Effects of methionine supplementing on intestine, liver and uterus morphology, and on positivity and expression of calbindin-D28k and TRPV6 calcium carriers in laying quails in thermoneutral conditions and under thermal stress

Calcium carries in laying quails under thermal stress and methionine supplementation

Lanuza Ribeiro de Moraes¹, Maria Eduarda Araújo Delicato², André da Silva Cruz², Hugo Thyares Fonseca Nascimento Pereira da Silva², Clara Virgínia Batista de Vasconcelos Alves²³, Danila Barreiro Campos¹, Edilson Paes Saraiva³, Fernando Perazzo da Costa³, Ricardo Romão Guerra¹³*

¹ Programa de Pós-Graduação em Ciência Animal, Universidade Federal da Paraíba, Areia, Paraíba, Brazil, ² Departamento de Ciências Agrárias, Universidade Federal da Paraíba, Areia, Paraíba, Brazil, ³ Programa de Pós-Graduação em Zootecnia, Universidade Federal da Paraíba, Areia, Paraíba, Brazil

*Corresponding author

*e-mail: rromaoguerra@gmail.com
Abstract

The aim of this study was to provide support for the performance, localization and expression of the epithelial calcium transporter channels, calbindin-D28k (Calb) and TRPV6, and of the morphology of the digestive and reproductive system of laying quails under heat stress, and with methionine supplementation. Therefore, the present study characterized the positivity (immunohistochemistry) and expression (real-time PCR) of calcium channels (Calb and TRPV6) in the kidneys, intestine and uterus of 504 laying quails that were submitted to different methionine supplementation (100, 110 and 120%) and temperatures (20, 24, 28 and 32°C). The animals under thermal stress had lower villus height, villus:crypt ratio, and goblet cell index in the duodenum and jejunum, fewer secondary and tertiary uterine folds, smaller hepatic steatosis, and increased number of distal convoluted renal tubules (CT) positive to Calb (protein), and increased positivity in proximal CTs. The deleterious effects of heat stress were minimized with methionine supplementation for the following variables: duodenal crypts, number of goblet cells of the jejunum, number of uterine folds, decreased Calb positivity in intestines and kidney, increased positivity of Calb in the uterus and increased TRPV6 gene expression in the kidney. Calcium transporters were altered due to less need for calcium absorption and reabsorption due to more calcium available with the supplementation, increasing egg production and quality. Methionine supplementation further increased intestinal villus absorption area and height, increased steatosis, decreased Calb positivity in the intestine and kidney, increased uterine positivity and Calb expression, and increased TRPV6 expression in the uterus under thermoneutrality. This is the first study that describes the gene and protein expression of calcium transporters in the intestine, kidney and uterus of laying quails, and concludes that the use of methionine supplementation is justifiable in order to partially reverse the deleterious effects of thermal stress on the production.
**Introduction**

Quail laying farming has been growing in Brazil, mainly in the northeast region [1]. The raising of quails is a very profitable activity and with broad perspectives, which induces the development of research aiming at better production, perfecting techniques and alternatives to reach quality standards and expansion throughout the territory.

In tropical climates, such as those found in most of Brazil, laying birds suffer a reduction in their zootechnical indexes as well as an increase in mortality as a result of thermal stress, leading to productive and economic losses in production [2]. This fact is mainly observed in laying quail farming, where the first restrictive factor for eggshell formation is calcium, and calcium is negatively influenced by temperature increase [3].

Calcium comes mainly from intestinal absorption and bone resorption, which is mobilized from the blood to the uterus very quickly [4,5]. Nascimento et al. [6] stated that 70% of the production cost is based on food, and for this reason there is a need to develop balanced diets according to the needs of the birds, enabling them to use the diet with maximum efficiency.

For birds subjected to heat stress, it is necessary to supplement the diets with glycogenic amino acids, such as methionine, cystine and others [7]. Under such conditions of thermal stress, physiological and behavioral changes occur in quails, which severely affect feed intake and cause structural changes in the intestinal epithelium, reducing nutrient digestibility and absorption [8].

Methionine, classified as an essential amino acid [9], is also the first limiting factor in poultry feed, and is essential for the maintenance, growth, production, and development of feathers [10]. In addition to the productive responses obtained with methionine supplementation in laying hens, studies of the morphological analyses of the laying digestive and reproductive system of broilers and light birds indicate favorable quantitative and qualitative changes, such as an increase in egg mass by 10%. [11,12], decreased laying fat [13], increased eggshell thickness [14], and increased intestinal villi [15], which provide and technically justify the improvement in zootechnical indexes of these animals with methionine use [15-18].
Evaluating the existing literature [15-18], diets supplemented with methionine at levels above NRC (National Research Council) recommendation, can be a nutritional strategy to minimize heat stress damage, by improving the performance of laying birds in warmer regions. Such studies are scarce in laying hens and even more so in laying quails.

Thus, due to the gap in research related to the subject described, the objective was to evaluate the effect of methionine supplementation on intestinal morphology; on the villus:crypt ratio; on the quantity of goblet cells; on liver glycogen storage and steatosis; on uterine morphology in thermoneutral laying quails, and under high temperature heat stress. Furthermore, the objective was to evaluate positivity and gene expression of calbindin-D28k and TRPV6 calcium channels in laying birds under the same conditions, in order to evaluate the alteration of these channels due to methionine supplementation in thermoneutral laying birds and under thermal stress, since they are described as the main calcium carriers, acting on laying quails intestines, kidneys and uterus [19,20].

**Material and Methods**

A total of 504 Japanese quails in production stage (second cycle) were used, distributed in a completely randomized design in a 3x4 factorial scheme, with three levels of methionine (100%, 110% and 120%) and four temperature ranges (20 24, 28 and 32°C), representing thermoneutral and thermal stress ranges, totaling twelve treatments. Diets were formulated according to NRC (National Research Council) recommendation (Table 1).
Table 01. Experimental diets containing three levels of methionine supplementation for laying quails.

| Ingredientes                  | T1  | T2  | T3  |
|-------------------------------|-----|-----|-----|
|                               | 100% Met+Cys (0.888%) | 100+10% Met+Cys (0.977%) | 100+20% Met+Cys (1.066%) |
| Corn, 788%                    | 58.466 | 58.466 | 58.466 |
| Soybean meal, 45%             | 30.071 | 30.071 | 30.071 |
| Soyabean oil                  | 0.906 | 0.906 | 0.906 |
| Calcitic limestone            | 7.041 | 7.041 | 7.041 |
| Dicalcium phosphate, 18.5%    | 1.142 | 1.142 | 1.142 |
| Salt                          | 0.326 | 0.326 | 0.326 |
| L-Lisin HCl                   | 0.342 | 0.342 | 0.342 |
| L-Threonine                   | 0.078 | 0.078 | 0.078 |
| L-Tryptophan                  | 0.040 | 0.040 | 0.040 |
| Choline chloride, 60%         | 0.070 | 0.070 | 0.070 |
| Mineral premix                | 0.050 | 0.050 | 0.050 |
| Vitaminic Premix              | 0.025 | 0.025 | 0.025 |
| Antioxidant                   | 0.010 | 0.010 | 0.010 |
| DL-Methionine                 | 0.441 | 0.537 | 0.633 |
| Starch                        | 0.673 | 0.536 | 0.400 |
| Inert                         | 0.319 | 0.360 | 0.400 |
| TOTAL, kg                     | 100.00 | 100.00 | 100.00 |

**Chemical composition**

|                  | T1       | T2       | T3       |
|------------------|----------|----------|----------|
| PB, %            | 18.80    | 18.80    | 19.00    |
| EM, kcal/kg      | 2800     | 2800     | 2800     |
| Met digestible, %| 0.685    | 0.779    | 0.870    |
| Met + Cis digestible, % | 0.942 | 1.036 | 1.130 |
| Lis digestible, %| 1.148    | 1.148    | 1.148    |
| Thre digestible, %| 0.701 | 0.701    | 0.701    |
| Val digestible, %| 0.785    | 0.785    | 0.785    |
| Trp digestible, %| 0.241    | 0.241    | 0.241    |
| Arg digestible, %| 1.152    | 1.152    | 1.152    |
| Ile digestible, %| 0.717    | 0.717    | 0.717    |
| Leu digestible, %| 1.485    | 1.485    | 1.485    |
| Calcium, %       | 2.99     | 2.99     | 2.99     |
| Available match, %| 0.31  | 0.31     | 0.31     |
| Sodium, %        | 0.15     | 0.15     | 0.15     |
| Chlorine, %      | 0.24     | 0.24     | 0.24     |
| Potassium, %     | 0.72     | 0.72     | 0.72     |
| BE, mEq/kg       | 179.00   | 179.00   | 179.00   |
Histological Processing

Histological processing was performed at the Histology Laboratory of the Center for Agrarian Sciences of the Federal University of Paraíba. Biological samples of intestine (duodenum and jejunum), liver, uterus and kidney from 8 randomly chosen animals from each treatment were collected and fixed in Metacarn [21] for 12h and embedded in paraffin. The cuts were made with 5μm thickness. Hematoxylin-eosin staining and Periodic Acid Schiff (PAS) were used depending on the analysis, and digitized images were captured on an Olympus BX-60 microscope and a Olympus camera coupled with a Olympus cellSens Dimension digital imaging program. Samples of the duodenum were collected 4 cm after the ventricle and samples of the jejunum were collected in the middle region of this segment. Both were included in a transverse direction, so that it was possible to visualize the intestinal villi as well as the lumen of the organ. Kidney and liver samples were collected so as never to exceed 0.5 cm³ for adequate tissue fixation. Uterine samples were collected in the middle lateral region with a dimension of 1 cm².

Histomorphometry analyses were performed by a single histologist to avoid misinterpretation. ANOVA and Tukey's post test at 5% significance level were used to evaluate the influence of methionine supplementation on different temperature types.

Duodenal and Jejunal Morphology

Five photomicrographs were digitized in eight animals from each treatment, and two measurements in each image, totaling 80 measurements (8 animals x 5 photomicrographs x 2 measurements) of intestinal villi height and their respective crypts from each treatment, by means of a Olympus cellSens Dimension image analyzer and a Olympus digital camera attached to an Olympus BX-60 microscope. Villus height measurements were taken from its base to its apex; width was measured at the middle portion of each villus, and the crypt was measured from the base of its respective villus. The Villus:Crypt Ratio was given by dividing the Villus Height by its respective Crypt.

To quantify the goblet cell index in the duodenum and jejunum epithelium, histological slides from eight animals per treatment stained with PAS staining magenta on the goblet cells were used.
Images were captured and digitized with the 20x objective of the intestinal villi. At least 2 images from each animal were randomly chosen and the intestinal epithelium was measured linearly to 2000 µm, and the number of goblet cells per 500 µm was counted, making a sample of 32 per treatment (8 animals x 4 areas of 500 µm). Based on the results, the number of goblet cells in 1,000 micrometers for each treatment was defined based on a rule of three.

**Measurement of hepatic glycogen storage and hepatic steatosis**

For the measurement of hepatic glycogen storage, after the aforementioned histological processing and PAS staining, 5 photomicrographs of 8 animals from each treatment, chosen at random, were analyzed by optical microscopy by the same histologist, without his previous knowledge about the group belonging to each bird, totaling a sample of 40 per treatment (8 animals x 5 micrographs). The photomicrographs were classified according to the degree of glycogen deposition due to the positivity to PAS histochemistry: Grade +: little hepatic glycogen deposition; Grade ++: moderate hepatic glycogen deposition; and Grade +++: marked hepatic glycogen deposition. For analysis of the hepatic glycogen deposition index, the crosses were transformed into corresponding numbers (+ = 1, ++ = 2, +++ = 3) to perform the statistics according to the modified Ishak Semi Quantitative Score [22].

To evaluate hepatic steatosis, an evaluation score was assigned to each liver analyzed by liver photomicrographs of each animal (8 animals per treatment), totaling a sample number of 40 per treatment (8 animals x 5 photomicrographs), considering the amount and the size of the hepatocyte lipid cytoplasmic vacuoles: 0 (absence of steatosis), 1 (low steatosis), 2 (moderate steatosis) and 3 (advanced steatosis), following the modified Ishak Semi Quantitative Score [22]. For each treatment an average was obtained, which was submitted to statistics.

**Uterine Morphology**

After histological processing and the PAS histochemical staining, digital images were captured. Photomicrographs, 5 of each one of the 8 animals per treatment, totaling a sample number of 40, were evaluated according to the presence and quantity of uterine folds, and were evaluated according to
modified Ishak Semi-Quantitative Score [22]. Score 1 was given for the presence of primary folds only, score 2 for primary and some secondary folds, score 3 for the presence of primary, secondary and some tertiary folds, and score 4 for uterus with numerous tertiary folds. The photomicrographs were analyzed under optical microscopy by the same histologist, without his previous knowledge about the group belonging to each bird.

**Immunohistochemistry for anti-Calbindin-D28k**

Histological slides containing duodenum, jejunum, uterus and kidney from 6 animals per treatment were chosen randomly. The antibody was calbindin-D28K (Sigma, Clone Cl3000). The slides were dehydrated, blocked with 3 hydrogen peroxide baths for 10 minutes each and washed with phosphate buffer (PBS) 3 times for 3 minutes. The unmasking was performed with citrate buffer (pH 6.0) for 10 minutes in microwave, waiting for the temperature to drop for another 20 minutes. The slides were again washed in PBS and incubated at 4°C overnight with antibody diluted in PBS (1:200). The following day, the the slides were placed biotinylated secondary antibody for 15 minutes, followed by incubation in streptavidin peroxidase complex (DAKO-LSAB) for 30 minutes. Positive cells were labeled by DAB chromogen (DAKO) for 5 minutes. The photomicrographs were performed by the KS-400 Zeiss programs under Olympus BX60 microscope and AxioCam camera. The more antibody-positive, the higher the protein production of calbindin-D28k.

**Real-Time PCR (qPCR) for TRPV 6 and Calbindin-D28k**

Duodenum and jejunum, kidney and uterus samples from 6 randomly selected animals per treatment were used. For RNA extraction, two protocols were used, PureLinkTMRNA Mini Kit - Thermi Fisher Scientific and ReliaPrep™ RNA TissueMiniprep System. RNA samples were kept in the Ultra Freezer at -80°C until cDNA Synthesis was performed using the Invitrogen™, Super Script™, IV Master Mix Vilo™ with Enzima ezDNase™.

QPCR was performed by using MxPro - Mx3005P v4.10 Build 389, Schema 85 (Stratagene®, United States). All qPCR reactions used SYBR® Brilliant III Ultra-Fast QBRR Green Prime Mix Low
ROX (Agilent Technologies). Beta Actin was used as an endogenous control, and all oligonucleotides were obtained from Japanese quail DNA sequences previously published in the PubMed gene bank, and primer sequences were obtained from Primer3Plus - Bioinformatics (Table 2). Amplification conditions were 3 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 20 seconds at 60°C; 1 minute at 95°C; 30 seconds at 55°C, and finally 30 seconds at 95°C.

Melting curve analysis allowed the evaluation of the specificity of qPCR products. Samples were run in triplicate for each sample and relative quantification (target gene/endogenous control) determined their expression. Data were normalized to a calibrator sample using the $\Delta\Delta C_t$ method with correction for amplification efficiency [23].

**Table 02. Sequence of primers used for quantitative PCR – real time for quails.**

| Genes      | qPCR Primers (5'-3')                                   | GenBank Number |
|------------|-------------------------------------------------------|----------------|
| Calbindin28 | >GACGGCAATGGGTACATGGA<br/><TCGGGTGTTAAGTCCAAGCC        | XM_015855985.1 |
| TRPV6      | >CCATCATTGCCACCCTCCTT<br/><AGCAACAATCTGGGCTCTCC        | XM_015873874.1 |
| Beta Actina| >CCACTGGCATTGTCATGGACTCT<br/><TCCCTGATGTCACGGACGATTTCC  | X00182.1       |

$\geq$ = forward, $\leq$ = reverse

**Statistical analysis**

Data were subjected to analysis of variance (ANOVA) and, according to the significance of the F test ($P \leq 0.05$), the means were compared by the SNK test (Student-Newman-Keuls) at up to 5% probability of error. These analyses were performed by using the SAS® University Edition [24]. Pearson correlation analysis was also performed using the JMP® Pro 13. Data were represented by means ± standard deviation.

**Results and Discussion**

**Histology**
Duodenal and Jejunal Morphology

From the histomorphometric analysis of the intestine, it was found that the villus height variable (AV) in laying quails is reduced during heat stress in the duodenum and jejunum (Table 3), corroborating Mitchell and Carlisle [25], who observed decrease in jejunal villus height of broilers kept under constant thermal stress compared to birds in thermoneutrality.

Table 3. Morphometry of the digestive and breeding system of laying Japanese quail (*Coturnix japonica*) supplemented (S) with methionine at levels of 100%, 110% and 120% submitted to different temperatures (20°, 24°, 28° and 32°C).

| Variable               | S %   | Temperature (°C)                       |
|------------------------|-------|----------------------------------------|
|                        |       | 20          | 24          | 28          | 32          |
| Duodenum VH            | 100   | 860.85±140.49aB | 808.93±73.59bB | 811.22±97.36aB | 822.56±107.17abA |
|                        | 110   | 673.9±106.13cC | 856.47±90.46aA | 829.90±127.27aA | 757.96±73.81bB  |
|                        | 120   | 937.65±113.96aA | 886.30±90.76bA | 760.13±67.55cB | 729.15±68.09dC  |
| Jejunum VH             | 100   | 631.19±95.47bB | 678.79±60.45aA | 653.87±83.03abA | 676.65±77.91aA  |
|                        | 110   | 628.78±98.38bB | 564.32±51.51bB | 578.69±129.69bB | 575.78±85.11bB  |
|                        | 120   | 691.92±66.93aA | 656.45±69.34bA | 547.94±113.41cB | 557.35±77.37cB  |
| Duodenum CD            | 100   | 46.37±9.69bB  | 54.43±11.33aA  | 49.97±9.60bB    | 45.25±7.04bcAB  |
|                        | 110   | 49.97±9.94aB  | 48.92±8.21aB   | 47.77±8.67aAB   | 44.17±6.88bB    |
|                        | 120   | 57.40±13.31aA | 48.93±8.91bB   | 44.84±7.73cB    | 47.57±7.85bcA   |
| Jejunum CD             | 100   | 37.27±5.89cB  | 40.60±7.68bB   | 45.79±7.53aA    | 44.59±6.70aA    |
|                        | 110   | 39.72±6.91bAB | 39.83±6.47bB   | 40.83±5.90abB   | 42.36±4.78aA    |
|                        | 120   | 41.12±7.64aB  | 44.84±7.33aA   | 41.11±8.03bB    | 44.13±6.02aA    |
| Villous: Duodenum      | 100   | 19.38±5.20aA  | 15.49±3.38bB   | 16.66±3.08aB    | 18.45±2.91abA   |
|                        | 110   | 14.05±3.69bC  | 17.93±3.20aA   | 17.89±4.01aA    | 17.58±3.28aA    |
|                        | 120   | 16.88±2.92bC  | 18.84±3.57aA   | 17.41±3.16bA    | 15.71±2.85bC    |
| Villous: Jejunum       | 100   | 17.16±2.79aA  | 17.18±2.93aA   | 14.61±2.74bA    | 15.52±2.94bA    |
|                        | 110   | 15.99±2.13aB  | 14.55±2.83abB  | 14.40±3.55abA   | 13.78±2.67bB    |
|                        | 120   | 17.35±3.41aA  | 14.98±2.68bB   | 13.46±2.24bcA   | 12.80±2.17bC    |
| GC Duodenum            | 100   | 27.71±8.56abB | 31.38±7.65aA   | 30.50±9.93aA    | 23.33±7.61bB    |
|                        | 110   | 42.79±15.33aA | 31.08±6.66bca  | 36.00±6.78bA    | 27.21±4.32bA    |
|                        | 120   | 33.08±9.05aB  | 25.13±8.36bB   | 35.58±7.92aA    | 30.46±6.09abA   |
| GC Jejunum             | 100   | 36.25±6.53abA | 32.67±7.88bB   | 40.13±11.16aA   | 43.42±11.99aA   |
|                        | 110   | 39.17±10.56bA | 49.04±12.16aA  | 38.42±8.80bA    | 39.75±7.89bA    |
|                        | 120   | 39.38±10.73aA | 42.88±11.22aA  | 40.21±8.06aA    | 44.67±8.84A     |
| Uterine folds          | 100   | 3.11±0.68aA   | 3.12±0.99abA   | 2.87±0.64bcB    | 2.28±0.83cA     |
|                        | 110   | 3.06±0.73aA   | 3.22±0.73aaA   | 3.06±1.06aA     | 2.39±0.61bA     |
|                        | 120   | 3.43±0.51bA   | 3.50±0.71aaA   | 2.28±0.75bB     | 2.83±0.71bA     |
| Hepatic steatosis      | 100   | 1.17±0.59abA  | 1.43±0.97aA    | 0.70±0.75bB     | 0.87±0.78abA    |
|                        | 110   | 1.07±0.64bA   | 0.87±1.07aB    | 0.60±0.67aB     | 0.90±0.80aA     |
|                        | 120   | 1.10±0.84abA  | 1.57±1.19aA    | 1.43±1.04abA    | 0.87±1.01bA     |
Hepatic glycogen

|   | 100  | 110  | 120  |
|---|------|------|------|
|   | 1.17±0.38aA | 1.30±0.53aB | 1.43±0.63aA |
|   | 1.17±0.38bA | 2.53±0.63aA | 1.03±0.18bB |
|   | 1.43±0.50aA | 1.00±0.00bB | 1.50±0.51aA |
|   | 1.43±0.63aA | 1.27±0.45bA | 1.43±0.68aA |

Averages followed by the same letter lowercase in the row and uppercase in the column do not differ by Tukey's test up to 5% probability; VH: villus height; CD: crypt depth; Villus: Crypt: Villus Relationship: Crypt; GC: Globet cells.

Supplementation with 120% methionine provided higher villus height (VH) at thermoneutral temperatures of 20 and 24°C, but not at higher temperatures, including thermal stress temperatures (32°C). Thus, methionine supplementation is ineffective at reversing the harmful effects of heat stress for VH. Supplementation with 120% methionine even led to decreased VHs at the temperature of 32°C. Such event, without contextualize the crypt depth (CD), leads to believe in the reduction of intestinal area and consequent lower contact with food, decrease of nutrient absorption and production.

In the jejunum, the effects were similar; supplementation with 120% methionine promoted VH increase at 20°C, maintained VH at 24°C, and also reduced VH at 28 and 32°C. Thus, methionine supplementation was also not effective in minimizing the deleterious effects of heat stress on this small intestine segment.

The negative effects of high ambient temperature are known and have been reported by Marchini et al. [27], where high ambient temperature up to the fourth week of age promoted reduction in digestive enzyme secretion and in the VH of broilers.

According to some authors, food digestibility and intestinal mucosal integrity are strongly related to ambient temperature variations. Thus, the low amount of food present in the gastrointestinal tract during thermal stress also impairs the trophic stimulation of the intestinal mucosa, besides decreasing the secretion of digestive enzymes [27]. At high temperatures, feed intake is decreased in an attempt to decrease endogenous heat production that could cause damage to intestinal morphology and integrity, compromising digestion and absorption mechanisms and thereby reducing bird performance [26]. Thus, such a reduction may have led to lower methionine consumption at 32°C, and compromising the expected
effect of methionine in reversing, at least in part, the deleterious effects of VH heat stress in both duodenum and jejunum. Animals kept at the lowest temperatures in this study maintained their feed intake, thus explaining the increase in duodenal villus means. However, the use of methionine supplementation for this variable would not be justified, and is therefore not recommended.

Although methionine supplementation (120%) has not been shown to increase duodenal and jejunal villi under heat stress, and thus reverse the deleterious effects on intestinal morphology, it can be effective in increasing the absorption area (increased VH) in thermoneutrality.

Intestinal crypts are related to the intestinal health of the animal, the greater the crypt depth (CD), the greater the villous regeneration due to possible injuries (mechanical and/or other pathogenic mechanisms) occurred, or due to villous growth related to animal growth [28], becoming an important variable to be analyzed. Thus, the increase in CD can also predict an increase in VH when a trophic agent is presented, because it is exactly in this region that the cells that will migrate are produced to ensure the maintenance and/or increase of VH.

The histomorphometric analysis of CD in the duodenum showed that at thermoneutral temperatures for quails, the CD was lower, that is, the temperature of thermal stress (32°C) and at the lowest temperature (20°C), close to the thermal stress by low temperature, there is a higher need for cell turnover. However, 120% methionine supplementation led to a decrease in CD at higher temperatures (28 e 32°C). It can be inferred that at high temperatures, methionine supplementation reduced the deleterious effects of stress, reducing the need for cell proliferation in this region. The same result found for duodenum in relation to methionine supplementation was found in the jejunum. Regarding temperature, CD was different only at 20°C; it was lower at this temperature. The results show that methionine supplementation at high temperatures leads to a decrease in CD, which leads to a greater villus:crypt ratio, a variable used as an important marker of intestinal health, as it reveals a larger area of contact with food, consequently increased absorption without the need for too much energy expenditure on crypt turnover.
Crypt epithelium hyperplasia found at 32°C must have been induced to reestablish villus height, and is considered a compensatory mechanism [29], since thermal stress by heat in broilers for four consecutive days causes negative alterations in duodenum and jejunum crypts, including reduction in villus height, in villus/crypt ratio, in absorption area, and increased crypt depth.

The villus:crypt ratio (VCR) is related to the intestinal health of the animal, the higher the ratio, the greater its intestinal health. The results showed that the reduction of CD found in methionine supplementation at high temperatures (28 and 32°C) in the duodenum and jejunum did not translate into higher VCR. In contrast, at 32°C, VCR was lower in both intestinal segments after methionine supplementation. The improvement in intestinal health seen from the increase in VCR was only observed at a temperature of 24°C at duodenal level. These results show that methionine supplementation in laying quails under thermal stress does not reverse the deleterious effects of heat on VCR. Regarding the different temperatures, the increase of this variable also led to a decrease in intestinal health, that is, lower VCR.

These results corroborate Wu et al. [30], which report that thermal stress by heat is detrimental to the integrity of the intestinal mucosa of broilers, where the villi become shorter and flatter, and consequently the crypt increases its activity by becoming deeper in an attempt to reverse this situation, with this the VCR decreases. According to the authors, high ambient temperature reduces feed intake of birds [30], justifying the reduction in VCR at high temperature. This leads to less energy available for maintenance and renewal of the intestinal mucosa and consequently for production.

Goblet cells (GC) play an important role in the digestive system of animals; the quantity of GC even indicates the degree of intestinal health. GC produce mucus, mucin, which protects the intestinal epithelium from infectious agents and mechanical agents, and forms the glycocalyx which also plays an important role in intestinal digestion [31]. It is well known, as happened in the present study at the duodenal level (reduction of GC at 32°C), that high temperatures decrease the quantity and production of GC, thereby reducing intestinal health. Sandikciet al. [32] reported significant reduction in GC in the three intestinal segments, in addition to villus height, in Japanese quails subjected to thermal stress.
According to the authors, it is especially possible to relate the damage observed in intestinal mucosa, including decreased GC, to low feed intake during thermal stress [33]. Unlike in the literature [32] for chickens, rising temperatures did not decrease jejunum GC, perhaps because quails are more heat tolerant than laying hens.

Methionine supplementation led to an increase in GC during thermal stress (32°C) in the jejunum. These results demonstrate that methionine supplementation in heat stress reverses the deleterious effects of heat on the jejunum, but not on the duodenum. The increase in GC found in the jejunum under conditions that mimic thermal stress by heat provides better protection of the intestinal mucosa and better digestion, leading to improved intestinal health [34], thus justifying the use of methionine supplementation in this case.

Climate warming has been causing concern for quail farming, since, as results show, thermal stress by heat promotes various alterations in behavior and physiological mechanisms of quails, culminating in harmful morphological alterations, poor bird performance, and economic losses for the sector [35].

**Uterine Histomorphometry**

For the first time in quails, morphometric results showed that the increase of temperature, that is, thermal stress (32°C), causes decrease in the uterine folds, mainly in the secondary and tertiary folds (Table 3), which implies in a smaller area for the production of calcium carbonate, the main eggshell compound [3], negatively influencing the egg production of the animals. High temperatures also decreased eggshell production and thickness (Table 4). The highest indexes of uterine folds were found in treatments at 20 and 24°C.
Table 4. Average productive performance and eggshell thickness of laying quails submitted to methionine supplementation at 3 levels (100, 110 and 120%) and 4 temperature ranges varying from thermoneutrality to heat stress.

| Level x Temp | Production % 2º Period | Shell thickness 2º Period |
|--------------|-------------------------|---------------------------|
|              | 20°C | 24°C | 28°C | 32°C | 20°C | 24°C | 28°C | 32°C |
| 100%         |      |      |      |      |      |      |      |      |
| 110%         |      |      |      |      |      |      |      |      |
| 120%         |      |      |      |      |      |      |      |      |

Table 4: Means followed by the same lowercase letter in the rows and uppercase in the columns do not differ by Tukey's test up to 5% probability.

These results corroborate the egg production results (Table 4); the highest productive performance was found at 24°C, and the lowest performance was at 32°C.

Although the literature mentions that methionine supplementation increases the amount of folds in the uterus in layers and light birds [15-18], this was not observed when heat stress was applied in this study on quails. There was an increase in the uterine fold index only at 28°C with 110% methionine supplementation; however, these results did not interfere with egg production (Table 4). We can infer that methionine supplementation, except 110% at 28°C, does not minimize the deleterious effects of heat stress on uterine folds in quails.

Measurement of hepatic glycogen and steatosis storage

The increase in ambient temperature decreased the hepatic steatosis index, with the highest indexes at 20 and 24°C. Methionine supplementation (120%) increased this rate only at 24°C. This result differs from that found by Bunchasak et al. [14], who describe that the higher the methionine supplementation the higher the fatty acid synthesis in laying hens, and thus the higher the rate of hepatic steatosis. This variable is important since the increase in hepatic steatosis is related to estrogen production, that is, the higher the steatosis, the higher the estrogen production and the higher the egg
production [14]. Thus, methionine supplementation (120%) at 24°C would not only increase hepatic steatosis but also egg production by these quails, which occurred in the present study, since the increase in hepatic steatosis was reflected in a significant increase in production (Table 4). In contrast, birds subjected to a temperature of 20°C, despite not showing an increase in steatosis, had increased egg production (Table 4).

Hepatic glycogen levels did not appear to change with the alteration in ambient temperature, corroborating studies on broilers by Lana et al. [36]. However, these studies were contrary to the results for broilers, which showed decreased hepatic glycogen stores, as well as reduced feed intake and weight gain [37], and reduced liver weight [38] with consequent reduction in metabolic activity under thermal stress.

However, only at 24°C, 110% methionine supplementation increased hepatic glycogen stores. These results demonstrate that at thermoneutrality, 110% supplementation maximizes energy storage in the form of glycogen in the liver. Such a surplus can be transferred to production, in this case in egg production. Thus, in heat stress methionine supplementation was not efficient.

**Immunohistochemistry**

In modern strains of laying hens, which can be extrapolated to laying quails, the equivalent of 10% of total body calcium is transferred daily for deposition as eggshells [39-41]; the major sources of calcium are through absorption from the diet at the intestinal level, renal resorption, and bone storage. Since calbindin-k28D is the carrier responsible for the absorption of calcium from the digestive system, it would have the ability to modulate its deposition in the womb [46], in addition to intestinal absorptive capacity [45], influencing the production and the eggshell quality.

**Intestine**

For all treatments (temperatures and methionine levels), anti-calbindin-k28D was positive throughout the duodenal epithelium; the lamina propria (connective tissue layer below the epithelium) was not positive (Fig 1). Positivity was more intense in the basal and more apical portion of the
epithelium, since the middle portion was an area that had many enterocyte nuclei and the present marking is cytoplasmic. The most positive area was the apical surface of the enterocytes. In contrast, goblet cells were not positive for anti-calbindin-k28D. These results corroborate the study carried out in layers, which states that in the intestine of layers, there is calbindin-D28k positivity (protein) in all segments, higher in the duodenum and jejunum, especially in the apical portions of the villi, but smaller in the ileum [41,47].

Anti-calbindin-k28D positivity in duodenal intestine epithelium corroborates calcium absorption in this region. The greater intensity of positivity in the apical portion of enterocytes corroborates previous studies [41] that cite calbindin-k28D as a cellular calcium transport. This transporter binds to calcium absorbed by the cell and diffuses it into the cytoplasm, which is finally extruded by CA2+-ATPase into the basolateral membrane, reaching the vascular system through lamina propria vessels [41]. The form present in birds is 28kDa molecular weight, or calbindin-D28k, present in the kidney, brain and intestine and uterus of birds [20,42-44].

Goblet cells are not positive, since these cells have no function of absorption, but of production; they are responsible for producing and releasing mucin on the intestinal surface, as well as in other organs.

Among the temperatures studied, there was lower positivity to the calcium transport at 24 and 28°C (S1D Fig), when compared to 20°C, and mainly to 32°C. At temperatures with lower positivity, the increase in methionine level had even lower positivity to anti-calbindin-k28D. At 32°C (heat stress), methionine supplementation (120%) also led to lower positivity for calbindin-D28k when compared to the 100% level.

The decrease in positivity could be explained by the increased availability of calcium and consequently less need for absorption, and increased eggshell quality, which actually occurred in the present study. Although the performance model of calbindin-D28k has already been described in layers, this is the first study in quails. In a study with methionine supplementation in diets with lower protein levels in Thailand, a country with thermal similarities to that of northeastern Brazil, there was an increase in laying production rates, including increased eggshell thickness [14]. In the aforementioned study, the
increase in methionine must also have minimized the deleterious effect of heat stress and increased calcium availability to improve production rates, as occurred in the present study. It can be imagined that in this study the positivity of calbindin-D28k must also have decreased.

**Fig 1. Photomicrographs of anti-calbindin-D28k immunohistochemistry in laying quail intestine at different magnifications.** Positive anti-calbindin-D28k intestinal epithelium (arrows) and non-positive goblet cells (arrowheads) (A and B) are observed. Non-antibody-positive crypts (asterisk) are also observed (C). Lower epithelial positivity is observed under 28°C (D) when compared to other temperature treatments. Chromogen staining diaminobenzidine+hematoxylin.

High temperature stress negatively affects laying performance, decreasing feed intake, live weight gain and efficiency [48,49]. It also decreases egg production and eggshell quality and thickness [50-53] due to decreased availability of calcium ions [53]. It is important to say that increasing dietary calcium does not improve the quality of the shell in heat stress conditions [54,55]. High temperature heat stress decreases the presence of calbindin-D28k in the ileum, cecum, colon, and uterus of birds, causing deterioration of eggshell quality [56]. However, in this study we observed that the effect on the duodenum is the opposite, heat stress decreases the positivity to calbindin-D28k.

In the present study, at temperatures considered to be of higher thermal comfort for the quails (24 and 28°C), these animals presented lower positivity of the cellular calcium transport in their duodenal epithelia, exactly because they were in better thermal comfort, and did not need a higher absorption of calcium (Fig 2). Literature provides studies [57] that show that the higher the ambient temperature and thermal stress, the greater the need to supplement dietary calcium, as the animals will need more calcium for their metabolism, since the thermal stress decreases the availability of calcium [53]. Within the 24 and 28°C treatments, methionine supplementation provided even lower positivity for anti-calbindin-k28D when compared to normal levels. The gene expression of this same gene followed a similar model in gross values; however, these results were not significant (Fig 5), perhaps due to the small sample size and/or the large standard deviation. Another similar fact was that methionine supplementation also
decreased gene expression of this gene (Fig 6). Therefore, it is assumed that methionine supplementation leaves more calcium available, which makes the need for lower intestinal calcium absorption necessary.

The greatest positivity was at the temperature of thermal stress (32°C), when it is thought to have less calcium available, which increases the need for calcium. Thus there were more calcium transporters (calbindin-k28D) (higher positivity) to provide greater absorption to maintain the production. The positivity at the temperature of 20°C is intermediate, because for quails, this temperature is already relatively low, thus, the animal already feels some result of thermal stress, in this case for low temperature, changing its physiology, and also needing more calcium. This explains the slightly higher positivity at 20°C than that found in the 24 and 28°C treatments.

This is the first study to cite calbindin-d28k protein expression in the intestine of laying quails, and it is also the first to demonstrate the influence of high temperature heat stress on this calcium transport.

**Kidney**

Calbindin exists in 2 major forms: with low molecular weight, a 9kDa protein (Calbindin-D9k) present in mammalian intestines, and another with high molecular weight with 28kDa (Calbindin-D28k), present in the kidney of birds [20,42] and mammalian kidneys [20]. In the present study, anti-calbindin-d28k positivity was found in the distal contorted tubules (DCT) of the nephrons, but there is practically no positivity in the proximal tubules. This positivity in DCT was intense in the region surrounding the large renal blood vessels (Fig 2). The renal corpuscle, as well as the glomerulus (capillaries), were not positive for anti-calbindin-d28k.

Calbindin-d28k positivity was higher in DCTs, as these are the sites of greatest mineral resorption, including calcium [58]. The proximal contoured tubule (DCT) showed little positivity by not resorbing this mineral normally and in the renal corpuscle there is no positivity, as this portion of the nephron does not absorb or reabsorb, only filters blood resulting in pre-urine.
The most antibody positive areas were exactly the areas of DCT around large vessels in the renal cortex. This feature can be explained by the fact that these areas have blood with a higher amount of calcium, which has not yet been reabsorbed.

The positivity is lower at 24 and 28°C compared to at 20 and 32°C. In the treatment at 32°C, the amount of positive DCT increased, always in greater numbers near the great renal veins (Fig 2). At 28 and 32°C, DCT were more positive when compared to previous temperatures.

**Fig 2. Photomicrographs of anti-calbindin-D28k immunohistochemistry on kidney of quail laying at different temperatures.** A) 20°C: Positivity occurs mainly in distal contorted tubules near large vessels (asterisks). B and C) 24 and 28°C: Lower antibody positivity. D) 32°C: There are more positive distal contorted tubules and slightly positive proximal contorted tubules as well. More positivity (brown color) is observed at temperatures 20 and 32°C. Chromogen staining diaminobenzidine+hematoxylin. Magnification 100x.

In the treatments in which the animals are theoretically in greater thermal comfort, that is, in the 24 and 28°C treatments, the anti-calbindin-d28k positivity was lower. Since stress by high temperatures negatively affects the performance of layers [48, 49] due to decreased availability of calcium ions [53], it is expected that the rate of renal resorption will have to increase under stress. Thus, animals in thermal comfort would have less need to reabsorb large amounts of calcium, as occurred in treatments with 24 and 28°C (lower positivity). In animals with some degree of thermal stress, such as at 32°C (high temperature) and at 20°C, theoretically because it is a temperature below the thermal conformation for the species, they would have greater need to reabsorb more calcium (higher positivity).

Although it is well known that thermal stress by high temperatures decreases the presence of calbindin-D28k in the ileum, cecum, colon and uterus of birds, causing deterioration of eggshell quality [56], there was no information in literature on the influence of this calcium transport at renal level for thermal stress by high or low temperatures, as seems to occur at a temperature of 20°C. Thus, this is the
first report on the influence of heat stress on such a transport in the kidney, which, like the intestine, has
the opposite effect to that found for other organs in other experiments with layers [56].

In the case of high temperature heat stress treatment, more DCT were positive for anti-calbindin-
d28k, which shows that under such a situation not even increased positivity was enough to reabsorb the
calcium needed for the production of these birds; in addition to the increase in the expression of this
transport, the increase in the number of DCT that expressed such transport was needed (Fig 3).

Fig 3. Immunohistochemistry photomicrographs of anti-calbindin-D28k in kidney of laying quail at
different temperatures. A) 24°C: Note poor positivity in distal contorted tubules (arrowheads) and no
positivity in proximal contorted tubules (asterisks). B) 32°C: In thermal stress there is intense positivity of
the distorted contorted tubules (arrowheads) and low intensity in the proximal contorted tubules
(asterisks). Chromogen staining diaminobenzidine + hematoxylin. Magnification 400x.

Most intriguing was that at the two higher temperatures (28 and 32ºC), the positivity of PCT also
increased, so it seems that the animal physiologically adapts to reabsorb calcium, not only by DCT but
also by PCT in case of high temperature stress.

Methionine supplementation does not appear to alter protein expression of the calbindin-d28k
calcium transport. Therefore, such supplementation would not improve calcium utilization in animals
under thermal stress conditions.

Uterus

Positivity to anti-calbindin-D28K was high in the uterine glands, since these are the sites of
calcium carbonate production and secretion, which is produced and released for eggshell production in
the uterus, and is influenced by increased circulating estrogen [46], and modulates eggshell production
and quality. Uterine gland cells transport calcium from their basal portion to the apical surface during
calcium carbonate production, the more calcium carbonate, the faster the egg production and/or better
eggshell quality. The epithelium (ciliated pseudostratified) is not positive for anti-calbindin-D28k except for a thin layer on the apical portion of this epithelium.

The positivity pattern was higher at 24 and 28°C, lower in the treatment in which the animals were submitted to 32°C, and intermediate at 20°C (Fig 4). Methionine supplementation by 120% increased anti-calbindin-D28k positivity.

**Fig 4. Photomicrographs of anti-calbindin-D28k immunohistochemistry in uterus of laying quails at different temperatures (20, 24, 28 and 32°C) and supplemented with 120% methionine at 32°C.**

A) 20°C: observe lower positivity in the uterine glands (asterisk). B) 24°C: observe greater positivity in the uterine glands (asterisk). C) 28°C: observe greater positivity in the uterine glands (asterisk). D) 32°C: observe lower positivity in the uterine glands (asterisk). E) 32°C: supplemented with 120% methionine. More positive than treatment without methionine supplementation. Arrowheads (uterine epithelium), asterisks (uterine glands). DAB + hematoxylin chromogen staining. 400x magnification.

Ebeid et al. [56] state that under conditions of thermal stress by high temperature there is a decrease in the presence of calbindin-D28K in the uterus of laying hens, which corroborates the present study, in which the anti-calbindin-D28K positivity was lower at 32°C. This is the first time this fact is observed in quails. Stress by high temperatures is already known to negatively affect the performance of layers [48,49] due to decreased availability of calcium ions [53]. This can be explained by heat stress reducing the conversion of vitamin D3 to its metabolically active form, 1,25 (OH) 2D3, which is essential for calcium absorption and utilization. In fact, the calcium requirement for layers increases with high ambient temperatures [57].

Methionine supplementation at high temperatures (32°C) promoted increased positivity in the uterine glands (Fig 5) reversing part of the deleterious effect of thermal stress. The increase in positivity of this transport under thermal stress conditions possibly increased uterine gland calcium carbonate excretion for eggshell production, improving egg quality, although not reaching thermal comfort values.
Given these facts, methionine supplementation is recommended under thermal stress conditions.

The fact that the positivity of the 20°C treatment was intermediate shows that perhaps for quails, which have high heat resistance, this temperature is already below the ideal temperature for them.

**PCR in Real Time (QPCR) for TRPV6 and Calbindin-D28k**

TRPV6 acts as an epithelial channel of calcium in organs and glands that are characterized by high demand for calcium transport [58-60]. According to some studies [61,62], this ion channel exerts a facilitator effect on calcium entry into epithelial cells, expressed in the intestinal and kidney absorption and resorption epithelia, but there is still little information about its expression pattern in laying hens [19], and none in laying quails. Calbindin, in turn, has been described in studies in its two main forms, Calbindin-D9k (low molecular weight protein) present in mammal intestines, and Calbindin-D28k (high molecular weight protein) in kidney, brain and intestine and uterus of birds [20,42] and kidney of mammals [20].

Calbindin-D28k gene expression (Fig 5) in the kidneys of laying quails without methionine supplementation was higher at 28°C, a temperature that is still within thermoneutrality. With methionine supplementation, the highest expression was at 24°C. By comparing the expression of this transport within each temperature (supplemented but not with methionine), it is possible to say that methionine supplementation only increases Calbindin-D28k gene expression at 24°C (thermoneutrality), that is, by supplementing methionine we can maximize calcium reabsorption at the renal level, which can increase egg production by producing thicker eggshells in less time. However, under conditions of heat stress, supplementation is not effective in reducing the deleterious effects of heat, at least for this gene at the renal level.

For the TRPV6 ion channel gene in the same organ, the highest gene expression in animals without supplementation occurred at a temperature of 20°C, already mentioned as a temperature below thermal comfort for laying quails. In animals submitted to methionine supplementation, the highest peak
of gene expression occurred at a temperature of 24°C (thermoneutral), coinciding with the result obtained
in the calbindin gene, followed by a temperature of 32°C. Unlike calbindin, when comparing within each
temperature (supplemented and not), we see that methionine supplementation increased TRPV6 gene
expression, not only at 24°C but also at 32°C, that is, under thermal stress conditions. This result
demonstrates physiologically and technically validates the use of methionine supplementation for laying
quails in cases of thermal stress, and its effectiveness in minimizing the deleterious effects of high
temperatures. Such an increase in this gene increases calcium reabsorption, making more of the mineral
available for egg production, specifically in the release by the uterus for eggshell production.

Fig 5. Graphs of the effects of methionine supplementation (100% and 120%) at different
temperatures on the Calb 28 (A) and TRPV6 (B) genes expressions in the kidneys; Calb 28 (C) and
TRPV6 (D) in the intestine; and Calb 28 (E) and TRPV6 (F) in the uterus of Japanese quails (Coturnix
japonica) in production phase. a,b,A,B Averages followed by the same lower case letter for 100%
supplementation and upper case for 120% supplementation do not differ from each other by the SNK test
by up to 5% error probability; **,* and ns Indicate, respectively, significant differences up to 1%, up to
5% and not significant by the F test.

These results corroborate studies that already cited the gene expression of both genes (TRPV6 and
Calbindin-D28k) in kidney tissue of the laying birds [20,42], and still stands out for being the first to
demonstrate the positive expression of their gene expression, in the kidneys of laying quails.

In the intestine there was gene expression for both genes in all treatments, corroborating another
study regarding the presence of calbindin-D28k in layers [63]. This is also the first study to demonstrate
TRPV6 gene expression in intestines of laying quails. However, it was not possible to observe gene
alteration of Calbindin-D28k or TRPV6 with increasing temperature, nor with methionine
supplementation. These results imply that there is no alteration in the absorption or cellular calcium
transport during thermal stress, nor is there any improvement with methionine supplementation.
The TRPV6 gene, when compared to calbindin, showed little expressiveness in intestinal tissue in all the treatments, however, contradicting and filling in the gap left by some authors [42,63-64], who claim that the presence of TRPV6 is still uncertain in birds, including layers or quails. Although the difference was not significant due to the high standard deviation, high temperatures seemed to decrease intestinal TRPV6 gene expression.

For uterine tissue, Calbindin-D28K and TRPV6 gene expression also occurred in all treatments, and as for intestine, it was poor for TRPV6. Temperature increase and methionine supplementation did not influence gene expression of calbindin-D28k or TRPV6. Thus, the uterine tissue is not altered under these conditions either, thus not justifying the use of methionine supplementation.

The results described and observed in this study show the gene expression of TRPV6 and Calbindin-D28k genes in the renal, intestinal and uterine tissues of laying quails. Corroborating studies in laying hens [45-47] indicating that calbindin-D28k would modulate the intestinal calcium absorption and deposition capacity in the uterus, influencing eggshell production and quality, as well as the significant presence of TRPV6 (protein and mRNA) in the intestines and kidneys of layers [19].

Through analysis of variance, it was possible to verify the interaction between temperature and supplementation for both genes in the kidney (p≤0.01), supplementation for calbindin-D28k in the intestine (p≤0.01) and temperature for TRPV6 in the uterus (Table 5).

### Table 5. Analysis of Variance Summary (Mean Squared) for the effects of different temperatures and methionine supplementation (100% and 120%) on Calb 28 and TRPV6 gene expressions in the kidneys, intestine and uterus of Japanese quails (Coturnix japonica) in the production stage

| Variation source       | G1 | Kidney |                      |                      |                      |                      |
|------------------------|----|--------|----------------------|----------------------|----------------------|----------------------|
|                        |    | Calb 28 | TRPV6 | Calb 28 | TRPV6 | Calb 28 | TRPV6 |
| Temperature            | 3  | 0.63ns  | 0.12ns | 22.85ns | 0.54ns | 2.29ns  | 61.66* |
| Supplementation        | 1  | 0.62ns  | 0.26ns | 197.63** | 0.93ns | 8.36ns  | 45.33ns |
| Temp. vs Suplem.       | 3  | 2.00**  | 0.79** | 16.57ns | 0.29ns | 9.22ns  | 54.33ns |

*, **, and ns indicate, respectively, significant differences up to 5%, up to 1%, and not significant by the F test.

In addition, the responses of calbindin-D28k and TRPV6 in the kidney, intestine and uterus of Japanese quails supplemented with methionine + cystine (100% and 120%) at different temperatures
showed a strong positive correlation ($r = 0.90^*$) in the kidneys and moderately positive ($r = 0.69^*$) in the intestines between Calb 28 and TRPV6 gene expressions (Fig 6), indicating that both calbindin-D28k and TRPV6 (mRNA) act synergistically, modulating resorptive (kidney) and absorptive (intestine) capacity, and subsequent calcium deposition by the uterus. The correlations between the other variables were weak and not significant.

Fig 6. Pearson correlation for alterations in Calb 28 and TRPV6 gene expressions in the rim, intestine and uterus of Japanese quail (*Coturnix japonica*) supplemented with methionine (100% and 120%) at different temperatures in the production phase.

**Conclusion**

For the first time, this study brings histomorphological and expression variations (mRNA and protein) of TRPV6 and Calbindin-D28k in organs involved with absorption, reabsorption and calcium deposition in quails. It still physiologically justifies the use of methionine supplementation (120%) in thermal stress, since it reduces the deleterious effect on intestine, kidney and uterus parameters, besides improving others in the same organs under thermoneutrality conditions.

**References**

1. Instituto Brasileiro de Geografia e Estatística, Produção da Pecuária Municipal, Rio de Janeiro. 2016;44:29-31.

2. Pereira DF, Vale MM, Zevolli BR, Salgado DD. Estimating mortality in laying hens as the environmental temperature increases. Rev Bras Cienc Avic. 2010;12:265-271.

3. Albino LFT, Carvalho BR, Maia RC, Barros VRSM. Galinhas Poedeiras: Criação e Alimentação. Viçosa, Minas Gerais: Aprenda Fácil; 2014.

4. Carvalho LSS. Nutrição de poedeiras em clima quente. Rev Cient Eletrônica Med Vet. 2012;9(18).
5. Mello JF. Influência dos níveis de cálcio e fósforo na dieta de matrizes de codornas japonesas, no desempenho produtivo e no desenvolvimento ósseo embrionário da progênie [dissertation]. Maringá (PR): Universidade Estadual de Maringá; 2015.

6. Nascimento DCN, Sakomura NK, Siqueira JC. Exigências de metionina + cistina digestível para aves de corte ISA Label criadas em semiconfinamento. R Bras Zootec. 2009;38(5):869-78.

7. Virden WS, Kidd MT. Estresse fisiológico em frangos de corte: Ramificações na digestibilidade e respostas de nutrientes. J Appl Poult Res 2009;18(2):338-47.

8. Alagawany M, Farag MR, El-Hack MA & Patra A. Heat stress: effects on productive and reproductive performance of quail. Worlds Poult Sci J. 2017;73(4):747-56.

9. D’Mello JPF. Amino Acids in Animal Nutrition. In: Felix JP, editors. Formerly of the Scottish Agricultural College Edinburgh. 2th ed. Edinburgh: CABI, 2003. P.143.

10. Klasing KC. Comparative Avian Nutrition. Davis: Cab international; 1998.

11. Summers JD, Atkinson JL, Spratt D. Supplementation of a low protein diet in an attempt to optimize egg mass output. Can J Anim Sci. 1991;71:211-20.

12. Harm RH, Russell GB. Optimizing egg mass with amino acid supplementation of a low-protein diet. Poult Sci. 1993;72:1892-6.

13. Bunchasak C, Santoso U, Tanaka K, Ohtni S, Collado CM. The effects of supplementing methionine plus cystine to a low-protein diet on the growth performance and fat accumulation of growing broiler chicks. Asian-Australas J Anim Sci. 1997;10(2):185-191.

14. Bunchasak C, Silapasorn T. Effects of adding methionine in low-protein diet on production performance, reproductive organs and chemical liver composition of laying hens under tropical conditions. Int J Poult Sci. 2005;4(5):301-8.

15. Júnior JPF. Níveis de metionina+cistina digestíveis para aves de reposição leves nas fases inicial e cria [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2013.

16. Lopes GM. Biodisponibilidade relativa e níveis de metionina para frangos de corte. [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2014.
17. Pinheiro SG. Relação energia metabolizável: aminoácidos sulfurosos para aves leves. [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2014.

18. Santana MHM. Níveis de metionina+cistina para galinhas poedeiras leves nas fases de recria e postura. [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2014.

19. Yang JH, Hou JF, Farquharson C, Zhou ZL, Deng YF, Wang L, Yu Y. Localisation and expression of TRPV6 in all intestinal segments and kidney of laying hens. Br Poult Sci. 2011;52(4):507-16.

20. Fleet JC, Schoch RD. Molecular mechanisms for regulation of intestinal calcium absorption by vitamin D and other factors. Crit Rev Clin Lab Sci. 2010;47(4):181-95.

21. Ramos AH, Santos LM, Miglino MA, Peres JA, Guerra R R. Biometria, histologia e morfometria do sistema digestório do cachorro-do-mato (Cerdocyon thous) de vida livre. Biotemas. 2011;24(4):111-9.

22. Ishak K, Baptista A, Bianchi L, Callea F, de Groote J, Gudat F, et al. Gradação histológica e estadiamento da hepatite crônica. J Hepatol. 1995;22(6):696-9.

23. Pfaffl MW. Um novo modelo matemático para quantificação relativa em RT-PCR em tempo real. Pesquisa de ácidos nucleicos. 2001;29(9):e45

24. Cody R. An Introduction to SAS University Edition. Cary. NC: SAS Institute. 2015. 366 p.

25. Mitchell MA, Carlisle AJ. The effects of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl (Gallus domesticus). Comp Biochem Physiol A Comp Physiol. 1992;101:137-142.

26. Lin H, Jeao HC, Buyse J, Decuypere E. Strategies for preventing heat stress in Poultry. Worlds Poult Sci J, Ithaca. 2006;62(1):71-86.

27. Marchini CFP, Silva PL, Nascimento MRBM, Beletti ME, Guimarães EC, & Soares HL. Morfometria da mucosa duodenal em frangos de corte submetidos à temperatura ambiente cíclica elevada. Arq Bras Med Vet Zootec. Belo Horizonte. 2009;61(2):491-7.

28. Araújo LF, Junqueira OM, Lopes EL, da Silva Araújo CS, Ortolan JH, & de Laurentiz AC. Utilização de levedura desidratada (Saccharomyces cerevisiae) para leitões na fase inicial. Cienc Rural. 2006;36(5):1576-1581.
29. Santos RR, Awati A, Roubos-Van Den Hil PJ, Tersteeg-Zijderveld MH, Koolmees PA, Fink-Gremmels J. Quantitative histo-morphometric analysis of heat-stress-related damage in the small intestines of broiler chickens. Avian Pathol. 2015;44(1):19-22.

30. Wu QJ, Liu N, Wu XH, Wang GY, Lin L. Glutamine alleviates heat stress-induced impairment of intestinal morphology, intestinal inflammatory response, and barrier integrity in broilers. Poult Sci. 2018;97(8):2675-2683.

31. Furlan RL, Macari M, Luquetti BC, editors. Como avaliar os efeitos do uso de prebióticos, probióticos e flora de exclusão competitiva. Anais 5º Simpósio Técnico de Incubação, Matrizes de Corte e Nutrição, 2004, Balneário Camboriú, SC, BRA. 2004.

32. Sandikci M, Eren U, Onol AG, Kum S. The effect of heat stress and the use of Saccharomyces cerevisiae or (and) bacitracin zinc against heat stress on the intestinal mucosa in quails. Revue Méd Vét. 2004;155(11):552-556.

33. Mehaisen GM, Ibrahim RM, Desoky AA, Safaa HM, El-Sayed OA, Abass AO. The importance of propolis in alleviating the negative physiological effects of heat stress in quail chicks. PLoS One. 2017;12(10):e0186907.

34. Nyoni NMB, Grab S, Arche ERM. Heat stress and chickens: climate risk effects on rural poultry farming in low-income countries. Clim Dev. 2019;11:83-90.

35. Figueiredo DF, Murakami AE, dos Santos Pereira MA, Furlan AC, Toral FLB. Desempenho e Morfometria da Mucosa de Duodeno de Frangos de Corte Alimentados com Farelo de Canola durante o Período Inicial. R Bras Zootec. 2003;32(6):1321-9.

36. Lana GRQ, Rostagno HS, Albino LFT, Lana AMQ. Efeito da temperatura ambiente e da restrição alimentar sobre o desempenho e a composição da carcaça de frangos de corte. R Bras Zootec. 2000;29:1117-23.

37. Rosa PS. Desempenho e concentrações de alguns componentes do metabolismo intermediário de frangos com potencial de crescimento diferenciado submetidos ao estresse por calor [dissertation]. Jaboticabal (SP): Universidade Estadual de São Paulo; 2005.
38. Plavnik I, Yahav S. Efeito da temperatura ambiente em frangos submetidos a restrição de crescimento em idade precoce. Avicultura. 1998;77(6):870-2.

39. Miller SC, editor. Calcium homeostasis and mineral turnover in the laying hen. Proceedings of the Poultry Science Symposium. 1992.

40. Sugiyama T, Kusuhara S. Avian calcium metabolism and bone function. Asian-Australas J Anim Sci. 2001;14:82–90.

41. Sugiyama T, Kikuchi H, Hiyama S, Nishizawa K, Kusuhara S. Expression and localisation of calbindin D28k in all intestinal segments of the laying hen. Br Poult Sci. 2007;48(2):233-8.

42. Bar A. Calcium transport in strongly calcifying laying birds: mechanisms and regulation. Comp Biochem Physiol A Comp Physiol. 2008;152,447–69.

43. Fullmer CS, Brindak ME, Bar A, Wasserman RH. The purification of calcium-binding protein from the uterus of the laying hen. Proc Soc Exp Biol Med. 1976;152(2):237-41.

44. Parmentier M. Calbindin D28k is essentially located in the colonic part of the toad intestine. Biol Cell. 1990;68(1):43-9.

45. Saki AA, Tivey DR. Immunohistochemical detection of calbindin D28k and oestrogen receptor in the small intestine of pre – and post – lay hens. Proc Aust Poult Sci. 1997;9.

46. Corradino RA, Smith CA, Krook LP, Fullmer CS. Tissue-specific regulation of shell gland calbindin D28K biosynthesis by estradiol in precociously matured, vitamin D-depleted chicks. Endocrinology. 1993;132(1):193-8.

47. Wu JC, Smith MW, Mitchell MA, Peacock MA, Turvey A, Keable SJ. Enterocyte expression of calbindin, calbindin mRNA and calcium transport increases in jejunal tissue during onset of egg production in the fowl (Gallus domesticus). Comp Biochem Physiol Comp Physiol. 1993;106(2):263-9.

48. Donkoh A. Ambient temperature: a factor affecting performance and physiological response of broiler chickens. Int J Biometeorol. 1989;33:259-65.

49. Siegel HS. Stress, strains and resistance. Br Poult Sci. 1995;36:3-22.
50. Deaton JW, Reece FN, Mcnaughton JL, Lott BD. Effect of differing temperature cycles on egg shell quality an layer performance. Poult Sci. 1981;60:733-737.

51. Emery DA, Vohra D, Ernst RA. The effect of cycle and constant ambient temperature on feed consumption, egg production, egg weight, and shell thickness of hens. Poult Sci. 1984;63:2027-35.

52. Campos EJ. Avicultura: razões, fatos e divergências. Belo Horizonte: Editora FEPMVZ; 2000.

53. Mack LA, Felver-Gant JN, Dennis RL. Genetic variations alter production and behavioral responses following heat stress in two strains of laying hens. Poult Sci. 2013;92:285-94.

54. De Andrade AN, Rogler JC, Featherston WR, Alliston CW. Interrelationships between diet and elevated temperatures (cyclic and constant) on egg production and shell quality. Poult Sci. 1977;56:1178-88.

55. Tanor MA, Lesson S, Summers JD. Effect of heat stress and diet composition on performance of white leghorn hens. Poult Sci. 1984;63:304-310.

56. Ebeid TA, Suzuki T, Sugiyama T. High ambient temperature influences eggshell quality and calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. Poult Sci. 2012;91(9):2282-7.

57. Plavnik I, editor. Nutrição de aves em climas quentes. Proceedings ot the Conferência Apinco; 2003. p. 235-245.

58. Christakos S, Dhawan P, Porta A, Mady LJ, & Seth T. Vitamin D and intestinal calcium absorption. Mol Cell Endocrinol. 2011;347(1-2):25-9.

59. Nijenhuis T, Hoenderop JG, Van Der KAW, Bindels RJ. Localization and regulation of the epithelial Ca2+ channel TRPV6 in the kidney. J Am Soc Nephrol. 2003;14:2731–40.

60. Hoenderop Joost GJ, Nilius Bernd, Bindels René JM. Calcium absorption across epithelia. Physiol Rev. 2005;85(1):373-422.

61. Brown AJ, Krits I, Armbrecht HJ. Effect of age, vitamin D, and calcium on the regulation of rat intestinal epithelial calcium channels. Arch Biochem Biophys. 2005;437:51–8.
62. Bianco SD, Peng JB, Takanaga H, Suzuki Y, Crescenzi A, Kos CH, et al. Marked disturbance of calcium homeostasis in mice with targeted disruption of the Trpv6 calcium channel gene. J Bone Miner Res. 2007;22(2):274-85.

63. Wasserman RH, Taylor AN. Vitamin D-induced calcium-binding protein in chick intestinal mucosa. Science. 1966;152:791–3.

64. Qin X, Klandorf H, Porter DW, Holt SB, Martin WG. Estrogen enhancement of calcium, magnesium, and calcium-magnesium stimulated adenosine triphosphatase activity in the chick shell gland. Gen Comp Endocrinol. 1993b;89:4–10.
Figure 1
Figure 3
Figure 5
