Effect of chronic heat stress on gastrointestinal histology and expression of feed intake-regulatory hormones in broiler chickens

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ABSTRACT

Heat stress (HS) dramatically impairs the growth performance of broiler chickens, mainly as a consequence of reduced feed intake due to the loss of appetite. This study was aimed at evaluating the alterations induced by chronic HS conditions on the morphological and morphometric features of the gastrointestinal (GI) tract and on the expression of some enteroendocrine cells (EECs) involved in the regulation of feed intake in chickens. Three hundred male chickens (Ross 308) were divided into two experimental groups and raised either in thermoneutral environment for the whole fattening period (0–41 days) (TNT group) or subjected to chronic HS conditions (30 °C for 24 h/day) from 35 to 41 days (HS group). Samples of proventriculus, duodenum, jejunum and cecum were collected from 24 broilers (12/group). Haematoxylin-eosin was used for the morphometric evaluations, while immunohistochemistry was applied for the evaluation of EECs expressing ghrelin (GHR), cholecystokinin (CCK), neuropeptide Y (NPY), glucagon-like peptide-1 (GLP-1), and serotonin (5-HT). In the proventriculus, HS reduced total wall thickness and mucous layer height ($P \leq 0.01$) as well as mean diameter, circumference, and area of the compound tubular glands ($P \leq 0.001$) with respect to TNT. The small intestine of HS birds was characterised by decreased villous height and total thickness (duodenum, $P \leq 0.01$; jejunum, $P \leq 0.001$), whereas crypt depth and width were reduced only in the jejunum ($P \leq 0.01$). HS had negligible effects on the morphological aspects of the cecum. In the proventriculus, an increase in GHR and NPY EECs was observed in response to HS ($P \leq 0.001$). Similarly, the small intestine villi of the HS group showed greater GLP-1 ($P \leq 0.05$), 5-HT ($P \leq 0.001$) and CCK ($P \leq 0.01$) EECs. Moreover, the expression of 5-HT EECs was higher in the duodenal ($P \leq 0.01$) and jejunal ($P \leq 0.01$) crypts of HS birds, whereas GLP-1 and CCK EECs increased only in jejunal crypts ($P \leq 0.05$). Finally, 5-HT EEC expression was increased in the cecum of HS group ($P \leq 0.01$). In conclusion, these outcomes demonstrate that chronic HS induces morphometric alterations not only in the small intestine but also in a key organ such as the proventriculus. Furthermore, HS conditions affect the presence and distribution of EECs, suggesting that some GI peptides and biogenic amine may be implicated in the regulation of appetite and voluntary feed intake in heat-stressed broiler chickens.

Implications

The alterations induced by heat stress on the histological and morphometric features of proventriculus, duodenum, jejunum and cecum in broiler chickens were investigated in this study. In the same gastrointestinal segments, the modifications of enteroendocrine cells expressing some hormones and biogenic amine involved in the regulation of feed consumption were evaluated. The results show that heat stress significantly altered several morphometric characteristics, as well as the number of enteroendocrine cells. Overall, these findings may provide the basis for a better comprehension of the biological mechanisms affecting feed intake and growth performance in heat-stressed broiler chickens.

Introduction

Heat stress (HS) is a major issue for the poultry industry due to its profound effects on animal health, welfare and productivity. HS results from unbalanced thermoregulation that verifies when animals produce or gain more heat with respect to its capacity to disperse it into the environment (Gonzalez-Rivas et al., 2020). Such a

condition affects broiler physiology determining multiple disorders that involve the immune and endocrine systems as well as electrolyte imbalance (Gonzalez-Rivas et al., 2020). The gastrointestinal (GI) tract is particularly sensitive to thermal stressors (Tellez et al., 2017), such that it could be likely considered the most vulnerable tissue in heat-stressed broiler chickens (Quinteiro-Filho et al., 2010). High temperatures have been shown to negatively affect the intestinal development (Garriga et al., 2006), resulting in mucosal epithelial cell damage and villous atrophy, as well as in impaired mucosal barrier function and altered microbiota composition (Santos et al., 2015). Moreover, HS determines relevant disorders in the antioxidant system, with oxidative stress constituting the main reason for intestinal mucosa damages and microflora variations (Zhang et al., 2012). These alterations are correlated with greater intestinal permeability to pathogens and their relative toxins, which promote intestinal barrier breakdown and phlogosis (Awad et al., 2017). Within this context, many studies were carried out to characterise the impact of HS on the functionality and health of the GI tract in broiler chickens. In particular, it has been reported that HS can affect some morphometric parameters of the small intestinal mucosa, such as modifications of the number of the enterocytes, villi height and width, and depth of the crypts (Garriga et al., 2006; Burkholder et al., 2008; Deng et al., 2012; Santos et al., 2015).

The importance of the small intestine for nutrient digestion and absorption is well acknowledged, yet it is important to consider the overall efficacy of the digestive processes is also influenced by a proper functionality of the other sections of GI tract (Scanes and Christensen, 2020). The proventriculus is the analogous of the glandular region of the mammalian stomach, presenting gastric glands with lobules and secretory conducts in which oxyntopeptic cells are located. The primary function of the proventriculus is to produce the gastric juice, which is mainly composed of hydrochloric acid and pepsinogen, the inactivated form of the endogenous protease pepsin (Scanes and Christensen, 2020). Therefore, it is widely accepted that the first steps of the enzymatic digestion of dietary proteins begin in the proventriculus. In addition, the proventriculus plays a crucial role in digesta acidification, which is important to preserve animal health by hindering pathogen development (Scanes and Christensen, 2020). Results from other species suggest that the gastric mucosa is highly sensitive to thermal stress (Laine et al., 2008) and the redistribution of bloodstream driven by HS can affect its health and functionality or increase the occurrence of gastric injuries *via*, for example, the secretion of ulcerogenic agents (Abdel-Salam et al., 2001). However, very limited attention has been reserved so far to as concern the effects of HS on the morphological features of the proventriculus in broiler chickens.

One of the first responses of broilers towards high environmental temperature is the drop in feed intake to limit metabolic heat production (Gonzalez-Rivas et al., 2020). The mechanisms controlling feed intake in avian species are complicated, involving the gut-brain axis as well as hormonal and non-hormonal signals acting through integration with the central nervous system (Tachibana and Tsutsui, 2016). Many studies have revealed that some GI hormones, such as ghrelin (**GHR**), gastrin (**GAS**), glucagon-like peptide−1 (**GLP-1**), cholecystokinin (**CCK**), peptide YY, neuropeptide Y (**NPY**) and biogenic amine (e.g. serotonin or **5-HT**), take part in the modulation of feed intake in both birds and mammals (Tachibana and Tsutsui, 2016; Honda et al., 2017). Several authors have reported that prolonged heat exposure alters the expression of brain–gut neuropeptides in poultry (Song et al., 2012; Lei et al., 2013; Chowdhury, 2019). Therefore, understanding the variations in the number of enteroendocrine cells (**EECs**) expressing some hormones and biogenic amines involved in feed intake regulation can reveal important physiological mechanisms by which

high environmental temperatures negatively influence feed consumption and growth performance of modern fast-growing broiler hybrids.

Thus, the aim of this research was to provide a comprehensive characterisation of the morphological and morphometric alterations induced by chronic HS conditions in the main sections of the chicken GI tract, such as proventriculus, duodenum, jejunum and cecum. In addition, the presence and distribution of EECs expressing some feed intake-regulatory hormones/biogenic amine were assessed in the different segments of the GI tract in an attempt to provide additional information on the mechanisms controlling feed intake under heat exposure.

Material and methods

Animals, diet, and environmental conditions

A detailed description of the experimental design is reported in Zampiga et al. (2021a). Briefly, 300-day-old male chicks (Ross 308) were divided into two experimental facilities showing identical features. Birds placed in the first facility were subjected to thermoneutral (**TNT**) conditions for the entire period of trial (0–41 days of bird age; TNT group, six replicates of 25 birds/each). In this facility, environmental temperatures were consistent with the current recommendations for fast-growing broilers (i.e. 0 day: 30 °C; 3 days: 28 °C; 6 days: 27 °C; 9 days: 26 °C; 12 days: 25 °C; 15 days: 24 °C; 18 days: 23 °C; 21 days: 22 °C; 24 days: 21 °C; 27 days-onwards: 20 °C). Birds placed in the second facility were raised in similar environmental conditions to those applied for the TNT group up to 35 days of age and then subjected to chronic HS (30 °C for 24 h/day) in the last 6 days of the rearing cycle (35–41 days of bird age; HS group, six replicates of 25 birds/each). Room temperatures were monitored during the HS period as shown in Zampiga et al. (2021a). Relative humidity fluctuated between 40 and 55 % in both the rooms during the trial period. Feed (commercial basal diet; mash form) and water were administered on *ad-libitum* basis. Stocking density and photoperiod were consistent with the EU legislation (Directive 2007/43/EC). Furthermore, all aspects concerning bird raising, handling and processing were in accordance with the EU legislation in force (Directive 2007/43/EC; Regulation 1099/2009/EC; Directive 2010/63/EU). The experimental protocol (ID: 1031/2019) was positively evaluated and approved by the Ethical Committee of the University of Bologna.

Sampling procedures

At 41 days, all birds were processed in a commercial plant and slaughtered according to the legislation in force using water-bath electrical stunning (200–220 mA, 1 500 Hz). After stunning and bleeding, the GI tract was gently removed from a total of 24 broiler chickens (12 birds per experimental group; two birds/replicate). For each experimental group, broilers were selected to be representative of the average BW of the corresponding group (TNT and HS). Specimens of the proventriculus (in the middle part of the *regio glandularis*), duodenum (*ansa duodeni*), jejunum (proximal to the *diverticulum vitellinum* or Meckel's diverticulum), and cecum (in the *corpus ceci*), were collected. After rinsing with 0.01 M phosphate buffer saline, tissues were fixed in 4 % paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) for 48 h at 4 °C. Fixed tissues were dehydrated in a graded series of ethanol and then embedded in paraffin. Paraffin sections (5 μm thick) were obtained by means of a microtome. To avoid counting the same microscopical characteristics, each seventh section was mounted

onto poly-L-lysine-coated slides for histology and immunohistochemistry.

Histology and immunohistochemistry

Haematoxylin and eosin stains were used for histological and morphometric evaluations. The avidin–biotin-peroxidase complex (ABC) method was applied for immunohistochemistry as previously reported by Mazzoni et al. (2018). The sections were subsequently incubated overnight at 4 °C with antisera directed to specific EECs markers such as GHR, GAS/CCK, GLP-1, NPY and 5-HT. Subsequently, the sections were incubated at room temperature for 1 h with the biotin-conjugated secondary antibody [goat anti-mouse IgG, goat anti-rabbit IgG and horse anti-goat IgG (Vector)] and then with ABC complex (Vector elite kit, Vector Laboratories, Burlingame, USA). Finally, a 3,3′-diaminobenzidine chromogen solution (Vector DAB kit, Vector Laboratories, Burlingame, USA) was used to visualise the immune reactions. The primary and secondary antibodies used in this study are summarised in Table 1.

Antibody specificity

Specificity of GHR, GLP-1, GAS/CCK and 5-HT has been previously demonstrated (Mazzoni et al., 2018). The NPY antibody specificity was proved by the lack of immunostaining when the antibodies were pre-adsorbed with an excess of the homologous peptide NPY1R antibody blocking peptide (BP18805c, Abcepta, San Diego, CA, USA). Since the antiserum is not able to discern the C-terminus homology of CCK and GAS, it was not possible to discriminate the presence of CCK-IR cells in the duodenum where both cells are simultaneously present.

Morphometrical analyses and enteroendocrine cell evaluation

The morphometrical evaluation was carried out on the stained sections using Nikon DS-Qi1Nc digital camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) software. and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada). For each sample, the morphological analysis has been carried out on four sections per 10 different slides (not the same sections or seriated sections) to avoid evaluating the same histological characteristics or number of cells. The morphometric measurements (expressed in μm) on the proventriculus included the total wall thickness (from the top of the villi/folds to the serous layer), the mucous layer height (from the top of the villi/folds to the deepest limit of the compound tubular glands at the *muscularis*

mucosae), the muscle layer thickness (inner circular muscle layer + outer longitudinal layer), the height of mucosal epithelium + *lamina propria*, and the compound tubular gland diameters (an "average diameter" was calculated from the ratio of the two diameters, the major diameter and minor diameter). In addition, based on the two diameters, the circumference and area of the compound tubular glands were calculated considering the shape of the glands as an ellipse. For each chicken, 20 proventricular glands where the central space was clearly evident were considered for the analysis. As for duodenum and jejunum, the morphometric measurements were focused on the total wall thickness (from the top of the villi to the serous layer), the height and width of the villi, the crypt depth and width, and the muscle layer thickness (inner circular muscle layer + outer longitudinal layer).

In the caecum, the total wall thickness (from the top of the mucosal surface to the serous layer), the gland depth and width and the muscle layer thickness (inner circular muscle layer + outer longitudinal layer) were considered in the analysis. Immunoreactive (-IR) EECs were counted in 20 random villi and 20 intestinal glands (crypts) in the GI tract. In the proventriculus, positive EECs were counted in 12 random microscope fields ($0.41 \ \text{mm}^2$ each field), for a total area of about $4.1 \ \text{mm}^2$. In the small intestine and large intestine, only the villi/crypts located perpendicularly to the *muscularis mucosae* were evaluated. The values obtained from morphometric evaluations and EECs number were grouped for each experimental group (HS and TNT), and the means were calculated.

Statistical analysis

Data were analysed through one-way ANOVA (Graph Prism 4, GraphPad Software, Inc., La Jolla, CA, USA). A $P \leq 0.05$ level was identified as statistically significant. The values were expressed as mean ± SEM. The bird represented the experimental unit for all the considered parameters.

Results

The results of the GI tract morphological features and morphometrical analysis are shown in Figs. 1-5, Tables 2-4. The morphometrical analysis of the proventriculus (Fig. 1 and Table 2) shows that chronic HS significantly reduced both the total wall thickness (3 288 vs 3 886 μm, respectively, for HS and TNT $P \leq 0.05$) and the mucous layer height (3 009 vs 3 578 μm, respectively, for HS and TNT; $P \leq 0.01$) in comparison to TNT conditions (Table 2). In addition, HS group was characterised by a significant reduction of the average diameter (1 761 vs 2 168 μm, respectively, for HS and TNT; $P \leq 0.001$) and the circumference (5 535 vs 7 005 μm, respectively, for HS and TNT; $P \leq 0.001$) of the compound tubular glands. Overall, the glandular area was significantly diminished in HS birds

Table 1
List and dilutions of primary and secondary antisera used for immunohistochemistry on the gastrointestinal tract of broiler chickens raised in either thermoneutral or heat stress conditions.

Primary antibodies	Code	Species	Dilution	Supplier
Gastrin/Cholecystokinin	CCK/GAS # 9303	mouse	1:1 000	CURE/DDRC
Ghrelin	AM26736PU-N	mouse	1:400	Acris
5-hydroxitryptamine	ab16007	mouse	1:200	Abcam
Neuropeptide Y	NBP1-46535	goat	1:1 200	Novus Biological
Glucagon-like peptide-1	GLP-1(1-36) # 9153	rabbit	1:200	CURE/DDRC
Secondary antibodies			Dilution	Supplier
Biotinylated goat anti-mouse immunoglobulins G			1:200	Vector
Biotinylated horse anti-goat immunoglobulins G			1:300	Vector
Biotinylated goat anti-rabbit immunoglobulins G			1:300	Vector

CURE/DDRC, UCLA, Los Angeles, CA, USA. Acris Antibodies GmbH OriGene Company, Schillerstraße 5, Herford, DE. Abcam, Cambridge, UK. Novus Biological, Centennial, CO, USA. Vector, Vector Laboratories, Burlingame, CA, USA.
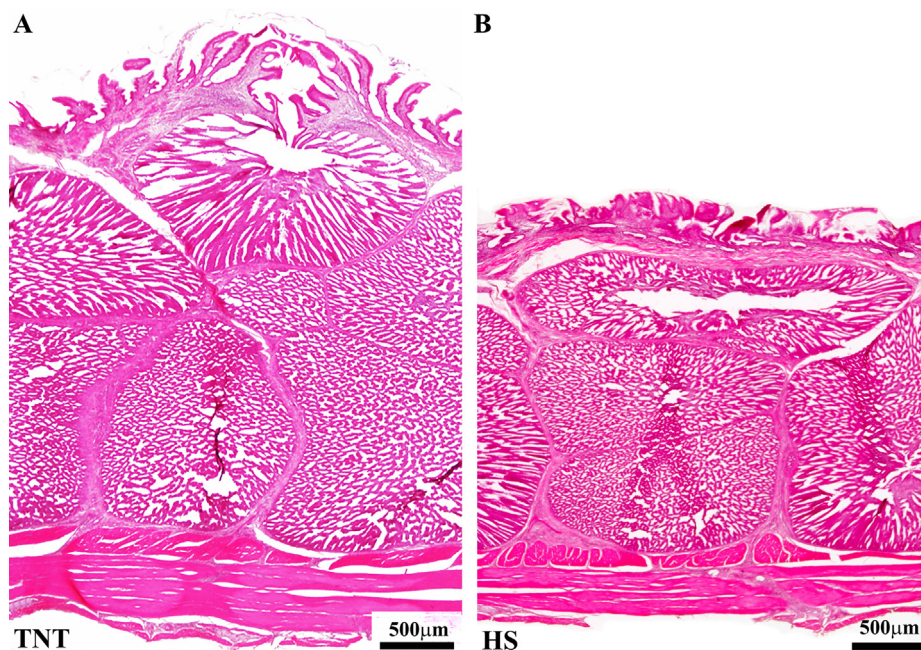
Fig. 1. Representative images of the proventriculus of broiler chickens reared in thermoneutral (A, TNT) or heat stress (B, HS) conditions from 35 to 41 d of age.

with respect to TNT ones (2.2 vs 3.4 μm², respectively; $P \leq 0.001$). No significant effect of the thermal challenge was observed on the muscle layer thickness and the height of mucosal epithelium + *lamina propria*.

In the duodenum (Fig. 2 A and B, Table 3), HS conditions determined a significant reduction of the total wall thickness (1 725 vs 2 067 μm, respectively, for HS and TNT; $P \leq 0.01$) and the villus height (1 341 vs 1 723 μm, respectively, for HS and TNT; $P \leq 0.01$). However, other parameters such as villus width, crypt depth and width, and muscle layer thickness were only marginally affected by the HS challenge applied in this study. Similarly, in the jejunum (Fig. 2 C and D, Table 3), HS induced a significant reduction in total wall thickness (1 070 vs 1v534 μm, respectively, for HS and TNT; $P \leq 0.001$), villus height (838 vs 1 117 μm, respectively; $P \leq 0.001$), crypt depth (154 vs 207 μm, respectively; $P \leq 0.001$) and width (40 vs 46 μm, respectively; $P \leq 0.01$), and muscular layer thickness (182 vs 231 μm, respectively; $P \leq 0.01$). In contrast, villus width was not significantly affected by chronic HS conditions. It also emerged that HS had no appreciable effects on the morphological aspects of the cecum (Fig. 3, Table 3).

According to their morphological appearance, the EECs showed an "open-type" aspect presenting cytoplasmic prolongation that tends to reach the luminal surface, and "closed type" which are confined to the basal lamina and did not reach the lumen (Figs. 4 and 5). NPY and GHR immunoreactivity were observed along the proventriculus gland intermingled with the epithelial cells. Most of the NPY- and GHR-IR cells showed round shape ("closed-type" EECs) feature, while some elongated shape cells exhibit a typical "open-type" EEC morphology (Fig. 4 A-E). Immunohistochemistry for GLP-1, CCK and 5-HT revealed that chicken EECs are scattered as single cells in the epithelium of duodenum, jejunum and cecum. EECs have comma-like shape or rounded profile in crypts ("closed-type" EECs, Fig. 5 A-F) and flask-like shape with long cytoplasmic processes reaching the intestinal lumen in the villous epithelium ("open-type" EECs, Fig. 5 A-F). In the cecum, many 5-HT-IR cells scattered in the intestinal glands were observed: some EECs exhibit "open-type" appearance while other EECs exhibit "closed type" (Fig. 5 E and F). Conversely, few CCK, GLP-1 and GHR-IR cells were found (about one cell every 30/40 crypts), while no NPY-IR cells were detected.

In the proventriculus, an increase in the mean number of the GHR-IR and NPY cells was observed in HS birds with respect to TNT ones (24 vs 15 and 29 vs 24, respectively; $P \leq 0.001$). As for the duodenum (Table 4), HS birds presented a higher number of 5-HT-IR cells in villi (7.3 vs 5.9, respectively, for HS and TNT; $P \leq 0.001$) and crypts (2.6 vs 2.1, respectively; $P \leq 0.01$). Similarly, the number of 5-HT-IR cells was greater in villi and crypts of the jejunum of heat-stressed birds compared to the TNT counterpart (5.8 vs 3.6, respectively, $P \leq 0.001$; and 1.2 vs 0.8, respectively, $P \leq 0.01$). An increase in the 5-HT-IR cells was also observed in the cecum crypts (2.5 vs 1.8 in the HS and TNT, respectively; $P \leq 0.001$) (Table 4). In the duodenum, the HS conditions affected the number of GLP-1-IR cells in the villi ($P \leq 0.05$) but not in the crypts, while in the jejunum, the HS groups showed more GLP-1 positive cells than TNT in both villi and crypts ($P \leq 0.05$) (Table 4). Regarding the CCK, the number of IR cells was greater in the jejunum of HS birds than in TNT ones both in the villi (1.1 vs 0.7, respectively; $P \leq 0.01$) and in the crypts (0.6 vs 0.3, respectively; $P \leq 0.05$) (Table 4).

Discussion

The validation of the HS model applied in this trial has been already reported in our companion paper (Zampiga et al., 2021a). Indeed, HS birds showed a significant decrease in BW and feed consumption (−14 and –33 %, respectively), coupled with higher rectal temperature and heterophil: lymphocyte ratio (Zampiga et al., 2021a).

The GI tract represents one of the most susceptible organs towards stressors, including thermal stress. HS can mimic epithelial surface receptors for pathogen binding, impairing the integrity of the epithelial tissue by decreasing villus height and crypt depth (Burkholder et al., 2008). A large number of studies showed macro and microscopical changes in the GI tract of poultry raised under HS conditions (Garriga et al., 2006; Burkholder et al., 2008; Deng et al., 2012). The findings obtained in the present research indicate that 6 days of chronic HS exposure can induce relevant damages in the duodenum and jejunum mucosa of fast-growing broilers, which can have contributed to the reduction of growth perfor-
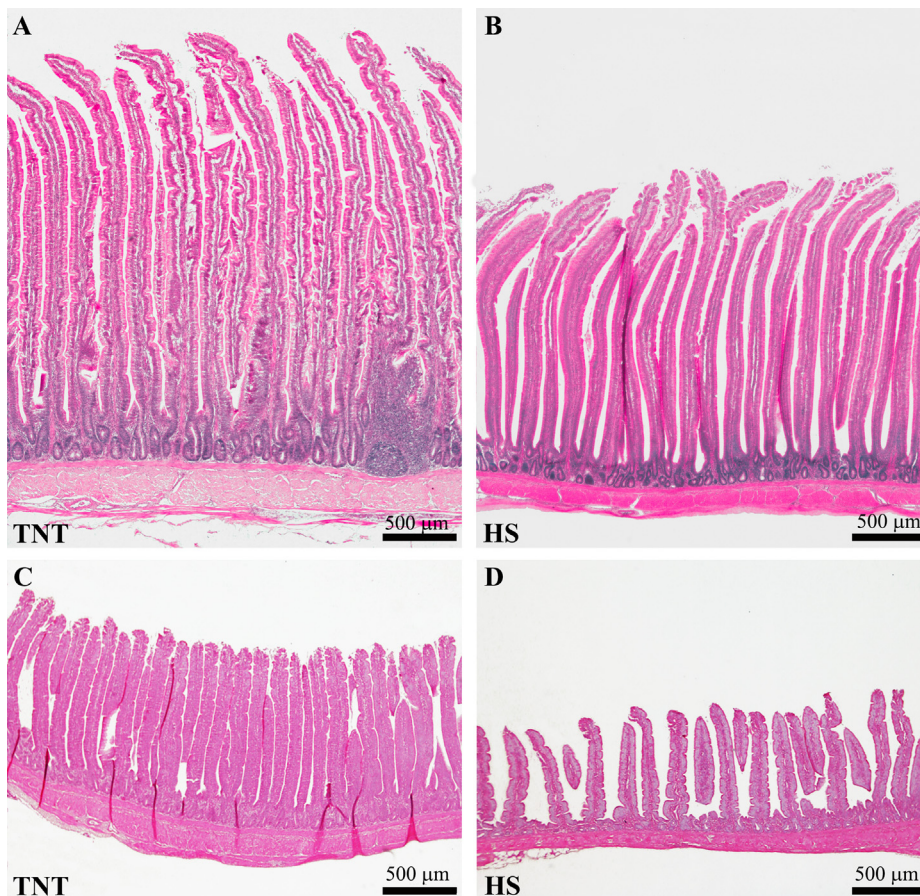
Fig. 2. Representative images of the duodenum (A and B) and jejunum (C and D) of broiler chickens reared in thermoneutral (TNT) and heat stress (HS) conditions from 35 to 41 days of age.
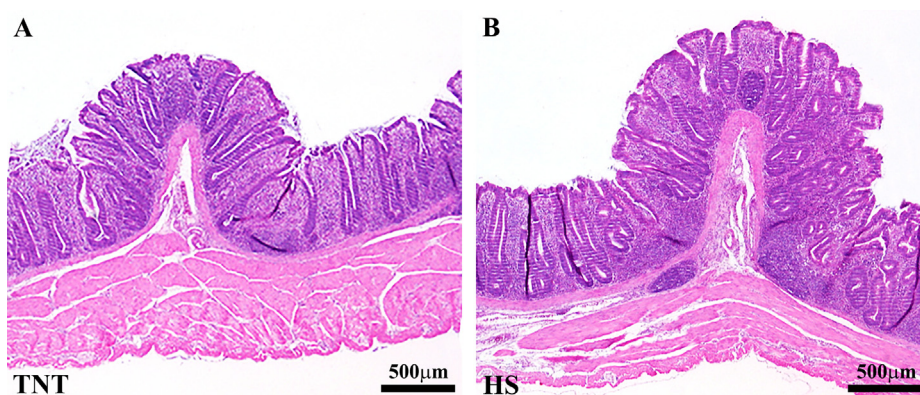

Fig. 3. Representative images of the caecum of broiler chickens reared in thermoneutral (A, TNT) and heat stress (B, HS) conditions from 35 to 41 days of age.

mance observed in HS group (Zampiga et al., 2021a). Considering the different sections of the GI tract, it could be stated that HS has dramatically affected the morphometric features of the proventriculus. Indeed, the HS conditions applied herein significantly reduced the average glandular diameter, the mucus layer height, the total wall thickness as well as the gland circumference and glandular area. Such alterations can remarkably impair the functionality of this organ, leading to a generalised impairment of the digestive processes. Indeed, the pepsin secreted with the gastric fluid initiates the digestion of dietary proteins (Scanes and Christensen, 2020), which is crucial to maximise nitrogen util-

isation while limiting environmental impact and uncontrolled fermentation in the hindgut (Zampiga et al., 2021b). In addition, a proper functionality of the proventriculus is crucial to ensure digesta acidification (Scanes and Christensen, 2020).

Significant changes in the morphometric characteristics of duodenum and jejunum of broiler chickens raised in HS conditions were also observed. Indeed, villus length was significantly reduced in both duodenum and jejunum by HS conditions applied herein. However, no significant effect of HS was detected on villus width. HS significantly reduced crypt depth and width in the jejunum but not in the duodenum. Previous study showed a general reduction
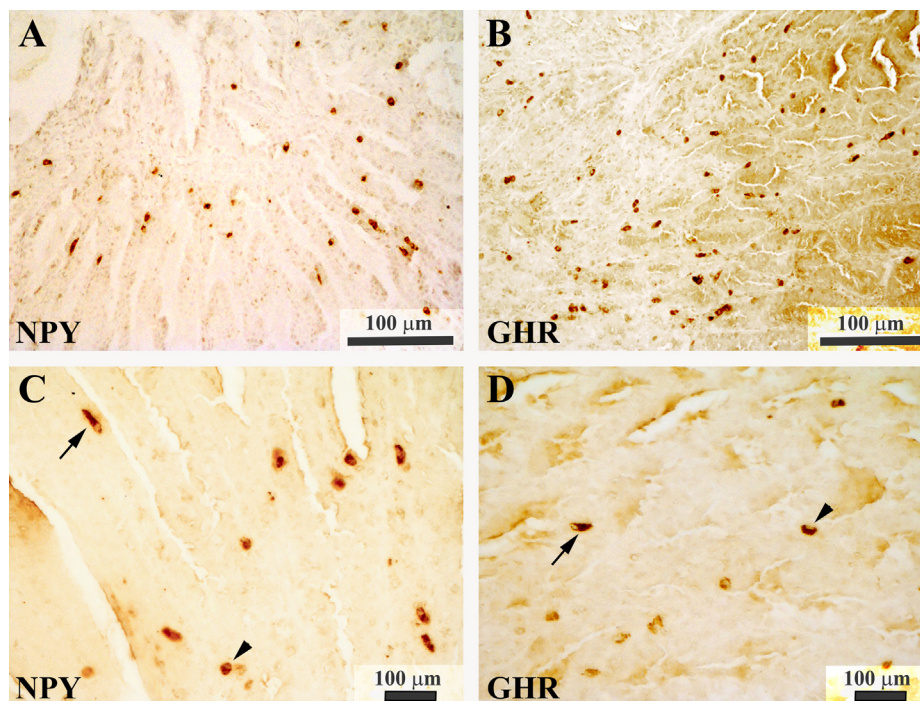
Fig. 4. Distribution of enteroendocrine cells (EECs) in the chicken proventriculus. Neuropeptide Y (A and C, NPY) and ghrelin (B and D, GHR) immunoreactive (-IR) cells in the mucosal gland show a typical "open-type" EEC morphology (arrow), while other EEC-IR cells were found lying close to the basal lamina of the glands and exhibit typical closed-type EEC morphology (arrowheads).

of villus height and the increase of crypt depth in response to the HS condition (Garriga et al., 2006; Burkholder et al., 2008; Deng et al., 2012). Santos et al. (2015) reported an increased width of villi base and decreased epithelial cell area in the duodenum, jejunum, and ileum mucosa of broilers exposed to HS. Interestingly, the morphometric features of cecum were only slightly affected by HS, corroborating the observations of Deng et al. (2012). Conversely, Adji et al. (2019) showed substantial alterations in the morphometric parameters of the cecum. Some of the discrepancies between the present research and previous studies could be attributed to the experimental HS conditions, with particular regard to the duration and the magnitude of the thermal stress. Hao et al. (2012) identified no damage in the jejunum mucosa of broiler chickens after 10 h of HS exposure. Intestinal injuries associated with HS were found after 24 h exposure by Burkholder et al. (2008). Quinteiro-Filho et al. (2010) detected no differences in villus height and crypt depth in the small intestine of chickens raised under HS for 6 days, which might suggest that the birds could adapt to this challenging environmental condition. However, when birds are exposed to prolonged HS, such compensatory mechanisms may not be sufficient to preserve intestinal integrity. Analogously, Ashraf et al. (2013) demonstrated that high environmental temperatures (35 °C) for 21 days were able to induce severe damage in the gut. Overall, it could be concluded that HS severely affects the morphological features of the different sections of the broiler GI tract, particularly in the upper part. Indeed, the most relevant alterations observed in this study are located from the proventriculus to the jejunum, whereas the cecum was only slightly affected. Such modifications can lead to a generalised worsening of the digestive functionality, resulting in lower productive performance and impaired health conditions because of reduced nutrient intake and increased mucosa permeability.

The findings of the present study indicate that EECs are scattered along the epithelium of the chicken GI tract from the glandular stomach to the cecum, corroborating previous observations reported by Mazzoni et al. (2018). Meal-related stimuli induce EECs to secrete GI hormones and peptides such as GHR, CCK, gastrointestinal peptide (**GIP**), GLP-1, peptide YY and NPY. These peptides have several functions, such as the secretion of neurotransmitters and hormones, the regulation of feeding behaviour, nutrient absorption, energy metabolism, as well as GI peristalsis and emptying (Gribble and Reimann, 2016).

NPY is considered one of the most powerful endogenous stimulators of feed consumption in mammals (Michel, 2012) and birds (Boswell and Takeuchi, 2005). Intracerebroventricular injection of chicken NPY increased feed intake and anti-chicken NPY antibody decreased feed intake in chicks (Chen et al., 2016). Similarly, intraperitoneal injection of recombinant NPY stimulated feed consumption in 9-d old chicks (Dhamad et al., 2021). For the first time, this study provides evidence that NPY-IR cells are largely distributed in the chicken proventriculus glands. In this regard, NPY-IR cells were previously observed in the digestive tract of the goat (De Felice et al., 2021) and in Teleosts (Barrios et al., 2020). In addition, a higher number of NPY-IR cells were observed in the HS group with respect to the TNT one. Chowdhury (2019) reported that the suppression of feed intake associated with HS was mirrored by the upregulation of a hypothalamic NPY precursor at the transcriptional level. In laying hens, HS stimulated a higher expression of hypothalamic NPY gene, although not significantly, with respect to TNT conditions (Song et al., 2012). It is plausible to hypothesise that the gastric regulation of NPY in response to HS conditions is similar to that observed in the hypothalamus.

In mammals, GHR is secreted by the EECs located in the gastric mucosa and acts as a modulator of several biological functions, including growth hormone release, feed intake, and GI motility (Yamazaki et al., 2002). Studies demonstrated that GHR can prompt feeding behaviour in Teleosts (Unniappan et al., 2002) and bullfrog larvae (Shimizu et al., 2014). GHR-IR cells have been observed in the mucosal layer of the broiler glandular stomach (Mazzoni et al., 2018). The peripheral administration of GHR was
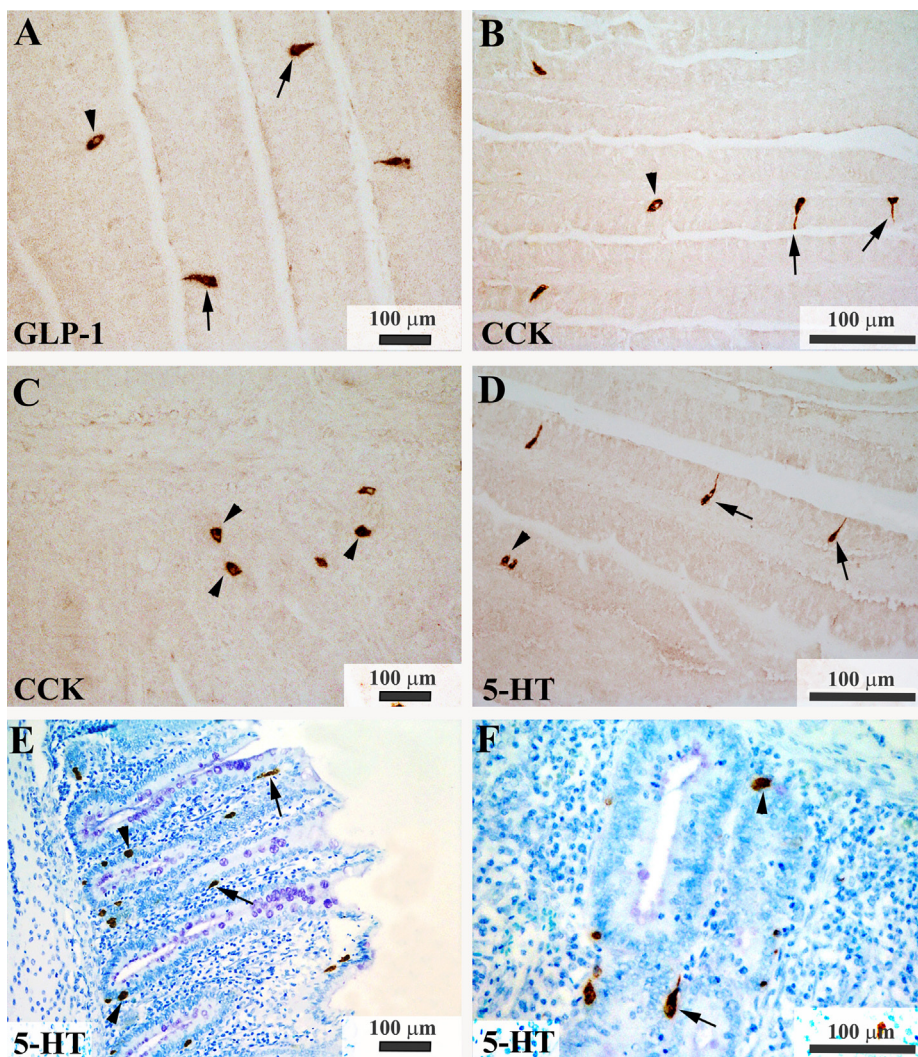
Fig. 5. Distribution of immunoreactive (-IR) enteroendocrine cells (EECs) in the chicken's small intestine and large intestine. EECs, with immunoreactivity for glucagon-like peptide−1 (GLP-1) (A, duodenum), cholecystokinin (CCK) (B and C, jejunum) and 5-hydroxytryptamine (5-HT) (D, jejunum; E and F cecum), are located in the area from crypts to the middle part of villi in each intestinal segment. EEC-IR cells show flask- or comma-like shape with long cytoplasmic processes reaching the endoluminal surface both in the villous and crypts ("open-type" EECs) (A, B, D, E and F, arrows), while some positive cells, mostly located in the crypts, show rounded profile without cytoplasmic prolongation (closed-type EECs) (A-F, arrowheads). In the cecum, the 5-HT-IR cells equally exhibit both "open-type" (arrows) and "closed-type" (arrowheads) appearance (E and F). In images E and F, the sections were contrasted with toluidine blue.

Table 2
Morphometric data obtained in the proventriculus of broiler chickens raised in either thermoneutral or heat stress conditions. Values (μm) are expressed as mean ± SEM.

Item	HS	TNT	P-value
Total wall thickness	3 288 ± 62.5	3 887 ± 173.5	≤0.05
Mucous layer height	3 009 ± 83.5	3 578 ± 141.9	≤0.01
Muscle layer thickness	280 ± 24.5	285 ± 23.9	NS
Mucosal epithelium + lamina propria	399 ± 23.7	383 ± 25.7	NS
Average glandular diameter	1 761 ± 51.6	2 168 ± 77.5	≤0.001
Gland circumference	5 535 ± 133.6	7 005 ± 321.9	≤0.001

Abbreviations: HS, heat stress group; TNT, thermoneutral group.

associated with the suppression of feed intake in broiler chicks (Ocloń and Pietras, 2011). Saito et al. (2002) demonstrated that the central administration of chicken GHR to neonatal chicks reduced feed intake. Intracerebroventricular injection of rat GHR also suppressed feed consumption in adult Japanese quail (Shousha et al., 2005). Therefore, differently from mammals and fish, GHR is considered as anorexigenic peptide in birds. In this study, heat-stressed chickens presented a higher number of GHR-IR cells compared to those raised in the TNT environment. Song et al. (2012) showed that the expression of GHR mRNA was increased in both proventriculus and hypothalamus of heat-stressed hens. The authors hypothesised that its effects on the hypothalamus might mediate one of the pathways for the anorexigenic effect triggered by high environment temperature in laying hens as well as GI GHR signals.

Serotonin or 5-Hydroxytryptamine (5-HT), an important GI regulatory factor deriving for tryptophan, plays a key role in several physiological functions in humans and animals (Martin et al., 2017). The majority of 5-HT is synthesised and secreted by EECs located in the GI tract. In chickens, 5-HT is involved in the regulation of various physiological activities, including inhibition of feed intake (Saadoun and Cabrera, 2002). In addition, serotonin activates neural pathways and modulates intestinal secretion, sensitivity, and peristaltic movements (Jonnakuty and Gragnoli, 2008). In this study, the expression of 5-HT-IR cells in the intestine of broiler chickens was characterised, confirming the results obtained

Table 3
Morphometric data obtained from the duodenum, jejunum and caecum of broiler chickens raised in either thermoneutral or heat stress conditions. Values (μm) are expressed as mean ± SEM.

Item	Duodenum			Jejunum			Caecum		
	HS	TNT	P-value	HS	TNT	P-value	HS	TNT	P-value
Total wall thickness	1 725 ± 93.9	2 067 ± 77.9	≤ 0.01	1 070 ± 63.4	1 534 ± 57.1	≤ 0.001	831 ± 29.9	876 ± 25.9	NS
Villus height	1 341 ± 82.7	1 723 ± 75.3	≤ 0.01	838 ± 29.5	1 117 ± 41.2	≤ 0.001	–	–	NS
Villus width	139 ± 2.8	137 ± 6.0	NS	126 ± 7.2	140 ± 6.1	NS	–	–	NS
Deep crypts	253 ± 7.2	257 ± 12.6	NS	154 ± 6.4	207 ± 12.3	≤ 0.001	386 ± 9.6	377 ± 13.6	NS
Crypts width	52.7 ± 1.6	55.1 ± 1.7	NS	40.0 ± 1.0	40.6 ± 1.9	≤ 0.01	71.7 ± 1.8	73.7 ± 1.7	NS
Muscle layer thickness	245 ± 10.5	229 ± 13.1	NS	182 ± 10.7	231 ± 10.8	≤ 0.01	349 ± 24.5	413 ± 38.7	NS

Abbreviations: HS, heat stress group; TNT, thermoneutral group.

Table 4
Number of enteroendocrine cells evaluated in the duodenum, jejunum and cecum of broiler chickens raised in either thermoneutral or heat stress conditions. Values are expressed as mean ± SEM.

Item		Duodenum			Jejunum			Caecum		
		HS	TNT	P-value	HS	TNT	P-value	HS	TNT	P-value
5-HT	Villi	7.3 ± 0.2	5.9 ± 0.2	≤ 0.001	5.8 ± 0.4	3.6 ± 0.4	≤ 0.001	–	–	–
	Crypts	2.6 ± 0.2	2.1 ± 0.1	≤ 0.01	1.2 ± 0.1	0.8 ± 0.1	≤ 0.01	2.5 ± 0.1	1.8 ± 0.1	≤ 0.001
GLP-1	Villi	0.7 ± 0.1	0.5 ± 0.1	≤ 0.05	1.1 ± 0.1	0.7 ± 0.1	≤ 0.05	–	–	–
	Crypts	0.5 ± 0.1	0.4 ± 0.1	NS	0.7 ± 0.1	0.5 ± 0.1	NS	–	–	–
GAS/CCK	Villi	–	–	–	1.1 ± 0.1	0.7 ± 0.1	≤ 0.01	–	–	–
	Crypts	–	–	–	0.6 ± 0.1	0.3 ± 0.1	≤ 0.05	–	–	–

Abbreviations: HS, heat stress group; TNT, thermoneutral group. 5-HT, 5-hydroxytryptamine; GLP-1, glucagon-like peptide-1; GAS/CCK, gastrin/cholecystokinin.

in our previous investigation (Mazzoni et al., 2018). Moreover, the HS condition increased the number of 5-HT-IR cells in the duodenum and jejunum. It has been reported that rats subjected to feed deprivation for 24 and 48 h presented lower serotonin level in the brain, but higher levels in the GI tract (Bubenik et al., 1992). In Japanese quails, Bayrakdar et al. (2017) reported that thermal conditioning increased the number of 5-HT-IR EECs in jejunum compared to a control group. Although traditionally considered a regulator of gastric motility (Spencer et al., 2015), and more recently, a mediator in the pathogenesis of inflammatory intestinal disorders (Spiller, 2008), mounting evidence highlights the role of gut-derived 5-HT as a modulator of metabolic functions in peripheral tissues (Martin et al., 2017). Given the high amounts of serotonin synthesised by EEC cells, it could be speculated that increases in peripheral serotonin can be considered an energy-saving body response (Namkung et al., 2015).

Cholecystokinin and gastrin are well-known GI peptides in vertebrates, which belong to the same family sharing the same 5-amino acid sequence at the C-terminus (Miyasaka and Funakoshi, 2003). Cholecystokinin exerts several effects in the gut, including gastric emptying, gallbladder contraction, GI motility, as well as gastric acid and pancreatic enzyme secretion (Miyasaka and Funakoshi, 2003). When administered centrally, CCK inhibits feeding behaviour in young and neonatal chicks (Denbow and Myers, 1982; Tachibana et al., 2012). The presence and distribution of CCK EECs in the chicken GI tract have been previously documented (Mazzoni et al., 2018). Our results indicate that the number of CCK-IR cells in the jejunum was significantly higher in HS birds compared to TNT ones. In broilers, Wang et al. (2021) observed, by means of ELISA, that the expression of CCK in the jejunum and serum was higher in heat-stressed broilers than in unstressed ones. On the contrary, the mRNA expression of the CCK was reported to be increased in the duodenum and decreased in the jejunum of

broilers raised under high environmental temperatures (Lei et al., 2013). A significant downregulation of CCK gene expression was also observed in the duodenum and jejunum of heat-exposed laying hens (Song et al., 2012). These variations among studies could be ascribed to the use of different analytical techniques (PCR-RT vs ELISA or IHC). Indeed, the presence of CCK mRNA transcripts does not necessarily imply subsequent protein synthesis, while IHC and ELISA quantify the protein. It is possible to hypothesise that the increased production of CCK in HS chickens could be involved in the reduction of the appetite stimulus. In addition, CCK causes greater intestinal motility in the duodenum and jejunum, and, consequently, an increase in the digesta transit rate. Such a phenomenon could have consequences on nutrient digestibility as well as on the amount of nutrients that can be fermented in the ceca or excreted with the faeces.

Glucagon-like peptide−1 (GLP-1) is released after feed ingestion from EECs distributed along the gut epithelium. In chickens, GLP-1 modulates crop emptying (Tachibana et al., 2003) and feed intake (Honda et al., 2017), while in mammals, it is acknowledged as a peripheral satiety hormone (Van Bloemendaal et al. 2014). Intraperitoneal injection of 3 nmol GLP-1/kg of BW did not stimulate feed consumption in layer chicks after prolonged fasting (Tachibana et al., 2003). Conversely, intraperitoneal injection of 0.5 nmol GLP-1 of rat GHR suppressed feed intake in Japanese quails (Shousha et al., 2007). In this study, immunohistochemistry for GLP-1 revealed that chicken L cells are arranged as single cells in the jejunum epithelium, but only rarely in the duodenum and cecum. Overall, this observation is consistent with Hiramatsu et al. (2005). The HS group showed an increase in the number of GLP-1-IR cells in the villi (but not in the crypts) compared to the TNT group, both in the duodenum and in the jejunum. Wang et al. (2021) did not notice any difference in the concentration of GLP-1 in response to high environmental temperature group in

the ileum and serum of broiler chickens. As previously mentioned, this difference with our results may be due to the different analytical methods used. In addition, the experimental conditions (e.g. time of exposure to heat, age of the chickens, etc.) can represent an additional source of variation among studies.

Conclusion

In conclusion, this study provides a detailed overview of the morphometric alterations induced by chronic HS conditions in different sections of the GI tract of broiler chickens, including proventriculus, duodenum, jejunum and cecum. For the first time, this study indicates that, besides the effects on duodenum and jejunum, HS dramatically affects the morphometric features of the proventriculus, determining alterations that can impair the functionality of this organ and the efficacy of the digestive processes. On the other hand, cecum morphological characteristics were only marginally affected by HS. Overall, such morphological alterations can likely be involved in the reduction of growth performance of HS birds, as reported in our companion paper (Zampiga et al., 2021a; 2021b). Furthermore, the characterisation of EECs in the above-mentioned gut sections revealed that HS remarkably influence the expression of these cells presenting important secretory functions. In particular, HS significantly increased the number of EECs expressing GHR and NPY in the glandular stomach, and GLP-1, CCK and 5-HT in the small intestine (duodenum and jejunum). Overall, these outcomes indicate that some GI peptides and biogenic amine might be implicated in the reduction of appetite and voluntary feed intake previously observed in HS broilers, although further insights are necessary to characterise in detail their physiological roles. Indeed, it should also be considered that, besides of feed intake regulation, these peptides and biogenic amine are involved in other important biological functions in peripheral tissues, which could remarkably affect homeostatic processes in broiler chickens exposed to HS.

Ethics approval

The study conforms with the European legislation regarding the protection of chickens kept for meat production (Directive 2007/43), the protection of animals at the time of killing (Regulation 1099/2009) and the protection of animals used for scientific purposes (Directive 2010/63). The Ethical Committee of the University of Bologna approved the experiment (ID: 928/2018).

Data and model availability statement

None of the data was deposited in an official repository. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Declaration of interest

None.

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