# Interactive effects of dietary amino acid density and environmental temperature on growth performance and expression of selected amino acid transporters, water channels, and stress-related transcripts

R. A. Alhotan,[*,1] A. A. Al-Sagan,[†] A. A. Al-Abdullatif,[*] E. O. S. Hussein,[*] I. M. Saadeldin,[*] M. M. Azzam,[*,‡] and A. A. Swelum[*]

[*]Department of Animal Production, King Saud University, King Abdullah Road, Riyadh 11451, Saudi Arabia; [†]King Abdulaziz City for Science & Technology, Riyadh 11442, Saudi Arabia; and [‡]Department of Poultry Production, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt

**ABSTRACT** Exposure to heat stress (**HS**) is one of the challenges facing the broiler industry worldwide. Various nutritional strategies have been suggested, such as altering dietary concentrations of some nutrients. Thus, we evaluated feeding different amino acid (**AA**) densities on live performance, *Pectoralis* (*P.*) muscles, and expression of selected AA transporters, water channels, and stress-related transcripts in a fast-growing broiler strain. Ross 308 chicks (n = 576) were randomly assigned to 4 dietary treatments (24 reps, 6 chicks per rep), differing in AA density (110, 100, 90, and 80% of a breeder's AA specifications). During 24 to 36 days of age, half of the birds were kept at a thermoneutral (**TN**) temperature of 20°C, whereas the other half were subjected to HS at 32° C for 8 h daily, making the treatment design a 4 × 2. The results revealed no interaction between housing temperature and AA density on growth performance or *P.* muscles weights. Feeding 80% AAs depressed BWG, FCR, and *P.* muscles at 36 d ($P < 0.001$). There was an interaction ($P < 0.001$) between AA density and temperature on the expression of all examined genes. Reducing the AA density beyond 100% upregulated the expression of AA transporter (CAT1, $B^0AT$, $b^{0,+}AT$, SNAT1, LAT1), HSP70, HSP90, glucocorticoid receptor (**GR**), and AQP3 in the TN birds' jejunum. Whereas in the HS birds, inconsistent expressions were observed in the jejunum, of which CAT1, $B^0AT$, and LAT1 were markedly downregulated as AA density was reduced. In *P. major* of TN birds, reducing AA density resulted in upregulating the expression of all AA transporters, HSP70, GR, and AQP1, while downregulating HSP90 and AQP9. In contrast, AA reduction markedly downregulated CAT1, $B^0AT$, and LAT1 in the *P. major* of HS birds. These findings indicate that the dietary AA level alters the expression of various genes involved in AA uptake, protein folding, and water transport. The magnitude of alteration is also dependent on the housing temperature. Furthermore, the results highlight the importance of adequate AA nutrition for fast-growing chickens under HS.

**Key words:** broiler, performance, amino acid, density, aquaporin, water channel

## INTRODUCTION

Amino acids (**AAs**) are vital nutrients in broiler chicken nutrition as they are needed for maintenance, growth and are even utilized as energy sources. Feed proteins contain 20 AAs, of which ten are essential and must be offered in the diet in adequate quantities. The other nonessential AAs are also required for the de novo synthesis of proteins, but they are not necessary to be present in the diet in adequate quantities (D'Mello, 2003). The process of protein synthesis demands an uninterrupted supply of all the 20 AAs at the appropriate site; otherwise, protein synthesis will be terminated. The AAs that are not utilized in protein synthesis for any reason will be catabolized, and this process is associated with high heat production, which can be an extra burden during times of heat stress (**HS**) conditions (Musharaf and Latshaw, 1999; Balnave, 2004).

In the broiler industry, feeding lower or higher AA density is a commonly applied practice to achieve production goals (Vieira and Angel, 2012). Previous research has shown that modern broiler strains responded very well to increasing dietary AA density under thermoneutral (**TN**) conditions (Dozier III et al., 2008). Under TN conditions, increasing dietary AA density resulted in

increased growth rate, increased breast meat yield, improved feed efficiency, and reduced body fat of broilers (Corzo et al., 2005; Dozier III et al., 2007; Lilly et al., 2011; Johnson et al., 2020). However, under HS conditions, the results are controversial and inconclusive (Furlan et al., 2004; Gonzalez-Esquerra and Leeson, 2006; Awad et al., 2019). In general, HS has been reported to depress the biological performance and alter the expression of nutrient transporters, including AA transporters, in poultry (Habashy et al., 2017; Al-Zghoul et al., 2019; Liu et al., 2020) and water channels (aquaporins) in rats (Wang et al., 2015). AA transporters and water channels are responsible for the cellular uptake of AAs and water, which are regarded as the main body components in fast-growing broiler chickens (Borgnia et al., 1999; Swennen et al., 2004; Habashy et al., 2017).

Water is the major component of the chicken body, varying between 64 and 76% of the body weight depending on age and sex (Peebles et al., 2001; Mitchell et al., 2011; Butzen et al., 2015; Schallier et al., 2019). Furthermore, water content varies among different body tissues. For instance, the abdominal fat pad is an adipose tissue that contains about 26% water and 72% of lipids (Yau et al., 1991). In contrast, breast muscles, *Pectoralis* (*P.*) *major* and *minor*, are lean tissues that contain about 74% moisture and 23% protein (Bianchi et al., 2007; Lilly et al., 2011; Koçer et al., 2018). Transport of water across biological membranes is undertaken by diffusion through lipid bilayers and osmosis (Haines, 1994; Reuss, 2012). Under osmotic gradient, water molecules pass rapidly in and out of the cells via water channels (Borgnia et al., 1999). Until now, 13 isoforms of water channels (AQP0 to AQP12) have been discovered in several species, including poultry (Orlowski et al., 2017; Michałek and Grabowska, 2019).

Currently, no previous research has assessed the interactive effects of dietary AA density and environmental temperature on growth performance and the expression of AA transporters and water channels in poultry. A deeper understanding of the interactive effects of AA density and rearing temperature on growth performance and nutrient transport is required when formulating broiler feeds. We hypothesized that increasing the dietary AA density could influence the growth performance of HS chickens differently. We also hypothesized that the mRNA expressions of AA transporters and water channels might be affected by the dietary AA density and environmental temperature. Therefore, this study's objectives were to examine the influence of supplementing various dietary AA densities on growth performance, breast muscle yield, and the transcripts expression of selected AA transporters, water channels, and stress-related proteins in broiler chickens raised under TN and cyclic HS conditions.

## MATERIALS AND METHODS

### Birds and Housing

All the experimental procedures followed regarding the care and use of birds were reviewed and approved by the Research Ethics Committee at King Saud University (Riyadh, Saudi Arabia; Ethics Reference No: KSU-SE-20-54). A total of 576 day-old male chicks belonging to the Ross 308 broiler strain were obtained from a commercial hatchery (Al Khumasia Hatchery, Al Majmah, Riyadh, Saudi Arabia). Vaccination against the most common diseases in the area (i.e., Newcastle, Infectious Bursal Disease, and Infectious Bronchitis) was done in the hatchery. The chicks were housed in 96 wire battery cages in 2 environmentally controlled rooms with similar conditions. The cages were identical in dimensions (0.6 m² each), and each cage was equipped with a heating system, a trough feeder, and a nipple watering system. A lighting program containing 24 h of light was provided for the entire 36 d experiment according to previous research (Almeida et al., 2018; Pestana et al., 2020; Ghanima et al., 2020). Initial room temperature was set at 31°C, and then the temperature was decreased gradually by 0.5 to reach 20°C at 24 days of age and was maintained thereafter until the experiment was terminated at 36 d. The cyclic HS protocol applied herein was previously validated in poultry (Cheng et al., 2019). During cyclic HS periods that started at 24 days of age in one room, the temperature was raised every day to reach a target of 32°C (recorded actual = 32°C ± 0.7), and this temperature was maintained for 8 h (from 8:00 to 16:00). The source of extra heat in the HS room was provided by a carbon fiber heater (Inter Heat, Seongnam, Gyeonggido, Korea) linked to a thermostat and a control unit. Besides, the HS room was equipped with humidifiers to raise the relative humidity during the cyclic HS (recorded actual = 66% ± 4). The temperature and humidity were monitored using EasyLog USB data loggers (Lascar Electronics, Whiteparish, Wiltshire, United Kingdom) with a temperature accuracy of ±0.5 and humidity accuracy of ±3%.

### Experimental Design and Treatment Design

Four dietary treatments varying in AA density were prepared as follows: 1) 110% of Aviagen AA recommendations (Aviagen Inc, 2019); 2) 100% of Aviagen AA recommendations, control; 3) 90% of Aviagen AA recommendations; and 4) 80% of Aviagen AA recommendations (Tables 1 and 2). Each dietary treatment was allocated to 24 cages in a completely randomized design with 6 chicks each. At 24 days of age, another factor, environmental temperature, was included in the treatment design, making the final design a 4 × 2 with 4 AA densities and 2 microclimate environments, TN and HS.

### Diet Preparation

The diets were formulated using WUFFDA 2.0 Formulation (Pesti et al., 2016) based on standardized ileal digestible AAs with ideal ratios (Table 3). The minimum CP constraint in the formulation software was removed during the formulation process. The reference AA, lysine, was increased by 10% or decreased by 10 and

**Table 1.** Ingredient compositions of the experimental diets.[1]

| Ingredient (%) | Starter phase (0−10 d) | | | | Grower phase (11−23 d) | | | | Finisher phase (24−36 d) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 80% | 90% | 100% | 110% | 80% | 90% | 100% | 110% | 80% | 90% | 100% | 110% |
| Yellow corn | 66.66 | 60.39 | 54.11 | 47.84 | 69.86 | 64.17 | 58.48 | 52.80 | 71.07 | 65.76 | 60.44 | 55.12 |
| Soybean meal (48%) | 27.58 | 32.96 | 38.34 | 43.72 | 23.78 | 28.65 | 33.53 | 38.41 | 21.50 | 26.06 | 30.63 | 35.20 |
| Vegetable oil | 1.30 | 2.18 | 3.07 | 3.95 | 2.28 | 3.08 | 3.89 | 4.69 | 3.59 | 4.34 | 5.09 | 5.84 |
| Dicalcium phosphate | 2.06 | 2.00 | 1.95 | 1.90 | 1.85 | 1.81 | 1.76 | 1.71 | 1.72 | 1.67 | 1.63 | 1.58 |
| Limestone | 1.10 | 1.09 | 1.08 | 1.07 | 1.01 | 1.00 | 0.99 | 0.98 | 0.95 | 0.94 | 0.93 | 0.92 |
| Dl-Methionine | 0.26 | 0.31 | 0.37 | 0.42 | 0.23 | 0.28 | 0.33 | 0.38 | 0.22 | 0.27 | 0.31 | 0.36 |
| L-Lysine HCL | 0.22 | 0.23 | 0.23 | 0.24 | 0.21 | 0.21 | 0.21 | 0.21 | 0.19 | 0.19 | 0.19 | 0.19 |
| L-Threonine | 0.11 | 0.13 | 0.15 | 0.17 | 0.09 | 0.11 | 0.12 | 0.14 | 0.07 | 0.09 | 0.10 | 0.12 |
| L-Valine | 0.00 | 0.01 | 0.03 | 0.04 | 0.00 | 0.00 | 0.01 | 0.03 | 0.00 | 0.00 | 0.00 | 0.01 |
| Common salt | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| Vitamin premix[2] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Mineral premix[3] | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| Choline CL (60%) | 0.11 | 0.09 | 0.07 | 0.06 | 0.10 | 0.09 | 0.07 | 0.06 | 0.10 | 0.09 | 0.07 | 0.06 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

[1]Amino acid specifications are based on the 2019 Aviagen Nutrition Guide for Ross broilers.

[2]Vitamin premix provided the following (per kg of diet): Vitamin B1, 2 mg; Niacin, 50 mg; Vitamin B2, 6 mg; Pantothenic Acid, 15 mg; Vitamin B12,16.0 $\mu$g; Vitamin B6, 3 mg; Biotin, 150 $\mu$g; Folic Acid, 1.75 mg; Vitamin K3 (MNB), 3 mg; Vitamin D3 (cholecalciferol), 5000 IU; Vitamin A (retinol acetate), 10,000 IU; Vitamin E (Dl-alpha-tocopheryl acetate), 50 IU; Total antioxidants, 50 mg.

[3]Trace mineral premix provides the following in milligrams per kilogram of diet: Manganese (Oxide), 120; Zinc (Oxide), 100; Iron (Sulphate), 40; Copper (Sulphate), 16; Iodine (Potassium Iodide), 1.25; Selenium (Sodium Selenite) 0.30.

**Table 2.** Calculated and analyzed compositions of the experimental diets.[1]

| | Starter phase (0−10 d) | | | | Grower phase (11−23 d) | | | | Finisher phase (24−36 d) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 80% | 90% | 100% | 110% | 80% | 90% | 100% | 110% | 80% | 90% | 100% | 110% |
| Calculated composition | | | | | | | | | | | | |
| Metabolizable energy, kcal/kg | 3,000 | 3,000 | 3,000 | 3,000 | 3,100 | 3,100 | 3,100 | 3100 | 3,200 | 3,200 | 3,200 | 3,200 |
| Crude protein, % | 18.55 | 20.70 | 22.85 | 25.01 | 16.92 | 18.87 | 20.82 | 22.77 | 15.89 | 17.70 | 19.53 | 21.35 |
| Calcium, % | 0.96 | 0.96 | 0.96 | 0.96 | 0.87 | 0.87 | 0.87 | 0.87 | 0.81 | 0.81 | 0.81 | 0.81 |
| Choline, mg/kg | 1,700 | 1,700 | 1,700 | 1,700 | 1,600 | 1,600 | 1,600 | 1,600 | 1,550 | 1,550 | 1,550 | 1,550 |
| Available phosphorus, % | 0.48 | 0.48 | 0.48 | 0.48 | 0.44 | 0.44 | 0.44 | 0.44 | 0.41 | 0.41 | 0.41 | 0.41 |
| Digestible lysine, % | 1.02 | 1.15 | 1.28 | 1.41 | 0.92 | 1.04 | 1.15 | 1.27 | 0.85 | 0.95 | 1.06 | 1.17 |
| Digestible methionine, % | 0.51 | 0.59 | 0.67 | 0.74 | 0.47 | 0.53 | 0.60 | 0.67 | 0.44 | 0.51 | 0.58 | 0.64 |
| Digestible total sulfur AA, % | 0.76 | 0.85 | 0.95 | 1.04 | 0.70 | 0.78 | 0.87 | 0.96 | 0.66 | 0.75 | 0.83 | 0.91 |
| Digestible threonine, % | 0.69 | 0.77 | 0.86 | 0.95 | 0.62 | 0.69 | 0.77 | 0.85 | 0.57 | 0.64 | 0.71 | 0.78 |
| Digestible tryptophan, % | 0.19 | 0.22 | 0.25 | 0.27 | 0.17 | 0.20 | 0.22 | 0.25 | 0.16 | 0.18 | 0.20 | 0.23 |
| Digestible arginine, % | 1.10 | 1.25 | 1.40 | 1.55 | 0.98 | 1.12 | 1.26 | 1.40 | 0.92 | 1.04 | 1.17 | 1.30 |
| Digestible valine, % | 0.77 | 0.86 | 0.96 | 1.06 | 0.70 | 0.78 | 0.87 | 0.96 | 0.66 | 0.73 | 0.81 | 0.89 |
| Digestible leucine, % | 1.43 | 1.55 | 1.67 | 1.79 | 1.33 | 1.44 | 1.55 | 1.66 | 1.27 | 1.37 | 1.47 | 1.57 |
| Digestible Isoleucine, % | 0.69 | 0.77 | 0.86 | 0.95 | 0.62 | 0.70 | 0.78 | 0.86 | 0.58 | 0.66 | 0.73 | 0.80 |
| Digestible histidine, % | 0.45 | 0.49 | 0.54 | 0.59 | 0.41 | 0.45 | 0.50 | 0.54 | 0.39 | 0.43 | 0.47 | 0.51 |
| Digestible phenylalanine, % | 0.82 | 0.91 | 1.01 | 1.10 | 0.74 | 0.83 | 0.92 | 1.00 | 0.70 | 0.78 | 0.86 | 0.94 |
| Analyzed composition | | | | | | | | | | | | |
| Dry matter,% | 95.48 | 95.48 | 96.18 | 96.03 | 94.54 | 94.76 | 91.47 | 94.80 | 94.99 | 95.09 | 95.19 | 95.16 |
| Crude protein,% | 17.81 | 19.75 | 21.85 | 24.14 | 16.19 | 19.02 | 19.83 | 22.96 | 15.06 | 17.84 | 19.02 | 20.79 |
| Gross energy, kcal/kg | 4,206 | 4,190 | 4,179 | 4,363 | 4,168 | 4,217 | 4,256 | 4,357 | 4,245 | 4,274 | 4,388 | 4,410 |
| Ether extract, % | 3.99 | 5.15 | 5.99 | 7.43 | 6.47 | 5.72 | 7.36 | 7.51 | 5.12 | 6.74 | 8.66 | 8.62 |
| Crude fiber, % | 1.59 | 2.70 | 1.75 | 1.76 | 1.51 | 2.36 | 1.78 | 2.70 | 1.26 | 1.78 | 2.49 | 1.61 |
| Ash, % | 6.41 | 6.20 | 6.37 | 6.48 | 4.86 | 5.24 | 5.65 | 6.12 | 5.09 | 4.97 | 5.95 | 5.56 |

[1]Amino acid specifications are based on the 2019 Aviagen Nutrition Guide for Ross broilers.

20% during feed formulation, depending on the dietary treatment. As a result, the other AAs were lifted up or down by approximately the same percentages. The percentages 80, 90, 100, and 110% represent the dietary AA densities on average. The diets were fortified with choline chloride to maintain the minimum choline specifications. About 500 g was sampled from each diet for the analysis of proximate analysis and gross energy (Table 2). The diets were offered in a mash form during the starting (0−10 d), growing (11−23 d), and finishing (24−36 d) periods. Feed analysis was determined based on AOAC International (2000) according to Azzam et al. (2020).

## Measurements and Sample Collection

Feed and chicks were weighed at 0, 11, 23, and 35 days of age to calculate body weight gain (**BWG**), feed intake and, feed conversion ratio (**FCR**). Mortality was counted as it occurred. At 23 days of age, 24 chicks per treatment (96 total) were humanely euthanized by cervical dislocation for *P. major* and *minor* collection. Cloaca temperature was taken at 30 and 36 d from one randomly selected bird per pen using digital thermometers. On both days, the rectal temperature was measured at 12:00 PM (in the middle of the heat exposure period). The thermometer probe was inserted in the rectum of

**Table 3.** Ideal amino acid ratios.[1]

| Amino acid (%) | Starter | Grower | Finisher |
|---|---|---|---|
| Lysine | 100 | 100 | 100 |
| Methionine + Cysteine | 74 | 76 | 78 |
| Methionine | 40 | 41 | 42 |
| Threonine | 67 | 67 | 67 |
| Valine | 75 | 76 | 76 |
| Isoleucine | 67 | 68 | 69 |
| Arginine | 107 | 107 | 107 |
| Tryptophan | 16 | 16 | 16 |
| Leucine | 110 | 110 | 110 |

[1]Amino acids are referenced to lysine.

the birds to a depth of about 3 cm. Then, stable readings were obtained when the device beeped. At 36 days of age, 2 birds were selected randomly from each cage for processing (24 per treatment, 192 total). *P. major* and *minor* muscles were extracted and weighed individually. Fresh samples were collected from the same location of the right *P. major* (~1 g) and intestinal jejunum (~2 cm segment proximal to Meckel's diverticulum) from each bird. The samples were taken by a well-trained technician to assure consistency in sampling and to minimize individual variability. The collected tissue samples were immediately washed with normal saline solution to remove any contaminants, placed in sterilized tubes, snap-frozen in liquid nitrogen, and then stored at $-80°C$ for further RNA analysis.

### Heterophil: Lymphocyte Ratio

Heterophil: Lymphocyte ratio (H: L ratio) was determined according to Al-Murrani et al. (2006). Briefly,

one fresh drop of blood from each bird (12 birds per group; 96 total) was collected immediately at slaughter. Each blood sample was then smeared on a glass slide with the canted edge of a second grass slide. Later, the smears were air−dried, fixed, and stained using the May-Grunwald-Giemsa stain. Then a total of 100 leucocytes per slide were counted using a microscope (oil immersion lens) at 100 × and the total number of H was divided by that of L to get the H: L ratio.

### RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction

Extraction of total RNA from the *P.* major muscle and intestinal jejunum samples was performed according to Hassan et al. (2019) using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) and following the manufacturer's protocol. A Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA) was used to determine the purity and concentration of the extracted RNA at 230 and 260 nm, with acceptable ratios being above 2.00. The reverse-transcription reaction was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions. The relative expression of $\beta$-actin (**ACTB**) as a housekeeping gene, glucocorticoid receptors (**GR**), heat shock proteins 70 and 90 (HSP70 and HSP90), aquaporins (AQP1, AQP3, and AQP9), AA transporters ($B^0AT$, $b^{0,+}AT$, SNAT1, LAT1, and CAT1) were determined by the relative quantitative Real-time PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems,). Briefly, specific amplicons were targeted and amplified by using

**Table 4.** Primers sequence and information used for real-time PCR analysis.

| Gene name | Solute carrier | Accession number | Sequence 5'—3' (forward/reverse) | Function |
|---|---|---|---|---|
| HSP90 | | NM_001109785 | GAGTTTGACTGACCCGAGCA TCCCTATGCCGGTATCCACA | Chaperone |
| HSP70 | | NM_001006685 | CTCTGAGCTCTTCCACGCAA ATCTCCTGGATCTGGCCCTT | Chaperone |
| GR | | NM_001037826 | TATGACAGCACGCTGCCCGA CTACCACTTGCCGTCCTCCTAACAT | |
| AQP1 | | NM_001039453.1 | AGCTGGTTTTGTGCGTTCTT TCTGGCTGGGTTAATTCCAC | Passive transport of water |
| AQP3 | | AB358970.1 | TGCTCCTGGTCCCTGACACT CTTTTGCCTTCCCATTGCA | Passive transport of water |
| AQP9 | | AB359226.1 | CACAATTCCTGGGAGCATTT TGTTGCGTAAGGTCCTGTGA | Passive transport of water |
| ACTB | | NM_205518 | CCATCTATGAAGGCTACGC CTCGGCTGTGGTGGTGAA | |
| $B^0AT$ | SLC6A19 | XM_419056 | TCCACACACTGCACTCATCC CCTGCTTCTGACCTTGCTCA | Apical resorption of neutral amino acids |
| $b^{0,+}AT$ | SLC7A9 | NM_001199133.1 | GCACCAATTATCACCGCACC CAACTTCCTCTGGTGGCACA | Apical exchange of extracellular cationic amino acids and cysteine for neutral |
| SNAT1 | SLC38A1 | NM_001199603.1 | TTTTCGGCATGGTACGTGGA CGCCGATTCGAGACAGAGAA | Transport of L-glutamine |
| LAT1 | SLC7A5 | KT876067.1 | CCACCTTGGCATCATGTCCT AAAGAACAGCCGGGAAGAGG | Uptake of large neutral amino acids |
| CAT1 | SLC7A1 | NM_001145490 | CCAGACATCTTCGCTGTGGT TTCACGAAGCCAGAGACCAC | Transport of cationic amino acids |

Abbreviations: ACTB, $\beta$-Actin; AQP, aquaporin; CAT, cationic amino acid transporter; GR, glucocorticoid receptor; HSP, heat shock protein; LAT, large neutral amino acid transporter; SNAT, sodium-coupled neutral amino acid transporter.

**Table 5.** Rectal temperature and Heterophils to Lymphocytes ratio of broiler chickens fed various amino acid densities and subjected to thermoneutral or heat stress conditions.

| Temperature | AA density[1] | n[2] | Rectal temperature °C | | H:L[3] |
|---|---|---|---|---|---|
| | | | D 30 | D 36 | |
| Thermoneutral | | | | | |
| | 110% | 12 | 41.5 | 41.4 | 0.26 |
| | 100% | 12 | 41.5 | 41.3 | 0.24 |
| | 90% | 12 | 41.6 | 41.3 | 0.25 |
| | 80% | 12 | 41.5 | 41.4 | 0.24 |
| Heat stress | | | | | |
| | 110% | 12 | 42.1 | 41.7 | 0.30 |
| | 100% | 12 | 42.1 | 41.8 | 0.35 |
| | 90% | 12 | 42.2 | 41.8 | 0.34 |
| | 80% | 12 | 42.1 | 41.8 | 0.31 |
| | SEM | | 0.08 | 0.06 | 0.02 |
| Main effect means | | | | | |
| Thermoneutral | | 48 | 41.5[b] | 41.4[b] | 0.25[b] |
| Heat stress | | 48 | 42.1[a] | 41.8[a] | 0.33[a] |
| SEM | | | 0.04 | 0.03 | 0.01 |
| | 110% | 24 | 41.8 | 41.6 | 0.28 |
| | 100% | 24 | 41.8 | 41.6 | 0.29 |
| | 90% | 24 | 41.9 | 41.5 | 0.30 |
| | 80% | 24 | 41.8 | 41.6 | 0.27 |
| | SEM | | 0.06 | 0.04 | 0.01 |
| Source of variation[4] | | | ——— P-values ——— | | |
| Temperature | | | <0.001 | <0.001 | <0.001 |
| AA density | | | 0.659 | 0.810 | 0.541 |
| Temperature× AA density | | | 0.989 | 0.486 | 0.218 |

[1]Amino acid specifications are based on the 2019 Aviagen Nutrition Guide for Ross broilers.

[2]Number of replicate pens with 1 chicken randomly selected from each pen for measurements.

[3]Heterophils to Lymphocytes ratio at 36 days of age.

[4]Means within a column with no common superscript differ significantly ($P \le 0.05$).

specific primer sequences (listed in Table 4). The qRT-PCR reaction comprises 20 $\mu$L that contained 10 $\mu$L of 2X Power SYBR Green PCR Master Mix, 1 $\mu$L of each primer (10 pmol), 6 $\mu$L of distilled water, and a 2 $\mu$L containing 100 ng of the cDNA template. The total reaction volume was subjected to 95°C for 15 min, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. The primers' specificity was determined through melt-curve analysis using Applied Biosystem 7500 real-time PCR machine and software (Applied Biosystems). Relative quantitative fold change of the target genes expression was determined according to the $2^{-\Delta\Delta Ct}$ method developed by Livak and Schmittgen (2001); where $\Delta\Delta Ct$ is to normalize the fold-change of the treatment group to the corresponding control group and to normalize the expression of the target gene to that of the ACTB for each sample.

## Statistical Analysis

Data analysis was conducted using the GLIMMIX procedure of SAS software 9.2 (SAS Institute, 2010). For the 23-day performance data, a one-way ANOVA was used for hypothesis testing, whereas for the rest of the data, a 2-way ANOVA was utilized to test the effects of AA density, microclimate environment, and any possible interactions. Differences were considered significant when $P \le 0.05$. When found significant, means were separated using Tukey's Studentized Range (HSD) Test for performance data and Scheffé Test for gene expression data. Correlation analysis was conducted between the relative expressions of the examined mRNA transcripts using the CORR procedure of SAS software. Only significant correlations (less than 0.05) were considered and discussed.

## RESULTS

### Chicken Rectal Temperature and H: L Ratio

The results in Table 5 revealed that there was no significant interaction between room temperature and AA density on chicken rectal temperature and H: L ratio. In addition, rectal temperature and H: L ratio was not influenced by the AA density of the diet. However, the main effect of room temperature was found to be significant as the rectal temperatures of the birds housed in the HS room were higher at 30 (42.1 ± 0.04 vs. 41.5 ± 0.04; $P < 0.001$) and 36 (41.8 ± 0.03 vs. 41.4 ± 0.03; $P < 0.001$) days of age than those housed in the TN room. Furthermore, HS broilers were found to have a higher ($P < 0.001$) H: L ratio than their counterparts housed in the TN room.

**Table 6.** Effect of various amino acid densities on live performance of broiler chickens from placement to 23 days of age.

| Variable | AA Density[1,2,3] | | | | SEM | ANOVA | L[4] | Q[4] |
|---|---|---|---|---|---|---|---|---|
| | 110% | 100% | 90% | 80% | | | | |
| 0 to 10 days | | | | | | | | |
| FI (g /chick) | 269.7 | 276.8 | 280.3 | 275.1 | 3.17 | 0.122 | 0.163 | 0.052 |
| BWG (g /chick) | 233.3[a] | 232.7[a] | 227.4[a] | 210.2[b] | 3.66 | <0.001 | <0.001 | 0.023 |
| FCR (g /g) | 1.16[c] | 1.19[bc] | 1.23[b] | 1.30[a] | 0.01 | <0.001 | <0.001 | 0.210 |
| 10 to 23 days | | | | | | | | |
| FI (g /chick) | 1117.1[ab] | 1116.6[ab] | 1137.1[a] | 1081.4[b] | 12.78 | 0.022 | 0.124 | 0.035 |
| BWG (g /chick) | 897.5[a] | 868.3[a] | 827.6[b] | 732.5[c] | 9.98 | <0.001 | <0.001 | 0.001 |
| FCR (g /g) | 1.23[d] | 1.28[c] | 1.37[b] | 1.48[a] | 0.01 | <0.001 | <0.001 | 0.005 |
| 0 to 23 days | | | | | | | | |
| FI (g /chick) | 1386.7[ab] | 1393.3[ab] | 1418.4[a] | 1358.7[b] | 14.48 | 0.041 | 0.368 | 0.026 |
| BWG (g /chick) | 1130.7[a] | 1100.8[a] | 1054.9[b] | 942.6[c] | 11.97 | <0.001 | <0.001 | 0.001 |
| FCR (g /g) | 1.23[d] | 1.27[c] | 1.34[b] | 1.44[a] | 0.01 | <0.001 | <0.001 | 0.005 |

[1]Amino acid specifications are based on the 2019 Aviagen Nutrition Specifications guide for Ross broilers.

[2]Values are means of 24 replicate pens per treatment.

[3]Means within a row with no common superscript differ significantly ($P \le 0.05$).

[4]$P$-values for Linear and quadratic regression responses.

**Table 7.** Effect of amino acid density and ambient temperature on live performance of broiler chickens from 24 to 35 days of age.

| Temperature | AA density[1] | n[2] | FI (g) | BWG (g) | FCR (g/g) |
|---|---|---|---|---|---|
| Thermoneutral | 110% | 12 | 1,809.9 | 1,123.3 | 1.61 |
| | 100% | 12 | 1,871.2 | 1,074.1 | 1.72 |
| | 90% | 12 | 1,835.9 | 1,082.4 | 1.73 |
| | 80% | 12 | 1,809.6 | 940.5 | 1.99 |
| Heat stress | 110% | 12 | 1,659.4 | 991.2 | 1.69 |
| | 100% | 12 | 1,726.4 | 967.1 | 1.77 |
| | 90% | 12 | 1,654.2 | 951.5 | 1.76 |
| | 80% | 12 | 1,576.0 | 850.9 | 1.88 |
| | SEM | | 24.0 | 23.6 | 0.04 |
| Main effect means | | | | | |
| Thermoneutral | | 48 | 1,831.7[a] | 1,055.1[a] | 1.76 |
| Heat stress | | 48 | 1,654.0[b] | 940.1[b] | 1.78 |
| SEM | | | 12.0 | 11.90 | 0.02 |
| | 110% | 24 | 1,734.6[b] | 1,057.2[a] | 1.65[b] |
| | 100% | 24 | 1,798.8[a] | 1,020.6[a] | 1.75[b] |
| | 90% | 24 | 1,745.1[ab] | 1,016.9[a] | 1.74[b] |
| | 80% | 24 | 1,692.8[b] | 895.6[b] | 1.93[a] |
| | SEM | | 17.0 | 16.7 | 0.03 |
| Source of variation[3] | | | | | |
| Temperature | | | <0.001 | <0.001 | 0.655 |
| AA density | | | <0.001 | <0.001 | <0.001 |
| Temperature × AA density | | | 0.242 | 0.776 | 0.099 |

[1]Amino acid specifications are based on the 2019 Aviagen Nutrition Guide for Ross broilers.

[2]Number of replicate pens.

[3]Means within a column with no common superscript differ significantly ($P \leq 0.05$).

## Live Performance

At the end of the starter phase, chicks that were fed the very low AA dense diet (−20% AAs) gained less weight (~10% less, $P < 0.001$) and had inferior FCR (11points more, $P < 0.001$) compared to the control 100% group (Table 6). Similarly, chicks fed very low AAs or low AAs (−10% AAs) gained less weight ($P < 0.001$), and had higher FCR ($P < 0.001$) than the control group at the end of the grower phase. In comparison, the high-density diet (+10% AAs) produced chicks with improved FCR (5 points less) compared to the control diet. A quadratic regression trend ($P = 0.023$) was observed in BWG response to AA density at d= 10, characterized by a plateau followed by a reduction in BWG as AA density decrease. On the same day, FCR linearly responded to AA density, increasing as the AA density decrease. Whereas at d 23, FI, BWG, and FCR all responded in a quadratic manner ($P < 0.05$) to AA density but varied in the response shape.

There was no interaction between room temperature and AA density on any of the live performance parameters during the finisher phase (Table 7). However, the main effects of temperature and AA density were significant on live performance. Heat-stressed chickens consumed ($P < 0.001$) about 10% less feed and had about 11% less ($P < 0.001$) gain than their counterparts housed in the TN room. Feeding either high or very low AA densities resulted in reduced ($P < 0.001$) FI by about 4 and 6%, respectively, compared to the control. In addition, feeding very low AA density reduced BWG by about 12% and increased FCR by 18 points compared to feeding the control diet that meets 100% of the AA specifications.

## Processing Performance

The absolute and relative weight of *P. major* at 23 days of age was significantly reduced when a very low AA density was fed (Table 8). Compared with the control group, chicks fed the very low AA density had reduced ($P < 0.001$) absolute *P. minor* weight. The relative and absolute weights of both *P. major* and *minor* muscles responded linearly ($P < 0.05$) to AA density, decreasing as the AA density decreased.

No significant interaction between room temperature and AA density was observed on the breast muscles at 36 days of age (Table 9). Additionally, the main effects of room temperature on breast muscles were not significant. In contrast, the main effects of AA density were found to be significant as the birds fed the very low AA diet were characterized by smaller *P. major* and *minor* muscles.

## Correlation Analysis of Gene Expression

The correlation analysis results in Table 10 revealed positive correlations between the mRNA expression levels of the AA transporter genes in the examined broiler tissues at 36 days of age. CAT1 and $b^{0,+}$AT expression levels in *P. major* muscle were positively correlated ($r = 0.85$, $P = 0.008$). Similarly, the expression levels of LAT1 and SNAT1 in *P. major* were positively correlated ($r = 0.86$, $P = 0.006$).

The expression level of AQP1 in *P. major* was found to be positively correlated with the expression levels of CAT1($r = 0.90$, $P = 0.002$) and $b^{0,+}$AT ($r = 0.83$,

**Table 8.** Effect of various amino acid densities on Pectoralis (P.) muscles of broiler chickens at 23 days of age.

| Variable | AA density[1,2,3] | | | | SEM | ANOVA | L[4] | Q[4] |
|---|---|---|---|---|---|---|---|---|
| | 110% | 100% | 90% | 80% | | | | |
| *P. major* (g) | 190.92[a] | 178.64[ab] | 167.30[b] | 143.38[c] | 5.92 | <0.001 | <0.001 | 0.327 |
| *P. minor* (g) | 41.82[a] | 38.35[ab] | 35.93[cb] | 32.26[c] | 1.16 | <0.001 | <0.001 | 0.930 |
| *P. major* (%)[5] | 15.94[a] | 15.29[ab] | 14.66[cb] | 14.08[c] | 0.26 | <0.001 | <0.001 | 0,881 |
| *P. minor* (%)[5] | 3.42[a] | 3.29[ab] | 3.16[b] | 3.18[b] | 0.06 | 0.014 | 0.003 | 0.256 |

[1]Amino acid specifications are based on 2019 Aviagen Nutrition Guide for Ross broilers.

[2]Values are means of 24 replicate pens per treatment with one chicken randomly selected from each pen for processing.

[3]Means within a row with no common superscript differ significantly ($P \leq 0.05$).

[4]P-values for Linear and quadratic regression responses.

[5]Calculated as a percentage of live body weight.

**Table 9.** Effect of amino acid density and ambient temperature on Pectoralis (P.) muscles of broiler chickens at 36 days of age.

| Temperature | AA density[1] | n[2] | P. major (g) | P. minor (g) | P. major[3] (%) | P. minor[3] (%) |
|---|---|---|---|---|---|---|
| Thermoneutral | 110% | 12 | 423.47 | 90.44 | 18.10 | 3.87 |
| | 100% | 12 | 413.56 | 88.03 | 18.21 | 3.83 |
| | 90% | 12 | 400.13 | 84.78 | 17.69 | 3.75 |
| | 80% | 12 | 347.88 | 72.69 | 16.69 | 3.49 |
| Heat stress | 110% | 12 | 422.68 | 90.58 | 18.25 | 3.91 |
| | 100% | 12 | 424.22 | 91.06 | 18.16 | 3.90 |
| | 90% | 12 | 407.03 | 85.34 | 17.91 | 3.76 |
| | 80% | 12 | 312.33 | 66.81 | 15.64 | 3.35 |
| | SEM | | 12.21 | 2.25 | 0.28 | 0.07 |
| Main effect means | | | | | | |
| Thermoneutral | | 48 | 391.56 | 83.98 | 17.67 | 3.73 |
| Heat stress | | 48 | 396.26 | 83.45 | 17.49 | 3.73 |
| SEM | | | 6.10 | 1.13 | 0.14 | 0.03 |
| | 110% | 24 | 423.07[a] | 90.51[a] | 18.18[a] | 3.89[a] |
| | 100% | 24 | 418.89[a] | 89.54[a] | 18.19[a] | 3.86[a] |
| | 90% | 24 | 403.58[a] | 85.06[a] | 17.80[a] | 3.76[a] |
| | 80% | 24 | 330.10[b] | 69.75[b] | 16.16[b] | 3.41[b] |
| | SEM | | 8.63 | 1.59 | 0.20 | 0.05 |
| Source of variation[4] | | | | | | |
| Temperature | | | 0.588 | 0.738 | 0.374 | 0.943 |
| AA density | | | <0.001 | <0.001 | <0.001 | <0.001 |
| Temperature × AA density | | | 0.221 | 0.245 | 0.098 | 0.422 |

[1]Amino acid specifications are based on 2019 Aviagen Nutrition Guide for Ross broilers.
[2]Number of replicate pens with two chickens randomly selected from each pen for processing.
[3]Calculated as a percentage of live body weight.
[4]Means within a column with no common superscript differ significantly ($P \leq 0.05$).

**Table 10.** Pearson correlation between amino acid transporters, aquaporins, and stress-related transcripts in the intestinal jejunum and pectoralis major of broiler chickens at 36 days of age.

| | Variable 1 | Variable 2 | Coefficient | P-value |
|---|---|---|---|---|
| 1 | CAT1 (P. major) | b$^{0,+}$AT (P. major) | 0.85 | 0.008 |
| 2 | HSP90 (P. major) | b$^{0,+}$AT (P. major) | −0.71 | 0.047 |
| 3 | LAT1 (P. major) | SNAT1 (P. major) | 0.86 | 0.006 |
| 4 | CAT1 (P. major) | AQP1 (P. major) | 0.90 | 0.002 |
| 5 | b$^{0,+}$AT (P. major) | AQP1 (P. major) | 0.83 | 0.011 |
| 6 | HSP70 (P. major) | AQP1 (P. major) | 0.74 | 0.035 |
| 7 | HSP70 (P. major) | AQP9 (P. major) | −0.73 | 0.038 |
| 8 | b$^{0,+}$AT (P. major) | AQP9 (P. major) | −0.85 | 0.007 |
| 9 | CAT1 (P. major) | AQP9 (P. major) | −0.77 | 0.026 |
| 10 | HSP70 (P. major) | HSP70 (jejunum) | 0.80 | 0.017 |
| 11 | AQP1 (P. major) | AQP9 (P. major) | −0.88 | 0.004 |
| 12 | GR (jejunum) | SNAT1 (jejunum) | 0.90 | 0.002 |
| 13 | AQP3 (jejunum) | SNAT1 (jejunum) | 0.78 | 0.023 |
| 14 | HSP70 (jejunum) | CAT1 (jejunum) | 0.75 | 0.032 |
| 15 | HSP70 (jejunum) | SNAT1 (jejunum) | 0.71 | 0.050 |
| 16 | HSP70 (jejunum) | GR (jejunum) | 0.90 | 0.003 |
| 17 | HSP70 (jejunum) | AQP3 (jejunum) | 0.72 | 0.042 |

Abbreviations: AQP, aquaporin; CAT, cationic amino acid transporter; GR, glucocorticoid receptor; HSP, heat shock protein; LAT, large neutral amino acid transporter; SNAT, sodium-coupled neutral amino acid transporter.

$P = 0.011$) in the same muscle. In the jejunum, the expression levels of AQP3 and SNAT1 were positively correlated ($r = 0.78$, $P = 0.023$). In contrast, the AQP9 expression level in P. major was negatively correlated with CAT1 ($r = −0.77$, $P = 0.026$) and b$^{0,+}$AT ($r = −0.85$, $P = 0.007$) expression levels in the same tissue. In addition, the expression of AQP1 and APQ9 in the P. major was found to be negatively correlated ($r = −0.88$, $P = 0.004$).

In the jejunum, positive associations were observed between HSP70 expression level and each of CAT1 ($r = 0.75$, $P = 0.032$), SNAT1($r = 0.71$, $P = 0.050$), GR ($r = 0.90$, $P = 0.003$), and APQ3 ($r = 0.72$, $P = 0.042$). In the P. major, the HSP70 expression was positively correlated with AQP1 ($r = 0.74$, $P = 0.035$) and negatively correlated with AQP9 ($r = −0.73$, $P = 0.038$). Moreover, the expressions of HSP70 in the jejunum and P. major were correlated positively ($r = 0.80$, $P = 0.017$). HSP90 expression in P. major correlated negatively with b$^{0,+}$AT ($r = −0.71$, $P = 0.047$) in the same tissue. Another significant correlation was observed between GR and SNAT1 in the jejunum ($r = 0.90$, $P = 0.002$).

## Gene Expression in the Intestinal Jejunum

The influence of AA density on the expression levels of the selected genes in the intestinal jejunum was dependent on the environmental temperature as the two factors

**Table 11.** Interactive effects between environmental temperature and amino acid density on the expression of selected genes in the intestinal jejunum of broiler chickens at 36 days of age.

| Temperature | AA density[1] | n[2] | CAT1 | B$^0$AT | b$^{0,+}$AT | SNAT1 | LAT1 | HSP90 | HSP70 | GR | AQP3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Thermoneutral | 110% | 6 | 0.90$^d$ | 0.95$^g$ | 1.14$^b$ | 1.16$^c$ | 1.16$^e$ | 0.94$^{cd}$ | 1.14$^e$ | 1.16$^d$ | 1.17$^e$ |
| | 100% | 6 | 1.00$^d$ | 1.00$^g$ | 1.00$^c$ | 1.00$^e$ | 1.00$^{ef}$ | 1.00$^{bc}$ | 1.00$^f$ | 1.00$^e$ | 1.00$^f$ |
| | 90% | 6 | 3.69$^b$ | 11.15$^d$ | 1.31$^a$ | 1.06$^d$ | 3.72$^a$ | 0.86$^d$ | 1.36$^b$ | 1.32$^c$ | 1.34$^c$ |
| | 80% | 6 | 4.01$^a$ | 12.18$^c$ | 1.17$^b$ | 1.61$^a$ | 1.16$^e$ | 1.14$^a$ | 1.53$^a$ | 1.62$^a$ | 1.64$^a$ |
| Heat stress | 110% | 6 | 0.69$^e$ | 2.71$^f$ | 1.25$^a$ | 1.16$^c$ | 0.86$^f$ | 0.96$^{bcd}$ | 1.25$^{cd}$ | 1.25$^c$ | 1.26$^d$ |
| | 100% | 6 | 2.25$^c$ | 18.11$^a$ | 1.26$^a$ | 1.06$^d$ | 2.45$^b$ | 1.15$^a$ | 1.27$^{bc}$ | 1.25$^c$ | 1.26$^d$ |
| | 90% | 6 | 0.51$^f$ | 6.62$^e$ | 1.27$^a$ | 1.45$^b$ | 1.80$^d$ | 1.06$^{ab}$ | 1.29$^{bc}$ | 1.52$^b$ | 1.44$^b$ |
| | 80% | 6 | 0.98$^d$ | 14.40$^b$ | 1.17$^b$ | 1.14$^c$ | 2.06$^c$ | 0.94$^{cd}$ | 1.17$^{de}$ | 1.25$^c$ | 1.16$^e$ |
| | SEM | | 0.02 | 0.08 | 0.01 | 0.01 | 0.03 | 0.02 | 0.02 | 0.01 | 0.01 |
| Main effect means | | | | | | | | | | | |
| Thermoneutral | | 24 | 2.40 | 6.32 | 1.16 | 1.21 | 1.76 | 0.98 | 1.26 | 1.27 | 1.29 |
| Heat stress | | 24 | 1.11 | 10.46 | 1.24 | 1.20 | 1.79 | 1.03 | 1.24 | 1.32 | 1.28 |
| SEM | | | 0.01 | 0.04 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| | 110% | 12 | 0.79 | 1.83 | 1.20 | 1.16 | 1.01 | 0.95 | 1.19 | 1.21 | 1.21 |
| | 100% | 12 | 1.63 | 9.55 | 1.13 | 1.03 | 1.73 | 1.08 | 1.14 | 1.23 | 1.13 |
| | 90% | 12 | 2.10 | 8.88 | 1.29 | 1.25 | 2.76 | 0.96 | 1.32 | 1.42 | 1.39 |
| | 80% | 12 | 2.50 | 13.29 | 1.17 | 1.37 | 1.61 | 1.04 | 1.35 | 1.44 | 1.40 |
| | SEM | | 0.02 | 0.06 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 |
| Source of variation[3] | | | | | | | | | | | |
| Temperature | | | <0.001 | <0.001 | <0.001 | 0.482 | 0.115 | 0.004 | 0.336 | <0.001 | 0.271 |
| AA density | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Temperature × AA density | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[1]Amino acid specifications are based on 2019 Aviagen Nutrition Guide for Ross broilers.
[2]Number of sampled chickens per treatment group.
[3]Means within a column with no common superscript differ significantly ($P \leq 0.05$).

interacted significantly (Table 11). For CAT1 and B$^0$AT genes in TN kept birds, lowering the AA density of the diet by 10% increased the expression levels of the genes significantly by 2.69 and 10.15 folds, respectively, compared to the 100% control, and the expression further increased at 20% reduction of the AA density. Increasing the AA density by 10% did not significantly change the expression levels of the two genes. For HS birds, reducing the AA density by 10% resulted in a significant reduction in the expression levels of CAT1 and B$^0$AT by 1.74 and 11.49 folds, respectively. Similarly, reducing the AA density by 20% decreases the expression levels of the two genes by 1.27 and 3.71 folds, respectively. Surprisingly, both genes' expression levels were reduced when the HS birds were fed 10% higher AA density. Heat stress upregulated the expression of CAT1 in the jejunum of the birds fed 100% compared to nonstressed birds, but downregulated its expression when the birds were fed with either lower or higher AA density.

In contrast, the expression of B$^0$AT was upregulated by HS at 110, 100, 80% AA density and was downregulated at 90% density. Feeding diets lower or higher in AAs than the 100% control increased the expression levels of b$^{0,+}$AT slightly but significantly in the jejunum of TN birds. When the birds were exposed to HS and fed either 100 or 110% AAs, the gene expression levels were increased ($P < 0.05$) slightly compared to feeding the same densities for TN birds. The expression of SNAT1 in TN birds was upregulated slightly, but significantly when feeding diets either lower or higher in AA density. Similarly, in the HS birds, the expression of SNAT1 was upregulated slightly when feeding either diet compared to the 100% control. Heat stress upregulated the expression of SNAT1 when the deficiency in AAs was 10% (1.06 vs. 1.45) but downregulated its expression at 20% deficiency

(1.61 vs. 1.14). For the LAT1 gene, lowering the AA density by 10% increased its expression level in the TN birds (1.00 vs. 3.72). In contrast, lowering the AA density by either 10 or 20% reduced the LAT1 expression in HS birds, and so did increasing the AA density by 10%. Comparison of feeding the same AA density in the two environmental conditions revealed that HS upregulated the expression of LAT1 at 100 and 80% but downregulated its expression at 110 and 90% AA density.

For TN birds, HSP90 responded differently to AA density as its expression was slightly reduced at 90% but slightly increased at 80% AA density. For HS birds, the expression of HSP90 was slightly reduced at both 80 and 110%. Heat stress increased HSP90 expression at 100 and 90 but reduced its expression at 80% density. Feeding lower AA densities upregulated the expression of HSP70, GR, and AQP3 in TN birds in a similar manner. In HS birds, a lower expression level of HSP70 was associated with feeding 80% AA density, whereas a higher expression level of GR was associated with a 90% density. Besides, the AQP3 expression level was found to be increased at 90% and decreased at 80% density. In general, HS upregulated the expression of HSP70, GR, and AQP3 at 110, 100, and 90% and downregulated the expression of the genes at 80% AA density.

## Gene Expression in the P. Major Muscle

There was a 2-way interaction ($P < 0.001$) between AA density and temperature on the expression of the selected genes in the *P. major* (Table 12). The expression of CAT1 in the *P. major* of the TN birds was greatly upregulated ($P < 0.05$) when reducing the AA density of the diets to 90% (9.19 vs. 1.00) and 80%

**Table 12.** Interactive effects between environmental temperature and amino acid density on the expression of selected genes in the pectoralis major muscle of broiler chickens at 36 days of age.

| Temperature | AA density[1] | n[2] | CAT1 | B⁰AT | b⁰,⁺AT | SNAT1 | LAT1 | HSP70 | HSP90 | GR | AQP1 | AQP9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Thermoneutral | 110% | 6 | 2.08$^c$ | 0.89$^g$ | 1.26$^b$ | 1.13$^{fg}$ | 1.06$^f$ | 1.44$^c$ | 1.41$^d$ | 1.38$^e$ | 0.98$^c$ | 0.79$^d$ |
| | 100% | 6 | 1.00$^f$ | 1.00$^g$ | 1.00$^c$ | 1.00$^g$ | 1.00$^f$ | 1.00$^e$ | 1.00$^f$ | 1.00$^f$ | 1.00$^c$ | 1.00$^b$ |
| | 90% | 6 | 9.19$^b$ | 1.74$^d$ | 1.47$^a$ | 8.10$^a$ | 2.44$^a$ | 1.66$^b$ | 0.97$^f$ | 1.32$^e$ | 1.73$^a$ | 0.47$^e$ |
| | 80% | 6 | 9.64$^a$ | 2.15$^b$ | 1.55$^a$ | 1.27$^f$ | 1.16$^e$ | 2.18$^a$ | 0.80$^g$ | 2.53$^a$ | 1.82$^a$ | 0.47$^e$ |
| Heat stress | 110% | 6 | 1.82$^d$ | 1.27$^f$ | 0.72$^e$ | 5.94$^b$ | 1.67$^c$ | 1.65$^b$ | 1.78$^a$ | 2.04$^c$ | 0.97$^c$ | 1.00$^b$ |
| | 100% | 6 | 1.72$^{de}$ | 3.58$^a$ | 0.86$^d$ | 1.98$^e$ | 0.38$^g$ | 1.17$^d$ | 1.23$^e$ | 2.24$^b$ | 0.87$^d$ | 1.36$^a$ |
| | 90% | 6 | 0.82$^f$ | 1.47$^e$ | 0.99$^c$ | 2.16$^d$ | 1.36$^d$ | 1.66$^b$ | 1.54$^c$ | 1.62$^d$ | 1.06$^c$ | 0.86$^c$ |
| | 80% | 6 | 1.51$^e$ | 1.90$^c$ | 1.03$^c$ | 5.57$^c$ | 2.04$^b$ | 1.72$^b$ | 1.67$^b$ | 1.35$^e$ | 1.37$^b$ | 0.77$^d$ |
| | SEM | | 0.04 | 0.03 | 0.02 | 0.03 | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.01 |
| Main effect means | | | | | | | | | | | | |
| Thermoneutral | | 24 | 5.48 | 1.44 | 1.32 | 2.87 | 1.41 | 1.57 | 1.04 | 1.56 | 1.38 | 0.68 |
| Heat stress | | 24 | 1.47 | 2.06 | 0.90 | 3.91 | 1.36 | 1.55 | 1.55 | 1.81 | 1.08 | 1.00 |
| SEM | | | 0.02 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 |
| | 110% | 12 | 1.95 | 1.08 | 0.99 | 3.43 | 1.36 | 1.55 | 1.60 | 1.71 | 0.98 | 0.90 |
| | 100% | 12 | 1.36 | 2.29 | 0.93 | 1.49 | 0.69 | 1.09 | 1.12 | 1.62 | 0.93 | 1.18 |
| | 90% | 12 | 5.00 | 1.60 | 1.23 | 5.14 | 1.90 | 1.66 | 1.25 | 1.47 | 1.39 | 0.66 |
| | 80% | 12 | 5.58 | 2.03 | 1.29 | 3.42 | 1.60 | 1.95 | 1.23 | 1.94 | 1.60 | 0.62 |
| | SEM | | 0.03 | 0.02 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 |
| Source of variation[3] | | | | | | | | | | | | |
| Temperature | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.047 | <0.001 | <0.001 | <0.001 | <0.001 |
| AA density | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Temperature × AA density | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

[1]Amino acid specifications are based on 2019 Aviagen Nutrition Guide for Ross broilers.
[2]Number of sampled chickens per treatment group.
[3]Means within a column with no common superscript differ significantly ($P \leq 0.05$).

(9.64 vs. 1.00) compared to the 100% control. Also, the elevation of the AA density by 10% upregulated the expression of the CAT1 but to a lesser extent (2.08 vs. 1.00). For the HS birds, the expression of the CAT1 was significantly downregulated at an AA density of 90% (0.82 vs. 1.72). Heat stress downregulated the CAT1 expression at 80% (1.51 vs. 9.64), 90% (0.82 vs. 9.19), and 110% (1.51 vs. 2.08) density. In contrast, the CAT1 was upregulated by HS at 100% density (1.72 vs. 1.00).

Similarly, the expression levels of B0AT and b⁰,⁺AT were increased when the birds were fed 90 and 80% AA density compared to the 100% control. However, the response of the two genes to AA density was variable in the HS birds as the B⁰AT expression was reduced at lower AA densities, whereas the b⁰,⁺AT expression was slightly increased. Heat stress upregulated the B⁰AT expression at 100% (3.58 vs. 1.00) and 110% (1.27 vs. 0.89) while downregulated its expression at 90% (1.47 vs. 1.74) and 80% (1.90 vs. 2.15) density. In contrast, HS downregulated the expression of b⁰,⁺AT at all the levels of AA densities examined, 110% (0.72 vs. 1.26), 100% (0.86 vs. 1.00), 90% (0.99 vs. 1.47), and 80% (1.03 vs. 1.55).

SNAT1 and LAT1 shared similarities in their expression in TN birds as the two genes were upregulated at 90 and 80%, and their expressions did not change at 110% density. In HS birds, both genes behaved similarly as their expression was upregulated at 110, 90, and 80% compared to the 100% control. Heat stress upregulated the SNAT1 expression at 110% (5.94 vs. 1.13), 100% (1.98 vs. 100), and 80% (5.57 vs. 1.27) but downregulated its expression at 90% (2.16 vs. 8.10) AA density. The LAT1 was upregulated by HS at 110% and 80% but downregulated by HS at 100% (0.38 vs. 1.00) and 90% (1.36 vs. 2.44) AA density.

HSP70 expression level increased in both stressed and nonstressed birds at any AA density compared to the 100% control. The expression of HSP70 was upregulated by HS at 110% (1.65 vs. 1.44), 100% (1.17 vs. 1.00), and was downregulated at 80% (1.72 vs. 2.18) AA density. The expression level of HSP90 decreased at 80% (0.80 vs. 1.00) but increased at 110% (1.41 vs. 1.00) AA density in TN birds. In HS birds, the HSP90 expression increased at any AA density. Heat stress upregulated the expression of the HSP90 at any AA density, 110% (1.78 vs. 1.41), 100% (1.23 vs.1.00), 90% (1.54 vs. 0.97), and 80% (1.67 vs. 0.80). The expression levels of the GR increased at any AA density in TN birds but decreased at any AA density in HS birds compared to the control. Heat stress upregulated the expression of the GR at 110% (2.04 vs. 1.38), 100% (2.24 vs. 1.00), and 90% (1.62 vs. 1.32) and downregulated its expression at 80% (1.35 vs. 2.53) AA density.

AQP1 and AQP2 expressions behaved differently in response to the various AA densities. In both stressed and nonstressed birds reducing the AA density increased the expression levels of AQP1 but reduced the expression levels of AQP9. Heat stress downregulated the expression of AQP1 at 100% (0.87 vs. 1.00), 90% (1.06 vs. 1.73), and 80% (1.37 vs. 1.82) density. In contrast, the expression of the AQP9 was upregulated by HS at all AA densities, 110% (1.00 vs. 0.79), 100% (1.36 vs. 1.00), 90% (0.86 vs. 0.47), and 80% (0.77 vs. 0.47) density.

## DISCUSSION

The response of fast-growing meat-type chickens to different dietary AA density regimens has been well

documented, but minimal research has examined the response of broilers subjected to cyclic HS to increasing AA density. Therefore, the first objective of this study was to examine whether environmental temperature and AA density interact on broiler performance and processing yield during the finishing period (24−36 d). To achieve this, broilers were exposed to 8 h of elevated temperature (32°C ± 0.7), which was sufficient to induce HS as evidenced by the visible heavy panting, increased rectal temperature, and high H: L ratio. H: L ratio is a reliable indicator of environmental stress, and high values reflect stressful conditions (Gross and Siegel, 1983). In addition, the HS birds consumed ~ 10% less feed and gained ~ 11% less weight during the finishing period, which was in good agreement with previous reports that confirmed HS as a detrimental factor to the growth performance of broilers (Gonzalez-Esquerra and Leeson, 2005; Quinteiro-Filho et al., 2010, Zhang et al., 2012).

Our results revealed no significant interaction between environmental temperature and dietary AA density on any of the broiler performance and processing parameters during the finishing period, suggesting that HS does not change the shape of bird response to AA density. In other words, neither feeding higher AA density than the recommended level depresses the growth performance, nor feeding lower levels improves the performance under cyclic HS. Indeed, the growth performance of the HS chickens was reduced linearly as the AA density of the diet was lowered. Our results are in agreement with those of Temim et al. (2000) and Soares et al. (2020), who found that lowering the CP content of the diet depressed the growth performance for broilers reared under constant and cyclic HS (32°C), respectively. In contrast, Cheng et al. (1997) reported that feeding broiler chickens higher protein diets than the recommended level depressed the growth performance during 3 to 7 wk of age when housed under constant HS (26.7−35°C).

The discrepancy in broiler responses to dietary protein (AAs) reported in the literature is most likely due to HS severity (e.g., 35 vs. 27°C), exposure duration (e.g., 8 vs. 24 h per day), timing of stress (third week vs. sixth week), dietary AA imbalance (Gonzalez-Esquerra and Leeson, 2005, 2006; Awad et al., 2019). Feeding a high protein diet with an unbalanced AA profile can lead to a substantial increase in heat increment (Naga Raja Kumari and Narendra Nath, 2018). However, current feed formulation practices (as applied herein) rely on synthetic AA fortifications, use the digestible AA coefficients, apply the ideal protein concept when adjusting the AA density, and remove the minimum CP constraint during formulation. Following these practices should theoretically minimize dietary-induced thermogenesis associated with AA breakdown since the unbalanced AA portion is minimal. In the current study, HS birds receiving the highest AA dense diet gained more than those receiving the lowest AA dense diet (991.2 g vs. 850.9 g) and were more feed efficient (1.69 vs. 1.88). Similarly, the HS birds had more breast muscle yield

(22.16% vs. 18.99%) when fed the highest AA dense diet compared to feeding the lowest AA dense diet. These observations suggest increasing the AA density of the diet to mitigate the reduced feed intake under cyclic HS.

As expected, feeding very low AA density (80% of the recommendations) depressed live performance and processing yield severely during 0 to 23 and 24 to 35 d for all birds in the present study, similar to the results reported by Lilly et al. (2011) and Pekel et al. (2020) who reported reduced performance and processing yield of broilers fed very low AA density. Broilers were more sensitive to AA density reduction during 0 to 23 d only in the present study as reducing the AA density by 10% depressed growth performance. This observation highlights the importance of feeding the appropriate AA density during the first three weeks of life (Kidd et al., 2004; Zhai et al., 2013). Conversely, feeding higher AA density than the manual brought about no clear benefits on the growth performance and processing yield, and this is maybe due to the higher AA levels being above the bird's requirements.

The second and major objective of our study was to assess any possible interactions between environmental temperature and AA density on the mRNA expression of selected genes encoding AA transporters, water channels, and stress-related transcripts in broiler chickens. AA transporters are vital to protein synthesis because they regulate the flow of AAs across plasma membranes into cells (Palacín et al., 1998). The $Na^+$ independent cationic AA transporter 1 (CAT1) transports cationic AAs (e.g., lysine, arginine, and histidine) across cellular membranes (Humphrey et al., 2008). The $Na^+$ dependent neutral AA transporter ($B^0AT$) mediates the transport of neutral AAs such as isoleucine, leucine, valine, and methionine (Jando et al., 2017). The $b^{0,+}$-type amino acid transporter 1 ($b^{0,+}$ **AT**) facilitates apical uptake of AAs such as lysine, arginine, and cysteine (Wang et al., 2009). The $Na^+$ coupled neutral AA transporter 1 (SNAT1) is involved in transporting small neutral AAs, mainly glutamine (Mackenzie et al., 2003). The large neutral AA transporter 1 (LAT1) transports large neutral AAs (e.g., tryptophan, phenylalanine, leucine, and histidine) across membranes (Prasad et al., 1999).

Interestingly, our results revealed that the expressions of the examined genes were not influenced by AA density only but also by environmental temperature as both factors were dependent on each other. Feeding suboptimal AA levels under TN conditions upregulated the expression of all AA transporter genes CAT1, $B^0AT$, $b^{0,+}AT$, SNAT1, and LAT1 in the jejunum as well as in the *P. major*. Previous studies have indicated altered expression of specific AA transporter encoding genes in broiler chickens fed either low protein diets or an essential AA-deficient diet. For instance, Corzo et al. (2011) found that feeding a low protein diet to chickens to upregulate the expression of the AA transporters $y^+LAT2$ and EAAT3. Barekatain et al. (2019) reported an increase in the expression of the AA transporter $y^+LAT1$ in the jejunum of chickens fed a low protein

diet. Additionally, Fagundes et al. (2020) showed that feeding a methionine deficient diet to chickens to upregulate $b^{0,+}$AT and LAT4 expressions in the ileum and kidney, and SNAT1, SNAT2, and CAT1 in the *P. major*. The upregulation of the AA transporter genes in the TN birds in the present study may be indicative of an adaptive mechanism to meet the needs of the ongoing protein synthesis that supports the maximum genetic potential for growth. In general, living bodies are able to adapt their digestive physiology to increase the provision of free AAs to meet the cellular needs through increasing the expression of AA transporters (Ganapathy et al. 2006; Dato et al. 2019; Morales et al. 2020). AA transporters are believed to act as sensors through sensing-signaling pathways to sense extracellular or intracellular AA concentrations (Taylor, 2014). In case of AA starvation, AA transporters may activate the general control non-derepressible pathways, leading to upregulation of AA transporter expression to increase AA supply for sustained growth (Taylor, 2014).

Our results demonstrated that HS strikingly upregulated CAT1, $B^0$AT, and LAT1 expressions in the jejunum and CAT1, $B^0$AT, and SNAT1 expressions in the *P. major* of broilers fed diets containing sufficient AAs (100%). On the other hand, HS markedly downregulated LAT1 expressions in the *P. major* when feeding the 100% AA diet. Our results partially agree with the recent findings of Habashy et al. (2017), who found HS to upregulate SNAT1 in the *P. major* of broiler chickens after 12-d of constant heat exposure at 35° C. However, these researchers reported downregulated CAT1, $B^0$AT, and LAT1 in the ilium and $B^0$AT in the *P. major* of broilers, which contradict our results. Several differences exist between the 2 studies, which could partially explain the different responses, including applying a high (35°C) constant temperature and starting HS challenge early at 14 d in the former study. Whereas in the present study, the birds were exposed to only 8 h of cyclic HS at 32° C and were started HS at 24 d. In the present study, the growth data revealed that chicks that were fed the 100% diet and subjected to HS consumed ~145 g (~8%) less feed but maintained almost similar breast weight (*P. major* 18.16% vs. 18.21%; *P. minor* 3.90% vs. 3.83%) compared to their counterparts in the TN environment. This observation suggests that HS birds were able to counteract the reduced feed intake by increasing the expression of the AA transporter genes to maintain the target level of protein deposition in the breast muscles. Unfortunately, AA digestibility was not evaluated in the present study to support this observation. However, Koelkebeck et al. (1998) evaluated the apparent digestibility of 14 AAs in laying hens and observed that the HS group to have almost always higher digestibility coefficients compared to the TN group but with only 2 significant coefficients for lysine and histidine. In addition, Habashy et al. (2017) compared the apparent ileal digestibility of 20 AAs in HS broiler chickens and found that the HS group was greater in the digestibility coefficients of all AAs compared to the TN group, but out of the 20 AAs tested, only one significant coefficient could be detected. In the present study, the consistent increase in the mRNA expressions of CAT1, B0AT, and SNAT1 in the jejunum and *P. major* suggests that birds fed sufficient AA dense diet increase transport of specific cationic and neutral AAs when exposed to HS.

Our results also show that feeding suboptimal AA levels under HS conditions downregulated the expression of CAT1, $B^0$AT, and LAT1 in the jejunum, and CAT1 and $B^0$AT in the *P. major* in a similar fashion, with the 90% AA group being more affected. In contrast, SNAT1 in both the jejunum and *P. major*, and $b^{0,+}$AT, and LAT1 in the *P. major* were upregulated in HS birds fed suboptimal AA levels. It is unclear how reducing the dietary AA density under HS influenced the expression of the aforementioned genes differently. However, downregulation of CAT1 and $B^0$AT in both the jejunum and *P. major* is expected to be unfavorable to protein synthesis in the breast muscles due to the reduced uptake of limiting AAs such as lysine. Lysine has been regarded as a principal AA controlling the growth of the breast muscles (Kerr et al., 1999; Tesseraud et al., 1999; Bernal et al., 2014). In the present study, HS birds on the 80% AA diet had less *P. major* weight than their counterparts under TN conditions (16.69% vs. 15.64%). Recently, HS has been reported to reduce protein synthesis and increase protein degradation in broiler chickens (Ma et al., 2018; Ma et al., 2020). Therefore, it is reasonable to assume that the profound upregulation of SNAT1 in the *P. major* of HS birds aimed to maintain glutamine homeostasis by increasing the uptake of glutamine for energy metabolism (Young and Ajami, 2001). Interestingly, SNAT1 expression in the intestinal jejunum was positively correlated with the GR ($r = 0.90$, $P = 0.002$), indicating that the glutamine uptake from SNAT-1 under stress conditions was improved; it is well known that glutamine is involved in the synthesis of proteins, amino acids, and gluconeogenesis under particularly with low protein diets (Watford, 2015). An early stress response is the rapid release of muscle glutamine and a resultant decrease in both muscle and plasma free glutamine levels (Watford, 2015), indicating stress that is detected by the increment in glucocorticoid receptor expression (Rimoldi et al. 2015).

In almost all living organisms, various stressors and conditions such as unfavorable environmental temperature, infections, heavy metals, ischemia, inflammation, hypertrophy, tissue development, and growth factors trigger the production of heat shock proteins that aim at ensuring the correct folding of new or damaged proteins (Kiang and Tsokos, 1998). Our research demonstrates that reducing or increasing the dietary AA density constantly upregulates the expression levels of HSP70 in the *P. major* of both the TN and HS birds. Surprisingly, HSP70 expression was higher in the *P. major* of the TN birds than the HS birds at 80% AA density (2.18 vs.1.72). Although HSP70 expression is typically

elevated under HS (Hao and Gu, 2014), the increased HSP70 expression in the TN birds may be related to the increased size of the *P. major* (16.69 vs. 15.64%) as a means to maintain muscle protein integrity. The HSP90 behaved similarly in the *P. major* of the HS birds, as its expression was increased with reducing or increasing the dietary AA density, but in the TN birds its expression was reduced with reducing the AA density. Our results also showed a strong positive correlation ($r = 0.90$, $P = 0.003$) between HSP70 and GR in the intestinal jejunum of broiler chickens at 36 day of age. This result might be attributed to the modulatory functions of GR on HSP70 expression in various cell lines, as revealed by Beck et al. (2013). Contrary to the muscle, intestinal expression of HSP70 and HSP90 after heat stress showed no change when AA was reduced. Recent findings showed that intestinal heat shock proteins including HSP70, HSP60, and HSP47 are expressed during the acute exposure to HS (6 h or less), but the long duration of heat stress (i.e., 24 h) can result in a relatively small expression or reduced the expression when compared to the acute one (Al-Zghoul, 2018, Hasan Siddiqui et al. 2020).

To our knowledge, the current study is the first investigating the effects of altering AA density on AQP expressions. Our results show that the expression levels of AQP 1 in the *P. major* increased as the AA density was reduced, with the TN birds having a more pronounced elevated expression level. This may suggest the importance of AQP1 in pumping water into muscle tissue for protein synthesis, as evidenced by the strong positive association between AQP1 and each of CAT1 ($r = 0.90$, $P = 0.002$) and $b^{0,+}AT$ ($r = 0.83$, $P = 0.011$). The expression levels of AQP1 in the *P. major* of TN and HS birds on 100 and 110% diets were either the same (1.0 vs. 0.98) or changed slightly (0.97 vs. 0.87), which may indicate that the maximum *P. major* growth rate of these birds was reached (18.10−18.25%) and the need for pumping water in is at minimum (hence, low AQP1 expression). In contrast, the increased AQP1 expression in the *P. major* of the birds fed suboptimal AA levels may suggest an ongoing need for pumping in more water to synthesize muscle proteins, and this is supported by the associated increase in AA transporter gene expressions of the same birds. Unlike AQP1, AQP9 does not seem to take a significant role in muscle protein synthesis as suggested by the negative correlations between AQP9 and each of CAT1 ($r = -0.77$, $P = 0.026$), $b^{0,+}AT$ ($r = -0.85$, $P = 0.007$) and AQP1 ($r = -0.88$, $P = 0.004$). However, it is unclear why the expression of AQP9 was downregulated at suboptimal AA density.

The current results highlight the modulatory responses of AA transporters, aquaporins, and HSPs expressions as a protection mechanism to reduce the severity of muscular mass reduction associated with HS and AA reduction in broilers. To summarize the possible interaction between HS and the expression of the transcripts, we proposed an interaction in the jejunum that involved increasing the expression of AQP3 and

SNAT1, possibly to conserve water and small neutral AAs (mainly glutamine) during HS, respectively (Supplementary Figure 1). In the *P. major* muscle, it is plausible that the complex changes in the *P. major* associated with increasing LAT1, SNAT1, and $b^{0,+}AT$ were to restore AAs and additionally to increase AQP1 expression to restore water as a means to minimize the catabolism associated with HS and AA loss (Supplementary Figure 2). However, further analyses are required to reveal the molecular mechanisms underlying the adaptive response of the birds against AA depletion and HS exposure through the involvement of the other heat shock proteins and aquaporins together with other AA transporters and metabolic enzymes during HS.

## CONCLUSIONS

The data suggest that broiler chickens raised under high cyclic temperature should be fed a diet sufficient in balanced AAs and that lowering the AA density as a means to reduced dietary-induced thermogenesis is detrimental to growth performance. Moreover, the mRNA expressions of the examined AA transporters, aquaporins, and heat-shock proteins depended on the dietary AA level and environmental temperature. Typically, reducing the dietary AA density upregulated the mRNA expression of these genes in the jejunum and *P. major* of the TN birds. However, the reduction in the AA density influenced the expression of these genes differently in HS birds. These results highlight the importance of adequate AA nutrition for fast-growing chickens under HS conditions. Further investigations are required to fully understand the complex interactions among all the genes encoding AA transporters, water channels, and heat-shock proteins in HS broiler chickens fed various dietary AA regimes.

## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi: https://doi.org/10.1016/j.psj.2021.101333.

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