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Comparison of Some Relative Gene Expressions in the Yolk Sac Membrane and Small Intestine of the Embryos in the Layer Hybrid and Pure Breeds

ABSTRACT

Pure breeds are important due to the fact that they adapt well to poor husbandry conditions. However, gene expression of digestive enzymes and nutrient transporters of pure breeds and layer hybrids have not been studied yet. Therefore, this study was directed to detect the gene expression of digestive enzymes and nutrient transporters in the yolk sac membrane and small intestine of the embryos in layer hybrid and pure breeds. A total of 354 hatching eggs were collected from 29 wk old Denizli, Gerze and Lohman White (LW) breeder flocks. Twelve eggs from each genotype were selected at embryonic day 19 (E19) and day 21 (E21). APN, SI, PepT1, EAAT3, CAT1, B⁰AT, SGLT1 and GLUT5 mRNA expressions were evaluated in the yolk sac membrane (YSM) and small intestine of the embryos from different genotypes. Expressions of APN and EAAT3 in the small intestine of Denizli embryo were upregulated from E19 to E21. B⁰AT, PepT1 and SGLT1 expressions were upregulated in the small intestine of pure breed and layer hybrid embryos with increasing embryonic age. SI expression in the small intestine of the LW embryos was upregulated from E19 to E21. APN, B⁰AT and EAAT3 expressions in the YSM of Denizli embryos were downregulated from E19 to E21. EAAT3 and SGLT1 expressions in the YSM of Gerze embryos were upregulated at E21 and compared to E19. Taken together, our results indicate that genotype and embryonic age influenced the expression of digestive enzymes and nutrient transporter genes in the small intestine and YSM of embryos.

INTRODUCTION

The embryonic development is important for posthatch growth. This is influenced by breeder age, egg storage and incubation (Chen *et al.*, 2016). Yolk sac provides the developing embryo with the nutrients required for growth and energy (Noble & Cocchi, 1990). During incubation, yolk nutrients are transported to the embryo via the endothelial cells of the yolk sac membrane (YSM). The nutrients pass from the yolk sac (YS) content to the YSM by receptor-mediated endocytosis of lipoproteins (Hermann *et al.*, 2000) and minerals (Yadgary *et al.*, 2010). Nutrients are then delivered into the blood vessels that protrude into the YSM and transported to the embryonic tissues (Yadgary & Uni, 2012). Nutrient transporters are responsible for nutrient absorption; so the expression of these nutrients transporters can influence overall nutrient status as well as the growth and development of embryos (Mott *et al.*, 2008; Shinde *et al.*, 2015; Speier *et al.*, 2012). Aminopeptidase N (APN) and sucrose Isomaltase (SI) are digestive enzymes. PepT1, EAAT3, B⁰AT, CAT1, SGLT1 and GLUT5 are important for transporting related nutrients (Spier *et al.*, 2012, Yadgary *et al.*, 2011).



Animal biodiversity has become an important issue because of the changes in the production systems (Zanetti *et al.*, 2010). The well-being of poultry and their rearing conditions are important (Rızzi & Chiericato, 2010). Pure breeds are preferred because of this idea and changed the rearing system (Rızzi & Chiericato, 2005). Pure breeds seem to have the advantage of being well adapted to the local stressful conditions in the rural areas such as high temperature, serious disease problems, poor farming hygiene and unbalanced diets (Bessadok *et al.*, 2003, Fotsa *et al.*, 2007, Kingori *et al.*, 2010). Denizli and Gerze are Turkish native chicken breeds which lay eggs with white color shells. Lohman White is a commonly used layer hybrid which also lays eggs with white color shells. Denizli and Gerze are reared primarily for egg and they have low egg production, whereas Lohman White is a hybrid for selection for high egg production. Knowledge including gene expression of purebreds and hybrids in embryonic period is lacking. Therefore, the objective of this study was to explore the developmental expression profiles of the APN, SI, PepT1, EAAT3, CAT1, B⁰AT, SGLT1 and GLUT5 in YSM and small intestine of embryos from different white layer genotypes during incubation.

MATERIALS AND METHODS

This study was approved by Ankara University Animal Care and Use Committee (2015/5/101). A total of 354 hatching eggs from 2 different pure breeds (Denizli and Gerze) and one layer hybrid (Lohman White) were used. Denizli and Gerze are Turkish pure breeds. Therefore, we used in this study 3 different genotypes. Flocks were at the same age (29 wk of age).

Eggs from each genotype were incubated in the same incubator (Çimuka Incubator, Ankara, Turkey) at 37.7°C, 53% relative humidity (RH) and were turned till 18 d of incubation. On day 18, all eggs were transferred to the hatcher (Çimuka Incubator, Ankara, Turkey) at 37.5°C and 70% RH until day 21

of the incubation. Twelve eggs from each genotype were selected at the beginning of the embryonic day 19 (E19) and E21 of incubation. The eggs were opened and the embryos were sacrificed by cervical dislocation and subsequently the YS and small intestines were dissected from each embryo and cleaned with PBS. Then the YSM and small intestine were flash frozen within cryogenic tubes separately in liquid nitrogen and stored at -80°C. The RNA was extracted from the yolk sac membrane and small intestine samples using the commercial kit (Gene Jet, Thermo K0732) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by Multiskan Go (Thermo Scientific) and also Qubit RNA HS Assay kit (Thermo Fisher, Q 32855) with Qubit 2.0 Fluorometer (Invitrogen). cDNA was synthesized from 1 µg total RNA using the Maxima First Strand cDNA kit (Thermo Scientific, K1672). To accurately determine the efficiency of PCR reactions, a 4-log dilution series was performed and then the synthesized cDNAs were diluted 1:30 before the addition to PCR reactions. GAPDH was used as a housekeeping gene. APN, B⁰AT, EAAT3, CAT1, PepT1, SI, SGLT, GLUT5 and GAPDH gene specific primers were designed using FastPCR 6.1.2 program (Kalendar *et al.*, 2009) (Table 1).

Real-time PCR was performed on LightCycler[®] 480 II (Roche Applied Science) instrument. For each 25 µL PCR reaction, 2 µL of the cDNA (diluted 1:30), 12.5 µL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, K0223), 0.5 µL of the forward and reverse primer (5µM) and 9.5 µL of water were added. The reaction was incubated at 95°C for 10 min followed by repeated 40 cycles with 95°C for 30 s and 60°C for 1 min. All PCRs were performed in triplicate.

Statistical analysis

The Relative Expression Software Tool V2.0.13 (REST 2009) was used for statistical analysis of relative gene expression results (Pfaffl *et al.*, 2002). Significant results are indicated by $p < 0.05$.

Table 1 – Primers used for real-time PCR

Gene	Forward primer 5'→3'	Reverse primer 5'→3'	Genbank No	T _m	cDNA bp
APN	CCTACCTGCTGGCCTTCATCG	GCCGTGTTGTAGTGCCTCTCAA	NM_204861	60	172
SI	TGGCATAACAGGTTTCGACGCAA	TACTGGGATGGCAAGCGGGTA	XM_422811	60	114
PepT1	TGGCTGTAAGCTCCATAAACG	GAGACACAAGGTTTGATCCCA	NM_204365	60	139
EAAT3	TGGATTCCAGTGTCTTCTGGGA	CCAGTTTAAATGGTCACAACC	XM_424930	60	112
CAT1	GTTATGTGACTGTTGGCGAGC	ATTTCATCGAACGTGGCGCTC	XM_417116	60	115
B ⁰ AT	TGTAGAAGGTGTGCTTGTAAC	AGTAATTGCCAGACCTCAGGA	XM_419056	60	137
SGLT1	TCTTCTGGCTGGACGGAGCA	CCCACAAAATGTCCACTGCCA	XM_415247	60	85
GLUT5	GGTGGGACCTCTGGTCAACAA	CCCCATGATAACACGGGAGACG	XM_417596	60	147
GAPDH	GGCCAAGGTCATCCATGACAA	TGCCATCTCTCCACAGCTTCC	NM_204305	60	119



RESULTS AND DISCUSSION

APN is a digestive enzyme that converts proteins into amino acids and provides substrate for amino acid transporter such as B⁰AT, CAT1 and EAAT3 (Drag *et al.*, 2010, Kania *et al.*, 1977). In the current study, expression of APN in the small intestine of Denizli embryo was upregulated from E19 to E21 (Table 2). The examined genotypes at E19, showed APN expression in the small intestine was 0.41 and 0.46-fold lower and those at E21 was 0.46 and 0.37-fold lower in Denizli and Gerze embryos compared to LW embryos, respectively (Table 3 and 4). This was downregulated in both Denizli and Gerze embryos compared to LW embryos in both at E19 and E21. This indicated that this gene is important in the small intestine of LW embryos both at E19 and E21 than in those of pure breeds. The examined expression of APN in the YSM, was downregulated with embryonic age in only Denizli

Table 2 – Relative expressions of some genes in the small intestine of different genotypes at E21 compared to E19.

Gene	Group	Relative expression	p-value	Result
APN	Lohman White	1.25±0.76	0.220	ND
	Denizli	1.40±0.68	0.035	UP
	Gerze	1.01±0.48	0.935	ND
B ⁰ AT	Lohman White	3.90±0.43	0.030	UP
	Denizli	4.51±0.75	0.001	UP
	Gerze	5.74±1.57	<0.001	UP
CAT1	Lohman White	1.41±0.76	0.068	ND
	Denizli	0.63±0.13	0.129	ND
	Gerze	0.75±0.11	0.450	ND
EAAT3	Lohman White	1.78±0.82	0.025	UP
	Denizli	2.08±0.79	<0.001	UP
	Gerze	1.17±0.46	0.493	ND
PepT1	Lohman White	3.98±2.13	<0.001	UP
	Denizli	2.05±0.63	0.004	UP
	Gerze	2.63±0.94	<0.001	UP
SI	Lohman White	1.94±1.05	0.001	UP
	Denizli	1.90±0.10	0.175	ND
	Gerze	1.29±0.09	0.587	ND
SGLT1	Lohman White	2.60±1.27	<0.001	UP
	Denizli	2.49±0.99	<0.001	UP
	Gerze	1.48±0.60	0.045	UP
GLUT5	Lohman White	1.23±0.72	0.266	ND
	Denizli	0.71±0.24	0.147	ND
	Gerze	1.38±0.61	0.080	ND

Means ±SEM are reported. ND, Sample group is not different to control group; UP, Upregulated.

Table 3 – Relative expressions of some genes in the small intestine of Denizli and Gerze embryos compared to LW embryos at E19.

Gene	Group	Relative expression	P-value	Result
APN	Denizli	0,41±0.24	0.001	DOWN
	Gerze	0,46±0.27	<0.001	DOWN
B ⁰ AT	Denizli	1.00±0.14	1.000	ND
	Gerze	0.56±0.08	0.253	ND
CAT1	Denizli	0.72±0.41	0.082	ND
	Gerze	0.72±0.47	0.023	DOWN
EAAT3	Denizli	0.59±0.33	0.003	DOWN
	Gerze	0.69±0.34	0.066	ND
PepT1	Denizli	0.96±0.43	0.868	ND
	Gerze	0.96±0.32	0.839	ND
SI	Denizli	1.00±0.66	0.972	ND
	Gerze	1.13±0.78	0.285	ND
SGLT1	Denizli	0.80±0.47	0.215	ND
	Gerze	1.04±0.63	0.815	ND
GLUT5	Denizli	0.92±0.49	0.620	ND
	Gerze	1.12±0.38	0.485	ND

Means ±SEM are reported. ND, Sample group is not different from control group; DOWN, Downregulated.

Table 4 – Relative expressions of some genes in the small intestine of Denizli and Gerze embryos compared to LW embryos at E21.

Gene	Group	Relative expression	p-value	Result
APN	Denizli	0.46±0.19	<0.001	DOWN
	Gerze	0.37±0.17	<0.001	DOWN
B ⁰ AT	Denizli	1.16±0.28	0.626	ND
	Gerze	0.82±0.16	0.574	ND
CAT1	Denizli	0.32±0.07	0.002	DOWN
	Gerze	0.38±0.08	0.010	DOWN
EAAT3	Denizli	0.69±0.21	0.123	ND
	Gerze	0.44±0.14	0.003	DOWN
PepT1	Denizli	0.49±0.15	0.006	DOWN
	Gerze	0.63±0.19	0.096	ND
SI	Denizli	0.98±0.09	0.961	ND
	Gerze	0.76±0.06	0.564	ND
SGLT1	Denizli	0.77±0.26	0.252	ND
	Gerze	0.59±0.18	0.045	DOWN
GLUT5	Denizli	0.53±0.16	0.019	DOWN
	Gerze	1.25±0.44	0.316	ND

Means ±SEM are reported. ND, Sample group is not different from control group; DOWN, Downregulated.

genotype (Table 5). This result indicates that the yolk contents were more quickly used up in Denizli embryos and APN activity diminished. Speier *et al.* (2012) also indicated that the highest level of APN occurred at E15 and then decreased in the YSM of Cobb and Leghorn embryos. APN expression level did not differ among genotypes at E19 or at E21.

Amino acid transporters such as B⁰AT mRNA expression in the small intestine increased from E19



Table 5 – Relative expressions of some genes in the YSM of different genotypes at E21 compared to E19.

Gene	Group	Relative expression	p-value	Result
APN	Lohman White	0.89±0.25	0.839	ND
	Denizli	0.39±0.15	0.027	DOWN
	Gerze	0.77±0.28	0.500	ND
B ⁰ AT	Lohman White	1.26±0.32	0.731	ND
	Denizli	0.30±0.10	0.024	DOWN
	Gerze	9.45±0.19	0.090	ND
CAT1	Lohman White	3.24±1.40	0.024	UP
	Denizli	3.18±0.37	0.205	ND
	Gerze	1.89±0.53	0.220	ND
EAAT3	Lohman White	1.15±0.42	0.708	ND
	Denizli	0.52±0.34	0.008	DOWN
	Gerze	6.13±1.55	<0.001	UP
PepT1	Lohman White	1.47±0.31	0.647	ND
	Denizli	0.89±0.52	0.613	ND
	Gerze	0.97±0.20	0.950	ND
SGLT1	Lohman White	1.92±0.44	0.399	ND
	Denizli	1.64±0.64	0.360	ND
	Gerze	2.43±0.89	0.040	UP
GLUT5	Lohman White	2.40±1.03	0.132	ND
	Denizli	0.96±0.42	0.938	ND
	Gerze	1.30±0.57	0.390	ND

Means ±SEM are reported. ND, Sample group is not different from control group; UP, Upregulated; DOWN, Downregulated.

to E21 in all examined genotypes. Expression of B⁰AT gene in the small intestine is thought to be more important for all embryos examined at E21 than at E19. Similarly, Chen *et al.* (2015) reported that B⁰AT was more important during late embryogenesis, because its level increased 220-fold from E9 to hatch time in their study. CAT1 gene expression in the small intestine did not differ in all genotypes at E21 compared to E19. However, EAAT3 gene expression in the small intestine of LW and Denizli embryos increased from E19 to E21. B⁰AT, EAAT3 and CAT1 genes are important in amino acid absorption (Chen *et al.*, 2016). B⁰AT and EAAT3 are brush-border membrane proteins. B⁰AT transports the neutral amino acids into the intestinal enterocyte. However, CAT1 is a basolateral protein that transports cationic amino acid out of the intestinal enterocyte (Speier *et al.*, 2012). EAAT3 has a high affinity for glutamate; glutamate as an oxidative fuel source, enters the various metabolic sequences in enterocytes, increasing the amount of energy needed for rapidly growing embryos (Chen *et al.*, 2015). mRNA expression of EAAT3 increased from E19 to E21. Similar results were found by Gilbert *et al.* (2007) and Chen *et al.* (2015). However, Chen *et al.* (2015) indicated that CAT1 reached higher levels at E9 and E11, then decreased with embryonic age, because it plays an important role in the early stages

of embryonic development. However, in the current study, CAT1 expression maintained similar levels at the end of incubation compared to E19. At E19, EAAT3 expression was downregulated in Denizli embryos and CAT1 expression was downregulated in Gerze embryos in the small intestine compared to LW embryos (Table 6). CAT1 was downregulated in both Denizli and Gerze embryos compared to LW embryos and EAAT3 expression was downregulated in Gerze embryos compared to LW embryos at E21 (Table 7). CAT1 expression in the YSM increased with embryonic age in LW embryos but EAAT3 increased only Gerze embryos with increasing embryonic age. B⁰AT and EAAT3 expressions in the YSM of Denizli embryos were lower than those at E19. CAT1 expression in the YSM of Denizli embryos was 3.24-fold lower than that of LW embryos at E19. EAAT3 expression was 0.27-fold lower than that of LW embryos at E19. B⁰AT, CAT1 and EAAT3 expression levels did not differ between genotypes at E21.

Table 6 – Relative expressions of some genes in the YSM of Denizli and Gerze embryos compared to LW embryos at E19.

Gene	Group	Relative expression	p-value	Result
APN	Denizli	0.89±0.25	0.834	ND
	Gerze	1.03±0.50	0.940	ND
B ⁰ AT	Denizli	1.26±0.32	0.736	ND
	Gerze	0.15±0.01	0.158	ND
CAT1	Denizli	3.24±1.40	0.024	UP
	Gerze	0.74±0.34	0.572	ND
EAAT3	Denizli	1.15±0.42	0.711	ND
	Gerze	0.27±0.09	0.029	DOWN
PepT1	Denizli	1.47±0.31	0.630	ND
	Gerze	1.38±0.25	0.753	ND
SGLT1	Denizli	1.92±0.44	0.396	ND
	Gerze	0.59±0.12	0.575	ND
GLUT5	Denizli	2.40±1.03	0.125	ND
	Gerze	0.69±0.31	0.610	ND

Means ±SEM are reported. ND, Sample group is not different from control group; UP, Upregulated; DOWN, Downregulated.

PepT1 is localized at the brush border membrane. Amino acids are introduced into the enterocyte as di or tri peptides via PepT1 (Chen *et al.*, 2016; Leibach & Ganapathy, 1996). PepT1 expression was regulated in the developmental stage during the embryonic growth and its mRNA level rose from E16 to hatch (Chen *et al.*, 2005, Gilbert *et al.*, 2007, Speier *et al.*, 2012). PepT1 expression in the intestine was upregulated in all the examined embryos from E19 to E21. This may be linked to the importance of proteins for embryonic development with increasing embryonic age. However, its expression levels did not differ in the YSM between



age groups. At E21, PepT1 expression in the small intestine was 0.49-fold, a decrease in the Denizli embryos compared to LW embryos.

Table 7 – Relative expressions of some genes in the YSM of Denizli and Gerze embryos compared to LW embryos at E21.

Gene	Group	Relative expression	p-value	Result
APN	Denizli	0.60±0.22	0.369	ND
	Gerze	0.88±0.25	0.812	ND
B ^o AT	Denizli	0.41±0.10	0.165	ND
	Gerze	1.11±0.05	0.891	ND
CAT1	Denizli	1.52±0.29	0.615	ND
	Gerze	0.56±0.23	0.224	ND
EAAT3	Denizli	0.50±0.27	0.075	ND
	Gerze	1.46±0.61	0.362	ND
PepT1	Denizli	1.13±0.51	0.722	ND
	Gerze	0.91±0.34	0.835	ND
SGLT1	Denizli	0.60±0.15	0.383	ND
	Gerze	0.74±0.37	0.475	ND
GLUT5	Denizli	0.51±0.30	0.051	ND
	Gerze	0.37±0.17	0.012	DOWN

Means ±SEM are reported. ND, Sample group is not different from control group; DOWN, Downregulated.

SI is a digestive enzyme and carbohydrates are separated from monosaccharides by this enzyme. SI expression in the small intestine cells of the LW embryos was upregulated at E21 compared with E19. Fold increase of SI was 1.94 from E19 to E21 in the LW embryos. This may be due to LW embryos need of high energy in this incubation period. However, expression of SI gene in the YSM of examined genotypes was not detected. Speier *et al.* (2012) reported that expression of SI gene in the YSM of Cobb and Leghorn breeds was detected at a very low level throughout the incubation. This study indicated that SI in the YSM may not be crucial at E19 and E21 for the embryos. Yadgary *et al.* (2010) reported that carbohydrates can be found in the yolk contents and their levels increase from midincubation to E19 and decrease from E19 to E21. Onbaşilar *et al.* (2017) indicated that Denizli and Gerze embryos consumed yolk composition quickly to the beginning of the E21. Therefore, APN, EAAT3 and SI expressions of these pure breeds were lower than those of the layer hybrids at E21.

SGLT1 is a brush border membrane protein (Speier *et al.*, 2012, Yadgary *et al.*, 2011). The SGLT1 gene expression in the YSM and small intestine of all examined embryos was upregulated from E19 to E21. Upregulation of SGLT1 in all genotypes at E21 would enhance the uptake of glucose for the energy needed to hatch. However, it was upregulated from E19 to

E21 in the YSM of Gerze embryos. No significant difference in the small intestine was found between genotypes at E19. While at E21, SGLT expression was downregulated in the small intestine of Gerze embryos compared to that of LW embryos.

GLUT5 is a brush border membrane protein that transports fructose into intestinal enterocyte (Speier *et al.*, 2012). Expression of GLUT5 was similar with that of SGLT1 and its expression in both small intestine and YSM were similar with E19 and E21. Increasing embryonic age, GLUT5 expression in the small intestine was 0.53-fold lower in Denizli embryos compared with LW embryos. While, at E21, GLUT5 expression in the YSM of Gerze embryos was 0.37-fold lower than that of LW embryos. This result showed that glucose transporter is more important in the hybrid embryos than pure breed embryos in the last embryonic stage.

CONCLUSION

Expressions of B^oAT, PepT1 and SGLT1 were upregulated in the small intestine of pure breed and layer hybrid embryos with increasing embryonic age. EAAT3, SI and APN expressions were higher in the hybrid embryos due to the higher quantity of protein and energy needed than for pure breed embryos at E21. Genotype and embryonic age have a changing effect on expression of gene in the YSM and small intestine. APN, B^oAT, EAAT3, CAT1, PepT1, SI, SGLT and GLUT5 didn't show similar expression sides in the YSM and small intestine of different layer genotypes.

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