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Body Weight, Intestinal Morphometry and Cell Proliferation of Broiler Chickens Submitted to Cyclic Heat Stress

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Abstract: To investigate the effects of heat stress on body weight, intestinal length, mucous area, crypt's depth, villus height and percentage of cells in proliferation activity in male broiler chickens, one hundred birds of Avian Farms strain were housed in cages and divided into two groups, the reared under heat stress and in thermoneutrality. The group reared under heat stress was submitted daily, from 12-13 h, to 38°C from first to 27th day old and 40°C from 28 to 42nd day. The group reared in thermoneutrality was maintained for 24 h in comfort temperature. The body weight of ten birds of each group was obtained weekly from the first until 42nd day old. Each week, five birds from each group were euthanased by cervical dislocation to obtain intestinal length, the mucous area, crypt's depth, villus height and percentage of cells in proliferation activity. A completely randomized design was used in a factorial schema 6 x 2 (six ages: one, seven, 14, 21, 28 and 42 days and two ambient temperatures: thermoneutral and heat stress). Data were analyzed by analysis of variance and means compared by Tukey test at 5%. The birds stressed by heat presented less crypt depth, mucous area and villus height of duodenum and lower intestinal length at 42 days old. However, heat stress did not influence the percentage of PCNA positive cells, the area of the mucosa, crypt's depth and villus height in jejunum and ileum.

Key words: Performance, intestinal development, thermal stress, ambience, bird

INTRODUCTION

Broiler chicken is very sensitive to high ambient temperatures, so reduction in feed intake caused by high ambient temperature, according to NRC (1994) creates deficiencies of, maybe of all the essential nutrients for optimal performance of the bird, resulting in reduction in their growth and low body weight (Silva *et al*., 2003).

Understanding the macroscopic development of the intestine is important because growth and maintenance of the digestive tract are factors that contribute to the birds have efficiency increasing of digestive processes (Furlan *et al*., 2001). The proper and rapid weight gain of chickens and food utilization are directly related to their nutrition and the morphofunctional integrity of the digestive system (Smith *et al*., 1990), especially in the small intestine where part of the digestive process and absorption of nutrients occur in enterocytes (Furlan *et al*., 2001). According to Teixeira *et al*. (2004) there is a positive correlation between the total length of the intestines and weight gain of broilers.

The development and differentiation of gastrointestinal mucosa, especially in the first week of chick is a relevant condition to the future development of the animal, as physiological processes like cell hyperplasia and hypertrophy will influence the animal body weight (Furlan, 2006). The development of the intestinal mucosa is a result of cell renewal given by the proliferation and differentiation in the crypt and along the villi (Uni *et al*., 1998a) and cell loss by desquamation, which occurs naturally at the apex of the villi (Pelicano *et al*., 2003). Several factors can influence intestinal maturation, among them include stress (Fairchild, 2002). Reduction in feed intake leads to a decrease in the proliferation of enterocytes, which will affect the ability of the birds to digest and absorb nutrients necessary for their maintenance and production (Furlan, 2006).

Considering that most studies on the development and intestinal cell dynamics was conducted in thermoneutrality and those related to heat stress in birds have been conducted in constat high temperatures, this study aimed to evaluate the effect of cyclic heat stress during one hour daily, from first to 42nd day old on body weight, morphometry and activity of intestinal cell proliferation in broilers.

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MATERIALS AND METHODS

One hundred male broilers chickens, Avian Farms strain were housed and randomly assigned on an initial density of 31.25 birds/m^2 in four cages, each measuring 160 x 50 x 60 cm, placed 50 cm from the ground. These ones were kept at thermoneutrality, 32-35°C in the first week of age; and after, rearing temperature was reduced by 2-3°C, weekly, up to 21°C from 35 days old. The ambient temperature was maintained for conventional equipment for heating and cooling. All birds were subjected to 24 h of light daily (13 h natural and 11 h artificial) in their rearing ambient during the experimental period. The birds received, *ad libitum*, water and food made with corn and soybean supplemented with minerals and vitamins according to NRC (1994) recommendations.

A completely randomized design in a factorial scheme 6 x 2 made up of six ages (one, seven, 14, 21, 28 and 42 days) and two ambients (cyclic heat stress and thermoneutral) was used, with five replicates, totaling 60 animals evaluated, each animal constituted oneself in an experimental unit. The birds in the thermoneutral group were kept at thermoneutral ambient during 24 h. Birds subjected to stress were exposed to 38°C, from 12 to 13 p.m. from the first to the 27th day and at 40°C, at the same time, from the 28th to 42nd day. The stress environment was heated by four 200-watt incandescent light bulbs in each cage and the temperature was monitored during the stress period placed the birds' back level.

In the first, seventh, 14th, 21st, 28th and 42nd days old, ten birds from each group were randomly selected and weighed on a digital scale accurate to 5 g. Of these, five from each group were euthanized by cervical dislocation, for the removal and separation of the intestine. The length of the intestines from the beginning of the duodenum from the pylorus to the cloaca was measured as Furlan *et al.* (2001). Ring fragments of approximately two centimeters of small intestine in the middle portion of the duodenum, jejunum and ileum were collected and sectioned longitudinally arranged open in cork with the intestinal lumen facing up and then fixed in buffered 4% formaldehyde solution for 24 h, embedded in paraffin and stained in HE. Morphometric analysis of the mucosa of the duodenum, jejunum and ileum were obtained in an Olympus BX 40 microscope with camera Olympus OLY 200, coupled to a microcomputer, the Data Translation 3150 frame grabber. Measurements of height and width of ten intestinal villi and ten crypts width were made automatically by the image analysis HLImage 97 (Western Vision Software®). The absorption surface area in the intestinal mucosa was obtained as Kisielinski *et al.* (2002).

In this study PCNA was used as a tool to visualize the S-phase cells in the cell cycle corresponding to the proliferation activity in the intestinal chicken mucosa. For immunohistochemistry, deparaffinized sections were put in citrate buffer and subjected to antigenic unmasking in a microwave oven during 7 min. The slides were placed in a humidified chamber and the endogenous peroxidase activity blocked with 3% hydrogen peroxide for 30 min, followed by incubation with horse serum diluted 1:100 in PBS (Novocastra Laboratories®, Newcastle upon Tyne, United Kingdom) for 60 min. Then, the sections were incubated with mouse monoclonal antibody to the proliferating cell nuclear antigen (PCNA, Novocastra Laboratories®, Newcastle, UK) diluted 1:100 for 60 min at 37°C. The slides were washed in 0.05 M Tris-HCl buffer for 2 min and incubated with secondary biotinylated anti-globulin G antibodies (Novocastra Laboratories®, Newcastle upon Tyne, United Kingdom) for 30 min at 37°C. After new wash in 0.05 M Tris-HCl buffer the sensitivity was improved with the avidin-biotin technique (Super ABC kit EP-ABCu, Novocastra Laboratories®, Newcastle upon Tyne, United Kingdom) diluted 1:100 in PBS. The reaction was visualized by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (DAB, NCL-DAB, Novocastra Laboratories®, Newcastle upon Tyne, United Kingdom). Control slides were incubated in the unlabeled mouse serum. The slides were counterstained with haematoxylin and eosin and studied with an Olympus microscope. One slide for each experimental unit was made and the quantification of cells in proliferation activity was performed in the crypts' region and along the villi of the mucosa of the duodenum, jejunum and ileum. With an optical microscope Olympus BX 40 with camera Olympus OLY 200, coupled to a microcomputer by Data Translation 3150 frame grabber, the images were segmented by threshold forming binary images where the black areas were marked and the white ones not. The measurement of the marked areas was made automatically by image analysis HLImage 97 (Western Vision Software).

Data obtained were subjected to analysis of variance and means compared by Tukey test at 5% using the statistical program SISVAR (Ferreira, 2000) and cells percentage data in cell proliferation transformed into square root.

RESULTS AND DISCUSSION

Intestinal body weight and intestinal length: There was no interaction ($p>0.05$) between age and environment for body weight (Table 1). However, the rearing ambient influenced this parameter. Birds subjected to heat stress showed means of body weight 4.38% lower ($p<0.05$) than those kept in thermoneutrality. This result may be due to the reduced food intake (Niu *et al.*, 2009), inefficient digestion of food (Har *et al.*, 2000) and ambient temperature itself (Abu-Dieyeh, 2006).

There was an interaction ($p<0.05$) between age and ambient for intestinal length. At 42d, stressed birds

Table 1: Means of body weight (g) and length of the intestine (cm) of Avian strain male broiler chickens, at different ages (days) submitted or not submitted to heat cyclic stress for an hour daily, 38°C to 1st to 14th day and 40°C from the 15th to 42nd

		Body weight (g)		Length of the intestine (cm)	
Age (days)	Stress	Thermoneutral	Mean	Stress	Thermoneutral
1	47.0	47.0	47.00^{F}	53.56^{aD}	48.82^{aD}
7	221.0	222.0	221.50^{E}	82.96^{aC}	82.06^{aC}
14	514.4	530.0	523.00^{D}	106.14^{aB}	108.42^{aB}
21	987.5	991.0	989.25^{C}	111.26^{aB}	110.02^{aB}
28	1413.0	1462.0	1437.75^{B}	130.76^{aA}	137.46^{aA}
42	2268.5	2470.0	2369.25^{A}	139.46^{bA}	154.94^{aA}
Mean	1045.98^{b}	1093.84^{a}			

^{a,b}Means within a row with different superscripts differ significantly ($p<0.05$).

^{A,D}Means within a column with different superscripts differ significantly ($p<0.05$) by Tukey test

Table 2: Means of Mucosal Area (AM), in square micrometers, length of the Villi (VI) and Crypt's Depth (CR), in micrometers (µm) in the duodenum's mucosa of Avian strain male broiler chickens, at different ages (days), submitted or not to cyclic heat stress for an hour daily, 38°C from 1st to 14th day and 40°C from the 15th to 42nd

		Age (days)						
Duodenum	Ambient	1	7	14	21	28	42	Mean
AM	Stress	12.37	20.34	24.80	25.17	26.84	26.21	23.01^{B}
µm^{2}	Thermoneutral	13.24	23.20	26.78	27.28	27.66	32.68	25.32^{A}
Mean		12.80^{c}	21.77^{b}	25.79^{ab}	26.22^{ab}	27.25^{a}	29.44^{a}	
VI	Stress	584.28	1053.35	1529.57	1571.94	1643.79	1635.70	1365.18^{B}
µm	Thermoneutral	619.51	1137.97	1636.27	1784.44	1632.78	1807.82	1474.19^{A}
Mean		601.90^{c}	1095.66^{b}	1582.92^{a}	1678.19^{a}	1638.29^{a}	1721.76^{a}	
CR	Stress	90.05	117.34	166.66	155.69	184.83	191.20	164.30
µm	Thermoneutral	98.68	102.59	161.09	160.05	189.78	192.99	156.96
Mean		94.37^{b}	109.96^{b}	163.87^{a}	157.87^{a}	187.31^{a}	192.09^{a}	

^{a,c}Means within a row with different superscripts differ significantly ($p<0.05$).

^{A,B}Means within a column with different superscripts differ significantly ($p<0.05$) by Tukey test

showed lower means of intestinal length ($p<0.05$) then those kept under thermoneutral conditions (Table 1). Birds reared under thermoneutral conditions showed a intestine 15.48 cm longer than stressed birds at 42nd day of life. The decrease in intestinal length in birds exposed to heat stress is a physiological adjustment in the attempt to reduce the body heat production. According to Mitchell and Carlisle (1992) the decrease of intestinal length in birds subjected to heat stress may reduce the intestinal absorption area, which could explain their lower body weight, once, this is correlated with the surface area of intestinal segments (Geyra *et al*., 2001a).

Small intestine morphometry: There was no interaction ($p>0.05$) between the environment and age to the mucosa's area, villus length and crypt depth in duodenum (Table 2). However, heat stress reduced the area of mucosa and the length of the villus ($p<0.05$) in this portion. The crypt depth was not influenced by rearing ambient. The area of mucosa increased up until the 28th day old and the length of villi and crypt depth increased until the 14th day. In this experiment, the mucosa's area was increased by 70% until the 7th day and 25% from 7th to 28th day; the length of the villus increased by 82% until the 7th day and 44% from 7th to 14th; crypt's depth increased by 73% until 14th day. These results evidence that the major duodenal development occurred in the initial rearing phase. This is in support with results obtained by Geyra *et al.* (2001a) who have shown that mucosa's area of the duodenum increases rapidly until the third day old in male chickens, of Ross strain, kept in thermoneutrality; and with (Uni *et al*., 1995a) who reported that the length of the villus increases until the seventh day and crypt depth increases from the first the fifth day old (Uni *et al*., 1995b). Also, Iji *et al.* (2001) reported that the area and length of the villi of the duodenal mucosa increased from first to seventh day old in Steggles x Ross broiler chickens reared in thermoneutral ambient. Thus, it can be inferred that the higher body weight of birds reared under thermoneutral was due to greater area of the duodenal mucosa, since Geyra *et al.* (2001b) have shown that the growth is directly related to the mucosa's area.

There was no interaction ($p>0.05$) between the ambient and age to the mucosa' area of the jejunum, villus' height and crypt's depth (Table 3). The thermal environment did not affect the development of the jejunum. These results disagree from Mitchell and Carlisle (1992) who observed that in female broiler chickens exposed to the ambient temperature of 35°C, from 18th to 32th day old, the mucosa's villi of the jejunum were 19% smaller compared with birds kept at

Table 3: Means of Mucosal Area (AM), in square micrometers, length of the Villi (VI) and Crypt's Depth (CR), in micrometers (μm) in the jejunum's mucosa of Avian strain male broiler chickens, at different ages (days), submitted or not to cyclic heat stress for an hour daily, 38°C from the 1st to 14th day and 40°C from the 15th to 42nd

		Age (days)						
Jejunum	Ambient	1	7	14	21	28	42	Mean
AM	Stress	7.02	10.53	11.79	15.12	14.14	18.07	14.44
μm^2	Thermoneutral	8.65	9.58	14.35	15.16	15.09	17.30	14.00
Mean		7.83^{c}	10.06^{bc}	13.07^{ab}	15.14^{a}	14.61^{a}	17.69^{a}	
VI	Stress	322.33	496.06	648.89	783.61	820.66	986.07	725.96
μm	Thermoneutral	366.37	444.79	777.22	770.92	792.73	1009.27	722.76
Mean		344.35^{d}	470.43^{c}	713.05^{b}	777.27^{b}	806.69^{b}	997.67^{a}	
CR	Stress	80.39	94.71	126.20	114.67	125.89	130.72	121.37
μm	Thermoneutral	84.44	109.54	121.46	125.24	122.07	132.87	122.28
Mean		82.42^{c}	102.12^{bc}	123.83^{ab}	119.95^{ab}	123.98^{ab}	131.79^{a}	

^{a,d}Means within a row with different superscripts differ significantly ($p<0.05$) by Tukey test

Table 4: Means of Mucosal Area (AM), in square micrometers, length of the Villi (VI) and Crypt's Depth (CR), in micrometers (μm) in the ileum's mucosa of Avian strain male broiler chickens, at different ages (days), submitted or not to cyclic heat stress for an hour daily, 38°C from the 1st to 14th day and 40°C from the 15th to 42nd

		Age (days)						
	Ambient	1	7	14	21	28	42	Mean
AM	Stress	6.31	8.77	9.07	10.40	12.34	14.13	11.34
μm^2	Thermoneutral	7.64	8.42	8.97	10.48	11.97	14.77	10.98
Mean		6.98^{d}	8.59^{cd}	9.02^{cd}	10.44^{bc}	12.16^{ab}	14.45^{a}	
VI	Stress	285.55	377.62	485.25	496.79	580.64	731.69	530.29
μm	Thermoneutral	321.88	422.45	544.00	509.91	610.88	692.81	547.58
Mean		303.72^{d}	400.04^{cd}	514.63^{b}	503.35^{bc}	595.76^{b}	712.25^{a}	
CR	Stress	75.02	95.94	108.58	97.81	130.78	144.64	117.41
μm	Thermoneutral	88.31	99.97	109.77	108.82	117.52	134.73	116.84
Mean		81.67^{d}	97.96^{cd}	109.18^{bc}	103.32^{c}	124.15^{ab}	139.68^{a}	

^{a,d}Means within a row with different superscripts differ significantly ($p<0.05$) by Tukey test

22°C and from Uni *et al.* (2001) who have shown that Cobb broiler chickens underwent 24 h at high ambient temperature and high relative humidity (36°C, 70% RH) from the first to third day old, showed mucosa villi less wide than the birds kept on thermoneutrality in the fifth day old. The results' divergences probably occurred because of duration and intensity of stress, the age at which birds were challenged and the strain studied.

The area of jejunum increased until 21st day old, the length of the villus and crypt's depth, until 42nd day. The area of mucosa increased 28% until the seventh day old and 50% from the seventh to 21st, the length of the villi increased 36.6% until the seventh day and 112% from seventh to 42nd day; crypt's depth increased 23, 9% from the first to seventh day and of 29% from the seventh to 42nd day. Comparing to duodenum which had higher development in rearing initial phase, it is observed that jejunum spread until the age close to slaughter. Similar results were obtained by Iji *et al.* (2001) who have shown that the length of the villi and crypt's depth of jejunum increased with age in Steggles x Ross broiler chickens reared under thermoneutral conditions. According to Uni *et al.* (2000), the size of the crypt of the jejunum mucosa of Ross broiler chickens increases rapidly after hatch, until 14 days old and Uni *et al.* (1998a) reported an increase of two to three times in 15th day old.

There was no interaction ($p>0.05$) between age and environment and not influence ($p>0.05$) of rearing ambiente on morphology of the ileum. However, the area of mucosa, villus' height and crypt's depth increased until 42nd day old. The results corroborate those obtained by Sklan and Noy (2003) in turkey poult which had increased villus' height of ileum until 19th day old. It has also to consider that the development of the crypt is crucial to the increase of cell renewal rate and maturation in the gut, because, with the increase in the size of the crypt there is an increase in the number of enterocytes and villus growth, increasing the area intestinal absorption (Geyra *et al.*, 2001a).

According to Uni *et al.* (1995a) and Uni *et al.* (1995b), the development of the gastrointestinal tract given by the increase in area of the small intestine may be the more important limiting factor for the initial growth of the bird than the increase in digestion and absorption of nutrients, once the mucosa's area of intestinal segments is correlated with the growth of the bird (Geyra *et al.*, 2001a). Macari and Furlan (2001) considered that the absorptive capacity of nutrients is proportional to the area of mucosa available for absorption and Geyra *et al.* (2001b) argued that the growth of birds is correlated with the absorption area of the duodenum.

Thus, in this study, the cyclic heat stress reduces body weight, total length of the intestine and the absorption area, especially in the duodenum. It can be inferred that possibly the lower body weight presented by the birds

Table 5: Means of cells percentage in cell cycle S faze in the duodenum, jejunum and ileum mucosa of Avian strain male broiler chickens, at different ages (days), submitted or not to cyclic heat stress for an hour daily, 38°C from the 1st to 14th day and 40°C from the 15th to 42nd

		Age (days)						
	Ambient	1	7	14	21	28	42	Mean
Duodenum	Stress	2.37	1.40	1.23	2.13	1.78	2.48	2.00
	Thermoneutral	2.88	1.71	1.84	1.72	2.07	2.07	2.13
	Mean	2.62^{a}	1.55^{c}	1.53^{c}	1.93^{bc}	1.92^{bc}	2.28^{ab}	
Jejunum	Stress	2.62	1.96	1.59	2.23	2.43	2.67	2.28
	Thermoneutral	3.27	1.91	2.00	2.23	1.98	2.81	2.49
	Mean	2.95^{a}	1.93^{c}	1.80^{c}	2.23^{bc}	2.21^{bc}	2.74^{ab}	
Ileum	Stress	2.52	2.13	1.77	2.07	2.14	2.88	2.31
	Thermoneutral	3.23	2.02	2.00	1.96	2.35	2.82	2.48
	Mean	2.87^{a}	2.08^{b}	1.88^{b}	2.02^{b}	2.25^{ab}	2.85^{a}	

^{a,c}Means within a row with different superscripts differ significantly ($p<0.05$) by Tukey test

subjected to stress may be due to lower absorption area of the duodenum compared to birds kept at thermoneutrality. Failure to preserve the morphometric integrity of the digestive system in stressed birds compromises the absorption of nutrients (Noy and Sklan, 1999), therefore, alters the growth, development and performance of birds that do not reach the expected standards that your genetics can express.

The change in shape, increase in size and number of villi in the mucosa occurred until 42nd day old and coincided with the end of the experiential period. Future studies are needed to verify ages over 42 days in order to determine the stabilization of the intestinal development in broiler chickens.

Percentage of cells in cell proliferation activity: There was no interaction between age and environment for the percentage of cells in S phase of cell cycle. The cyclic heat stress did not influence the percentage of cells in cell proliferation activity in the mucosa of the duodenum, jejunum and ileum ($p>0.05$) (Table 5). The obtained results corroborate those found by Hori *et al.* (1998), using the technique of BrdU observed no difference in cell proliferation rates in the duodenum, jejunum and ileum of rats submitted to stress caused by space restriction and immersion in water when compared with non stressed. However, Uni *et al.* (2001) reported that Cobb male broiler chickens conditioned on the third day of age to 36°C and 70% relative humidity for 24 h, had a decrease of 25% in the proliferation of enterocytes in the crypts of the jejunum, when compared with birds raised at thermoneutral environment. In this study, the crypt's region and the villus were evaluated together to obtain the percentage of cells in cell proliferation activity evaluation, which could explain the difference between the results obtained by Uni *et al.* (2001), who have reported that cell proliferation, in broiler chickens, occurs both in the crypt and along the villus, 50% of the cells in proliferation were found in the crypt, 32% in the middle portion of the villus and 8% in the apical region of the villus (Uni *et al.*, 1998b).

The development of the intestinal mucosa is a result of cell renewal, given the proliferation and differentiation in the crypt and along the villi (Uni *et al.*, 1998a) and the cell loss by desquamation, which occurs naturally at the apex of the villi (Pelicano *et al.*, 2003). Thus, the largest area of absorption observed in the duodenum may have been due to lower cell loss by desquamation, once there was no difference between the rate of cell proliferation between the environments.

Means of cells proliferation activity percentage were affected by bird age. The highest means were obtained in the first days of age and lowest on 14th day in all segments of the small intestine. Uni *et al.* (2000) also reported that, in Ross broiler chickens reared in a thermoneutral environment, the rate of cell proliferation in the crypt and along the villi of the jejunal mucosa decreased from first to 14th days old.

The percentage of cells in cell proliferation activity has changed from 100%, in the first day old, to 58%, 61% and 65.5% in the duodenum, jejunum and ileum, respectively until 14th day old and the crypts along the villi. According to Uni *et al.* (2000), the proliferation rate decreases rapidly in the jejunum of Ross male broiler chickens reared in thermoneutrality. These authors reported that this rate is 100% at the time of hatching and change to less than 10% in the villi on 14th day and 100% to 60% in the crypts at 4th day after hatching. Also Geyra *et al.* (2001a) reported that in Ross broiler chickens at the time of hatching, almost all cells are proliferating in the crypt and along the villi in the three intestinal segments, however, with age, the percentage of PCNA-cells positive decreases, reaching 50% of proliferating cells in the crypts and 10-40% in the villi, three days after hatching. Uni *et al.* (1999) also consider that, immediately after hatching, growth occurs mainly by intestinal cell hyperplasia and not by hypertrophy.

In this study, the cyclic heat stress has not influenced the rate of cell proliferation of intestinal mucosa.

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