*Animal* (2017), **11:10**, pp 1791–1797 © The Animal Consortium 2017
doi:10.1017/S1751731117000404

animal

# Effect of egg storage duration and brooding temperatures on chick growth, intestine morphology and nutrient transporters

S. Yalcin[1†], I. Gursel[2†], G. Bilgen[1], B. H. Horuluoglu[2], G. Gucluer[2] and G. T. Izzetoglu[3]

[1]*Animal Science Department, Faculty of Agriculture, Ege University, 35100 Izmir, Turkey;* [2]*THORLAB, Molecular Biology and Genetic Department, Science Faculty, Bilkent University, 06800 Ankara, Turkey;* [3]*Biology Department, Faculty of Science, Ege University, 35100 Izmir, Turkey*

(Received 29 March 2016; Accepted 19 January 2017; First published online 21 February 2017)

*The effects of egg storage duration (ESD) and brooding temperature (BT) on BW, intestine development and nutrient transporters of broiler chicks were investigated. A total of 396 chicks obtained from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation were exposed to three BTs. Temperatures were initially set at 32°C, 34°C and 30°C for control (BT-Cont), high (BT-High) and low (BT-Low) BTs, respectively. Brooding temperatures were decreased by 2°C each at days 2, 7, 14 and 21. Body weight was measured at the day of hatch, 2, 7, 14, 21, 28 and 42. Cloacal temperatures of broilers were recorded from 1 to 14 days. Intestinal morphology and gene expression levels of $H^+$-dependent peptide transporter (*PepT1*) and Na-dependent glucose (*SGLT1*) were evaluated on the day of hatch and 14. Cloacal temperatures of chicks were affected by BTs from days 1 to 8, being the lowest for BT-Low chicks. BT-High resulted in the heaviest BWs at 7 days, especially for ESD14-14°C chicks. This result was consistent with longer villus and larger villus area of ESD14-14°C chicks at BT-High conditions. From 14 days to slaughter age, BT had no effect on broiler weight. ESD3-18°C chicks were heavier than ESD14-14°C chicks up to 28 days. The PepT1 and SGLT1 expression levels were significantly higher in ESD3-18°C chicks than ESD14-14°C on the day of hatch. There was significant egg storage by BT interaction for PepT1 and SGLT1 transporters at day 14. ESD14-14°C chicks had significantly higher expression of PepT1 and SGLT1 at BT-Low than those at BT-Cont. ESD14-14°C chicks upregulated PepT1 gene expression 1.15 and 1.57-fold at BT-High and BT-Low, respectively, compared with BT-Cont, whereas PepT1 expression was downregulated 0.67 and 0.62-fold in ESD3-18°C chicks at BT-High and BT-Low. These results indicated that pre-incubation egg storage conditions and BTs affected intestine morphology and PepT1 and SGLT1 nutrient transporters expression in broiler chicks.*

**Keywords:** broiler chicks, egg storage, brooding temperature, nutrient transporters, growth

## Implications

The chicks are sensitive to brooding temperatures (BT) which have been known to be a critical aspect of broiler management. On the other hand, longer egg storage durations (ESD) before incubation affect chick weight and posthatch growth negatively. Providing the correct BT will influence the chick growth. From the day of hatch to 7 days posthatch, BTs above the optimum would increase BW and improve villus development in chicks from egg stored for 14 days at 14°C. At low BTs, expression of Na-dependent glucose transporter increases in jejunum in order to provide necessary energy for chicks.

## Introduction

Although a chick is anatomically complete on the day of hatch, its digestive system which is critical for development and growth is not fully developed (Uni *et al.*, 1998). A shift from the yolk-based diet to a carbohydrate–protein diet after the hatch requires dramatic changes in morphology of the intestine with a nutrient transport mechanism (Sklan, 2001). Indeed, active nutrient transport system is already present in chicks' prenatal small intestine to prepare the chicks for exogenous feeding. Di- and tripeptide transporters seem to be represented by the *PepT1* ($H^+$-dependent peptide transporter), which provides a major mechanism for protein absorption. Increase in *PepT1* messenger RNA (mRNA) from embryonic day 20 to 14 days posthatch was reported by Gilbert *et al.* (2007) and Zwarycz and Wong (2013). The transporter Na-dependent glucose (*SGLT1*) of which expression is likely to influence the development and absorptive function of digestive system also develops prenatally. Gilbert *et al.* (2007) and Li *et al.* (2008) showed that intestinal *SGLT1* was upregulated from 18 days of incubation to 14 days posthatch.

Previous studies reported that feed restriction and dietary protein influence *PepT1* and *SGLT1* expression (Chen *et al.*, 2005;

† E-mail: servet.yalcin@ege.edu.tr

Gilbert *et al*., 2008). Our recent study (Yalcin *et al*., 2016) showed that long-term egg storage depressed expression of *PepT1* and *SGLT1* nutrient transporters on the day of hatch. Depending on the supply of hatching eggs, variable market demands for broiler chicks and hatchery capacity, eggs are stored for 3 to 18 days before incubation. But storage longer than 7 days adversely affects embryonic development by slowing embryo metabolism and growth (Christensen *et al*., 2001; Yalcin and Siegel, 2003; Fasenko, 2007) and chick weight at hatch (Reis *et al*., 1997; Ruiz and Lunam, 2002). Long-term egg storage also affects the postnatal growth (Tona *et al*., 2003), however the mechanisms behind have not been clearly understood.

It is questionable if BTs and ESD interacts to influence the broiler growth. Chicks are not able to control their body temperature at the day of hatch. After hatching, the transition into a warm-blooded organism takes place about 3 and 4 days, depending on the chick size (Molenaar, 2012). Therefore, it is important to maintain the chicks at optimum BTs during the first 2 weeks of postnatal period. In order to maintain cloacal temperatures between 40°C and 41°C, the optimum BT for broiler chicks should be around 32°C during the first few days of life (Scott and Washburn, 1985). Because of the differences in growth performance of chicks between short- and long-term stored eggs, BTs may influence intestine development and expression of nutrient transporters. Thus, the objective of the present study was to evaluate the effect of BT on BW, intestine development and nutrient transporters of chicks from eggs stored for short or long periods before incubation. For this purpose, chicks were brooded at three different temperatures from 1 to 21 days.

## Material and methods

*Eggs, incubation and rearing conditions*

All animal care and use were approved by the Ege University Animal Care and Use Committee. Eggs were obtained from a Ross 308 broiler breeder flock aged 38 weeks and stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C), with 75% relative humidity. Different storage temperatures were chosen, as these temperatures emulate current industry conditions to optimize hatchability (Meijerhof, 1992; Schulte-Drüggelte, 2011). In order to incubate all eggs at the same time, eggs were collected in 11-day interval. Average egg weights were 62.89 and 61.19 g (±0.38) for ESD3-18°C and ESD14-14°C eggs before incubation ($P < 0.001$). All eggs were warmed to room temperature before setting in the same incubator. Incubation temperature was set at 37.6°C with a relative humidity of 58%.

On the day of hatch, 396 chicks from each ESD (a total of 792 chicks) were wing banded and weighed individually. Chicks from each storage duration were placed into 36 environmentally controlled pens and assigned to 1 of 3 BTs. Temperatures were initially set at 32, 34 and 30°C for control (BT-Cont), high (BT-High) and low (BT-Low) BTs. Brooding temperatures were decreased by 2°C each at day 2, 7, 14 and 21. Temperature was maintained at 21°C from day 22 to slaughter age. Heaters were turned on 24 hours before the placement. During the experiment, the temperature was recorded continuously at chick height to be certain that brooding conditions are uniform. Relative humidity was between 50 and 70% through the experiment. There were 6 replicated pens with 22 chicks (14 chicks/$m^2$) for each egg storage and BT duration.

All pens were covered with pine shavings and had 2 hanging feeders and one bell-type drinker. Chicks were reared at 23:1 (hours, light : dark) from day-old to 7 days; at 16:8 from 8 to 42 days. The feed and water were provided *ad libitum*. Chicks were fed with a commercial starter diet consisted of 23% protein and 3100 kcal/kg ME from day 1 to 10, a grower diet with 22% protein and 3150 kcal/kg ME from day 11 to 22, and a finisher diet with 20% protein and 3200 kcal/kg ME from day 23 to 42.

*Data collection*

On the day of hatch, 12 chicks were randomly selected from each storage, weighed, and killed by *cervical dislocation.* The residual yolk sac and whole intestine were dissected and weighed after the intestine contents were emptied by gentle pressure. Jejunum was also excised and weighed. Weights were calculated relative to chick weight. A 2 cm of jejunum tissue near *Meckel's diverticulum* was removed from 8 chicks for histological measurements. About 2 cm of the jejunum sample from 4 randomly selected chicks was immediately rinsed in PBS, frozen in liquid nitrogen and stored at −80°C until the RNA extraction and analysis. The same procedure was repeated with 12 chicks from each egg storage and BT group at 14 days. Individual BWs were determined at day 2, 7, 14, 21, 28 and 42. Cloacal temperatures of the 12 chicks from each ESD and BT were recorded from 1 to 14 days by inserting a thermocouple to a depth of 3 cm into the cloaca.

*Histological measurements*

The sampled jejunum was gently flushed with 0.9% NaCl to remove the intestinal contents, Bouin's solution for 24 h, wash in 70% alcohol until no more yellow comes out, serially dehydrated and embedded in paraffin. Three serial sections from each bird were taken at 5μm, stained with Mayer's hematoxylin (Merck 1.09249) and eosin (Merck 1.09844), and then dehydrated quickly through 70%, 96% and absolute alcohols, cleared in xylene and mounted. Sections were examined for villus length, and villus width with light microscopy (Uni *et al*., 1998) using a computer software (Sigma Scan, Point Richmond, CA, USA). The crypt depth was also measured at day 14. The villus length was from the tip to the base of the lamina propria, villus width was in the middle of the villi, and the crypt depth was the distance from the lamina propria to invagination between adjacent villi. The villus area was calculated as the length multiplied by the width. Values used were means of 12 villi/chick.

*Real-time Polymerase Chain Reaction (PCR) analysis*

Polymerase chain reaction reaction was done as described previously (Yalcin *et al*., 2016). Briefly total *RNA* was

**Table 1** *Chicken primer sequences and their expected product size*

| Primers | Primer sequences (5'-3') | PCR (product size, bp) | Annealing temperature (°C) |
|---|---|---|---|
| *GAPDH* | F – GCCGTCCTCTCTGGCAAAGT | 273 | 56 |
| | R – CAGATGAGCCCCAGCCTTCT | | |
| *PepT1* | F – CTATGCAGATTCAGCCAGAC | 165 | 56 |
| | R – AAGCCAGACCAGCAAGGAAC | | |
| *SGLT1* | F – CGGAGTATCTGAGGAAGCGT | 183 | 56 |
| | R – GAGCAGTAATAGCAAGCAGG | | |

*GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *PepT1* = $H^+$-dependent peptide transporter; *SGLT1* = sodium-glucose co-transporter.

extracted from jejunum and complimentary DNA (cDNA) was synthesized with a cDNA synthesis kit (NEB, Ipswich, MA, USA). PCR reaction was prepared with Quick-load Taq 2X Master Mix (NEB). Conditions for PCR reaction were; 10 min 95°C for denaturation, and 34 cycles of denaturation 95°C for 10s, annealing 56°C for 30 s, extension 72°C 30 s and final extension of 10 min at 72°C. *PepT1* and *SGLT1* primers were designed by Primer 3 software and gene expression levels were calculated using the ΔΔCt method to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression as the endogenous control (Table 1). ΔΔCt values were generated by quantitative PCR system for calculation. The values represent the expression levels of each primer in the samples (*PepT1* and *SGLT1* primers in this case). The ΔΔCt values were normalized against *GAPDH*. The fold inductions were calculated comparing BT-Cont for each gene for each storage duration.

*Statistical analyses*

The data collected on the day of hatch were subjected to a one-way ANOVA to evaluate the effect of the ESD. The data collected during the rearing period were subjected to a two-way ANOVA to estimate main effects of the ESD and BTs and interaction between them. Chick weight was used as covariate to analyze BW measurements. *T*-test was used to analyze the magnitude of fold change. Means were considered significant at $P < 0.05$ unless otherwise indicated.

## Results

*Cloacal temperatures and body weights*

Cloacal temperatures were 39.70°C and 39.54°C on the day of hatch and 40.31°C and 40.11°C on day 2 for ESD3-18°C and ESD14-14°C chicks, respectively ($P < 0.05$). Thereafter, ESD3-18°C and ESD14-14°C chicks had similar cloacal temperatures. Cloacal temperatures increased from 1 to 10 days. Brooding temperatures had significant effect on the cloacal temperatures from 1 to 8 days. For the chicks kept at BT-Low, the cloacal temperature was lower compared with BT-Cont and BT-High (Figure 1). After 8 days, there was no effect of BT on cloacal temperatures.

ESD3-18°C chicks were heavier than ESD14-14°C chicks from 1 to 28 days, except at 21 days, BW difference between

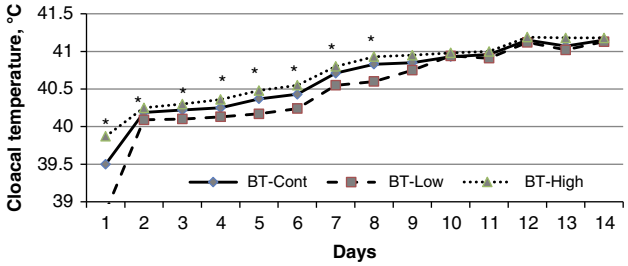


**Figure 1** Cloacal temperatures of chicks from 1 to 14 days. Temperatures were initially set at 32°C, 34°C and 30°C for control (BT-Cont), high (BT-High) and low (BT-Low) brooding temperatures (BT). Brooding temperatures were decreased by 2°C each at 2, 7, 14 and 21 days. *Means differ significantly ($P < 0.05$).

ESD3-18°C and ESD14-14°C chicks was only 26 g, being non-significant ($P = 0.077$) (Table 2). No effect of ESD was detected at 42 days. Chicks maintained at the BT-Low had lower BW at day 2 than broilers maintained at BT-Cont and BT-High. At day 7, ESD by BT interaction was significant, implicating that the highest BW for ESD14-14°C chicks were obtained at BT-High. The ESD3-18°C chicks had similar BW at BT-Cont and BT-High, but BW of chicks maintained at BT-Low was lighter than the others at day 7 (Figure 2). On the day 14, chicks at BT-High conditions were slightly but not significantly heavier than BT-Cont and BT-Low ($P = 0.071$). From day 14 to slaughter age, there was no effect of BT on BW (Table 2).

*Yolk sac and intestinal histological and morphological measurements*

On the day of hatch, relative residual yolk sac weight was similar for ESD3-18°C and ESD14-14°C chicks (Table 3). At 14 days, the effect of BT on the residual yolk sac weight was not significant. The ESD3-18°C chicks had numerically heavier residual yolk sac than ESD14-14°C chicks ($P = 0.057$), however this result existed only at the BT-Low conditions (residual yolk sac weight was 0.044 *v.* 0.007%, for ESD3-18°C and ESD14-14°C, respectively) (data not shown in Tables). The weights of whole intestine and jejunum were similar for ESD3-18°C and ESD14-14°C chicks on the day of hatch (Table 3). At day 14, BT had no effect on the weights of whole intestine and jejunum. The ESD14-14°C chicks had significantly heavier whole intestine weight than the ESD3-18°C chicks, whereas jejunum of ESD14-14°C chicks

**Table 2** *BWs (g) of broilers by egg storage duration (ESD) and brooding temperature (BT)*

| | ESD[1] | | | BT[2] | | | | ANOVA (*P*-values) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Days | ESD3-18°C | ESD14-14°C | SEM | Control | High | Low | SEM | ESD | BT | ESD × BT | Hatch weight |
| Hatch | 44.9[a] | 43.3[b] | 0.38 | – | – | – | – | <0.001 | – | – | – |
| 2 | 62[a] | 54[b] | 0.6 | 58[b] | 60[a] | 55[c] | 0.6 | <0.001 | <0.001 | 0.917 | <0.001 |
| 7 | 170[a] | 142[b] | 1.5 | 156[a] | 164[a] | 149[b] | 1.9 | <0.001 | <0.001 | <0.001 | <0.001 |
| 14 | 453[a] | 407[b] | 5.5 | 424 | 442 | 423 | 6.3 | 0.001 | 0.071 | 0.118 | <0.001 |
| 21 | 956 | 930 | 9.2 | 931 | 965 | 933 | 12.8 | 0.077 | 0.107 | 0.552 | <0.001 |
| 28 | 1658[a] | 1615[b] | 15.4 | 1606 | 1655 | 1649 | 19.5 | 0.044 | 0.130 | 0.332 | <0.001 |
| 42 | 3042 | 3012 | 30.1 | 2992 | 3043 | 3044 | 39.1 | 0.529 | 0.567 | 0.352 | <0.001 |

[a,b]Means within the same row and treatment with no common superscript letter differ significantly ($P<0.05$).
[1]Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation.
[2]Temperatures were initially set at 32°C, 34°C and 30°C for control, high and low BTs. BTs were decreased by 2°C each at 2, 7, 14 and 21 days. Temperature was maintained at 21°C from 22 days to slaughter age.

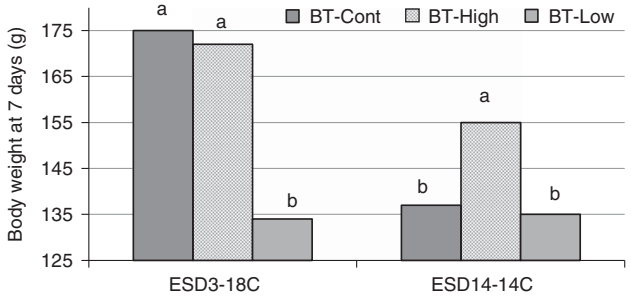


**Figure 2** Egg storage duration (ESD) and brooding temperature (BT) interaction for BW of chicks at 7 days, [a,b]Means within the same egg storage group with no common superscript letter differ significantly ($P<0.05$).Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation. Temperatures were initially set at 32°C, 34°C and 30°C for control (BT-Cont), high (BT-High) and low (BT-Low) BTs. Brooding temperatures were decreased by 2°C each at 2, 7, 14 and 21 days.

was slightly but not significantly heavier than ESD3-18°C chicks ($P = 0.076$).

On the day of hatch, there was no effect of ESD on villus length, width and surface area (Table 3). At 14 days post-hatch, BT-High increased villus length, width and area compared with BT-Low, but was not different from BT-Cont. Interaction between ESD and BT showed that ESD3-18°C chicks had similar villus length, width and area and crypt depth under three BTs. ESD14-14°C chicks had the highest villus length at BT-High while smallest villus width at BT-Low thus villus area (Table 4, marked with superscripts). Compared to ESD14-14°C chicks, ESD3-18°C chicks had shorter villus length at BT-High, but larger villus width and area at BT-Low (Table 4, marked with asterisk). At BT-Cont conditions, ESD3-18°C chicks had deeper crypts than ESD14-14°C chicks while for ESD14-14°C chicks the highest crypt depth was obtained at BT-High.

*Gene expression of nutrient transporters*

Expression of *PepT1* and *SGLT1* was higher in ESD3-18°C chicks than in ESD14-14°C on the day of hatch (Table 5). At day 14, ESD effect on expression of *PepT1* was not significant, however, ESD14-14°C chicks had numerically greater *PepT1* expression than ESD3-18°C chicks ($P = 0.077$). There was a significant ESD by BT interaction on jejunum *PepT1* gene expression. Expression of *PepT1* in ESD3-18°C chicks was greater under BT-Cont than under BT-Low conditions ($P < 0.01$). Indeed, compared with BT-Cont, downregulation in *PepT1* expression of ESD3 chicks was not significant at BT-High conditions but *PepT1* expression was downregulated 0.52-fold at BT-Low conditions ($P < 0.01$) (Figure 3). ESD14-14°C chicks had greater *PepT1* expression at BT-Low than at BT-Cont conditions. Fold increase of *PepT1* in ESD14-14°C chicks was 1.57 at BT-Low condition compared with BT-Cont ($P < 0.01$), being significantly different from those at BT-High ($P = 0.017$).

At day 14, higher expression of *SGLT1* was observed in ESD14-14°C chicks compared with ESD3-18°C (Table 5). The interaction between ESD and BT implicated that the greatest expression of *SGLT1* in ESD3-18°C and ESD14-14°C chicks was under BT-Cont and BT-Low conditions, respectively. Notably, compared with BT-Cont, *SGLT1* expression in ESD3-18°C chicks decreased 0.53- and 0.81-fold at BT-High and BT-Low conditions ($P < 0.01$ and <0.01, respectively), respectively, being significantly different from each other ($P = 0.01$) (Figure 3). Compared with BT-Cont, *SGLT1* expression level in ESD14-14°C chicks showed 2.77-fold increase ($P < 0.01$) at BT-Low condition, being significantly different from those at BT-Low ($P < 0.001$).

## Discussion

It is very well known that longer ESD before incubation associated with lower chick quality and postnatal performance in broilers (Christensen *et al.*, 2001 and 2002; Ruiz and Lunam, 2002; Tona *et al.*, 2003 and 2004). The present experiment was conducted to evaluate the interaction between ESD and BTs. To mimic industrial conditions, different storage temperatures were used for 4 and 14 days stored eggs. Because growth is associated with

**Table 3** *Residual yolk sac weights and intestinal parameters of broilers by egg storage duration (ESD) and brooding temperature (BT) at 14 days*

| | ESD[1] | | | BT[2] | | | | ANOVA (*P*-values) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Traits | ESD3-18°C | ESD14-14°C | SEM | Control | High | Low | SEM | ESD | BT | ESD × BT |
| Residual yolk sac (%) | | | | | | | | | | |
| Day 0 | 11.42 | 9.94 | 0.630 | – | – | – | – | 0.122 | – | – |
| Day 14 | 0.031 | 0.017 | 0.0051 | 0.029 | 0.019 | 0.026 | 0.0062 | 0.057 | 0.497 | 0.075 |
| Intestine weight (%) | | | | | | | | | | |
| Whole | | | | | | | | | | |
| Day 0 | 4.65 | 4.88 | 0.220 | – | – | – | – | 0.365 | – | – |
| Day 14 | 5.93[b] | 6.38[a] | 0.121 | 6.11 | 6.07 | 6.29 | 0.146 | 0.008 | 0.487 | 0.361 |
| Jejunum | | | | | | | | | | |
| Day 0 | 1.238 | 1.119 | 0.0682 | – | – | – | – | 0.226 | – | – |
| Day 14 | 1.914 | 2.026 | 0.0423 | 1.891 | 1.993 | 2.026 | 0.0533 | 0.076 | 0.187 | 0.149 |
| Villus | | | | | | | | | | |
| Length (μm) | | | | | | | | | | |
| Day 0 | 214 | 213 | 2.5 | – | – | – | – | 0.914 | – | – |
| Day 14 | 363 | 368 | 3.3 | 365[ab] | 373[a] | 358[b] | 3.3 | 0.216 | 0.006 | <0.001 |
| Width (μm) | | | | | | | | | | |
| Day 0 | 31.3 | 30.0 | 0.63 | – | – | – | – | 0.206 | – | – |
| Day 14 | 34.4[a] | 32.1[b] | 0.42 | 33.9[a] | 33.9[a] | 32.0[b] | 0.52 | <0.001 | 0.021 | <0.001 |
| Area ($\mu m^2 \times 10^{-2}$) | | | | | | | | | | |
| Day 0 | 67.0 | 64.2 | 0.99 | – | – | – | – | 0.275 | – | – |
| Day 14 | 125[a] | 118[b] | 2.5 | 123[a] | 127[a] | 116[b] | 1.9 | 0.015 | 0.004 | <0.001 |
| Crypt depth (μm) | | | | | | | | | | |
| Day 14 | 54.7[a] | 52.9[b] | 0.60 | 53.6 | 54.9 | 53.0 | 0.73 | 0.041 | 0.144 | <0.001 |

[a,b]Means within the same row and treatment with no common superscript letter differ significantly ($P<0.05$).
[1]Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation.
[2]Temperatures were initially set at 32°C, 34°C and 30°C for control, high and low BTs. BTs were decreased by 2°C each at 2, 7, 14 and 21 days.

**Table 4** *Means for interaction between egg storage duration (ESD) and brooding temperature (BT) for villus length, width, area and crypt depth*

| | | ESD[1] | |
|---|---|---|---|
| Traits | BT[2] | ESD3-18°C | ESD14-14°C |
| Villus length (μm) | Control | 365 | 365[b] |
| | High | 358* | 389[a] |
| | Low | 365 | 350[b] |
| Villus width (μm) | Control | 34.2 | 33.4[a] |
| | High | 33.8 | 33.9[a] |
| | Low | 35.2* | 28.8[b] |
| Villus area ($\mu m^2 \times 10^{-2}$) | Control | 125 | 122[a] |
| | High | 121* | 132[a] |
| | Low | 130* | 101[b] |
| Crypt depth (μm) | Control | 56.2* | 50.9[b] |
| | High | 53.3 | 56.6[a] |
| | Low | 54.6 | 51.4[b] |

[a,b]Means within the same column and measurement with no common superscript letter differ significantly ($P<0.05$).
*Means within the same row differ significantly ($P<0.05$).
[1]Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation.
[2]Temperatures were initially set at 32°C, 34°C and 30°C for control, high and low BTs. BTs were decreased by 2 °C each at 2, 7, 14 and 21 days.

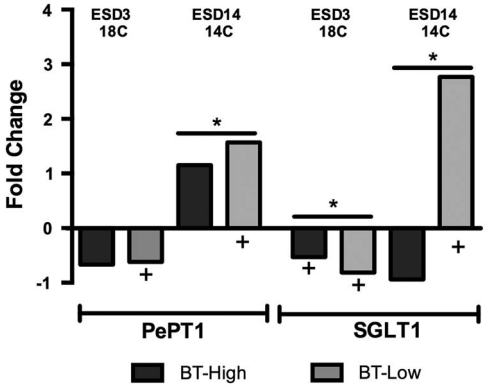


**Figure 3** Relative *PepT1* and *SGLT1* gene expression compared with control brooding (BT-Cont) for ESD3-18°C and ESD14-14°C chicks at 14 days. Gene expression was calculated using the ΔΔCt method. *GAPDH* was used as the endogenous control. Temperatures were initially set at 32°C, 34°C and 30°C for control (BT-Cont), high (BT-High) and low (BT-Low) brooding temperatures (BT). Brooding temperatures were decreased by 2°C each at 2, 7, 14 and 21 days. Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation. +Means that are significantly different compared with that of BT-Cont ($P<0.05$). *Means that are significantly different between the BT-High and BT-Low within the same gene expression and egg storage duration ($P<0.05$).

gastrointestinal tract development and maturation, the intestinal morphology and the expression of *PepT1* and *SGLT1* in jejunum were investigated.

As expected, longer ESD before incubation decreased chick weight at the day of hatch. Goliomytis *et al.* (2015) reported that the lower hatch weight from longer storage duration could be attributed to the water losses during egg

**Table 5** *Expression of* PepT1 *and* SGLT1 *in jejunum of broilers at 14 days by egg storage duration (ESD) and brooding temperature (BT)*

| | ESD[1] | | | BT[2] | | | | ANOVA (*P*-values) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Traits | ESD3-18°C | ESD14-14°C | SEM | Control | High | Low | SEM | ESD | BT | ESD × BT |
| *PepT1* | | | | | | | | | | |
| Day 0 | 0.0419[a] | 0.0156[b] | 0.00651 | – | – | – | – | 0.018 | – | |
| Day 14 | 0.0652 | 0.0793 | 0.00541 | 0.0729 | 0.0671 | 0.0767 | 0.00663 | 0.077 | 0.604 | 0.005 |
| *SGLT1* | | | | | | | | | | |
| Day 0 | 0.0028[a] | 0.0008[b] | 0.00044 | – | – | – | – | 0.016 | – | – |
| Day 14 | 0.0134[b] | 0.0188[a] | 0.00135 | 0.0139[b] | 0.0099[b] | 0.0245[a] | 0.00166 | 0.007 | <0.001 | <0.001 |

*PepT1* = $H^+$-dependent peptide transporter; *SGLT1* = sodium-glucose co-transporter.
[a,b]Means within the same row and treatment with no common superscript letter differ significantly ($P<0.05$).
[1]Chicks from eggs stored at 18°C for 3 days (ESD3-18°C) or at 14°C for 14 days (ESD14-14°C) before incubation.
[2]Temperatures were initially set at 32°C, 34°C and 30°C for control, high and low BTs. BTs were decreased by 2°C each at 2, 7, 14 and 21 days.

storage which led to lighter egg weights before incubation. They found no effect of ESD on BW at days 7 and 35 when BW was corrected for egg weight before incubation (Goliomytis *et al*., 2015). In our study, we used chick weight as covariate and showed that the ESD effect on BW was significant up to 28 days. This result was persistent when egg weight was used as covariate, as well (data not shown in the text). Therefore, the observed lighter BWs of ESD14-14°C chicks up to 28 days might be attributed to growth potential of those chicks in our study. However, the chicks compensated the growth retardation between 28 and 42 days. The lack of ESD effect at slaughter age was in disagreement with Tona *et al*. (2004) who found broilers from fresh eggs were heavier at day 42 than those from stored eggs. This difference could be explained by our statistical analyses where hatch weight was used as covariate for BW of broilers.

Regardless of ESD, highest BW was obtained at BT-High conditions at day 2. Our results showed that BT requirement of ESD14-14°C chicks was higher than ESD3-18°C chicks between 2 and 7 days while BT-High had no advantage on BW of ESD3-18°C chicks. Body weight differences between ESD3-18°C and ESD14-14°C at day 7 were lowest for chicks at BT-High conditions. This result indicated that BT-High triggered growth of ESD14-14°C chicks. This result was consistent with longer villus, larger villus area and crypt depth of ESD14-14°C chicks at BT-High conditions. The increased crypt depth may indicate continued mucosal growth and would allow more enterocytes to develop and migrate to increase villus length (Barri *et al*., 2011) which would play a fundamental role in meeting ESD14-14°C chicks' nutrient demands. Malheiros *et al*. (2000) reported a decline in the BW when the chicks were kept at 20°C. In our study, BT-Low reduced ESD3-18°C chick weight at day 7, whereas it was not different from ESD14-14°C chicks under the same conditions. This result indicated that lower critical temperature for broiler chicks should be higher than the BT-Low temperatures used in this study. Indeed, BT-Low chicks had the lowest cloacal temperatures until 8 days. Therefore, the lower BW of BT-Low chicks might be related to energy saving, avoid of moving for feed and water (Malheiros *et al*., 2000). However, this growth retardation of BT-Low chicks was compensated at 14 days.

This study showed that expression of *PepT1* and *SGLT1* increased from day-old to 14 days. While *PepT1* is a low-affinity/high capacity transporter and maximize amino acid assimilation, *SGLT1* is a high affinity/low capacity transporter and responsible for glucose absorption (Leibach and Ganapathy, 1996; Wood and Trayhurn, 2003). Up regulation of *PepT1* and *SGLT1* would enhance the uptake of small peptides and glucose, respectively. The increase in *PepT1* expression of ESD14-14°C chicks at BT-High compared to the chicks at BT-Cont associated with villus length and crypt depth of chicks being agreed with Barri *et al*. (2011) and suggested increased nutrient utilization for mucosal development. In spite of the higher *PepT1* and *SGLT1* expression of ESD14-14°C chicks compared with ESD3-18°C under BT-Low conditions, their BW was low which indicated that changes in nutrient transporters may not result in BW differences (Barri *et al*., 2011). The highest fold increase in *PepT1* and *SGLT1* that was obtained for ESD14-14°C chicks at BT-Low may indicate (1) adaptation to cold temperature to maximize amino acid utilization and to provide necessary glucose transport for energy expenditure under cold conditions, and (2) their low feed consumption, however feed consumption was not measured in the present study. An increase in *PepT1* and *SGLT1* expression following a feed restriction was reported (Gilbert *et al*., 2007; Duarte *et al*., 2011; Madsen and Wong, 2011). In our study, all birds were on *ad libitum* feeding. Therefore, this increase in *PepT1* and *SGLT1* in ESD14-14°C chicks BT-Low may be due to their behavior, to avoid moving for feeding to conserve heat, as indicated above. Thus, the increase in these transporters would have a role in the assimilation of nutrients when luminal concentration is lower than in blood. On the other hand, the absorption of nutrients from residual yolk sac has a crucial role in intestinal development (Noy and Sklan, 1999) and growth (Murakami *et al*., 1992). The highest fold increases in nutrient transporters for ESD14-14°C chicks at BT-Low was accompanied with lower residual yolk sac weights of chicks and suggested exhaustion of yolk reserves necessitates the surge in glucose transport for growth (Obst and Diamond, 1992). Indeed, BT effect was disappeared at day 14.

In conclusion, pre-incubation egg storage conditions affected growth performance of broilers until 28 days post-hatch. The results indicated that higher BTs used in this study had no advantage on BW of chicks from shorter ESD (ESD3-18°C); however, chicks from longer ESD (ESD14-14°C) could grow better under higher BTs by maximizing nutrient absorption capacity and expression of *PepT1*. Under lower BTs, chicks from longer ESDs (ESD14-14°C) increased expression of *SGLT1* nutrient transporters to supply necessary energy. Although protein amount may not be deduced by measuring mRNA, our results clearly demonstrated that pre-incubation egg storage conditions and BTs affected intestine morphology and relative gene expression of *PepT1* and *SGLT1*. Further studies on the other nutrient transporters would be valuable to understand the interaction between the BT and prolonged egg storage periods.

## Acknowledgement

This research was supported by TUBITAK (Project no: 112 0 220).

## References

Barri A, Honaker CF, Sottosanti JR, Hulet RM and McElroy AP 2011. Effect of incubation temperature on nutrient transporters and small intestine morphology of broiler chickens. Poultry Science 90, 118–125.

Chen H, Pan Y-X, Wong EA and Webb KE Jr 2005. Dietary protein level and stage of development affect expression of an intestinal peptide transporter (cPepT1) in chickens. Journal of Nutrition 135, 193–198.

Christensen VL, Wineland MJ, Fasenko GM and Donaldson WE 2001. Egg storage effects on plasma glucose and supply and demand tissue glycogen concentrations of broiler embryos. Poultry Science 80, 1729–1735.

Christensen VL, Wineland MJ, Fasenko GM and Donaldson WE 2002. Egg storage alters weight of supply and demand organs of broiler chicken embryos. Poultry Science 81, 1738–1743.

Duarte CRAM, Vicentini-Paulino LM, Buratini J, Castilho ACS and Pinheiro DF 2011. Messenger ribonucleic acid abundance of intestinal enzymes and transporters in feed-restricted and refed chickens at different ages. Poultry Science 90, 863–868.

Fasenko GM 2007. Egg storage and the embryo. Poultry Science 86, 1020–1024.

Gilbert ER, Li H, Emmerson DA, Webb KE and Wong EA 2008. Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. Journal of Nutrition 138, 262–271.

Gilbert ER, Li H, Emmerson DA, Webb KE and Wong EA 2007. Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. Poultry Science 86, 1739–1753.

Goliomytis M, Tsipouzian T and Hager-Theodorides A 2015. Effects of egg storage on hatchability, performance and immunocompetence parameters of broiler chickens. Poultry Science 94, 2257–2265.

Leibach FH and Ganapathy V 1996. Peptide transporters in the intestine and kidney. Annual Review of Nutrition 16, 99–119.

Li H, Gilbert ER, Zhang Y, Crasta O, Emmerson DA, Webb KE Jr and Wong EA 2008. Expression profiling of the solute carrier gene family in chicken intestine from the late embryonic to early post-hatch stages. Animal Genetics 39, 407–424.

Madsen SL and Wong EA 2011. Expression of the chicken peptide transporter 1 and the peroxisome proliferator-activated receptor α following feed restriction and subsequent refeeding. Poultry Science 90, 2295–2300.

Malheiros RD, Moraes VMB, Brunu LDG, Malheiros EB, Furlan RL and Macari M 2000. Environmental temperature and cloacal and surface temperatures of broiler chicks in first week post-hatch. Journal of Applied Poultry Research 9, 111–117.

Meijerhof R 1992. Pre-incubation holding of hatching eggs. World's Poultry Science Journal 48, 57–68.

Molenaar R 2012. The importance of brooding period. XXIV World's Poultry Congress. 5–9 August 2012, Salvador, Brazil. Retrieved on 27 November 2015 from http://www.facta.org.br/wpc2012-cd/pdfs/plenary/ Roos_Molenaar.pdf.

Murakami H, Akiba Y and Horiguchi M 1992. Growth and utilization of nutrients in newly-hatched chick with or without removal of residual yolk. Growth Development and Aging 56, 75–84.

Noy Y and Sklan D 1999. Energy utilization in newly hatched chicks. Poultry Science 78, 1750–1756.

Obst BS and Diamond J 1992. Ontogenesis of intestinal nutrient transport in domestic chickens (Gallus gallus) and its relation to growth. The Auk 109, 451–464.

Reis LH, Gama LT and Soares MC 1997. Effects of short storage conditions and broiler breeder age on hatchability, hatching time and chick weights. Poultry Science 76, 1459–1466.

Ruiz J and Lunam CA 2002. Effect of pre-incubation storage conditions on hatchability, chick weight at hatch and hatching time in broiler breeders. British Poultry Science 43, 374–383.

Schulte-Drüggelte R 2011. Recommendations for hatching egg handling and storage. Lohmann Information 46, 55–58.

Scott TR and Washburn KW 1985. Evaluation of growth, hormonal, and hematological responses of neonatal chickens to reduced temperature brooding. Poultry Science 64, 777–784.

Sklan D 2001. Development of digestive tract of poultry. World's Poultry Science Journal 57, 415–428.

Tona K, Onagbesan O, De Ketelaere B, Decuypere E and Bruggeman V 2004. Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick posthatch growth to forty-two days. Journal of Applied Poultry Research 13, 10–18.

Tona K, Bamelis F, De Ketelaere B, Bruggeman V, Moraes VMB, Buyse J, Onagbesan O and Decuypere E 2003. Effects of egg storage time on spread of hatch, chick quality, and chick juvenile growth. Poultry Science 82, 736–741.

Uni Z, Ganot S and Sklan D. 1998. Posthatch development of mucosal function in broiler small intestine. Poultry Science 77, 75–82.

Yalcin S, Gursel I, Bilgen G, Horuluoglu BT, Gucluer G and Izzetoglu GT 2016. Egg storage duration and hatch window affect gene expression of nutrient transporters and intestine morphological parameters of early hatched broiler chicks. Animal 10, 805–811.

Yalcin S and Siegel PB 2003. Developmental stability of broiler embryos in relation to length of egg storage prior to incubation. Japanese Poultry Science 40, 298–308.

Wood IS and Trayhurn P 2003. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. British Journal of Nutrition 89, 3–9.

Zwarycz B and Wong EA 2013. Expression of the peptide transporters *PepT1*, *PepT2*, and *PHT1* in the embryonic and posthatch chick. Poultry Science 92, 1314–1321.