRNA expression at d 16 of incubation. These results indicate that the in ovo injected pigeon may hatch with more mature enterocytes and greater intestinal digestive and absorptive capacity than the conventional hatchling. Therefore, the in ovo injected pigeons may become more precocial at hatch and easier to handrear during the immediate posthatch period.

**Figure 5.** The mRNA expression of aminopeptidase-N (A) and oligopeptide transporter 1 (B) on d 16 of incubation and day of hatch (within 2 h after hatch) in the pigeon jejunum. Relative gene expression ± SE (n = 12) was calculated using the 2−ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt (ΔCt = Ct gene of interest - Ct endogenous reference gene) value of jejunum in the control group at d 16 of incubation as the calibrator. Different letters within the same time points indicate significant differences between the 2 treatments (P < 0.05). The in ovo treatment involved injection with a carbohydrate solution containing 2.5% maltose (wt/vol) + 2.5% sucrose (wt/vol) dissolved in 0.75% (wt/vol) saline. Controls were not injected.

**LITERATURE CITED**


In ovo injection of carbohydrate solution


