1 **Full title**

2 **Effects of methionine supplementing on intestine, liver and uterus morphology, and on positivity**

3 **and expression of calbindin-D28k and TRPV6 calcium carriers in laying quails in thermoneutral**

4 **conditions and under thermal stress**

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6 **Short title**

7 **Calcium carries in laying quails under thermal stress and methionine supplementation**

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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 32ºC). 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].

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73 Evaluating the existing literature [15-18], diets supplemented with methionine at levels above

74 NRC (National Research Council) recommendation, can be a nutritional strategy to minimize heat stress

75 damage, by improving the performance of laying birds in warmer regions. Such studies are scarce in

76 laying hens and even more so in laying quails.

77 Thus, due to the gap in research related to the subject described, the objective was to evaluate the

78 effect of methionine supplementation on intestinal morphology; on the villus:crypt ratio; on the quantity

79 of goblet cells; on liver glycogen storage and steatosis; on uterine morphology in thermoneutral laying

80 quails, and under high temperature heat stress. Furthermore, the objective was to evaluate positivity and

81 gene expression of calbindin-D28k and TRPV6 calcium channels in laying birds under the same

82 conditions, in order to evaluate the alteration of these channels due to methionine supplementation in

83 thermoneutral laying birds and under thermal stress, since they are described as the main calcium carriers,

84 acting on laying quails intestines, kidneys and uterus [19,20].

Material and Methods

86 A total of 504 Japanese quails in production stage (second cycle) were used, distributed in a

87 completely randomized design in a 3x4 factorial scheme, with three levels of methionine (100%, 110%

88 and 120%) and four temperature ranges (20 24, 28 and 32°C), representing thermoneutral and thermal

89 stress ranges, totaling twelve treatments. Diets were formulated according to NRC (National Research

90 Council) recommendation (Table 1).

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97 **Table 01. Experimental diets containing three levels of methionine supplementation for laying**

98 **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			
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

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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.

126   Images were captured and digitized with the 20x objective of the intestinal villi. At least 2 images from

127   each animal were randomly chosen and the intestinal epithelium was measured linearly to 2000 μm, and

128   the number of goblet cells per 500 μm was counted, making a sample of 32 per treatment (8 animals x 4

129   areas of 500 μm). Based on the results, the number of goblet cells in 1,000 micrometers for each

130   treatment was defined based on a rule of three.

Measurement of hepatic glycogen storage and hepatic steatosis

132   For the measurement of hepatic glycogen storage, after the aforementioned histological processing

133   and PAS staining, 5 photomicrographs of 8 animals from each treatment, chosen at random, were

134   analyzed by optical microscopy by the same histologist, without his previous knowledge about the group

135   belonging to each bird, totaling a sample of 40 per treatment (8 animals x 5 micrographs). The

136   photomicrographs were classified according to the degree of glycogen deposition due to the positivity to

137   PAS histochemistry: Grade +: little hepatic glycogen deposition; Grade ++: moderate hepatic glycogen

138   deposition; and Grade +++: marked hepatic glycogen deposition. For analysis of the hepatic glycogen

139   deposition index, the crosses were transformed into corresponding numbers (+ = 1, ++ = 2, +++ = 3) to

140   perform the statistics according to the modified Ishak Semi Quantitative Score [22].

141   To evaluate hepatic steatosis, an evaluation score was assigned to each liver analyzed by liver

142   photomicrographs of each animal (8 animals per treatment), totaling a sample number of 40 per treatment

143   (8 animals x 5 photomicrographs), considering the amount and the size of the hepatocyte lipid

144   cytoplasmic vacuoles: 0 (absence of steatosis), 1 (low steatosis), 2 (moderate steatosis) and 3 (advanced

145   steatosis), following the modified Ishak Semi Quantitative Score [22]. For each treatment an average was

146   obtained, which was submitted to statistics.

Uterine Morphology

148   After histological processing and the PAS histochemical staining, digital images were captured.

149   Photomicrographs, 5 of each one of the 8 animals per treatment, totaling a sample number of 40, were

150   evaluated according to the presence and quantity of uterine folds, and were evaluated according to

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151   modified Ishak Semi-Quantitative Score [22]. Score 1 was given for the presence of primary folds only,

152   score 2 for primary and some secondary folds, score 3 for the presence of primary, secondary and some

153   tertiary folds, and score 4 for uterus with numerous tertiary folds. The photomicrographs were analyzed

154   under optical microscopy by the same histologist, without his previous knowledge about the group

155   belonging to each bird.

Immunohistochemistry for anti-Calbindin-D28k

157   Histological slides containing duodenum, jejunum, uterus and kidney from 6 animals per

158   treatment were chosen randomly. The antibody was calbindin-D28K (Sigma, Clone Cl3000). The slides

159   were dehydrated, blocked with 3 hydrogen peroxide baths for 10 minutes each and washed with

160   phosphate buffer (PBS) 3 times for 3 minutes. The unmasking was performed with citrate buffer (pH 6.0)

161   for 10 minutes in microwave, waiting for the temperature to drop for another 20 minutes. The slides were

162   again washed in PBS and incubated at 4°C overnight with antibody diluted in PBS (1:200). The following

163   day, the the slides were placed biotinylated secondary antibody for 15 minutes, followed by incubation in

164   streptavidin peroxidase complex (DAKO-LSAB) for 30 minutes. Positive cells were labeled by DAB

165   chromogen (DAKO) for 5 minutes. The photomicrographs were performed by the KS-400 Zeiss

166   programs under Olympus BX60 microscope and AxioCam camera. The more antibody-positive, the

167   higher the protein production of calbindin-D28k.

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

169   Duodenum and jejunum, kidney and uterus samples from 6 randomly selected animals per

170   treatment were used. For RNA extraction, two protocols were used, PureLinkTMRNA Mini Kit - Thermi

171   Fisher Scientific and ReliaPrep ™ RNA TissueMiniprep System. RNA samples were kept in the Ultra

172   Freezer at -80ºC until cDNA Synthesis was performed using the Invitrogen™, Super Script™, IV Master

173   Mix VILO™ with Enzima ezDNase™.

174   QPCR was performed by using MxPro - Mx3005P v4.10 Build 389, Schema 85 (Stratagene ®,

175   United States). All qPCR reactions used SYBR® Brilliant III Ultra-Fast QBRR Green Prime Mix Low

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176 ROX (Agilent Technologies). Beta Actin was used as an endogenous control, and all oligonucleotides

177 were obtained from Japanese quail DNA sequences previously published in the PubMed gene bank, and

178 primer sequences were obtained from Primer3Plus - Bioinformatics (Table 2). Amplification conditions

179 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

180 95°; 30 seconds at 55º, and finally 30 seconds at 95º.

181   Melting curve analysis allowed the evaluation of the specificity of qPCR products. Samples were

182 run in triplicate for each sample and relative quantification (target gene/endogenous control) determined

183 their expression. Data were normalized to a calibrator sample using the $\Delta\Delta$Ct method with correction for

184 amplification efficiency [23].

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

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

186   > = forward, < = reverse

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Statistical analysis

189   Data were subjected to analysis of variance (ANOVA) and, according to the significance of the F

190 test ($P \leq 0.05$), the means were compared by the SNK test (Student-Newman-Keuls) at up to 5%

191 probability of error. These analyses were performed by using the SAS® University Edition [24]. Pearson

192 correlation analysis was also performed using the JMP® Pro 13. Data were represented by means ±

193 standard deviation.

Results and Discussion

Histology

Duodenal and Jejunal Morphology

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197   From the histomorphometric analysis of the intestine, it was found that the villus height variable

198 (AV) in laying quails is reduced during heat stress in the duodenum and jejunum (Table 3), corroborating

199 Mitchell and Carlisle [25], who observed decrease in jejunal villus height of broilers kept under constant

200 thermal stress compared to birds in thermoneutrality.

201 **Table 3. Morphometry of the digestive and breeding system of laying Japanese quail (*Coturnix***

202 ***japonica*) supplemented (S) with methionine at levels of 100%, 110% and 120% submitted to**

203 **different temperatures (20º, 24º, 28º and 32ºC).**

Variable	S %	Temperature (°C)			
		20	**24**	**28**	**32**
Duodenum VH	100	860.85±140.49aB	808.93±73.59bB	811.22±97.36bA	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±98.38aB	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±9.60abA	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±7.64bA	44.84±7.33aA	41.11±8.03bB	44.13±6.02aA
Villous: Crypt Duodenum	100	19.38±5.20aA	15.49±3.38bB	16.66±3.08bA	18.45±2.91abA
	110	14.05±3.69bC	17.93±3.20aA	17.89±4.01aA	17.58±3.28aA
	120	16.88±2.92bcB	18.84±3.57aA	17.41±3.16bA	15.71±2.85cB
Villous: Crypt 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±2.17cB
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±4.23cAB
	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±8.84aA
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±0.73aA	3.06±1.06aA	2.39±0.61bA
	120	3.43±0.51bA	3.50±0.71aA	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±0.64aA	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	1.17±0.38aA	1.30±0.53aB	1.43±0.63aA	1.43±0.63aA
	110	1.17±0.38bA	2.53±0.63aA	1.03±0.18bB	1.27±0.45bA
	120	1.43±0.50aA	1.00±0.00bB	1.50±0.51aA	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 hight (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 32ºC. 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

230 effect of methionine in reversing, at least in part, the deleterious effects of VH heat stress in both

231 duodenum and jejunum. Animals kept at the lowest temperatures in this study maintained their feed

232 intake, thus explaining the increase in duodenal villus means. However, the use of methionine

233 supplementation for this variable would not be justified, and is therefore not recommended.

234   Although methionine supplementation (120%) has not been shown to increase duodenal and

235 jejunal villi under heat stress, and thus reverse the deleterious effects on intestinal morphology, it can be

236 effective in increasing the absorption area (increased VH) in thermoneutrality.

237   Intestinal crypts are related to the intestinal health of the animal, the greater the crypt depth (CD),

238 the greater the villous regeneration due to possible injuries (mechanical and/or other pathogenic

239 mechanisms) occurred, or due to villous growth related to animal growth [28], becoming an important

240 variable to be analyzed. Thus, the increase in CD can also predict an increase in VH when a trophic agent

241 is presented, because it is exactly in this region that the cells that will migrate are produced to ensure the

242 maintenance and/or increase of VH.

243   The histomorphometric analysis of CD in the duodenum showed that at thermoneutral

244 temperatures for quails, the CD was lower, that is, the temperature of thermal stress (32°C) and at the

245 lowest temperature (20°C), close to the thermal stress by low temperature, there is a higher need for cell

246 turnover. However, 120% methionine supplementation led to a decrease in CD at higher temperatures (28

247 e 32°C). It can be inferred that at high temperatures, methionine supplementation reduced the deleterious

248 effects of stress, reducing the need for cell proliferation in this region. The same result found for

249 duodenum in relation to methionine supplementation was found in the jejunum. Regarding temperature,

250 CD was different only at 20°C; it was lower at this temperature. The results show that methionine

251 supplementation at high temperatures leads to a decrease in CD, which leads to a greater villus:crypt

252 ratio, a variable used as an important marker of intestinal health, as it reveals a larger area of contact with

253 food, consequently increased absorption without the need for too much energy expenditure on crypt

254 turnover.

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255 Crypt epithelium hyperplasia found at 32°C must have been induced to reestablish villus height,

256 and is considered a compensatory mechanism [29], since thermal stress by heat in broilers for four

257 consecutive days causes negative alterations in duodenum and jejunum crypts, including reduction in

258 villus height, in villus/crypt ratio, in absorption area, and increased crypt depth.

259 The villus:crypt ratio (VCR) is related to the intestinal health of the animal, the higher the ratio,

260 the greater its intestinal health. The results showed that the reduction of CD found in methionine

261 supplementation at high temperatures (28 and 32°C) in the duodenum and jejunum did not translate into

262 higher VCR. In contrast, at 32°C, VCR was lower in both intestinal segments after methionine

263 supplementation. The improvement in intestinal health seen from the increase in VCR was only observed

264 at a temperature of 24°C at duodenal level. These results show that methionine supplementation in laying

265 quails under thermal stress does not reverse the deleterious effects of heat on VCR. Regarding the

266 different temperatures, the increase of this variable also led to a decrease in intestinal health, that is, lower

267 VCR.

268 These results corroborate Wu et al. [30], which report that thermal stress by heat is detrimental to

269 the integrity of the intestinal mucosa of broilers, where the villi become shorter and flatter, and

270 consequently the crypt increases its activity by becoming deeper in an attempt to reverse this situation,

271 with this the VCR decreases. According to the authors, high ambient temperature reduces feed intake of

272 birds [30], justifying the reduction in VCR at high temperature. This leads to less energy available for

273 maintenance and renewal of the intestinal mucosa and consequently for production.

274 Goblet cells (GC) play an important role in the digestive system of animals; the quantity of GC

275 even indicates the degree of intestinal health. GC produce mucus, mucin, which protects the intestinal

276 epithelium from infectious agents and mechanical agents, and forms the glycocalyx which also plays an

277 important role in intestinal digestion [31]. It is well known, as happened in the present study at the

278 duodenal level (reduction of GC at 32°C), that high temperatures decrease the quantity and production of

279 GC, thereby reducing intestinal health. Sandikciet al. [32] reported significant reduction in GC in the

280 three intestinal segments, in addition to villus height, in Japanese quails subjected to thermal stress.

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281 According to the authors, it is especially possible to relate the damage observed in intestinal mucosa,

282 including decreased GC, to low feed intake during thermal stress [33]. Unlike in the literature [32] for

283 chickens, rising temperatures did not decrease jejunum GC, perhaps because quails are more heat tolerant

284 than laying hens.

285 Methionine supplementation led to an increase in GC during thermal stress (32°C) in the jejunum.

286 These results demonstrate that methionine supplementation in heat stress reverses the deleterious effects

287 of heat on the jejunum, but not on the duodenum. The increase in GC found in the jejunum under

288 conditions that mimic thermal stress by heat provides better protection of the intestinal mucosa and better

289 digestion, leading to improved intestinal health [34], thus justifying the use of methionine

290 supplementation in this case.

291 Climate warming has been causing concern for quail farming, since, as results show, thermal

292 stress by heat promotes various alterations in behavior and physiological mechanisms of quails,

293 culminating in harmful morphological alterations, poor bird performance, and economic losses for the

294 sector [35].

Uterine Histomorphometry

296 For the first time in quails, morphometric results showed that the increase of temperature, that is,

297 thermal stress (32°C), causes decrease in the uterine folds, mainly in the secondary and tertiary folds

298 (Table 3), which implies in a smaller area for the production of calcium carbonate, the main eggshell

299 compound [3], negatively influencing the egg production of the animals. High temperatures also

300 decreased eggshell production and thickness (Table 4). The highest indexes of uterine folds were found in

301 treatments at 20 and 24°C.

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305 **Table 4. Average productive performance and eggshell thickness of laying quails submitted to**

306 **methionine supplementation at 3 levels (100, 110 and 120%) and 4 temperature ranges varying**

307 **from thermoneutrality to heat stress.**

Level x Temp	Production % 2º Period			
	20ºC	24ºC	28ºC	32ºC
100%	89.55±3.70aA	92.2±4.42aA	85.24±4.41aA	83.81±14.94aA
110%	91.67±3.40aA	91.27±6.02aA	85.32±5.21abA	76.98±11.35bA
120%	93.52±8.54abA	98.23±9.78aA	85.3±6.77bcA	74.78±6.78cA
	Shell thickness 2º Period			
	20ºC	24ºC	28ºC	32ºC
100%	0.27±0.02aA	0.25±0.01bA	0.26±0.02abA	0.25±0.01bA
110%	0.27±0.01aA	0.25±0.01aA	0.26±0.01aA	0.26±0.02aA
120%	0.27±0.01aA	0.26±0.01aA	0.27±0.01aA	0.26±0.01aA

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

310 These results corroborate the egg production results (Table 4); the highest productive performance

311 was found at 24ºC, and the lowest performance was at 32°C.

312 Although the literature mentions that methionine supplementation increases the amount of folds in

313 the uterus in layers and light birds [15-18], this was not observed when heat stress was applied in this

314 study on quails. There was an increase in the uterine fold index only at 28ºC with 110% methionine

315 supplementation; however, these results did not interfere with egg production (Table 4). We can infer that

316 methionine supplementation, except 110% at 28ºC, does not minimize the deleterious effects of heat

317 stress on uterine folds in quails.

318 ## Measurement of hepatic glycogen and steatosis storage

319 The increase in ambient temperature decreased the hepatic steatosis index, with the highest

320 indexes at 20 and 24ºC. Methionine supplementation (120%) increased this rate only at 24ºC. This result

321 differs from that found by Bunchasak et al. [14], who describe that the higher the methionine

322 supplementation the higher the fatty acid synthesis in laying hens, and thus the higher the rate of hepatic

323 steatosis. This variable is important since the increase in hepatic steatosis is related to estrogen

324 production, that is, the higher the steatosis, the higher the estrogen production and the higher the egg

15

325 production [14]. Thus, methionine supplementation (120%) at 24ºC would not only increase hepatic

326 steatosis but also egg production by these quails, which occurred in the present study, since the increase

327 in hepatic steatosis was reflected in a significant increase in production (Table 4). In contrast, birds

328 subjected to a temperature of 20ºC, despite not showing an increase in steatosis, had increased egg

329 production (Table 4).

330 Hepatic glycogen levels did not appear to change with the alteration in ambient temperature,

331 corroborating studies on broilers by Lana et al. [36]. However, these studies were contrary to the results

332 for broilers, which showed decreased hepatic glycogen stores, as well as reduced feed intake and weight

333 gain [37], and reduced liver weight [38] with consequent reduction in metabolic activity under thermal

334 stress.

335 However, only at 24ºC, 110% methionine supplementation increased hepatic glycogen stores.

336 These results demonstrate that at thermoneutrality, 110% supplementation maximizes energy storage in

337 the form of glycogen in the liver. Such a surplus can be transferred to production, in this case in egg

338 production. Thus, in heat stress methionine supplementation was not efficient.

Immunohistochemistry

340 In modern strains of laying hens, which can be extrapolated to laying quails, the equivalent of

341 10% of total body calcium is transferred daily for deposition as eggshells [39-41]; the major sources of

342 calcium are through absorption from the diet at the intestinal level, renal resorption, and bone storage.

343 Since calbindin-k28D is the carrier responsible for the absorption of calcium from the digestive system, it

344 would have the ability to modulate its deposition in the womb [46], in addition to intestinal absorptive

345 capacity [45], influencing the production and the eggshell quality.

Intestine

347 For all treatments (temperatures and methionine levels), anti-calbindin-k28D was positive

348 throughout the duodenal epithelium; the lamina propria (connective tissue layer below the epithelium)

349 was not positive (Fig 1). Positivity was more intense in the basal and more apical portion of the

16

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

376 increase in methionine must also have minimized the deleterious effect of heat stress and increased

377 calcium availability to improve production rates, as occurred in the present study. It can be imagined that

378 in this study the positivity of calbindin-D28k must also have decreased.

379

380 **Fig 1. Photomicrographs of anti-calbindin-D28k immunohistochemistry in laying quail intestine at**

381 **different magnifications**. Positive anti-calbindin-D28k intestinal epithelium (arrows) and non-positive

382 goblet cells (arrowheads) (A and B) are observed. Non-antibody-positive crypts (asterisk) are also

383 observed (C). Lower epithelial positivity is observed under 28°C (D) when compared to other

384 temperature treatments. Chromogen staining diaminobenzidine+hematoxylin.

385

386 High temperature stress negatively affects laying performance, decreasing feed intake, live weight

387 gain and efficiency [48,49]. It also decreases egg production and eggshell quality and thickness [50-53]

388 due to decreased availability of calcium ions [53]. It is important to say that increasing dietary calcium

389 does not improve the quality of the shell in heat stress conditions [54,55]. High temperature heat stress

390 decreases the presence of calbindin-D28k in the ileum, cecum, colon, and uterus of birds, causing

391 deterioration of eggshell quality [56]. However, in this study we observed that the effect on the duodenum

392 is the opposite, heat stress decreases the positivity to calbindin-D28k.

393 In the present study, at temperatures considered to be of higher thermal comfort for the quails (24

394 and 28ºC), these animals presented lower positivity of the cellular calcium transport in their duodenal

395 epithelia, exactly because they were in better thermal comfort, and did not need a higher absorption of

396 calcium (Fig 2). Literature provides studies [57] that show that the higher the ambient temperature and

397 thermal stress, the greater the need to supplement dietary calcium, as the animals will need more calcium

398 for their metabolism, since the thermal stress decreases the availability of calcium [53]. Within the 24 and

399 28ºC treatments, methionine supplementation provided even lower positivity for anti-calbindin-k28D

400 when compared to normal levels. The gene expression of this same gene followed a similar model in

401 gross values; however, these results were not significant (Fig 5), perhaps due to the small sample size

402 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.

427   The most antibody positive areas were exactly the areas of DCT around large vessels in the renal

428   cortex. This feature can be explained by the fact that these areas have blood with a higher amount of

429   calcium, which has not yet been reabsorbed.

430   The positivity is lower at 24 and 28ºC compared to at 20 and 32ºC. In the treatment at 32ºC, the

431   amount of positive DCT increased, always in greater numbers near the great renal veins (Fig 2). At 28

432   and 32ºC, DCT were more positive when compared to previous temperatures.

433

434   **Fig 2. Photomicrographs of anti-calbindin-D28k immunohistochemistry on kidney of quail laying at**

435   **different temperatures.** A) 20°C: Positivity occurs mainly in distal contorted tubules near large vessels

436   (asterisks). B and C) 24 and 28ºC: Lower antibody positivity. D) 32°C: There are more positive distal

437   contorted tubules and slightly positive proximal contorted tubules as well. More positivity (brown color)

438   is observed at temperatures 20 and 32°C. Chromogen staining diaminobenzidine+hematoxylin.

439   Magnification 100x.

440

441   In the treatments in which the animals are theoretically in greater thermal comfort, that is, in the

442   24 and 28ºC treatments, the anti-calbindin-d28k positivity was lower. Since stress by high temperatures

443   negatively affects the performance of layers [48, 49] due to decreased availability of calcium ions [53], it

444   is expected that the rate of renal resorption will have to increase under stress. Thus, animals in thermal

445   comfort would have less need to reabsorb large amounts of calcium, as occurred in treatments with 24

446   and 28ºC (lower positivity). In animals with some degree of thermal stress, such as at 32ºC (high

447   temperature) and at 20ºC, theoretically because it is a temperature below the thermal conformation for the

448   species, they would have greater need to reabsorb more calcium (higher positivity).

449   Although it is well known that thermal stress by high temperatures decreases the presence of

450   calbindin-D28k in the ileum, cecum, colon and uterus of birds, causing deterioration of eggshell quality

451   [56], there was no information in literature on the influence of this calcium transport at renal level for

452   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

478   eggshell quality. The epithelium (ciliated pseudostratified) is not positive for anti-calbindin-D28k except

479   for a thin layer on the apical portion of this epithelium.

480   The positivity pattern was higher at 24 and 28°C, lower in the treatment in which the animals were

481   submitted to 32°C, and intermediate at 20°C (Fig 4). Methionine supplementation by 120% increased

482   anti-calbindin-D28k positivity.

483

484   **Fig 4. Photomicrographs of anti-calbindin-D28k immunohistochemistry in uterus of laying quails at**

485   **different temperatures (20, 24, 28 and 32°C) and supplemented with 120% methionine at 32°C.** A)

486   20°C: observe lower positivity in the uterine glands (asterisk). B) 24°C: observe greater positivity in the

487   uterine glands (asterisk). C) 28°C: observe greater positivity in the uterine glands (asterisk). D) 32°C:

488   observe lower positivity in the uterine glands (asterisk). E) 32°C: supplemented with 120% methionine.

489   More positive than treatment without methionine supplementation. Arrowheads (uterine epithelium),

490   asterisks (uterine glands). DAB + hematoxylin chromogen staining. 400x magnification.

491

492   Ebeid et al. [56] state that under conditions of thermal stress by high temperature there is a

493   decrease in the presence of calbindin-D28K in the uterus of laying hens, which corroborates the present

494   study, in which the anti-calbindin-D28K positivity was lower at 32°C. This is the first time this fact is

495   observed in quails. Stress by high temperatures is already known to negatively affect the performance of

496   layers [48,49] due to decreased availability of calcium ions [53]. This can be explained by heat stress

497   reducing the conversion of vitamin D3 to its metabolically active form, 1.25 (OH) 2D3, which is essential

498   for calcium absorption and utilization. In fact, the calcium requirement for layers increases with high

499   ambient temperatures [57].

500   Methionine supplementation at high temperatures (32°C) promoted increased positivity in the

501   uterine glands (Fig 5) reversing part of the deleterious effect of thermal stress. The increase in positivity

502   of this transport under thermal stress conditions possibly increased uterine gland calcium carbonate

503   excretion for eggshell production, improving egg quality, although not reaching thermal comfort values

504 (Table 4). Given these facts, methionine supplementation is recommended under thermal stress

505 conditions.

506   The fact that the positivity of the 20°C treatment was intermediate shows that perhaps for quails,

507 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

509   TRPV6 acts as an epithelial channel of calcium in organs and glands that are characterized by high

510 demand for calcium transport [58-60]. According to some studies [61,62], this ion channel exerts a

511 facilitator effect on calcium entry into epithelial cells, expressed in the intestinal and kidney absorption

512 and resorption epithelia, but there is still little information about its expression pattern in laying hens [19],

513 and none in laying quails. Calbindin, in turn, has been described in studies in its two main forms,

514 Calbindin-D9k (low molecular weight protein) present in mammal intestines, and Calbindin-D28k (high

515 molecular weight protein) in kidney, brain and intestine and uterus of birds [20,42] and kidney of

516 mammals [20].

517   Calbindin-D28k gene expression (Fig 5) in the kidneys of laying quails without methionine

518 supplementation was higher at 28ºC, a temperature that is still within thermoneutrality. With methionine

519 supplementation, the highest expression was at 24ºC. By comparing the expression of this transport

520 within each temperature (supplemented but not with methionine), it is possible to say that methionine

521 supplementation only increases Calbindin-D28k gene expression at 24ºC (thermoneutrality), that is, by

522 supplementing methionine we can maximize calcium reabsorption at the renal level, which can increase

523 egg production by producing thicker eggshells in less time. However, under conditions of heat stress,

524 supplementation is not effective in reducing the deleterious effects of heat, at least for this gene at the

525 renal level.

526   For the TRPV6 ion channel gene in the same organ, the highest gene expression in animals

527 without supplementation occurred at a temperature of 20ºC, already mentioned as a temperature below

528 thermal comfort for laying quails. In animals submitted to methionine supplementation, the highest peak

529 of gene expression occurred at a temperature of 24ºC (thermoneutral), coinciding with the result obtained

530 in the calbindin gene, followed by a temperature of 32ºC. Unlike calbindin, when comparing within each

531 temperature (supplemented and not), we see that methionine supplementation increased TRPV6 gene

532 expression, not only at 24ºC but also at 32ºC, that is, under thermal stress conditions. This result

533 demonstrates physiologically and technically validates the use of methionine supplementation for laying

534 quails in cases of thermal stress, and its effectiveness in minimizing the deleterious effects of high

535 temperatures. Such an increase in this gene increases calcium reabsorption, making more of the mineral

536 available for egg production, specifically in the release by the uterus for eggshell production.

537

538 **Fig 5. Graphs of the effects of methionine supplementation (100% and 120%) at different**

539 **temperatures** on the Calb 28 (A) and TRPV6 (B) genes expressions in the kidneys; Calb 28 (C) and

540 TRPV6 (D) in the intestine; and Calb 28 (E) and TRPV6 (F) in the uterus of Japanese quails (*Coturnix*

541 *japonica*) in production phase. [a,b,A,B] Averages followed by the same lower case letter for 100%

542 supplementation and upper case for 120% supplementation do not differ from each other by the SNK test

543 by up to 5% error probability; **,* and [ns] Indicate, respectively, significant differences up to 1%, up to

544 5% and not significant by the F test.

545

546 These results corroborate studies that already cited the gene expression of both genes (TRPV6 and

547 Calbidin-D28k) in kidney tissue of the laying birds [20,42], and still stands out for being the first to

548 demonstrate the positive expression of their gene expression, in the kidneys of laying quails.

549 In the intestine there was gene expression for both genes in all treatments, corroborating another

550 study regarding the presence of calbindin-D28k in layers [63]. This is also the first study to demonstrate

551 TRPV6 gene expression in intestines of laying quails. However, it was not possible to observe gene

552 alteration of Calbindin-D28k or TRPV6 with increasing temperature, nor with methionine

553 supplementation. These results imply that there is no alteration in the absorption or cellular calcium

554 transport during thermal stress, nor is there any improvement with methionine supplementation.

555   The TRPV6 gene, when compared to calbindin, showed little expressiveness in intestinal tissue in

556   all the treatments, however, contradicting and filling in the gap left by some authors [42,63-64], who

557   claim that the presence of TRPV6 is still uncertain in birds, including layers or quails. Although the

558   difference was not significant due to the high standard deviation, high temperatures seemed to decrease

559   intestinal TRPV6 gene expression.

560   For uterine tissue, Calbindin-D28K and TRPV6 gene expression also occurred in all treatments,

561   and as for intestine, it was poor for TRPV6. Temperature increase and methionine supplementation did

562   not influence gene expression of calbindin-D28k or TRPV6. Thus, the uterine tissue is not altered under

563   these conditions either, thus not justifying the use of methionine supplementation.

564   The results described and observed in this study show the gene expression of TRPV6 and

565   Calbindin-D28k genes in the renal, intestinal and uterine tissues of laying quails. Corroborating studies in

566   laying hens [45-47] indicating that calbindin-D28k would modulate the intestinal calcium absorption and

567   deposition capacity in the uterus, influencing eggshell production and quality, as well as the significant

568   presence of TRPV6 (protein and mRNA) in the intestines and kidneys of layers [19].

569   Through analysis of variance, it was possible to verify the interaction between temperature and

570   supplementation for both genes in the kidney ($p \leq 0.01$), supplementation for calbindin-D28k in the

571   intestine ($p \leq 0.01$) and temperature for TRPV6 in the uterus (Table 5).

572   **Table 5. Analysis of Variance Summary (Mean Squared) for the effects of different temperatures**

573   **and methionine supplementation (100% and 120%) on *Calb* 28 and TRPV6 gene expressions in the**

574   **kidneys, intestine and uterus of Japanese quails (*Coturnix japonica*) in the production stage**

Variation source	Gl	Kidney		Intestine		Uterus	
		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

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

576   In addition, the responses of calbindin-D28k and TRPV6 in the kidney, intestine and uterus of

577   Japanese quails supplemented with methionine + cystine (100% and 120%) at different temperatures

578 showed a strong positive correlation (r = 0.90*) in the kidneys and moderately positive (r = 0.69 *) in the

579 intestines between Calb 28 and TRPV6 gene expressions (Fig 6), indicating that both calbindin-D28k and

580 TRPV6 (mRNA) act synergistically, modulating resorptive (kidney) and absorptive (intestine) capacity,

581 and subsequent calcium deposition by the uterus. The correlations between the other variables were weak

582 and not significant.

583

584 **Fig 6. Pearson correlation for alterations in Calb 28 and TRPV6 gene expressions in the rim,**

585 **intestine and uterus of Japanese quail (*Coturnix japonica*) supplemented with methionine (100%**

586 **and 120%) at different temperatures in the production phase.**

587

Conclusion

589 For the first time, this study brings histomorphological and expression variations (mRNA and

590 protein) of TRPV6 and Calbindin-D28k in organs involved with absorption, reabsorption and calcium

591 deposition in quails. It still physiologically justifies the use of methionine supplementation (120%) in

592 thermal stress, since it reduces the deleterious effect on intestine, kidney and uterus parameters, besides

593 improving others in the same organs under thermoneutrality conditions.

References

595 1. Instituto Brasileiro de Geografia e Estatística, Produção da Pecuária Municipal, Rio de Janeiro.

596 2016;44:29-31.

597 2. Pereira DF, Vale MM, Zevolli BR, Salgado DD. Estimating mortality in laying hens as the

598 environmental temperature increases. Rev Bras Cienc Avic. 2010;12:265-271.

599 3. Albino LFT, Carvalho BR, Maia RC, Barros VRSM. Galinhas Poedeiras: Criação e Alimentação.

600 Viçosa, Minas Gerais: Aprenda Fácil; 2014.

601 4. Carvalho LSS. Nutrição de poedeiras em clima quente. Rev Cient Eletrônica Med Vet. 2012;9(18).

602 5. Mello JF. Influência dos níveis de cálcio e fósforo na dieta de matrizes de codornas japonesas, no

603 desempenho produtivo e no desenvolvimento ósseo embrionário da progênie [dissertation]. Maringá

604 (PR): Universidade Estadual de Maringá; 2015.

605 6. Nascimento DCN, Sakomura NK, Siqueira JC. Exigências de metionina + cistina digestível para aves

606 de corte ISA Label criadas em semiconfinamento. R Bras Zootec. 2009;38(5):869-78.

607 7. Virden WS, Kidd MT. Estresse fisiológico em frangos de corte: Ramificações na digestibilidade e

608 respostas de nutrientes. J Appl Poult Res 2009;18(2):338-47.

609 8. Alagawany M, Farag MR, El-Hack MA & Patra A. Heat stress: effects on productive and reproductive

610 performance of quail. Worlds Poult Sci J. 2017;73(4):747-56.

611 9. D'Mello JPF. Amino Acids in Animal Nutrition. In: Felix JP, editors. Formerly of the Scottish

612 Agricultural College Edinburgh. 2th ed. Edinburgh: CABI, 2003. P.143.

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

614 11. Summers JD, Atkinson JL, Spratt D. Supplementation of a low protein diet in an attempt to optimize

615 egg mass autput. Can J Anim Sci. 1991;71:211-20.

616 12. Harm RH, Russell GB. Optimizing egg mass with amino acid supplementation of a low-protein diet.

617 Poult Sci. 1993;72:1892-6.

618 13. Bunchasak C, Santoso U, Tanaka K, Ohtni S, Collado CM. The effects of supplementing methionine

619 plus cystine to a low-protein diet on the growth performance and fat accumulation of growing broiler

620 chicks. Asian-Australas J Anim Sci. 1997;10(2):185-191.

621 14. Bunchasak C, Silapasorn T. Effects of adding methionine in low-protein diet on production

622 performance, reproductive organs and chemical liver composition of laying hens under tropical

623 conditions. Int J Poult Sci. 2005;4(5):301-8.

624 15. Júnior JPF. Níveis de metionina+cistina digestíveis para aves de reposição leves nas fases inicial e

625 cria [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2013.

626 16. Lopes GM. Biodisponibilidade relativa e níveis de metionina para frangos de corte. [dissertation].

627 Areia (PB): Universidade Federal da Paraíba; 2014.

628   17. Pinheiro SG. Relação energia metabolizável: aminoácidos sulfurosos para aves leves. [dissertation].

629   Areia (PB): Universidade Federal da Paraíba; 2014.

630   18. Santana MHM. Níveis de metionina+cistina para galinhas poedeiras leves nas fases de recria e

631   postura. [dissertation]. Areia (PB): Universidade Federal da Paraíba; 2014.

632   19. Yang JH, Hou JF, Farquharson C, Zhou ZL, Deng YF, Wang L, Yu Y. Localisation and expression of

633   TRPV6 in all intestinal segments and kidney of laying hens. Br Poult Sci. 2011;52(4):507-16.

634   20. Fleet JC, Schoch RD. Molecular mechanisms for regulation of intestinal calcium absorption by

635   vitamin D and other factors. Crit Rev Clin Lab Sci. 2010;47(4):181-95.

636   21. Ramos AH, Santos LM, Miglino MA, Peres JA, Guerra R R. Biometria, histologia e morfometria do

637   sistema digestório do cachorro-do-mato (*Cerdocyon thous*) de vida livre. Biotemas. 2011;24(4):111-9.

638   22. Ishak K, Baptista A, Bianchi L, Callea F, de Groote J, Gudat F, et al. Gradação histológica e

639   estadiamento da hepatite crônica. J Hepatol. 1995;22(6):696-9.

640   23. Pfaffl MW. Um novo modelo matemático para quantificação relativa em RT-PCR em tempo

641   real. Pesquisa de ácidos nucleicos. 2001;29(9):e45

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

643   25. Mitchell MA, Carlisle AJ. The effects of chronic exposure to elevated environmental temperature on

644   intestinal morphology and nutrient absorption in the domestic fowl (*Gallus domesticus*). Comp Biochem

645   Physiol A Comp Physiol. 1992;101:137-142.

646   26. Lin H, Jeao HC, Buyse J, Decuypere E. Strategies for preventing heat stress in Poultry. Worlds Poult

647   Sci J, Ithaca. 2006;62(1):71-86.

648   27. Marchini CFP, Silva PL, Nascimento MRBM, Beletti ME, Guimarães EC, & Soares HL. Morfometria

649   da mucosa duodenal em frangos de corte submetidos à temperatura ambiente cíclica elevada. Arq Bras

650   Med Vet Zootec. Belo Horizonte. 2009;61(2):491-7.

651   28. Araújo LF, Junqueira OM, Lopes EL, da Silva Araújo CS, Ortolan JH, & de Laurentiz AC. Utilização

652   de levedura desidratada (*Saccharomyces cerevisiae*) para leitões na fase inicial. Cienc Rural.

653   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 [dissertion]. Jaboticabal (SP): Universidade Estadual de São Paulo; 2005.

680   38. Plavnik I, Yahav S. Efeito da temperatura ambiente em frangos submetidos a restrição de crescimento

681   em idade precoce. Avicultura. 1998;77(6):870-2.

682   39. Miller SC, editor. Calcium homeostasis and mineral turnover in the laying hen. Proceedings of the

683   Poultry Science Symposium. 1992.

684   40. Sugiyama T, Kusuhara S. Avian calcium metabolism and bone function. Asian-Australas J Anim Sci.

685   2001;14:82–90.

686   41. Sugiyama T, Kikuchi H, Hiyama S, Nishizawa K, Kusuhara S. Expression and localisation of

687   calbindin D28k in all intestinal segments of the laying hen. Br Poult Sci. 2007;48(2):233-8.

688   42. Bar A. Calcium transport in strongly calcifying laying birds: mechanisms and regulation. Comp

689   Biochem Physiol A Comp Physiol. 2008;152,447–69.

690   43. Fullmer CS, Brindak ME, Bar A, Wasserman RH. The purification of calcium-binding protein from

691   the uterus of the laying hen. Proc Soc Exp Biol Med. 1976;152(2):237-41.

692   44. Parmentier M. Calbindin D28k is essentially located in the colonic part of the toad intestine. Biol Cell.

693   1990;68(1):43-9.

694   45. Saki AA, Tivey DR. Immunohistochemical detection of calbindin D28k and oestrogen receptor in the

695   small intestine of pre – and post – lay hens. Proc Aust Poult Sci. 1997;9.

696   46. Corradino RA, Smith CA, Krook LP, Fullmer CS. Tissue-specific regulation of shell gland calbindin

697   D28K biosynthesis by estradiol in precociously matured, vitamin D-depleted chicks. Endocrinology.

698   1993;132(1):193-8.

699   47. Wu JC, Smith MW, Mitchell MA, Peacock MA, Turvey A, Keable SJ. Enterocyte expression of

700   calbindin, calbindin mRNA and calcium transport increases in jejunal tissue during onset of egg

701   production in the fowl (*Gallus domesticus*). Comp Biochem Physiol Comp Physiol. 1993;106(2):263-9.

702   48. Donkoh A. Ambient temperature: a factor affcting perfomance and pysiological response of broiler

703   chickers. Int J Biometorol. 1989;33:259-65.

704   49. Siegel HS. Stress, strains and resistance. Br Poult Sci. 1995;36:3-22.

705   50. Deaton JW, Reece FN, Mcnaughton JL, Lott BD. Effect of differing temperature cycles on egg shell

706   quality an layer performance. Poult Sci. 1981;60:733-737.

707   51. Emery DA, Vohra D, Ernst RA. The effect of cycle and constant ambient temperature on feed

708   consumption, egg production, egg weight, and shell thickess of hens. Poult Sci. 1984;63:2027-35.

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

710   53. Mack LA, Felver-Gant JN, Dennis RL. Genetic variations alter production and behavioral responses

711   following heat stress in two strains of laying hens. Poult Sci. 2013;92:285-94.

712   54. De Andrade AN, Rogler JC, Featherston WR, Alliston CW. Interrelationships between diet and

713   elevated temperatures (cyclic and constant) on egg production and sheel quality. Poult Sci. 1977;56:1178-

714   88.

715   55. Tanor MA, Lesson S, Summers JD. Effect of heat stress and diet composition on performance of

716   white leghorn hens. Poult Sci. 1984;63:304-310.

717   56. Ebeid TA, Suzuki T, Sugiyama T. High ambient temperature influences eggshell quality and

718   calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. Poult Sci.

719   2012;91(9):2282-7.

720   57. Plavnik I, editor. Nutrição de aves em climas quentes. Proceedings ot the Conferência Apinco; 2003.

721   p. 235-245.

722   58. Christakos S, Dhawan P, Porta A, Mady LJ, & Seth T. Vitamin D and intestinal calcium absorption.

723   Mol Cell Endocrinol. 2011;347(1-2):25-9.

724   59. Nijenhuis T, Hoenderop JG, Van Der KAW, Bindels RJ. Localization and regulation of the epithelial

725   Ca2+ channel TRPV6 in the kidney. J Am Soc Nephrol. 2003;14:2731–40.

726   60. Hoenderop Joost GJ, Nilius Bernd, Bindels René JM. Calcium absorption across epithelia. Physiol

727   Rev. 2005;85(1):373-422.

728   61. Brown AJ, Krits I, Armbrecht HJ. Effect of age, vitamin D, and calcium on the regulation of rat

729   intestinal epithelial calcium channels. Arch Biochem Biophys. 2005;437:51–8.

730   62. Bianco SD, Peng JB, Takanaga H, Suzuki Y, Crescenzi A, Kos CH, et al. Marked disturbance of

731   calcium homeostasis in mice with targeted disruption of the Trpv6 calcium channel gene. J Bone Miner

732   Res. 2007;22(2):274-85.

733   63. Wasserman RH, Taylor AN. Vitamin D-induced calcium-binding protein in chick intestinal mucosa.

734   Science. 1966;152:791–3.

735   64. Qin X, Klandorf H, Porter DW, Holt SB, Martin WG. Estrogen enhancement of calcium, magnesium,

736   and calcium-magnesium stimulated adenosine triphosphatase activity in the chick shell gland. Gen Comp
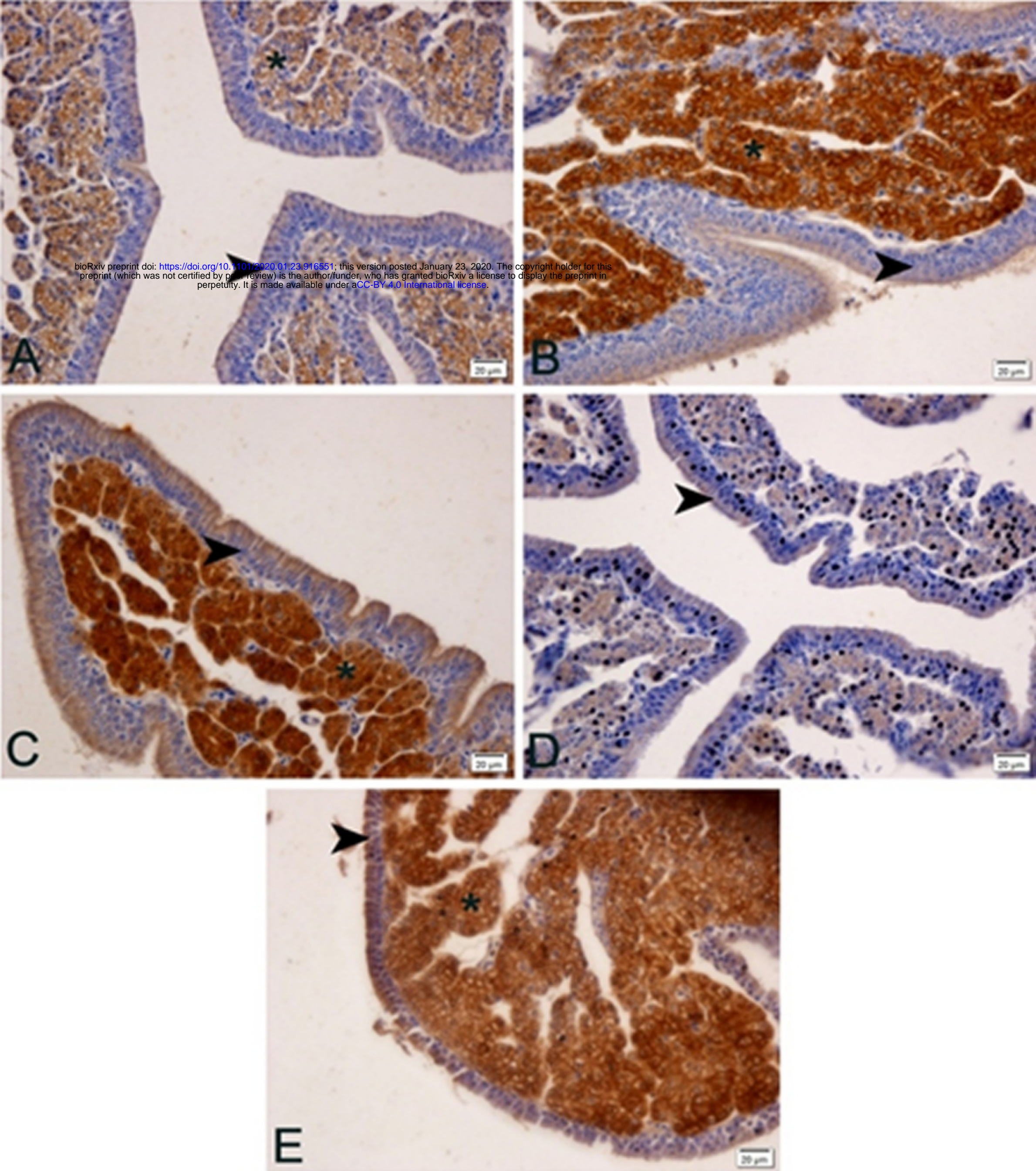
737   Endocrinol. 1993b;89:4–10.
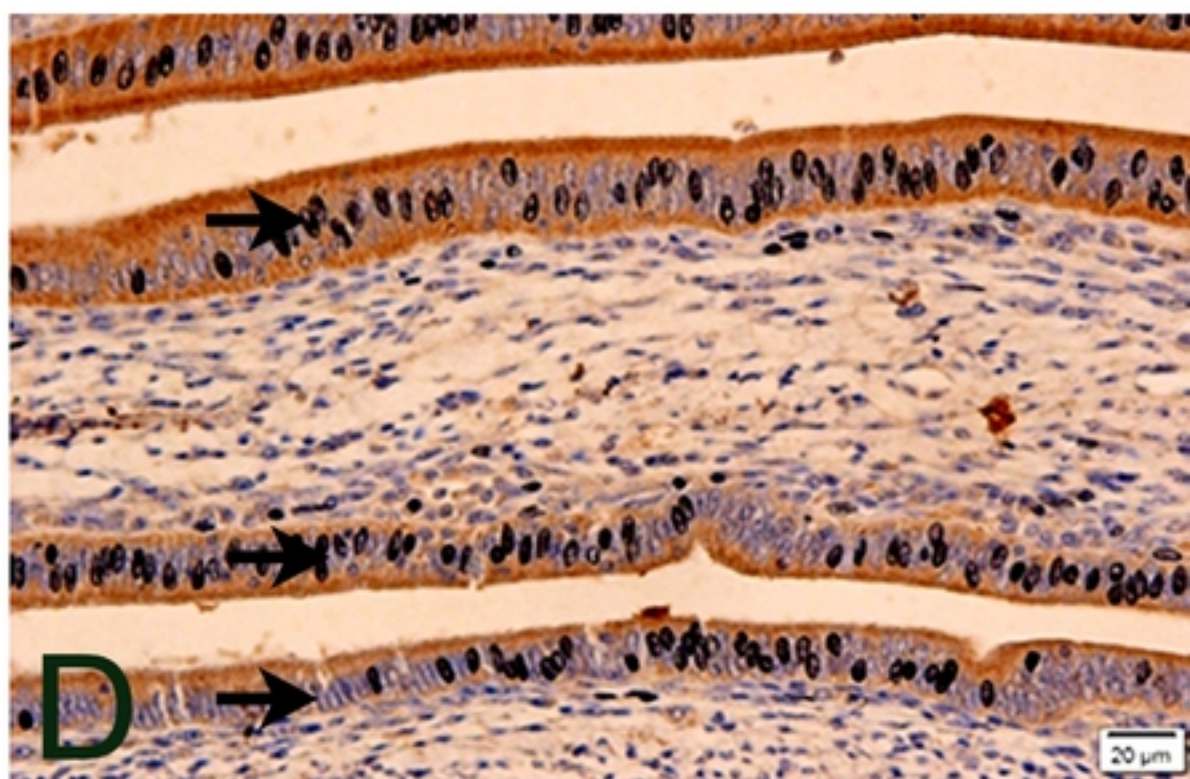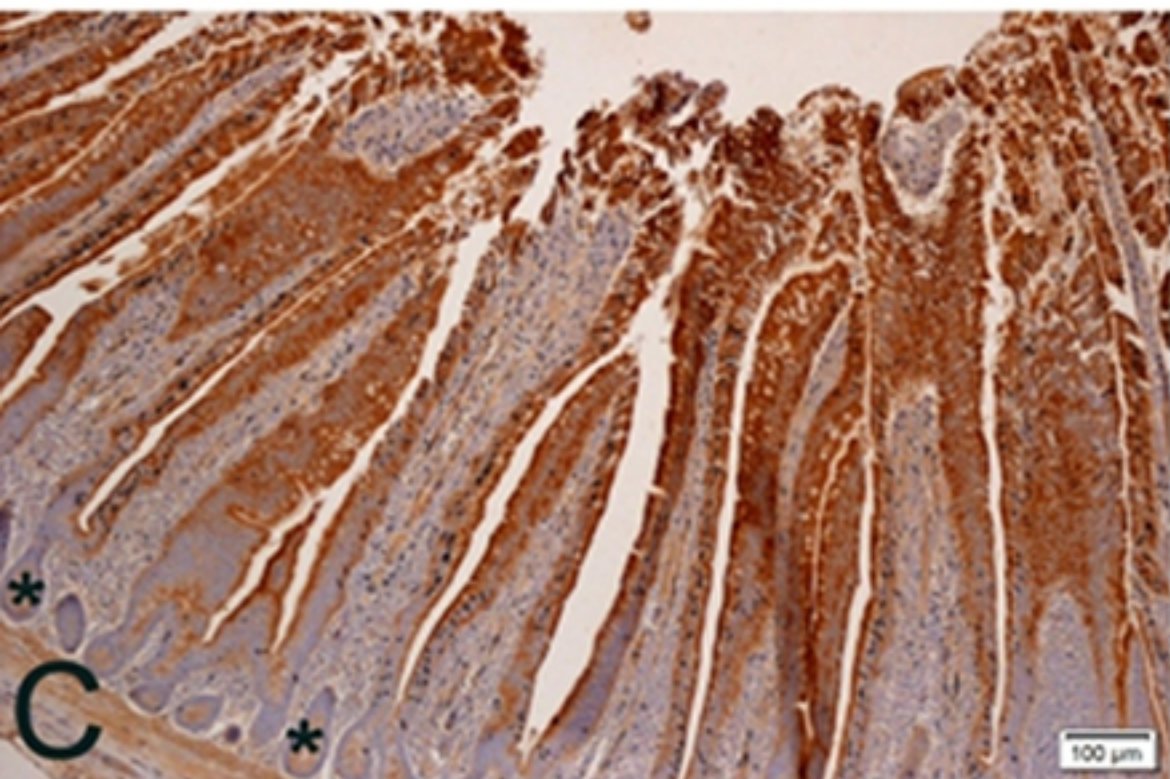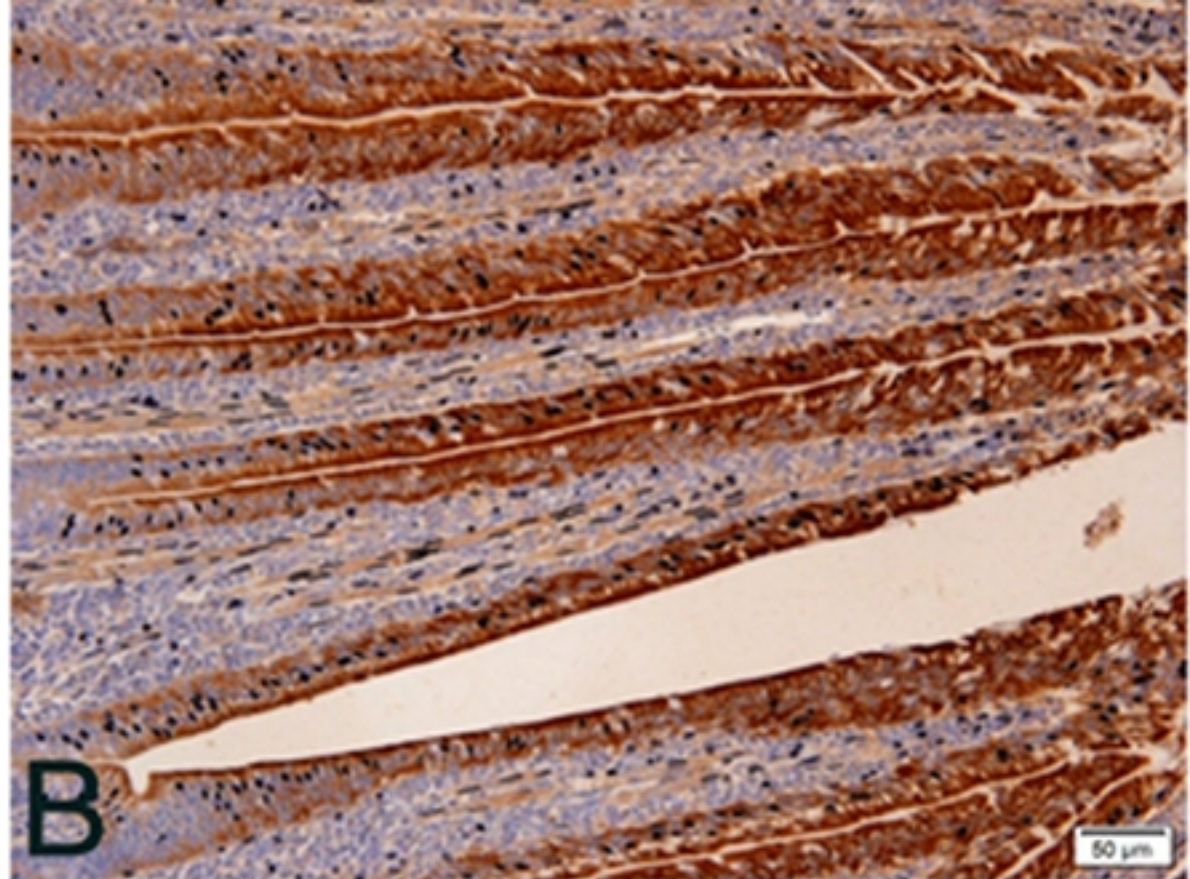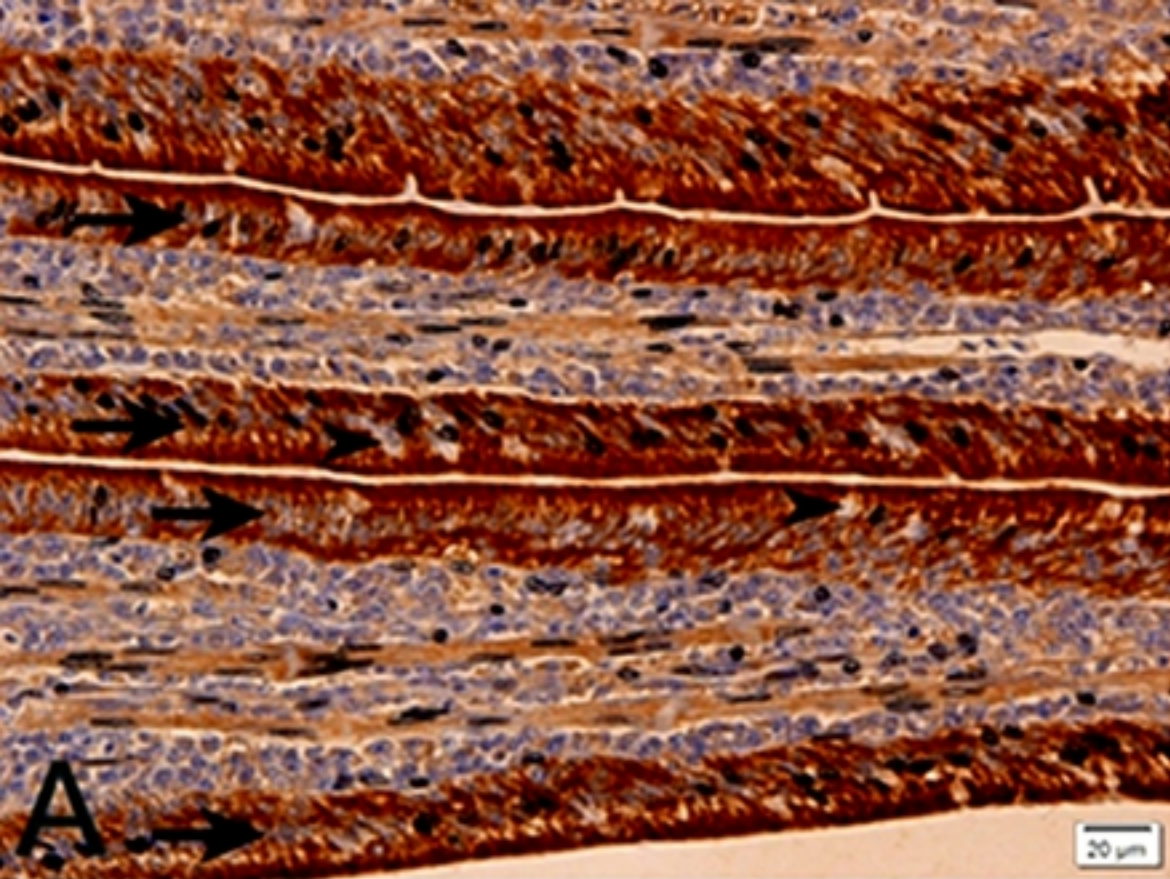
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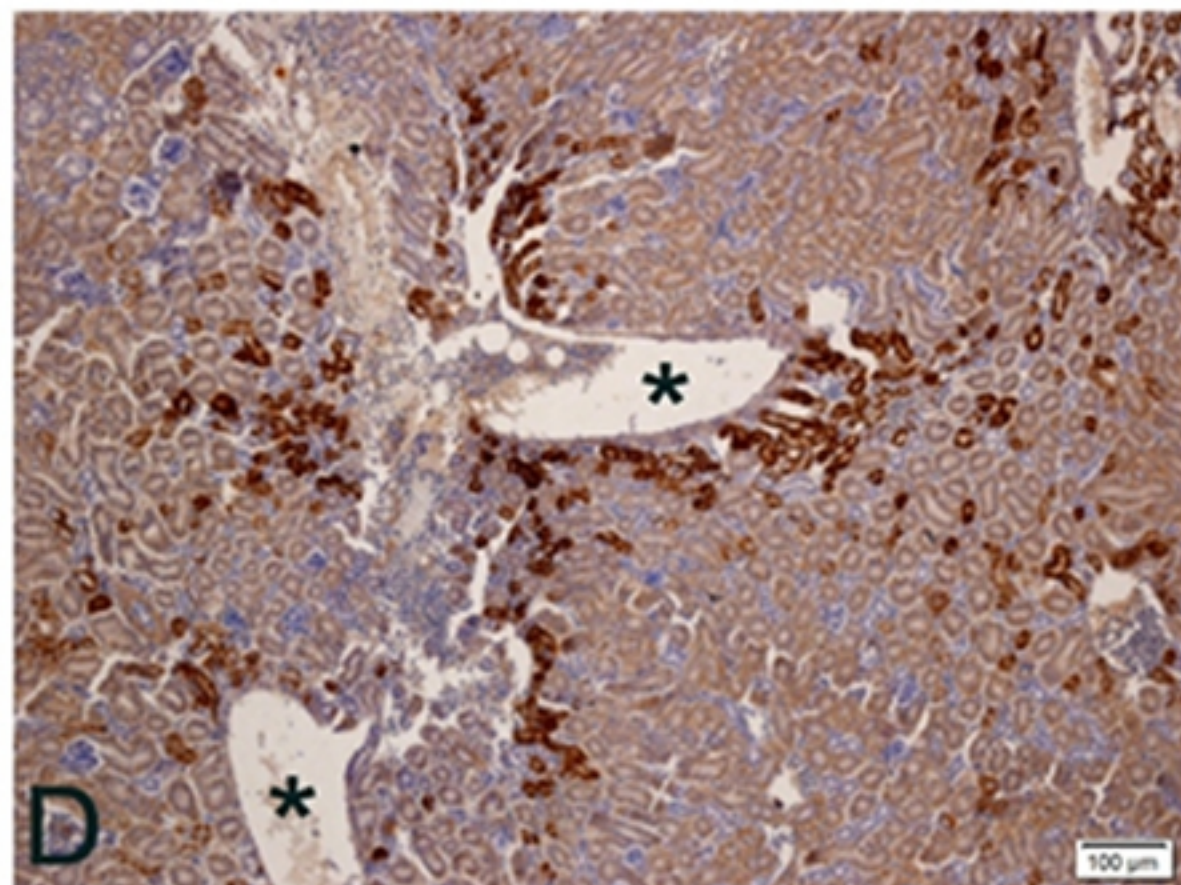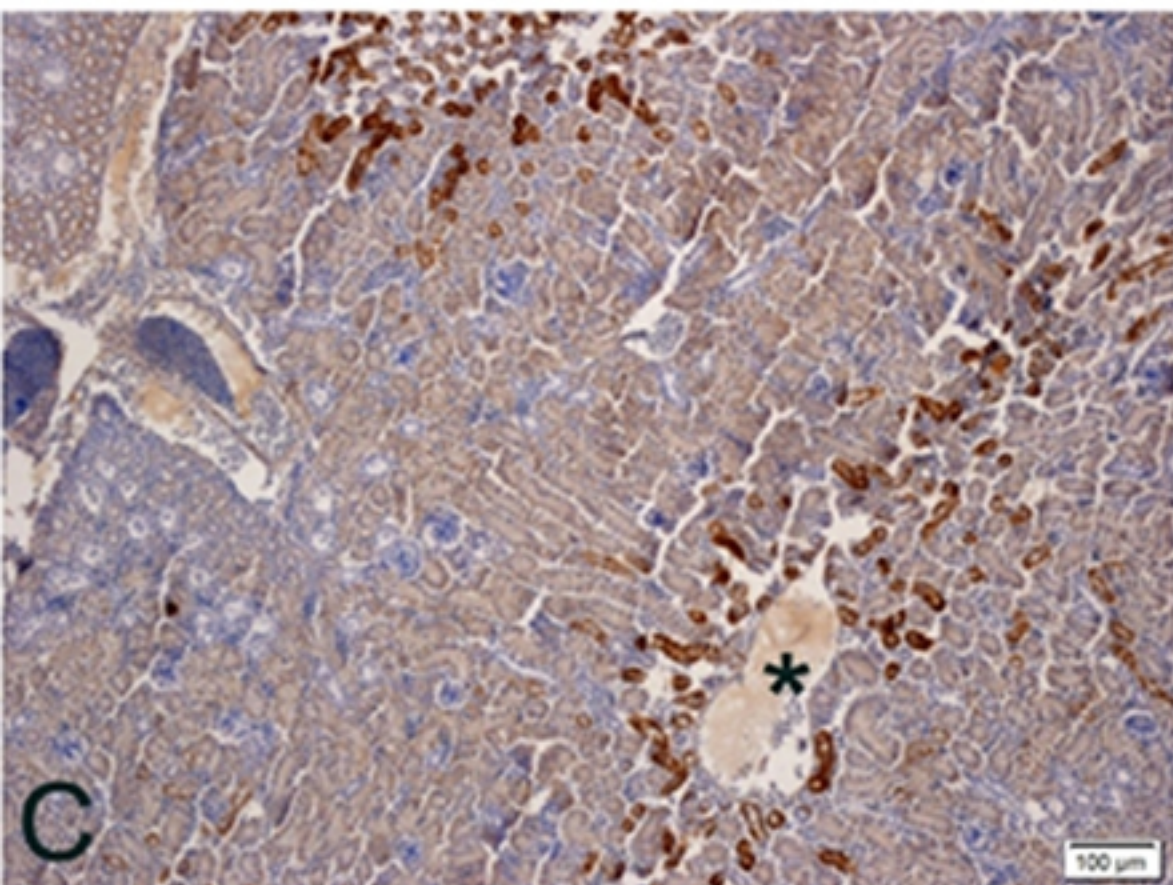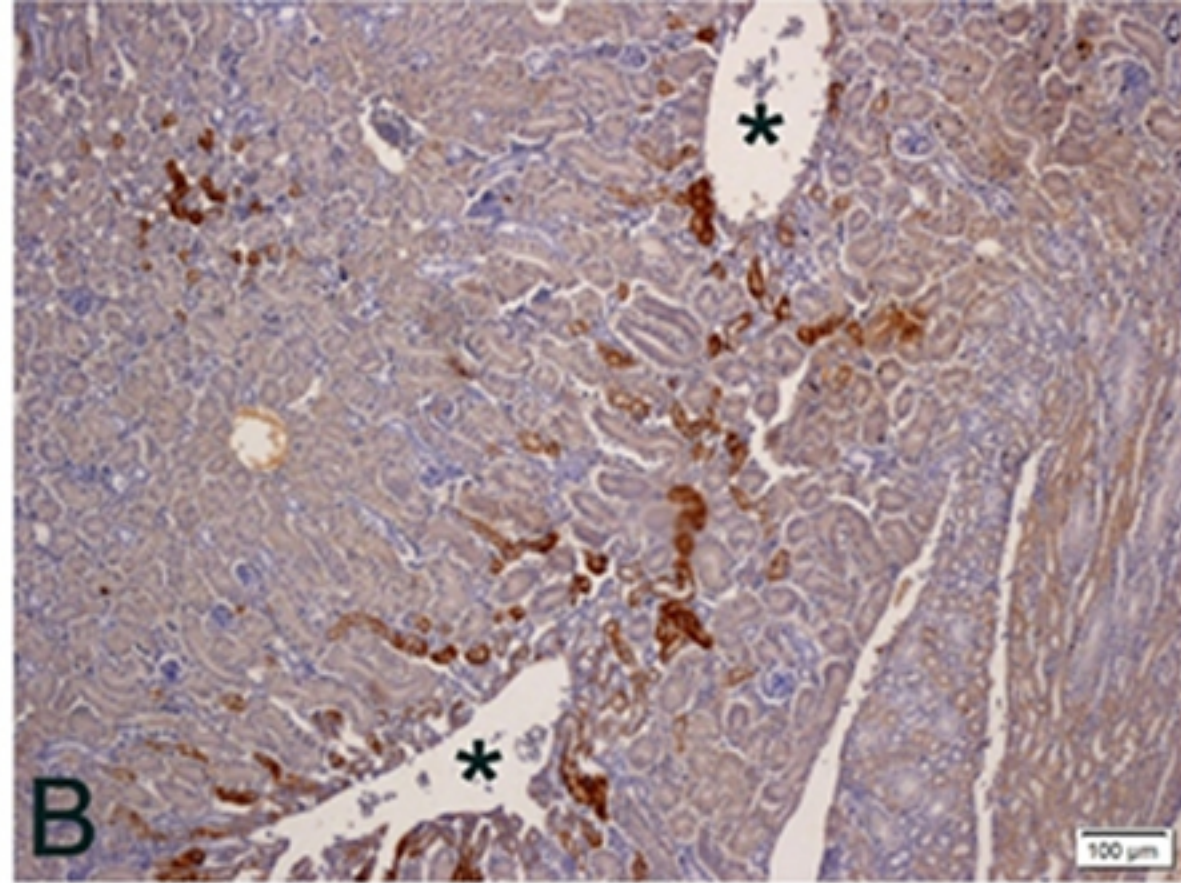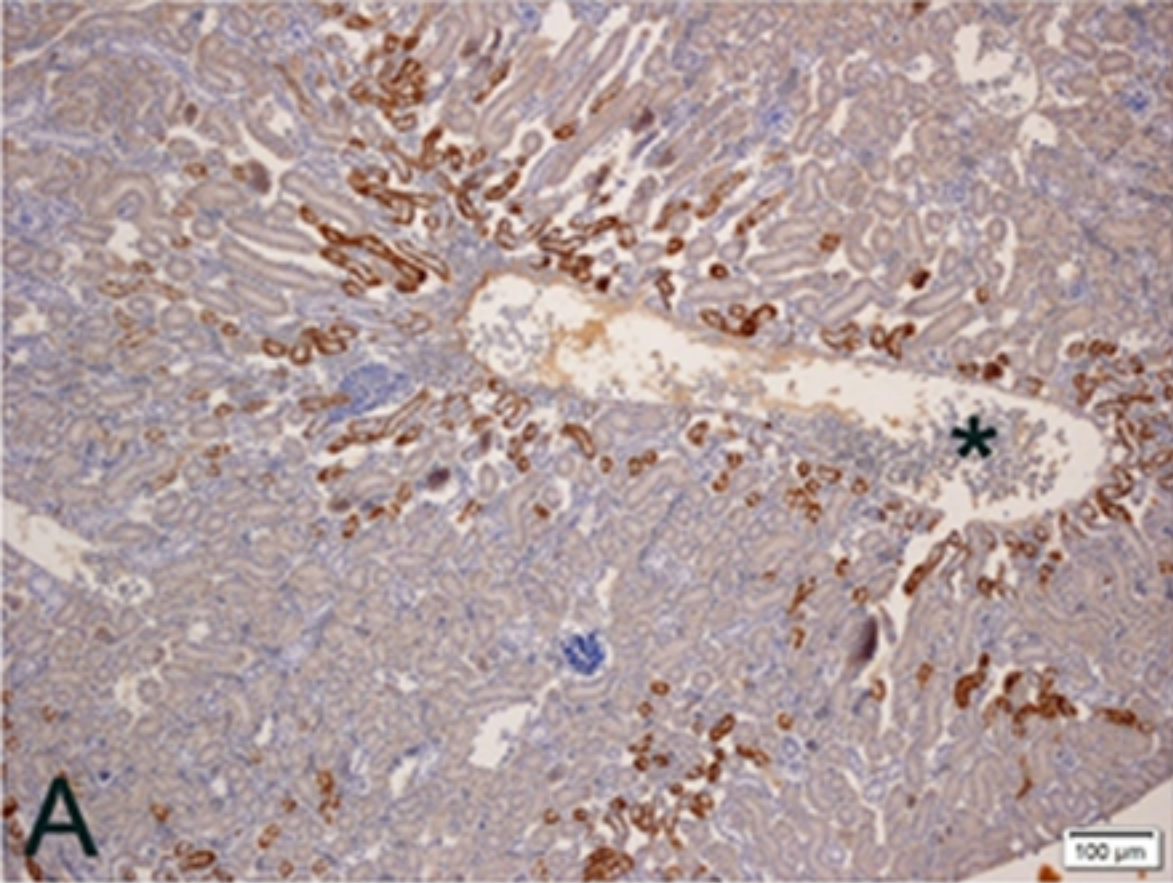
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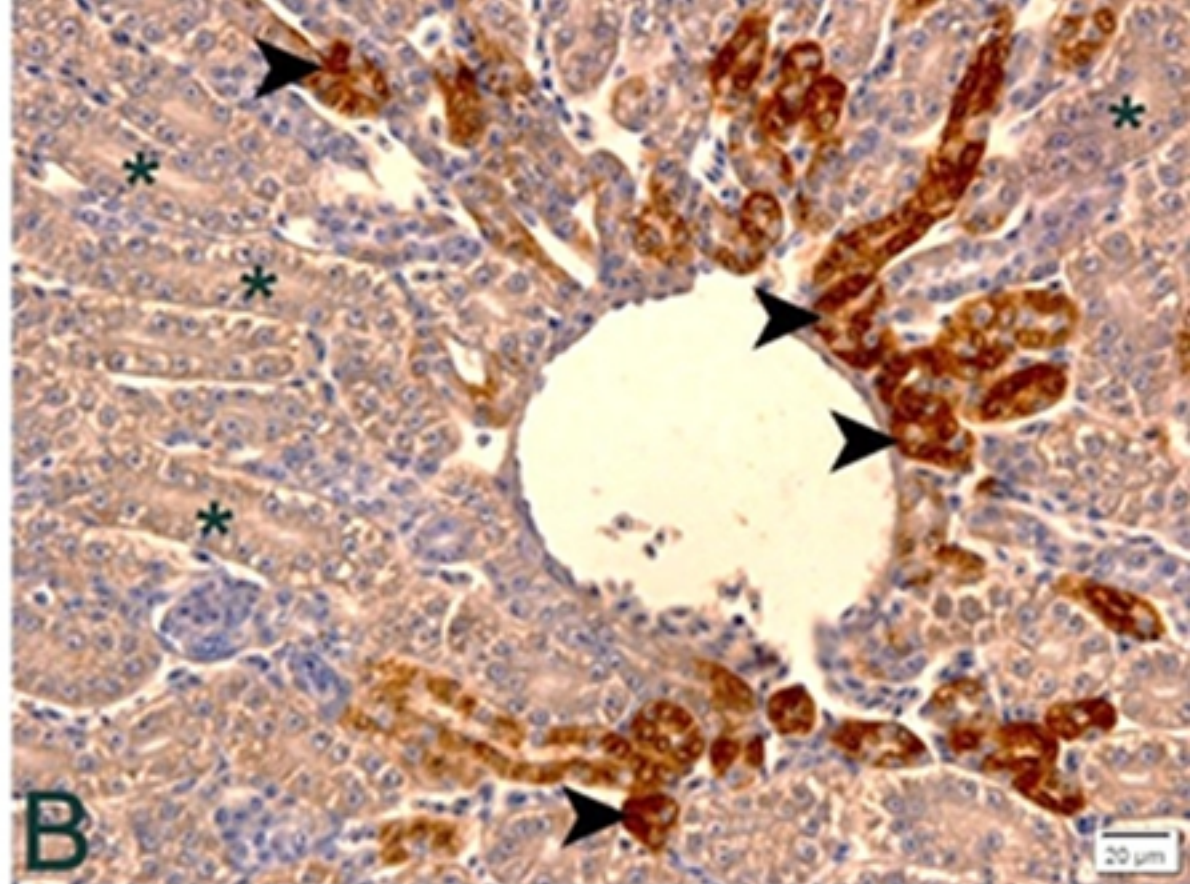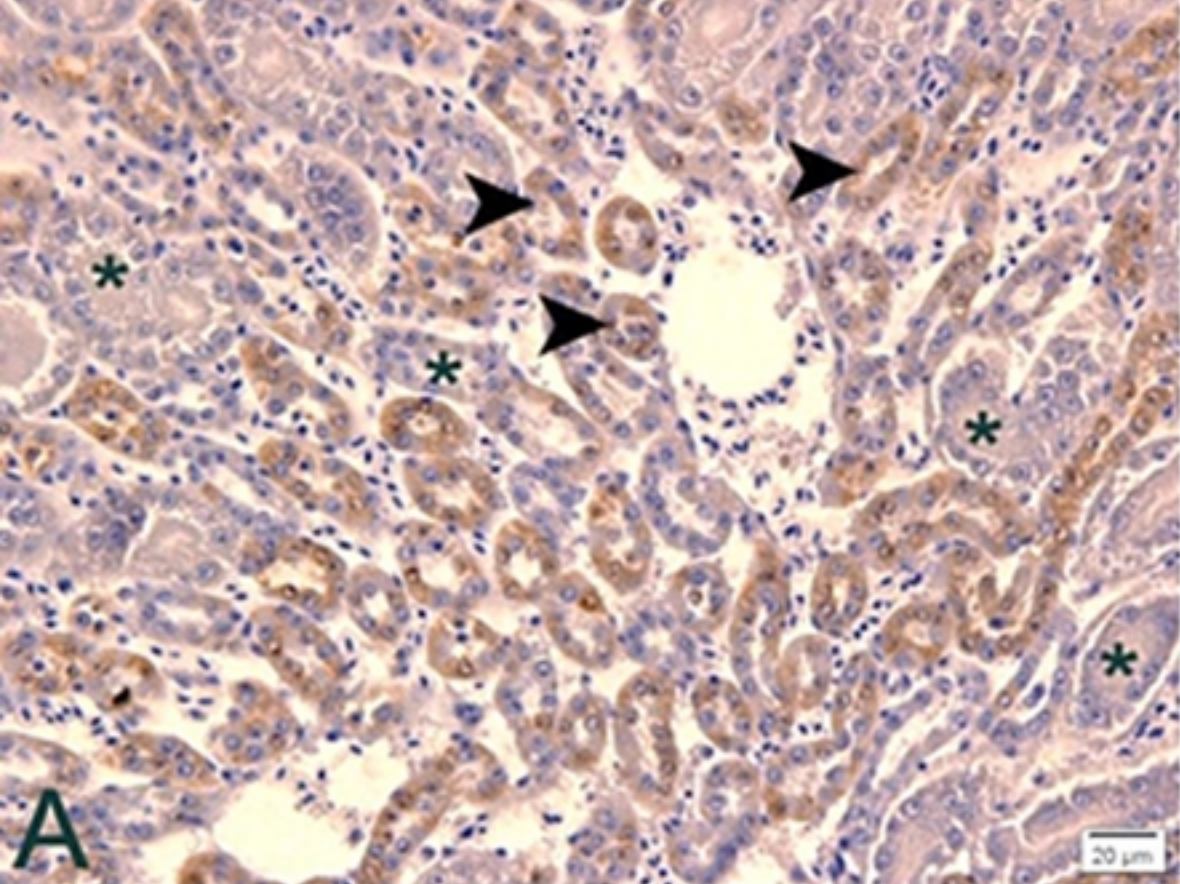
Figure3

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