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The Effects of Intra-Amnionic Feeding of Arginine And/or ß-Hyroxy-ß-Methylbutyrate on Jejunal Gene Expression in the Turkey Embryo and Hatchling

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Abstract: *In-ovo* Feeding (IOF), injecting nutrients into the amnion of the developing embryo may enhance post-hatch growth by enhancing intestinal expression and function prior to hatch. This hypothesis was evaluated with IOF solutions of Arginine (ARG), HMB and Egg White Protein (EW) in turkeys. Four treatments were arranged as a factorial of 2 levels of ARG (0 and 0.7%) and HMB (0 and 0.1%). An IOF solution of EW (18%) was evaluated for contrast. At 23 d of incubation (23E) each IOF solution was injected into the amnion. Upon hatch all poults were fed *ad libitum*. Intestinal mRNA of the digestion/absorption related genes Sodium Glucose Transporter (SGLT), Peptide transporter (Pept), Sucrase-isomaltase (SI) and Aminopepdiase (AP) were determined at 25E, hatch, 3 and 7 d by real-time PCR analysis. The data was analyzed as a 2X2 factorial and 1-way ANOVA for contrast. There were significant ARG X HMB effects on Pept, SGLT, SI and AP mRNA levels at hatch. IOF HMB alone enhanced Pept, SGLT, SI and AP intestinal mRNA expression at hatch, whereas inclusion of ARG depressed expression. There were main and independent effects of HMB or ARG on mRNA expression of SI and AP at 25E, in which ARG alone depressed expression, while IOF HMB alone had no effect on SI or AP expression. These results suggest that IOF may enhance early growth by improving intestinal capacity to digest and absorb nutrients at hatch which may fuel more rapid post-hatch growth.

Key words: *In ovo* feeding, intestinal gene expression, nutrient digestion, nutrient absorption, Turkey

INTRODUCTION

At hatch, the Gastrointestinal Tract (GIT) of the avian neonate must readily adapt from an *in ovo* lipid (yolk) nutriture to an external corn-based diet (Noble and Ogunyemi, 1989; Noy and Sklan, 1998). During the first two weeks post-hatch, the small intestine undergoes rapid morphological changes (Uni *et al*., 2000; Geyra *et al*., 2001; Uni *et al*., 2003) and steadily increases the capacity to digest and absorb nutrients (Uni *et al*., 1998, 1999, 2000; Noy *et al*., 2001; Sklan, 2001). Digestion occurs as macronutrients are chemically degraded into smaller molecules (disaccharides, peptides and free amino acids) by the intestinal brush border enzymes and subsequently, the nutrients are absorbed across the intestinal epithelium by the nutrient transporters. Digestion of nutrients by the brush border enzymes provide the substrates needed for growth, as the GIT is the primary supply organ and must function optimally very early in life to ensure survival. Consumed and digested nutrients are utilized to meet the metabolic requirements needed for rapid growth and development in young hatchlings (Uni *et al*., 2003; Uni and Ferket, 2003).

However, during this critical post-hatch period, the brush border enzymes have varying developmental timetables (Ferraris, 2001; Sklan *et al*., 2003; Uni *et al*., 2003; Uni and Ferket, 2004) and therefore, may constrain post-hatch growth. Many of the nutrient transporters and brush border enzymes show increased levels of expression just prior to hatching (Uni *et al*., 2003; Sklan *et al*., 2003). In the chick embryo, maltase, Aminopeptidase (AP), sodium-glucose co-transporter (SGLT-1) and ATPase activities begin to increase at 19 days of embryonic development and increases further at the day of hatch (Uni *et al*., 2003), while transcripts are first detected at 15 days of embryonic development. Thus, young hatchlings may experience mal-absorption of nutrients during this time frame in which the gut is reaching functional maturity.

Under standard hatchery practices, the avian neonate may be denied access to feed and water from 48-72 h post-hatch (Moran and Reinhart, 1980) that may be a hindrance to gut maturation and function. Studies have shown that orally consumed nutrients help accelerate enteric development, function and maturation (Zarling and Mobarhan, 1987; Butzner and Gall, 1990). Studies by Geyra *et al*. (2001) demonstrated that delaying access to feed up to 48 h after hatch adversely affected the GIT due to a reduction in the intestinal surface area, number of cells/crypt and the number of proliferating cells, particularly in the duodenum and jejunum. Additionally, Geyra *et al*. (2002) demonstrated that the expression of the transcriptional factors CdxA and CdxB needed for expression of intestinal genes responsible

for intestinal digestion and absorption were depressed by <48 h of starvation in comparison to fed chicks post-hatch. Hence, denied or delayed access to feed may developmentally delay post-hatch enteric maturation and limit subsequent growth.

We hypothesize that *in ovo* feeding (US Patent 6,592,878 Uni and Ferket, 2003), the administration of exogenous nutrients into the amnion of the late-term embryo of oviparous species, may serve as a tool to overcome growth constraints imposed by limited digestive capacity in hatchlings by enhancing intestinal expression of the digestive and absorptive related genes. Naturally, the embryo orally consumes the amniotic fluid prior to pipping the air cell, thus consuming the supplemented nutrients which are presented to the enteric tissues and stimulate nutrient digestion and absorption. Therefore, *in ovo* Feeding (IOF) may increase the GIT capacity to absorb and digest nutrients prior to hatching by up-regulating the expression of the nutrient transporters and brush border enzymes responsible for digestion of carbohydrates and proteins. Consequently, young hatchlings may have enhanced digestive and absorptive capacity during the critical post-hatch period.

Earlier studies (Tako *et al*., 2004) have demonstrated that IOF of carbohydrates and/or ß-Hydroxy-ß-Methyl-Butyrate (HMB) enhanced the intestinal absorptive surface area in comparison to control broiler chicks at hatch. Chicks *in ovo* fed HMB, a leucine metabolite, had a 45% increase in the jejunal villus surface area in comparison to the controls, while hatchlings *in ovo* fed carbohydrate alone or in combination with HMB had a 33% increase in the jejunal surface area in comparison to the controls at 3 days post-hatch. Other studies have shown that IOF HMB and/or protein or HMB and/or arginine enhance hatchling bodyweights (Foye *et al*., 2006a-b), hepatic glycogen reserves (Foye *et al*., 2006a-b) and jejunal activity of the brush border enzymes (Foye *et al*., 2007) responsible for carbohydrate and protein digestion.

Other studies have demonstrated enhanced expression and activity of intestinal absorption/digestion-related proteins and genes in response to diet in young and adult animals (Matsushita 1985; Ferraris *et al*., 1988, Ferraris and Diamond, 1997; Buddington *et al*., 1991; Erickson *et al*., 1995; Caviedes-Vidal *et al*., 2000; Ferraris, 2001; Jiang and Ferraris, 2001). However, there is a paucity of studies which address the affects of nutrients on early (embryonic to post-hatch) intestinal development of the absorption/digestion-related genes. The avian egg serves as an adequate animal model for experimentation to elucidate nutrient-gene interactions within the intestine due to a closed environment and known nutrient content. Therefore, nutrient manipulations in the egg of the developing avian embryo may be more closely correlative to intestinal gene expression, whereas the mammalian embryo develops in an environment with constant maternal influences and thus, it is more difficult to identify the inducers of intestinal cellular change. Therefore, the goal of this experiment was to elucidate the effects of IOF solutions containing HMB and/or arginine on the mRNA expression of the jejunal brush border enzymes, Sucrase-Isomaltase (SI), Leucine Aminopeptidase (LAP) and jejunal nutrient transporters, SGLT-1 and Peptide transporter (PEPT-1) in the late term embryo thru two weeks post-hatch.

MATERIALS AND METHODS

Incubation and *In Ovo* Feeding (IOF): Viable Hybrid® turkey eggs were obtained at 19 days of incubation from a commercial hatchery (Prestage Farms, Clinton NC) and incubated according to standard hatchery practices (99.9-100.0°F). At 21 days of incubation 500 eggs were individually weighed and distributed among 4 5-gram weight categories ranging from 65-85 g per egg. These eggs were evenly distributed among five treatment groups of 100 eggs each, such that the weight distribution profile among all 5 treatment groups was identical. At 23 days of incubation, each egg was candled to identify the location of the amnion. A hole was incised using a 23 gauge needle and 1.5 milliliters of IOF solution was injected into the amnion using a 23 gauge needle to a depth of about 15 millimeters. The injection site was disinfected with ethyl alcohol, sealed with cellophane tape and the eggs were transferred to hatching baskets. The IOF solutions contained the following: A) 0.1% [1]HMB in 0.4% saline (HMB); B) 0.7% ARG in 0.4% saline (ARG); D) 0.1% HMB + 0.7% ARG in 0.4% saline (HMB+ARG) and E) 18% egg white protein (EW) + 0.1% HMB + 0.7% ARG in 0.4% saline (HMB+ARG+EW). The controls were not injected with a solution, but they were subjected to the same handling procedures as the IOF treatment groups. Preliminary experimentation conducted in our laboratory indicated that *in ovo* injection of 2.0 milliliters of 0.4% saline did not affect embryo and poult bodyweights, breast yield, glycogen status or jejunal brush border enzyme activity.

Animal husbandry: Upon hatching, each poult was identified by neck tag and the body weights were recorded at hatch, 3, 7, 10 and 14 days post-hatch. Hatch of viable eggs exceeded 95% and did not differ significantly among treatment groups. Poults were randomly assigned to four rooms of approximately two hundred square feet each at Dearstyne Avian Research Facility, North Carolina State University. Twenty-five poults from each treatment were randomly assigned to each of four rooms. Each room was equipped with manual self-feeders and drinkers. The concrete floor was bedded with wood shavings and supplemental heat was provided to a spot brooding temperature of 40°C. Poults were given a turkey starter diet (2935 kcal/kg,

27.5% protein and 5.6% fat), which met or exceed the National Research Council (1994) requirements for turkeys. At hatch 3, 7 and 14 days post-hatch, 10 poults were randomly selected for sampling from each treatment (≈2 poults/room/treatment). The 10 poults per treatment were euthanized by cervical dislocation and jejunal samples were flash frozen in liquid nitrogen. The samples were stored in nuclease-free microfuge tubes at -80°C for later analysis. All experimental protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

RNA extraction, cDNA production and real-time polymerase chain reaction: Total RNA was isolated from the jejunal segment using TRI® reagent (MRC Molecular Research Center, Cincinnati, OH, USA catalog #TR-118) per manufacturer's protocol. Total RNA was dissolved in 0.1% (v/v)-diethyl pyrocarbonate-treated water, digested with DNase I (DNA-free kit, Ambion, Austin, TX cat #1906). Total RNA quality and quantity was determined by gel electrophoresis and spectrophotometric analysis. First strand cDNA was synthesized with 1 μg of DNase digested total RNA from the jejuna according to procedures detailed by Bio-Rad iScript™ cDNA synthesis kit® (Bio-Rad Laboratories, Hercules, CA catalog #170-8891). A primary cDNA standard was prepared by pooling 20 ng of cDNA from each experimental sampling treatment (1000 ng). Working cDNA standards were prepared using the following serial dilutions with DEPC-treated water: 1:2 (500 ng), 1:4 (250 ng), 1:8 (125 ng), 1:16 (75 ng), 1:32 (37.5 ng) and 1:64 (18.75 ng) of the pooled cDNA primary standard.

Gene/species-specific primers for PEPT-1, SGLT-1, SI and LAP (Table 1) were designed using software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA), with beta-actin as the endogenous housekeeping gene. Real-time PCR analysis was performed using the iCycler iQ Multi-Color Real-time PCR detection system (Bio-Rad Laboratories) following the manufacturer's instructions with each set of gene-specific primers.

Linear standard curves (cycle threshold vs. logarithm of cDNA concentration) were plotted to calculate the Amplification Efficiency (AE) of each primer pair by using the serially diluted working standard of pooled cDNA as a template. The Cycle Threshold (Ct) was determined as the cycle number associated with the linear increase in fluorescence (cDNA) above the set threshold; therefore lower Ct values are indicative of higher levels of mRNA expression. The AE was calculated using the following formula $10^{(-1/\text{slope of standard curve})}$. The correlation coefficient for all standard curves was greater than 0.990 and the calculated AE for all primer sets was greater than 95%. Real-time PCR analysis was run on all experimental samples in triplicate using 20 ng of cDNA template.

Calculations and statistical analysis: Relative jejunal gene expression was calculated as the ratio of the Ct value (gene of interest)/ Ct value (endogenous housekeeping gene). All data were statistically analyzed using general linear models procedures for ANOVA (SAS, 1986). Each bird served as an experimental unit for statistical analysis. Data from *in ovo* treatments 0.1% HMB, 0.7% ARG, 0.1% HMB + 0.7% ARG and the non-injected controls were analyzed as a 2 X 2 factorial arrangement, with two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%).

An additional statistical analysis was conducted to contrast the effects of the addition of 18 % dietary protein (EW) to the 0.1% HMB + 0.7% ARG IOF solution. These data were analyzed as a one-way ANOVA (SAS, 1996) comparing treatments 0.1% HMB + 0.7% ARG in 0.4% saline (HMB + ARG), 18% EW + 0.1% HMB + 0.7% ARG in 0.4% saline (EW + HMB + ARG) and the controls. All data were sorted by age and treatment. Variables having different F-test were compared using the least-squares-means function in SAS (1986) and the treatment effects were considered significant at $p<0.05$. All experiments were conducted with an equal frequency of variables within each treatment.

RESULTS

The effects of IOF of two levels of arginine (0% and 0.7%) and two levels of HMB (0% and 0.1%): At 25 days of incubation, there were no interactive effects of HMB and ARG on relative jejunal PEPT-1, SGLT-1, SI, and AP mRNA expression, but there were main and independent effects of ARG and HMB on these genes of interest. Only poults *in ovo* fed HMB alone or in combination with ARG had relative jejunal PEPT-1 mRNA expression levels that were significantly different, while the relative jejunal PEPT-1 mRNA expression levels of all other treatments were similar ($p<0.05$, Table 1). Relative gene expression levels of jejunal SGLT-1 were similar between the controls and poults *in ovo* fed HMB alone or HMB + ARG at 25 days of incubation with a main effect of HMB ($p<0.05$, Table 2). Conversely, poults *in ovo* fed ARG alone had significantly depressed relative jejunal SGLT-1 mRNA expression levels in comparison to poults *in ovo* fed HMB at 25 days of incubation ($p<0.05$, Table 2). Sucrase-isomaltase (SI) gene expression was significantly depressed in poults *in ovo* fed ARG alone, in comparison to the controls, HMB and HMB + ARG treatments at 25 days of incubation with independent and main effects of ARG and HMB ($p<0.05$, Table 3). Jejunal relative AP mRNA expression levels were similar between the controls and poults *in ovo* fed HMB or HMB + arginine at 25 days of incubation, while poults *in ovo* fed ARG alone had significantly depressed relative AP mRNA expression levels in comparison to poults *in ovo* fed HMB alone with main effects of ARG ($p<0.05$, Table 4).

Table 1: Custom Primer Sets designed for Real-time PCR Analysis

Gene	Accession #*	Primer	Sequence (5'-3')	Product (bp)†	Annealing Temp (°C)
Peptide transporter -1 (PEPT1)	AY029615	Sense	CAG GGA TCG AGA TGG ACA CT	243	58
		Antisense	CAC TTG CAA AAG AGC AGC AC		
Sodium glucose co-transporter1 (SGLT-1)	AJ236903	Sense	CAT CTT CCG AGA TGC TGT CA	220	57
		Antisense	AAT TCG GCT GAT CAT TCC AG		
Aminopeptidase (AP)	AP Y17105	Sense	TGG AAT GAC CTG TGG TTG AA	218	54
		Antisense	GCA ATG GAG TCG AAG ACC		
Sucrose-isomaltase (SI)	Y08960	Sense	TAC GGC CAT CAA ACA TCC TT	159	53
		Antisense	TAT GCT GGC ATT GCT GGT AG		
ß-actin	XM_139732	Sense	CTT CAC CAA CAT GGC TGA GA	198	57
		Antisense	AAG GAA GGC TGG AAA AGA GC		

*NCBI GeneBank Accession number. †PCR product size (base pairs).

Table 2: The effects of *in ovo* feeding (IOF) of arginine and Beta-hydroxy- Beta-methylbutyrate (HMB) on the on relative jejunal PEPT-1 gene expression of turkeys at 25 days of incubation (25E), hatch and 3, 7 and 14 days post-hatch[1]

	% of IOF						
IOF Treatment[2]	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.39^{ab}	1.22^{b}	1.99^{b}	1.11^{a}	1.37^{a}
HMB	0.1	0	1.36^{b}	1.09^{c}	2.12^{a}	1.12^{a}	1.27^{a}
ARG	0	0.7	1.42^{ab}	1.21^{b}	2.18^{a}	1.08^{a}	1.30^{a}
HMB+ARG	0.1	0.7	1.47^{a}	1.42^{a}	1.88^{b}	1.16^{a}	1.30^{a}
Source of variation			---------- P-value ----------				
ARG			0.093	0.0006	0.589	0.825	0.609
HMB			0.775	0.378	0.134	0.172	0.993
ARG X HMB			0.315	0.0003	0.0002	0.364	0.979
SEM(36)[3]			0.040	0.040	0.050	0.040	0.060

[1]All data represents the mean of 10 sample birds per treatment. [2]Treatment HMB *in ovo* feeding solution contained 0.1% in 0.4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in 0.4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained 0.1% HMB + 0.7% arginine in .4% saline. [3]SEM (36) = pooled standard error of the mean with 36 degrees of freedom. [a,b]Means within a column with different superscripts are significantly different ($p<0.05$).

Table 3: The effects of *in ovo* feeding (IOF) of arginine and Beta-hydroxy-Beta-methylbutyrate (HMB) on the on relative jejunal sodium glucose transporter (SGLT-1) gene expression of turkeys at 25 days of incubation (25E), hatch and 3, 7 and 14 days post-hatch[1]

	% of IOF						
IOF treatment[2]	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.41^{ab}	1.13^{b}	1.25^{b}	0.981^{a}	1.15^{b}
HMB	0.1	0	1.33^{b}	1.02^{c}	1.28^{b}	0.954^{b}	1.16^{b}
ARG	0	0.7	1.44^{a}	1.13^{b}	1.30^{b}	0.913^{b}	1.16^{b}
HMB+ARG	0.1	0.7	1.37^{b}	1.36^{a}	1.82^{a}	1.02^{a}	1.33^{a}
Source of variation			---------- P-value ----------				
ARG			0.424	<0.0001	0.512	0.949	0.079
HMB			0.057	0.050	0.261	0.097	0.075
ARG X HMB			0.979	<0.0001	0.058	0.007	0.131
SEM(36)[3]			0.040	0.030	0.040	0.020	0.050

[1]All data represents the mean of 10 sample birds per treatment. [2]Treatment HMB *in ovo* feeding solution contained 0.1% in 0.4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in 0.4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained 0.1% HMB + 0.7% arginine in .4% saline. [3]SEM (36) = pooled standard error of the mean with 36 degrees of freedom. [a,b]Means within a column with different superscripts are significantly different ($p<0.05$)

At the day of hatch, there were significant interactive effects of ARG and HMB on relative jejunal PEPT-1 (Table 1), SGLT-1 (Table 2), SI (Table 3) and AP (Table 4) mRNA expression levels ($p<0.05$). Poults *in ovo* fed HMB alone had significantly enhanced relative jejunal mRNA expression of PEPT-1, SGLT-1, SI and AP in comparison to the other treatments, while the inclusion of ARG significantly reduced relative PEPT-1, SGLT-1, SI and AP jejunal mRNA expression at hatch. Relative jejunal PEPT-1, SGLT-1, SI and AP mRNA expression levels of poults *in ovo* fed ARG alone were similar to the controls at hatch.

At 3 days post-hatch there were significant ARG X HMB effects on mRNA expression levels of all the genes of interest. Poults *in ovo* fed a combination of HMB + ARG had a significantly enhanced relative jejunal SI and AP mRNA expression in comparison to the other treatments ($p<0.05$), while relative mRNA expression levels were

Table 4: The effects of *in ovo* feeding (IOF) of arginine and Beta-hydroxy-Beta-methylbutyrate (HMB) on the on relative jejunal sucrase-isomaltase (SI) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch[1]

	% of IOF						
IOF treatment[2]	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.56^{b}	1.52^{b}	1.76^{b}	1.35^{b}	1.76^{b}
HMB	0.1	0	1.50^{b}	1.36^{c}	1.94^{a}	1.37^{b}	1.94^{a}
ARG	0	0.7	1.71^{a}	1.63^{b}	1.97^{a}	1.32^{b}	1.72^{b}
HMB+ARG	0.1	0.7	1.59^{b}	2.04^{a}	1.45^{c}	1.49^{a}	1.75^{b}
Source of variation			P-value				
ARG			0.0011	<0.0001	0.0001	0.193	0.091
HMB			0.0103	0.0149	<0.0001	0.007	0.135
ARG X HMB			0.3722	<0.0001	<0.0001	0.036	0.265
SEM(36)[3]			0.030	0.050	0.030	0.030	0.070

[1]All data represents the mean of 10 sample birds per treatment. [2]Treatment HMB *in ovo* feeding solution contained 0.1% in 0.4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in 0.4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained 0.1% HMB + 0.7% arginine in .4% saline. [3]SEM (36) = pooled standard error of the mean with 36 degrees of freedom. [a,b]Means within a column with different superscripts are significantly different ($p<0.05$)

similar between poults *in ovo* fed either HMB or ARG alone. Also, poults *in ovo* fed ARG or HMB alone had relative jejunal SI mRNA expression levels that were significantly lower than the controls (Table 3, $p<0.05$), while relative jejunal AP mRNA expression levels were similar to the controls at 3 days post-hatch. However, PEPT-1 and SGLT-1 relative jejunal mRNA expression levels were similar or lower than the expression levels of the controls at day 3 post-hatch, respectively.

At 7 days post-hatch, there were significant ARG X HMB effects on jejunal SGLT-1 (Table 2) and SI (Table 3) relative mRNA expression levels ($p<0.05$). Poults *in ovo* fed HMB or ARG alone had similar relative jejunal SGLT-1 mRNA expression levels which were significantly greater than the controls or poults *in ovo* fed a combination of HMB and ARG at 7 days post-hatch ($p<0.05$, Table 2), while poults *in ovo* fed HMB + ARG had SI ($p<0.05$, Table 3) relative jejunal mRNA expression levels that were significantly lower than all other treatments at one week post-hatch. There were no main or interactive effects of IOF ARG and/or HMB on relative jejunal PEPT-1 (Table 1) or AP (Table 4) mRNA expression levels at one week post-hatch. Additionally, there were no significant main or interactive effects of HMB and/or ARG on relative jejunal PEPT-1, SGLT-1, SI or AP mRNA expression at two weeks post-hatch.

Contrast between IOF of HMB with arginine versus HMB with arginine and 18% egg white protein (EW): Additional analysis was conducted to determine the effects of the inclusion of egg white protein to HMB + ARG on jejunal mRNA expression of the genes of interest. A one-way ANOVA was conducted to compare jejunal gene expression levels in the controls, HMB + ARG and 18% egg white protein (EW) + HMB + ARG *in ovo* treatments (Table 5). At 25 days of incubation, there were no significant differences in the relative jejunal PEPT-1, SI and AP mRNA expression levels between the three treatments. Conversely, the relative jejunal SGLT-1 mRNA expression levels of poults *in ovo* fed HMB + ARG and EW + HMB + ARG were similar, while the relative jejunal SGLT-1 expression in poults *in ovo* fed EW + HMB + ARG was significantly enhanced in comparison to the controls at 25 days of embryonic development ($p<0.05$, Table 5).

Yet, *in ovo* feeding of EW + HMB + ARG or HMB + ARG did not enhance the relative jejunal SGLT-1, SI or AP mRNA expression levels over the controls at hatch (Table 5). Jejunal PEPT-1 mRNA expression levels of poults *in ovo* fed HMB + ARG were significantly higher than the controls ($p<0.05$), however the inclusion of EW in the HMB + ARG treatment did not have an additive effect on relative PEPT-1 mRNA expression and PEPT-1 mRNA expression levels were similar to the controls at hatch.

At 3 days post-hatch, there were no significant differences in the relative jejunal SGLT-1 and AP mRNA expression levels between the three treatments. Relative PEPT-1 expression was similar between both treatments (HMB + ARG and EW + HMB + ARG), while relative PEPT-1 mRNA jejunal expression was significantly greater in poults *in ovo* fed EW + HMB + ARG in comparison to the controls at 3 days post-hatch ($p<0.05$, Table 5). Conversely, both treatments (HMB + ARG and EW + HMB + ARG) had significantly greater relative jejunal SI mRNA expression levels than the controls at 3 days post-hatch ($p<0.05$, Table 5).

Interestingly, the relative jejunal mRNA expression levels of SGLT-1 and SI were significantly higher in the controls in comparison to the other treatments (HMB + ARG and EW + HMB + ARG) at one week post-hatch (Table 5, $p<0.05$). Poults *in ovo* fed either HMB + ARG or EW + HMB + ARG had relative jejunal PEPT-1 mRNA expression levels that were similar, while PEPT-1 mRNA expression levels between the HMB + ARG *in ovo* fed group and the controls were significantly different with the PEPT-1 mRNA expression of the controls greater. Nonetheless, there were no significant differences in relative jejunal AP mRNA expression levels between the three treatments at one week post-hatch.

Table 5: The effects of *in ovo* feeding (IOF) of arginine and Beta-hydrox- Beta-methylbutyrate (HMB) on the on relative jejunal aminopeptidase (AP) gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7, and 14 days post-hatch[1]

	% of IOF						
IOF treatment[2]	HMB	ARG	25E	Hatch	Day 3	Day 7	Day 14
Control	0	0	1.74[b]	1.46[b]	1.61[a]	1.51[a]	1.65[a]
HMB	0.1	0	1.70[b]	1.41[c]	1.74[a]	1.49[a]	1.70[a]
ARG	0	0.7	1.86[a]	1.52[b]	1.75[a]	1.44[a]	1.63[a]
HMB+ARG	0.1	0.7	1.75[b]	1.74[a]	1.55[b]	1.51[a]	1.74[a]
Source of Variation			P-value				
ARG			0.037	0.0007	0.548	0.393	0.804
HMB			0.091	0.1116	0.336	0.374	0.248
ARG X HMB			0.403	0.0125	0.0003	0.158	0.659
SEM(36)[3]			0.040	0.050	0.040	0.030	0.070

[1]All data represents the mean of 10 sample birds per treatment. [2]Treatment HMB *in ovo* feeding solution contained 0.1% in 0.4% saline. Treatment ARG *in ovo* feeding solution contained 0.7% arginine in 0.4% saline. Treatment C is the non-injected control. Treatment HMB + ARG *in ovo* feeding solution contained 0.1% HMB + 0.7% arginine in .4% saline. [3]SEM (36) = pooled standard error of the mean with 36 degrees of freedom. [a,b]Means within a column with different superscripts are significantly different ($p<0.05$)

Table 6: The contrast of *in ovo* feeding (IOF) of arginine and Beta-hydroxy-Beta-methylbutyrate (HMB) and protein on the relative jejunal gene expression of turkeys at 25 days of incubation (25E), hatch, 3, 7 and 14 days post-hatch[1]

	SGLT-1				
IOF treatment[2]	25E	Hatch	Day 3	Day 7	Day 14
Control	1.41[a]	1.13[b]	1.25[a]	0.981[b]	1.15[b]
HMB+ARG	1.37[ab]	1.36[a]	1.18[a]	1.02[a]	1.33[a]
EW+HMB+ARG	1.29[b]	1.34[a]	1.15[a]	1.02[a]	1.28[a]
p-value	0.099	0.0001	0.231	0.089	0.027
SEM(27)[3]	0.030	0.040	0.040	0.020	0.050
	PEPT-1				
Control	1.39[a]	1.22[b]	1.99[a]	1.11[b]	1.39[a]
HMB+ARG	1.47[a]	1.42[a]	1.88[ab]	1.16[a]	1.47[a]
EW+HMB+ARG	1.38[a]	1.35[ab]	1.80[b]	1.14[ab]	1.28[a]
p-value	0.212	0.0278	0.072	0.050	0.928
SEM(27)[3]	0.040	0.050	0.050	0.020	0.050
	SI				
Control	1.56[a]	1.52[b]	1.76[a]	1.35[b]	1.76[a]
HMB+ARG	1.59[a]	2.04[a]	1.45[b]	1.49[a]	1.75[a]
EW+HMB+ARG	1.50[a]	2.00[a]	1.44[b]	1.50[a]	1.53[b]
p-value	0.249	<0.0001	<0.0001	<0.0001	0.018
SEM(27)[3]	0.030	0.040	0.030	0.030	0.060
	AP				
Control	1.74[a]	1.46[b]	1.61[a]	1.51[a]	1.66[a]
HMB+ARG	1.75[a]	1.74[a]	1.55[a]	1.51[a]	1.74[a]
EW+HMB+ARG	1.71[a]	1.73[a]	1.51[a]	1.53[a]	1.61[a]
p-value	0.744	0.0001	0.208	0.013	0.238
SEM(27)[3]	0.030	0.040	0.040	0.010	0.050

[1]All data represents the mean of 10 sample birds per treatment. [2]Treatment = Control (non-injected), 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG), and 18% egg white protein + 0.1% HMB + 0.7% arginine in 0.4% saline (HMB + ARG + EWP). [3]SEM (27) = pooled standard error of the mean with 27 degrees of freedom. [a,b]Means within a column with different superscripts are significantly different ($p<0.05$)

Inclusion of EW in the HMB + ARG *in ovo* feeding treatment enhanced relative jejunal SI mRNA expression levels over the controls and HMB + ARG treatment groups at two weeks post-hatch ($p<0.05$, Table 5). In contrast, relative jejunal SGLT-1 mRNA expression was lower in both the HMB + ARG and EW + HMB + ARG treatments in comparison to the controls at two weeks post-hatch, while there was no difference in relative jejunal PEPT-1 and AP mRNA expression levels at two weeks post-hatch.

DISCUSSION

Our data implies that gene up-regulation of the nutrient transporters and digestive enzymes occurs within 48 h post *in ovo* feeding in turkey embryos and remains elevated after hatch, which parallels other studies demonstrating rapid intestinal adaptation to diet (Diamond *et al*., 1984; Diamond and Karasov, 1987; Ferraris and Diamond, 1992; Sharp *et al*., 1996). Intestinal nutrient transport proteins show adaptive regulation to diet within approximately 24 h *ex vivo* in

mice fed a high or low carbohydrate diet (Diamond *et al*., 1984), with SGLT-1 transporters responding to increased luminal glucose concentration within approximately 1 h (Sharp *et al*., 1996). In parallel other feeding studies have demonstrated a similar time course increase in the site density (Ferraris and Diamond, 1992, 1993) and activity (Ferraris and Diamond, 1989) of the intestinal glucose transporters in response to increased dietary carbohydrate.

Intestinal genes are enhanced specifically for the production of proteins of the brush border membrane that digest and absorb the diverse nutrient molecules of the diet (Ferraris, 2001). Intestinal gene expression is age and diet dependent (Ferraris, 2001; Sklan *et al*., 2003). Intestinal phenotype is determined by pre-wired developmental patterns, which predict the appearance of specific sets of digestive and absorptive proteins within the gut correlative with age (Traber, 1997). The adult intestinal phenotype is established following a series of developmental transitions defined by gene expression of various specific sets of genes in each cell (Traber, 1997). Many of the epithelial nutrient digestion and absorption related genes of the intestine express pre-programmed timetables (Sklan *et al*., 2003), with their expression levels being influenced by dietary components within the lumen (Traber, 1997). A change in secondary phenotype in response to dietary stimulus is advantageous due to increased nutrient absorption and digestion (Ferraris and Diamond, 1989, 1992, 1993) and is correlated to increased expression of the absorptive and digestive related intestinal genes (Miyamoto *et al*., 1993; Kishi *et al*., 1999).

Disaccharidases are present in the 12-day old chick embryo in low concentrations and show marked increase during the last few days of incubation, at a time when there is elongation and increase in the number of jejunal villi (Sell, 1989, Uni *et al*., 2000, 2003). Siddons (1969) and Matsushita (1985) found that the activities of maltase and sucrase increased rapidly from Days 19-21 of incubation and 21 post-hatch, before reaching a plateau. The high disaccharidase activity present in the intestine of the young chick ensures that rapid digestion of dietary carbohydrates occurs in the young hatchling and develops in a way that allows utilization of dietary carbohydrate with the appearance of grains and starches in the diet.

However, our data suggest that gene expression of the nutrient transporters and digestive enzymes are regulated independently of their dietary substrates, demonstrated as increased mRNA expression of SGLT-1 and SI levels in poults *in ovo* fed amino HMB at the day of hatch. In contrast, other studies have demonstrated that intestinal nutrient transporters and digestive enzymes are up-regulated in response to their dietary substrates (Karasov *et al*., 1987; Karasov and Debnam, 1987; Diamond and Karasov, 1987; Ferraris and Diamond, 1993). This effect maybe explained as a non-specific adaptive mechanism in which the presence of food in the lumen of intestine may enhance the digestive and absorptive surface area and the ratio of nutrient transporting to non-transporting cells (Ferraris and Diamond, 1997), implying that IOF HMB may more specifically enhance the jejunal digestive and absorptive surface area. This data parallels other studies by Tako *et al*. (2004) which demonstrate approximately a 45% increase in the jejunal absorptive and digestive surface area in chicks *in ovo* fed HMB alone at 3 days post-hatch, which correlated with an increased capacity to digest and absorb nutrients.

Secondly, our data implies that gene expression of the nutrient transporters and digestive enzymes are regulated independently of each other. In contrast, other studies have shown that functionally related genes (i.e. carbohydrate digestion and absorption processes) show the same patterns of regulation in response to their dietary substrates (Miyamoto *et al*., 1993; Yasutake *et al*., 1995). Yet, we show that SGLT-1 and SI are regulated independently of each other, demonstrated by the up-regulation in SI mRNA expression levels in poults *in ovo* fed HMB + ARG, while SGLT-1 mRNA expression levels of poults *in ovo* fed HMB + ARG were similar to the controls at 3 days post-hatch. Interestingly, only the digestive enzymes (sucrase-isomaltase and leucine aminopeptidase) had enhanced mRNA expression levels in poults *in ovo* fed HMB + ARG, while the mRNA expression levels of the nutrient transporters (SGLT-1 and PEPT-1) were similar to the controls at 3 days post-hatch. Furthermore, SI was the only gene of interest that demonstrated enhanced mRNA expression levels in response to the inclusion of egg white protein to the HMB + ARG IOF solution at two weeks post-hatch. Sklan *et al*. (2003) also demonstrated that SI and SGLT-1 transcripts show differing developmental expression patterns in the chick embryo, suggesting that expression of each is controlled by different mechanisms.

We demonstrate that IOF HMB may enhance mRNA expression of the intestinal nutrient transporters and digestive enzymes only at the day of hatch, while the inclusion of ARG depresses expression. Even though this effect was lost by one and two week post-hatch, poults *in ovo* fed HMB had significantly enhanced bodyweights (data not shown) through two weeks post-hatch relative to the controls, which may signify that enhanced mRNA expression of the nutrient transporters and digestive enzymes may be correlated with improved nutrient acquisition. Thus, the *in ovo* fed poult may hatch with a greater intestinal digestive and absorptive capacity than the conventional hatchling, which may correlate to improved post-hatch growth performance (Smith *et al*., 1990), during the critical post-hatch period. This parallels other IOF studies which have demonstrated that the inclusion of HMB significantly

enhances bodyweight during the critical post-hatch period in chicks (Uni *et al*., 2005) and poults (Foye *et al*., 2006a,b). Hence, IOF may serve as a tool to enhance the expression of the digestion/absorption-related intestinal genes during embryonic development. Therefore, young hatchlings have an increased capacity to digest and absorb incoming nutrients form an external diet, which may persist through two weeks post-hatch and provide the energy and nutrients needed to fuel more rapid subsequent post-hatch growth.

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