RESEARCH NOTE

RELATIVE mRNA EXPRESSION OF TWO NUTRIENT TRANSPORTERS BETWEEN THE PHILIPPINE DARAG CHICKEN AND COMMERCIAL LAYER CHICKEN *(Gallus gallus domesticus)*

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ABSTRACT

Darag chicken is one of the Philippine native chicken strains originating from the Western Visayas region. It is common for poultry farmers to raise native chicken breeds due to their adaptability and resistance to diseases, thus most are produced through free-management systems. The majority of Darag chicken farmers use layer diets that are available on the market and are designed to meet the nutritional needs of commercial layer chickens. This study characterized the nutrient transporters in the small intestines of the Philippine Darag chicken and commercial layer chicken (CLC). Specifically, the experiment compared the mRNA expression levels of *EAAT3* and *B^{0}AT* between Darag chicken and commercial layer chicken fed with commercial ration. Tissue samples were collected from the three main intestinal segments of the Darag and CLC. Total RNA extraction from the intestinal tissue samples was done followed by cDNA synthesis and real-time quantitative polymerase chain reaction to determine the relative expression levels of *EAAT3* and *B^{0}AT* in the two bird groups. Philippine Darag chicken displayed an increase in relative mRNA expression levels of both genes, *EAAT3* and *B^{0}AT*, in the three (3) intestinal segments. The observed expression levels of both genes in Darag were statistically comparable with CLC.

Keywords: *B^{0}AT*, Darag chicken, *EAAT3*, mRNA expression analysis, Small intestine

INTRODUCTION

Darag chicken is one of the native Philippine chicken strains from Western Visayas. Along with other domesticated chickens, its genetic lineage can be traced to the Red Jungle fowl *(Gallus gallus)* (Contreras *et al.*, 2014). Due to their adaptability and resistance to disease, it is common for poultry farmers to raise native chicken breeds; consequently, most are produced through free-management systems (Padhi, 2016).

Amino acids (AA) are the building blocks of proteins and are responsible for a variety of important physiological functions throughout the body. Genetic advancements and better performance of laying hens have altered the interdependent optimal inputs of amino acids and energy.

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This study focused on two amino acid transporters and a housekeeping gene. Excitatory amino acid transporter 3 *(EAAT3)* is a Na+ -dependent transporter of anionic amino acids such as aspartate and glutamate located at the brush border membrane of enterocytes (Gilbert *et al.*, 2007). The *EAAT3* transports aspartate and glutamate, which are the main energy sources for intestinal epithelial cells and are used by enterocytes for other amino acid synthesis (Iwanaga *et al.*, 2005; Kanai *et al.*, 2013). *EAAT3* expression is highest in the ileum, indicating increased glutamate uptake in the lower section of the small intestine (Gilbert *et al.*, 2007).

The Na+-dependent neutral amino acid transporter *(B^0AT)* transfers a wide variety of neutral amino acids into cells. *B^0AT* is used in place of cationic amino acids and cysteine. (Fotiadis *et al.*, 2013). The transport of amino acids via *B^0AT* is driven by the membrane potential. This type of amino acid transporter is abundant in the small intestine's brush border membrane. (Terada *et al.*, 2005).

Studies of genes involved in protein digestion and absorption are vital steps in exploring native chickens because, until now, native chicken raisers still rely on the feed ingredients designed for commercial layer chickens. This study aimed to compare the mRNA expression levels of EAAT3 and B0AT between Philippine Darag chicken and commercial layer chicken. Moreover, the study will provide a critical analysis that can be useful during the formulation of nutrient specifications of feed ingredients established for the native chickens that optimally fit the Darag chicken's digestive potential.

MATERIALS AND METHODS

The study was done in accordance with the Institutional Care and Use Committee of UPLB (CAFS-2022-001). Individual metabolic cages were used to raise four (4) Philippine Native Darag hens from Samui Organic Farm in Silang, Cavite, and four (4) H&N commercial layer chickens (CLC) from IAA Agri Farm in Brgy. San Marcos, San Pablo City, Laguna. Each cage represented a single replica. All animals were fed a limited diet according to their body weight, applying the CLC-specific dietary requirements. The Darag hens and CLC received 110 g of crumbled commercial layer feed per day (Sustamina Agri-Industrial Corporation brand). All chickens were given free access to water. For efficient egg production, an artificial light source (20-W fluorescent lamp) was placed to provide the layers with 16 hours of light exposure. Three (3) Darag and three (3) H&N CLCs that had reached uniform egg laying were fasted for 16 hours and slaughtered at the Animal Production Science and Technology Division, Meat Science Building, IAS, UPLB, for small intestinal segment tissue sample collection.

Tissue samples were collected from the three main intestinal segments of the chosen Darag and H&N CLC (Figure 1). Particularly, 20 mm of the duodenum from the mid-duodenal loop; 20 mm of jejunum at the start of the mesenteric artery towards it, and 20 mm of ileum after measuring 50 mm from the ileocecal junction towards the jejunum were measured and obtained in duplicates (Pinca *et al.*, 2019).

Using the PureLink™ RNA Mini Kit, total RNA was extracted from intestinal tissue samples (i.e., duodenum, jejunum, and ileum). This followed the manufacturer's protocol for small tissue samples (≤ 100 mg). The collected total RNA concentration was quantified spectrophotometrically at 260/280 nm using UV absorbance or fluorescence microplate reader with Epoch 2 Microplate Spectrophotometer- Biotek at the Animal Biotechnology

Laboratory, Villegas Hall, IAS, UPLB. TaqMan® Reverse Transcription Reagents were used and run using the Applied BiosystesmTM VeritiTM Thermal Cycler for cDNA synthesis. To see if the primers bind to the cDNA synthesized, the cDNA samples were put through a polymerase chain reaction. The reaction was used for agarose gel electrophoresis immediately. The PCR of the produced cDNA was performed using the Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific).

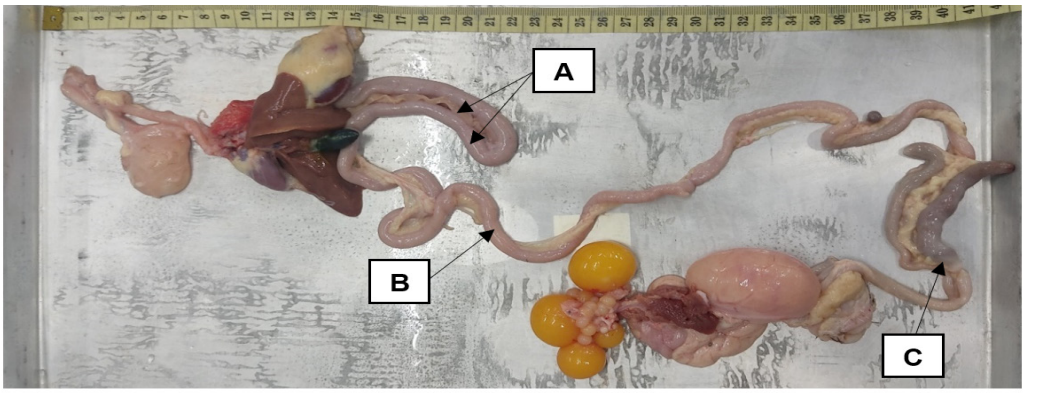

Figure 1. Gross morphology of the internal organs of a layer chicken displaying the three small intestinal segments (A- Duodenum, B- Jejunum, and C- Ileum) from which tissue samples for total RNA extraction were obtained.

Real-time quantitative polymerase chain reaction quantified the number of RNA molecules per nanogram of total RNA starting template for each gene of interest. Real-time quantification was done using the designed specific primers shown in Table 1 for the housekeeping and the target genes by Gilbert *et al.* (2007).

Table 1. Forward and reverse primer sequences for *EAAT3, B^0AT,* and *β-actin.*

Gene	Accession Number	Forward sequence	Reverse sequence
EAAT3	XM_046936555.1	TGC TGC TTT GGA TTC CAG TGT	AGC AAT GAC TGT AGT GCA GAA GTA ATA TAT G
B^0AT	XM_419056.8	GGG TTT TGT GTT GGC TTA GGA A	TCC ATG GCT CTG GCA GAT
β-actin	NM_205518.2	CTG GCA CCT AGC ACA ATG AA	CTG CTT GCT GAT CCA CAT CT

Analysis and collection of the data were done following the instructions of the QuantStudio® 3 System Real-Time PCR System (Thermo Fisher Scientific). Five-point standard curves for *EAAT3, B^0AT, and β-actin* were created with total RNA concentrations of 50.0, 10.0, 2.0, 0.4, and 0.8 ng/µL (1:5 dilution series). Using 10 ng/µL cDNA samples

from Darag and CLC's duodenum, jejunum, and ileum, target and housekeeping genes were amplified. The duplicate per gene with 10 ng/μL cDNA samples and negative control created a dissociation curve.

A mathematical model by Pfaffl (2001) (Equation 1) quantifies target genes relative to reference genes.

$$\text{Relative Expression Ratio} = [\, E_{target}^{\Delta Ct\,(\text{Gene of Interest})} \,] \,/\, [\, E_{ref}^{\Delta Ct\,(\text{Reference Gene})} \,] \qquad \text{[Equation 1]}$$

The E_{target} is the real-time PCR efficiency of the target gene. The E_{ref} is the real-time PCR efficiency of a reference gene. The ΔCP_{target} is the CP deviation of the control – sample of the target gene. The ΔCP_{ref} is the difference between the CP deviation of the control and sample of the reference gene. The reference gene can be a housekeeping gene that is characterized as stable and secure.

The mRNA expression levels were normalized using a housekeeping gene (βactin) expression level. SAS University Edition Software was used for the statistical analysis of three independent experiments. The Student t-test at 0.05 (95% confidence level) was used to compare intestinal nutrient transporter expression levels in Darag and H&N CLC.

RESULTS AND DISCUSSION

All A260/280 values are around 2.0. The total RNA isolated from each of the three segments from the two animal samples that were quantified spectrophotometrically at 260/280 nm is pure and contains no contaminants. High-resolution gel electrophoresis, as seen in Figure 2, showed the specificity of RT-PCR products and produced a single product with the appropriate length (β-actin, 123 bp; *EAAT3*, 79 bp; B^0AT, 60 bp).

The melt curves or dissociation curves for the three genes were created using the previously specified qPCR concentrations and conditions. It can be observed in Figure 3 that all three genes had a single melting point, revealing the expected amplicon (Tm). The melting curve ™ is the temperature at which 50% of DNA is double-stranded and 50% is single-stranded. The primers produce a particular PCR product without non-specific amplification. thus improving real-time quantitative polymerase chain reaction results.

The regression line equation for the standard curve for three genes—two target genes and a housekeeping gene—was calculated using one 20 μL. The regression line's slope (Equation 2) in the standard curve was used to calculate the amplification and percent efficiencies of *β-actin, EAAT3,* and B^0AT:

$$\%E = \left(\frac{-1}{10^{Slope}} - 1\right)(100) \qquad \text{[Equation 2]}$$

Serial dilution detection defined the assay's experimental linear dynamic range. Table 2 shows the line equation and amplification efficiency.

Figures 4A, 4B, and 4C compared *EAAT3* and B^0AT mRNA expression levels in the duodenum, jejunum, and ileum. Philippine Darag chicken intestine segments showed increased relative mRNA expression of *EAAT3* and B^0AT genes. The duodenum, jejunum, and ileum upregulate both genes, matching their respective expression levels in commercial layer chicken, the control in this study. Both genes may upregulate to take advantage of

substrate abundance or compensate for substrate deficiency (Gilbert *et al.*, 2007).

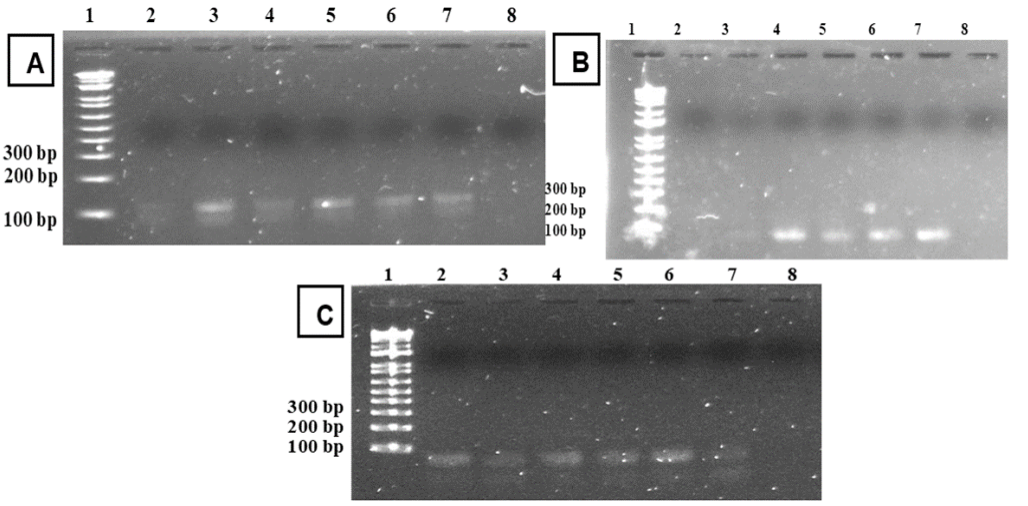

Figure 2. Agarose gel electrophoresis showing the amplification of (A) β-actin, (B) *EAAT3,* and (C) *B^{0}AT* (Lane 1 – 1000 bp DNA ladder for size determination; Lane 2 – duodenum of CLC; Lane 3 – jejunum of CLC; Lane 4 – ileum of CLC; Lane 5 – duodenum of Darag; Lane 6 – jejunum of Darag; Lane 7- ileum of Darag; Lane 8 – negative control.

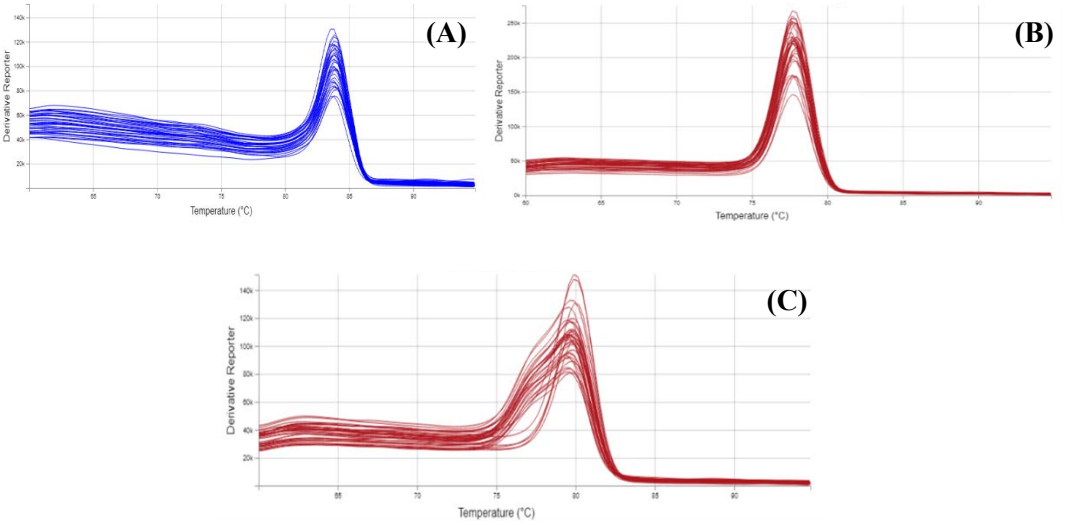

Figure 3. Melt curves from qPCR of (A) *Beta Actin,* (B) *EAAT3*, and (C) *B^{0}AT.*

Table 2. Regression line and amplification efficiencies of *β-actin*, *EAAT3,* and *B⁰AT.*

Gene	R^2 (≥0.98)	Slope	Y-intercept	Amplification Efficiency E	% Efficiency (90%-110%)
β-actin	0.999	-3.35	36.04	1.99	98.68
EAAT3	0.984	-3.14	29.76	2.08	107.98
B⁰AT	0.997	-3.29	29.07	2.01	101.28

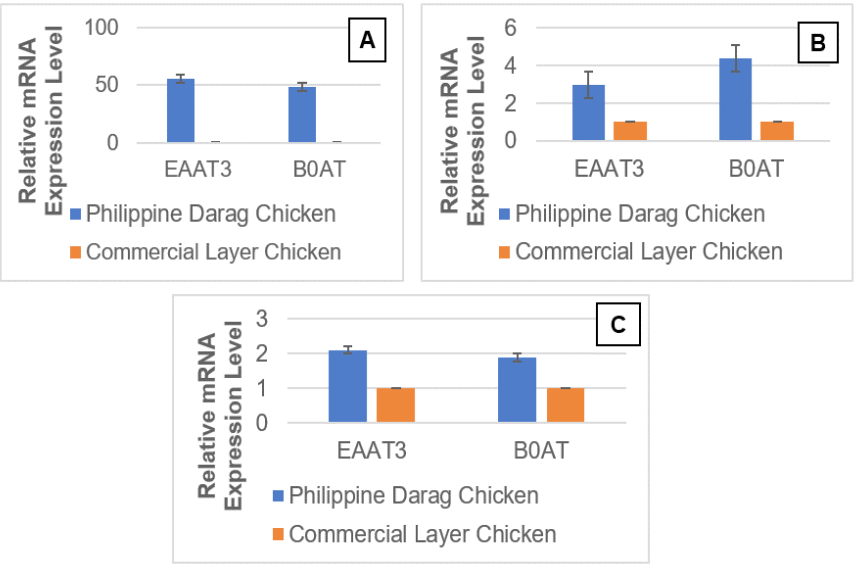

Figure 4. Expressions of *EAAT3* and *B⁰AT* in three intestinal segments (A- duodenum, B-jejunum, C-ileum) of Philippine Darag chicken.

In this study, if mRNA expression levels in the duodenum will be considered an outlier due to high CT values of the *β-actin* that affected the standard deviation, it can be stated that both genes were found to be the greatest in the jejunum and ileum. According to Gilbert *et al.* (2007), the abundances of *EAAT3* and *B⁰AT* were greatest in the ileum. Table 3 showed that the quantities of *EAAT3* mRNA were greatest in the jejunum ($\bar{x}$ = 2.9772) and intermediate in the ileum ($\bar{x}$ = 2.1020). Quantities of *B⁰AT* mRNA were of a similar magnitude to the quantities of mRNA observed for *EAAT3*; greatest in the jejunum ($\bar{x}$ = 4.3904) and intermediate in the ileum ($\bar{x}$ = 1.8726). Moreover, the findings of the experiment supported the report of Tauqir (2016) that the active sites for amino acid absorption are the ileum and jejunum.

Brush border membrane transporters *EAAT3* and *B⁰AT* govern intestinal lumen-to-epithelial free amino acid absorption. These genes' downregulation might limit critical amino acid input into infected cells. For example, decreasing *EAAT3* expression depletes intestinal cell glutamate energy. Even though there was an upregulation of both genes in the three intestinal segments, the expression levels of both genes were found to be not significantly different from the control. However, this study used minimal sampling due to the limited

Table 3. Pfaffl gene expression ratio and T-test values of *EAAT3 and B^0AT* in the duodenum, jejunum, and ileum of Philippine Darag chicken and commercial layer chicken samples.

Genes	Ratio S1	Ratio S2	Ratio S3	Ave	SD	N	SEM	Ctrl 1	Ctrl 2	Ctrl 3	Ave	SD	N	SE	T-Test
Duodenum															
EAAT3	30.87	4.93	129.95	55.25	53.87	3	38.09	1	1	1	1	0	3	0	0.15
B^0AT	25.29	8.06	111.38	48.24	45.19	3	31.96	1	1	1	1	0	3	0	0.14
Jejunum															
EAAT3	5.05	0.45	3.430	2.98	1.90	3	1.35	1	1	1	1	0	3	0	0.14
B^0AT	5.60	0.39	7.19	4.39	2.91	3	2.05	1	1	1	1	0	3	0	0.12
Ileum															
EAAT3	2.10	3.65	0.56	2.10	1.26	3	0.89	1	1	1	1	0	3	0	0.17
B^0AT	1.89	3.39	0.34	1.87	1.24	3	0.88	1	1	1	1	0	3	0	0.21

reagents available. Increasing the sample size is highly recommended to further demonstrate relative expressions of both genes between groups.

Tissue protein, enzyme, and hormone production and deamination can use absorbed amino acids. Laying hen diet amino acid levels and optimal dietary concentration are economically significant for egg-laying enterprises. If one critical amino acid is lacking or excessive, an egg production system cannot be maximized economically. The poultry feedstuff industry needs information that allows them to calculate the economic level of the addition of amino acids. Important for the economic evaluation is a precise description that describes the increasing egg mass output, egg numbers, and egg weights to increase the amino acid supply.

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