ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 12 (8): 464-472, 2013
ISSN 1682-8356

Egg Injection of Ascorbic Acid Stimulates Leucocytosis and Cell Proliferation in the Bursa of Fabricius

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Abstract: Broiler chicks with high immune defense potential at hatching are of great value to the poultry industry. Heat stress can have immunosuppressive or immune stimulatory effect and Ascorbic Acid (AA) acts as anti-stressor and has important physiological functions in bird immune system development. This study aimed to assess the effect of intra egg injection of AA before incubation on development of the bursa of Fabricius and leukocyte parameters in chicks from eggs incubated at thermoneutral and hot temperatures. Fertile eggs from broiler breeder chickens were used in a 5 x 2 factorial design with 5 treatments (non-injected and thus non-perforated and injected with 0, 2, 4 and 6% AA in 100 µL of water) and 2 incubation temperatures (thermoneutral: 37.5°C and hot: 39.0°C). The total leukocyte count; percentages of thrombocytes, basophils, eosinophils, monocytes and heterophils; weight; and bursal index remained unaffected by incubation temperature and AA dose. Bursal follicle area decreased in chicks from non-injected eggs when incubated at hot temperatures. With intra egg injection of 4 and 6% AA, bursal follicle size increased in eggs incubated at thermoneutral temperature and decreased at hot temperature compared to no AA injection. Cell proliferation in follicles increased by injecting 2% AA, whereas the percentage of lymphocytes increased by injecting 4 and 6% AA. The data show that AA acts as a dose- and temperature-dependent immunomodulator and that 4 and 6% AA doses stimulate egg bursal development and lymphocytosis. These findings suggest that intra egg injection of AA produces chicks with higher potential for immune defense against challenges in the field.

Key words: Cloacal sac, chicks, leucocytes, mitosis, vitamin C

INTRODUCTION

Starting from external pipping, chicks become exposed to new antigens which act against the frontline innate immune system, since their acquired immune system depends on development of post-hatched lymphoid organs (bursa of Fabricius, thymus and spleen) and these organs interacting with cell- and antibody-mediated antigens. The poultry industry selects strains with faster growth and better food conversion efficiency as this reduces the time and nutrient availability for bird immune system development, since their lymphoid organs develop and are active during the fast growth phase, regressing afterwards (Szember, 1976; Kincade and Moore, 1977). Thus, broiler chicks with high innate immune defense potential and increased lymphoid organ development are of great value to the poultry industry.

Heat stress can depress the bird immune system during incubation and post-hatching (Rosales *et al*., 1989; Puvadolpirod and Thaxton, 2000), thus altering potential immune response. Stress affects immunity according to stressor stimulus intensity and duration and varies between lineages and individuals (Morgulis, 2002) which may cause immunosuppression (Thaxton and Siegel, 1970; Brake, 1989; Miller and Qureshi, 1991) or immunostimulation (Coles, 1986; Ruckebush *et al*., 1994; Cunningham, 1999). Heat stress during incubation has been assessed in developing epigenetic adaptations which provide thermotolerance in birds during breeding; however, it should not affect chick immune potential.

Vitamins involved in metabolism, such as immunomodulators, improve immunity function and infection resistance in poultry and other domestic animals (Rutz, 2000). Research has shown that birds under stress require more vitamins and minerals (El-Boushy, 1988; Coelho and Mcnaughton, 1995; Miltenburg, 1999). Ascorbic Acid (AA) can reduce the effect of heat stress (Mahmoud *et al*., 2004), as it compensates for low vitamin C absorption that occurs at high temperatures which reduces its concentration in the blood and compromises immune function (Thornto, 1961; Briggs *et al*., 1994). Performance traits and immune function in birds that have experienced heat

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stress significantly improve with increasing vitamin C levels (Pardue and Thaxton, 1984; Pardue *et al*., 1985; Campo and Davila, 2002; Furlan and Macari, 2002).

A number of management mechanisms aiming at enhancing chick immunological resistance during incubation have been proposed (Kidd, 2004; Yahav *et al*., 2004; Yalcin *et al*., 2005, 2008; Ghonim *et al*., 2009; Selim *et al*., 2012). Intra egg injection of AA before incubation can be a viable nutritional management technique in improving chick immune potential and/or minimizing immunosuppressive effects of heat stress during incubation. Furthermore, it eliminates the need to handle and remove eggs from incubators or hatchers which can jeopardize egg hatchability.

The effects of stressor or modulating agents on bird lymphoid organs can be evaluated by weight and organ/body weight index (Wyatt *et al*., 1986; Rosales *et al*., 1989; Revidatti *et al*., 2002), by morphology of epithelial lining (inside bursae), bursal wall and lymphoid follicles (Cheville *et al*., 1978; Donker and Beuving, 1989), cell proliferation (Motoyama *et al*., 1995; Vasconcelos *et al*., 1996) and lymphocyte count (Thaxton *et al*., 1968; Gross and Siegel, 1983; Compton *et al*., 1990; Altan *et al*., 2000). Lymphoid organ weight reflects the body's ability to produce lymphoid cells during immune response (Ribeiro *et al*., 2008) and, together with relative bird organ: body weight body, enables us to evaluate a bird's potential immune defense. In this study, we investigated whether intra egg injections of AA before incubation improves the immune-related traits in broiler chicks exposed to continuous heat stress during incubation.

MATERIALS AND METHODS

Eggs and experimental design: The procedures used in this study were approved by the Animal Use Ethics Committee-CEUA (protocol No. 7377/10), at the Faculty of Agricultural and Veterinary Sciences, São Paulo State University- UNESP, Jaboticabal.

In this study, we used fertile eggs from 47-week-old broiler breeder (Cobb®) obtained from a commercial hatchery (Globoaves, Itirapina, São Paulo). Eggs were weighed and homogeneously distributed by weight (67g±2g) in a completely randomized 5 x 2 factorial design with five treatments (non-injected and thus non-perforated and injected with 0, 2, 4 and 6% AA in 100 µL of water) and two incubation temperatures (thermoneutral: 37.5°C and hot: 39.0°C), using two incubators per temperature and 50 eggs/treatment/incubator with automatic temperature control and eggs turned every two hours until day 18 of incubation (Premium Ecológica IP200). Relative humidity inside the incubator was 60% until day 18 of incubation. From day 19, the relative humidity was 70% and the temperature was lowered to 1°C for both incubation temperatures used.

The eggs were injected with Ascorbic Acid (AA) liquid solution (Synth, 99% purity), using the previously mentioned doses at the onset of incubation, except for control eggs which remained non-perforated. After cleaning the site with 100% ethanol, 100 µL of solution was injected 6-mm deep into the albumen near each egg's tapered end while in the horizontal position, using syringe with a sterile needle (13 x 0.38, 27.5 G1/2"). After injection, the hole was sealed with a label identifying treatment and replication. The AA was dissolved in autoclaved Mili-Q water in the dark due to its photosensitivity.

Total and leukocyte-specific counts and heterophil/lymphocyte ratio: After weighing the newly-hatched chicks, eight chicks per treatment were used to analyze leukocytes. Blood was collected from the jugular and kept in plastic tubes with 15 µL of anticoagulant/1 ml of blood (Glistab, cat. 29, Labtest Diagnóstica) on ice. For differential leukocyte counts, one blood smear was taken per bird immediately after collection. Next, the smears were stained with Panótico kit, rapidly dehydrated in an increasing ethanol series, diaphanized in xylene and mounted with entelan. The percentages of different types of leukocytes (lymphocytes, monocytes, heterophils, eosinophils, basophils and thrombocytes) and heterophil/lymphocyte (H/L) ratio were calculated from assessing 100 leucocytes per bird. The total leukocyte counts (number of leukocytes/mm^3) were taken in a Neubauer chamber immediately after collecting blood samples and diluting (1:100) with solution by Natt and Herrick (1952).

Bursa weight, histology and morphometry: After blood collection, the eight chicks per treatment were euthanized by cervical dislocation, necropsied and then decapitated. The bursae were dissected and immediately weighed. Relative weight of each bursa was calculated as percentage relative to each chick's body weight. We used bursae from eight chicks per treatment for histological observation and measuring follicle area, inner epithelial thickness and outer wall thickness. Once collected, the bursae were fixed in 10% formaldehyde solution. Next, they were processed by routine light microscopy method. Semi-serial 6-µm thick histological sections were stained with hematoxylin-eosin. The data were obtained using a system for image capturing and analysis (Leica), where 60 morphometric measurements were taken for each variable per bird.

Cell proliferation in bursa: Mitotic cells in the bursal follicles were immunohistochemically stained for protein Proliferating Cell Nuclear Antigen (PCNA) which are synthesized during interphase and are present in the cell until the end of the mitosis process (Miayachi *et al*., 1978; Celis and Celis, 1985). We used the Avidin-Biotin

complex[1] for this reaction. We obtained 6-µm thick sections from bursae fixed in 10% formaldehyde, embedded in histosec and mounted on electrically-charged slides (Starfrost® Green, tissue-tek® auto write). Sections were deparaffinized in xylene and rehydrated in decreasing ethanol series until water. To block endogenous peroxidase, the slides were immersed in hydrogen peroxide (3%) in methanol solution for 10 minutes at room temperature. Next, the solution was removed and the sections were covered with nonspecific serum (equine, 2% in 0.19M PBS, pH7.2) for 1 hour at room temperature to block nonspecific bindings. After blocking, the sections were incubated with mouse anti-PCNA[2] primary antibody diluted in 0.05 M Tris-HCl pH 7.4 (1:100) at 4°C for one hour in a humid chamber. Next, the sections were incubated for 10 minutes with biotinylated secondary antibody and then for 20 minutes with streptavidin-peroxidase complex at room temperature. The samples were washed in 0.05 M Tris-HCl buffer solution (pH 7.4). Betazoid diaminobenzidine chromogen (DAB)[2] solution was used for revealing the antigen-antibody reaction. The slides were dehydrated with increasing ethanol series, cleared with xylene and mounted with entelan. Negative controls were not incubated with primary antibody. Cell proliferation rate corresponded to the number of $PCNA^+$ cells present in a 2,700-μm^2 area of 25 follicles per bird. The number of cells undergoing mitosis was counted using a system for image capturing and analysis (Leica Q Win V3) coupled with a stereo microscope (Leica DM-2500).

Statistical analysis: Data were subjected to analysis of variance using the General Linear Model (GLM) in the SAS® program (SAS Institute, 2002), according to the previously described 5 x 2 factorial randomized design (5 treatments x 2 incubation temperatures). If a significant difference between means was obtained by F-test then they were also compared by Tukey test at 5% significance level. Using the contrasts technique, we also compared the control treatment mean against the combined mean of the treatments with 0, 2, 4 and 6% AA and tested regression models (linear, quadratic and cubic) in terms of effect of AA doses on these variables.

RESULTS

Bursa weights: The absolute and relative weights of bursae of Fabricius were not significantly ($p>0.05$) affected by ascorbic acid (AA) percentages injected intra egg or by the incubation temperature (Table 1).

Cell proliferation in bursa: Table 2 shows that treatments but not temperature had a significant effect ($p\leq0.05$) on the number of mitotic cells in bursal follicles, suggesting that 2 and 6% AA injection increased and decreased the number of mitoses, respectively. There was a significant ($p = 0.0004$) quadratic effect of the AA percentages on the number of mitotic cells in bursal follicles (Fig. 1), indicating that the number of cells increased with 2% AA dose injected, decreasing thereafter.

Table 1: Weight and index from the bursa of Fabricius of newly-hatched chicks from eggs injected with different Ascorbic Acid (AA) levels and incubated at 37.5°C or 39°C

	Bursa weight (g)	Bursa index* (%)
Treatments (T)		
Control	0.058	0.114
AA-0%	0.051	0.100
AA-2%	0.055	0.109
AA-4%	0.061	0.120
AA-6%	0.056	0.109
Temperatures (TP)		
37.5°C	0.056	0.110
39°C	0.056	0.110
Probability		
T	0.3670	0.3945
TP	0.7730	0.9073
T x TP	0.4504	0.4812
CV (%)	26.45	26.65

CV: coefficient of variation
*Calculated in relation to body weight

Bursa histology and morphometry: There was a significant ($p\leq0.05$) interaction between treatments and incubation temperatures for follicle area, epithelial lining thickness (inside bursae) and bursal wall thickness (Table 2). This interaction for follicle area (Table 3) showed no significant ($p>0.05$) difference in this variable between chicks from injected and non-injected eggs under thermoneutral temperature conditions. However, under high temperatures, the difference was significant ($p\leq0.05$), where the smallest follicle area occurred in chicks from eggs injected with 4% AA. Bursal follicle area was lower ($p\leq0.05$) in chicks after incubation at warm temperature than at thermoneutral temperature for both non-injected eggs, as for those injected with 4% and 6% AA. However, bursal follicle areas in chicks from eggs injected 0% AA (only water) and 2% AA remained unaltered by hot incubation temperature ($p>0.05$). Additionally, there was a cubic effect ($p\leq0.05$) of AA doses on bursal follicle area in chicks from eggs incubated at thermoneutral and hot temperatures (Table 3, Fig. 1), where 4% AA injection resulted in the highest follicle area in chicks from eggs incubated at thermoneutral temperature and the smallest follicle area in those incubated at hot temperature. The interaction between treatments and incubation temperature for bursal epithelial lining thickness (Table 4) showed that this trait in chicks from eggs injected with different AA percentages was higher ($p\leq0.05$) than in chicks from eggs not injected at thermoneutral temperature and lower ($p\leq0.05$) at hot temperature. Bursal epithelial lining thickness was significantly higher ($p\leq0.05$) in

Table 2: Size of follicles, thickness of internal epithelium and external wall and follicular cell proliferation from the bursa of Fabricius of newly-hatched chicks from eggs injected with different ascorbic acid (AA) levels and incubated at 37.5°C or 39°C

	Follicle size (μm²)[1]	Epithelium thickness (μm)[1]	Wall thickness (μm)[1]	Cell proliferation (n°)[1]
Treatments (T)				
Control	217.485 (12.17)	69.11 (4.17)	193.24 (5.20)	12.52 (2.57A)
AA-0%	225.457 (12.22)	66.65 (4.12)	196.70 (5.22)	11.08 (2.52B)
AA-2%	225.865 (12.21)	65.16 (4.13)	203.49 (5.21)	13.67 (2.65A)
AA-4%	213.185 (12.13)	61.06 (4.08)	193.12 (5.22)	12.33 (2.55A)
AA-6%	224.999 (12.30)	66.53 (4.15)	157.01 (4.98)	10.57 (2.47B)
Temperatures (TP)				
37.5°C	239.992 (12.29)	70.10 (4.20)	184.97 (5.17)	12.40 (2.57)
39°C	202.805 (12.09)	61.30 (4.06)	192.58 (5.17)	11.70 (2.54)
Probability				
T	0.1143	0.1196	<0.0001	0.0008
TP	<0.0001	<0.0001	0.8456	0.2923
T x TP	<0.0001	<.0001	0.0002	0.0892
CV (%)	3.84	9.04	7.56	13.44
Polynomial				
Control x AA levels	-	-	-	0.5693
Linear for AA levels	-	-	-	0.0601
Quadratic for AA levels	-	-	-	0.0004
Cubic for AA levels	-	-	-	0.068

A-B: means fallowed by distinct letters differ significantly by Tukey test ($p<0.05$).
[1]Comparison from transformed data by log (values between commas)

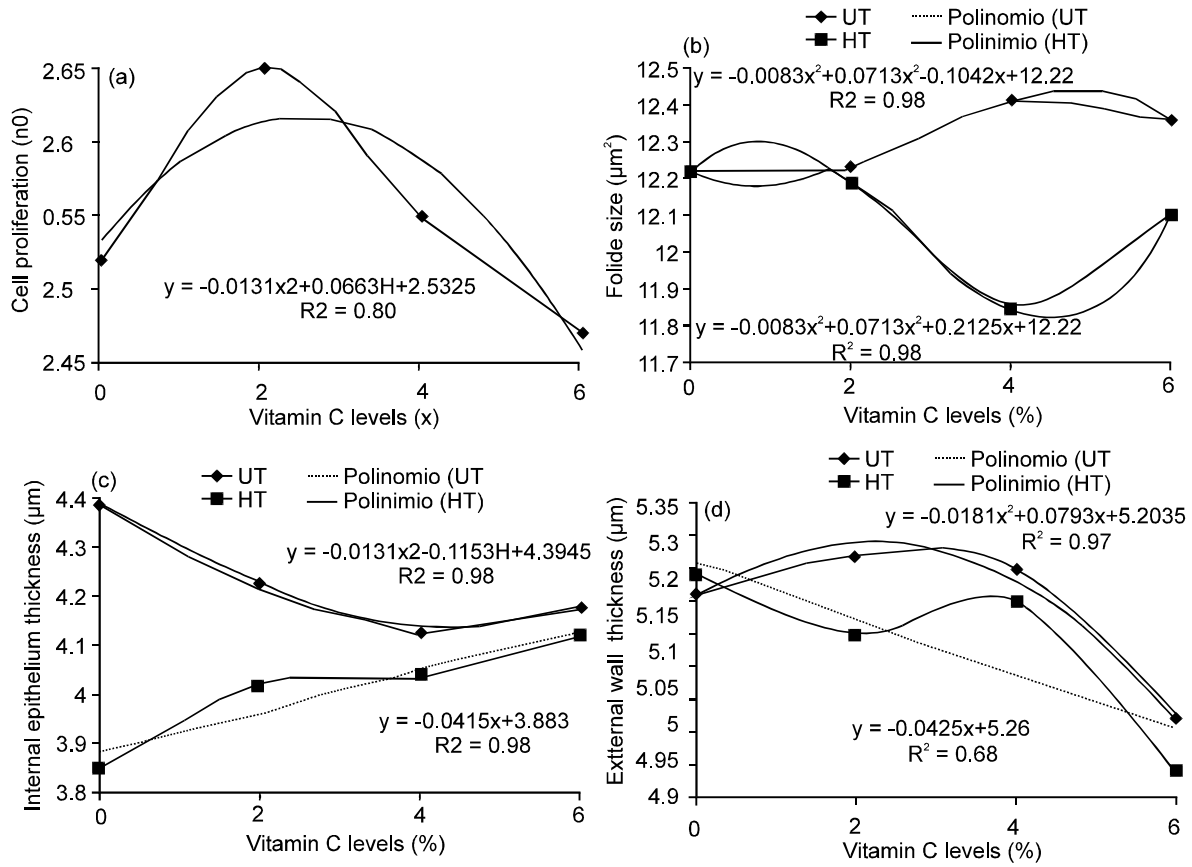

Fig. 1(a-d): Better equations for development of bursa of Fabricius. (a) Cell proliferation, (b) Follicle size, (c) Internal epithelium thickness and (d) External wall thickness. According to incubation temperature, as a function of the intra eggs injected ascorbic acid levels

chicks from eggs injected with 0% AA when incubated at thermoneutral temperature and in chicks from non-injected eggs when incubated at hot temperature and was significantly lower ($p\leq0.05$) in chicks from eggs

Table 3: Interaction between intra-eggs injected Ascorbic Acid (AA) levels and incubation temperature for follicle size (μm^2)[1] from the bursa of Fabricius of newly-hatched chicks

	Temperatures (T)		
Treatments	37.5°C	39°C	P
Control	229.518 (12.24) a	207.345 (12.11) Ab	0.0311
AA-0%	225.414 (12.22)	225.501 (12.22) A	0.9800
AA-2%	223.596 (12.23)	228.135 (12.19) A	0.4693
AA-4%	269.959 (12.41) a	157.718 (11.85) Bb	<0.0001
AA-6%	253.366 (12.36) a	196.632 (12.10) Ab	<0.0001
P	0.2221	<0.0001	
Polynomial	P		
Control vs. AA levels	0.1290	0.7180	
Linear for AA levels	0.0008	0.0004	
Quadratic for AA levels	0.4867	0.0017	
Cubic for AA levels	0.0246	<0.0001	

a-b, A-B: Means fallowed by distinct letters (lines and columns) differ significantly by Tukey test (P<0.05).
[1]Comparison from transformed data by log (values between commas).

Table 4: Interaction between intra eggs injected Ascorbic Acid (AA) levels and incubation temperature for internal epithelium thickness (µm)[1] from the bursa of Fabricius of newly-hatched chicks

	Temperatures (T)		
Treatments	37.5°C	39°C	P
Control	61.72 (4.07) Bb	76.49 (4.28) Aa	<0.0001
AA-0%	84.62 (4.39) Aa	49.01 (3.85) Cb	<0.0001
AA -2%	71.98 (4.23) ABa	58.33 (4.02) Bb	<0.0001
AA -4%	63.99 (4.13) Ba	58.13 (4.04) Bb	0.0481
AA -6%	69.19 (4.18) B	64.87 (4.12) B	0.2626
P	<0.0001	<0.0001	
Polynomial	P		
Control vs. AA levels	<0.0001	<0.0001	
Linear for AA	<0.0001	<0.0001	
Quadratic for AA levels	0.0021	0.2418	
Cubic for AA levels	0.5983	0.1543	

a-b, A-B: Means fallowed by distinct letters (lines and columns) differ significantly by Tukey test (p<0.05).
[1]Comparasion from transformed data by log (values between commas).

Table 5: Interaction between intra eggs injected Ascorbic Acid (AA) levels and incubation temperature for external wall thickness (µm)[1] from the bursa of Fabricius of newly-hatched chicks

	Temperatures (T)		
Treatments	37.5°C	39°C	P
Control	168.63 (5.10) BCb	218.05 (5.29) Aa	<0.0001
AA-0%	191.89 (5.21) ABa	201.51 (5.24) Ab	<0.0001
AA-2%	205.54 (5.27) Aa	201.45 (5.15) Ab	<0.0001
AA-4%	199.33 (5.25) ABa	186.91 (5.20) Ab	0.0485
AA-6%	159.94 (5.02)C	154.55 (4.94) B	0.2555
P	<0.0001	<0.0001	
Polynomial	P		
Control vs. AA levels	<0.0001	<0.0001	
Linear for AA levels	<0.0001	<0.0001	
Quadratic for AA levels	0.0030	0.1974	
Cubic for AA levels	0.6238	0.165	

a-b, A-B: means fallowed by distinct letters (lines and columns) differ significantly by Tukey test (P<0.05).
[1]Comparasion from transformed data by log (values between commas).

injected with 0, 4 and 6% AA. There was a significant ($p \leq 0.05$) negative linear effect of AA percentages on the trait in question, in chicks from eggs incubated at 39°C and a significant ($p \leq 0.05$) quadratic effect in chicks from eggs incubated at 37.5°C (Table 4, Fig. 1).

Bursal wall thickness in chicks from eggs injected with different AA doses was higher ($p \leq 0.05$) than in chicks from non-injected eggs incubated at thermoneutral temperature and lower ($p \leq 0.05$) at hot temperature (Table 5). Compared to chicks from non-injected eggs,

Table 6: Leucogram of newly-hatched chicks from eggs injected with different ascorbic acid (AA) levels and incubated at 37.5 or 39°C

Treatments (T)	TCLeu[1] (mm^3)	T (%)	Basophils[1] (%)	Eosinophils[1] (%)	H (%)	M (%)	Lymphocytes[1] (%)	H/L
Control	53.280 (10.77)	72.78	1.22 (0.89)	1.33 (0.90)	7.33	14.89	2.44 (1.19AB)	2.73
Ascorbic acid-0%	53.733 (10.71)	75.73	0.55 (0.67)	1.00 (0.85)	6.73	14.46	1.27 (0.77B)	1.45
Ascorbic acid-2%	36.480 (10.34)	65.10	1.10 (0.89)	2.10 (1.23)	7.40	15.80	1.75 (0.93B)	1.05
Ascorbic acid-4%	39.200 (10.34)	70.20	1.30 (0.92)	1.00 (0.81)	4.80	15.00	5.00 (1.48AB)	1.50
Ascorbic acid-6%	42.500 (10.58)	70.36	1.09 (0.86)	1.36 (0.88)	3.73	12.73	7.30 (1.78A)	0.37
Temperatures (TP)								
37.5°C	45.890 (10.58)	70.67	1.25 (0.92)	1.33 (0.94)	6.83	12.96	5.13 (1.49A)	1.31
39°C	44.573 (10.52)	71.07	0.85 (0.78)	1.39 (0.44)	5.15	15.93	2.08 (0.98B)	1.83
Probability								
T	0.2339	0.2220	0.7081	0.3444	0.1086	0.9432	0.0185	0.4920
TP	0.8648	0.8888	0.2943	0.9247	0.1414	0.2134	0.0217	0.7216
T x TP	0.0582	0.0701	0.9802	0.8811	0.1366	0.6687	0.1237	0.7390
CV (%)	5.33	14.83	53.22	52.52	61.77	60.51	59.33	99.31
Control x vit. C levels	-	-	-	-	-	-	0.7812	-
Linear for vit. C levels	-	-	-	-	-	-	0.0008	-
Quadratic for vit. C levels	-	-	-	-	-	-	0.5427	-
Cubic for vit. C levels	-	-	-	-	-	-	0.8623	-

TCLeu: total counts of leucocytes. AA: ascorbic acid. A-B: means fallowed by distinct letters differ significantly by Tukey test (P<0.05).
[1]Comparasion from transformed data by log (values between commas). T: Thrombocytes, H: Heterophils, M: Monocytes

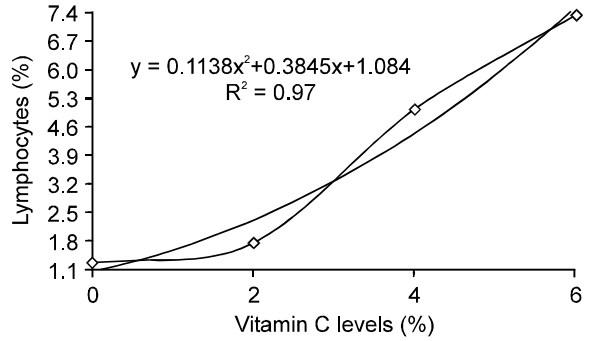

Fig. 2: Percentage of lymphocytes of newly-hatched chicks, as a function of intra eggs injected ascorbic acid levels. This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability

this trait was higher ($p \leq 0.05$) in chicks from eggs injected with 0% AA (only water) and lower ($p \leq 0.05$) in chicks from eggs injected with 6% AA. When eggs were incubated at 39 and 37.5°C, bursal wall thickness was higher ($p \leq 0.05$) in chicks from eggs injected with 2% AA and lower ($p \leq 0.05$) in chicks from eggs injected with 6% AA. Bursal wall thickness was higher ($p \leq 0.05$) when incubated at hot temperature in chicks from non-injected eggs and lower ($p \leq 0.05$) in chicks from eggs injected with 0, 4 and 6% AA. There was quadratic and significantly ($p \leq 0.05$) decreasing linear effect of AA levels on this trait when incubating eggs at 37.5 and 39°C, respectively (Table 5, Fig. 1), exhibiting reduced bursal wall thickness with increasing AA percentage injection in the case of the linear effect.

Total and differential leukocyte counts and heterophil/lymphocyte (H/L) ratio: There were no significant (P>0.05) effects of treatments and incubation temperatures on the total leukocyte count; percentages of thrombocytes, basophils, eosinophils, monocytes and heterophils; and H/L ratio (Table 6). However, there was a significant ($p \leq 0.05$) effect of incubation temperatures on percentage of lymphocytes which was lower in chicks from eggs incubated under hot temperature conditions. There was significant ($p \leq 0.0008$) quadratic effect of AA levels on percentage of lymphocytes (Table 6, Fig. 2), according to which percentage of lymphocytes increased with increasing AA doses.

DISCUSSION

The present study shows that heat stress during incubation and intra egg injection of AA did not significantly affect weight and bursal index. Alloui *et al.* (2005) classified the bursal index (x) in broiler chickens as excellent (if x>0.20%), good (0.18% <x≥0.20%), medium (up 0.15% <x≤0.18%) and bad (if x≤0.15%). The bursal rate at hatching obtained in this study did not reach 0.15% which can be considered a bad index for broiler chickens according to the classification by Alloui *et al.* (2005). This low bursal index shows that regardless of incubation temperature and AA injection, newly-hatched chicks have low protection ability due to acquired immunity which may be related to the fact that this lymphoid organ develops after hatching (Paramithiotis and Ratclffe, 1994; Glick, 2000). Although weight and bursal index indicate the absence of immunosuppressive or immunostimulatory effect of incubation temperature and AA on bursal immune response, follicle area, epithelial lining and bursal wall were altered due to both variables. This strengthens the bursal morphometric indices as good indicators of stress action, as mentioned by Donker and Beuving (1989), or immunomodulators. Non-injected eggs continually incubated at hot temperature exhibited reduced bursal development, decreased lymphoid follicle size, epithelial lining thickness and bursal wall

thickness compared to those incubated at thermoneutral temperature. According to Paramithiotis and Ratcliffe (1994), less bursal development at hatching caused by stress can interfere at the onset of B lymphocyte migration from the bursa to peripheral lymphoid organs, a process that normally begins around hatching and continues in postnatal life. Morphometric data of the present study (bursal follicle area, epithelial lining thickness and bursal wall thickness), considering the relationship between morphology and function, indicate the immunosuppressive effect of heat stress on the bursal acquired immune response which should have caused a lower percentage of leukocytes in chick blood, recorded in the leucogram. Reduced bursal follicle size was also recorded by Torres (2006) under heat stress at birth. This indicates that reduced follicle size is an effect of heat stress at incubation or birth.

Intra egg injection of AA before incubation at thermoneutral or hot temperatures exerted dose-dependent effects on morphometric variables assessed in bursae which were not always similar and at times even contradictory. Follicle area was affected by 4 and 6% AA injection and increased when incubated at thermoneutral temperature and decreased when incubated at hot temperature. This result is very interesting, as it suggests that AA exerted adverse effects on the acquired immune response, i.e., immunostimulatory when incubated at thermoneutral temperature and immunosuppressive when incubated under heat stress conditions.

Lymphocytosis from injecting increasing AA doses was another indicator of the stimulatory effect of intra egg injection of AA on chick innate immune response. The effects of intra egg injection of 4 and 6% AA on follicles were accompanied by reduced bursal wall thickness and altered epithelial lining thickness (inside bursae) when incubated at hot temperature. The bursal epithelium undergoes pinocytotic activity from 19-20 days incubation (Dieterlen-Lièvre, 1994; Glick, 1995), where intensity can accentuate epithelial thickness. The effect of AA injection on bursal epithelial lining thickness may be indicative of stimulation of epithelial pinocytic activity.

The bursa is colonized by lymphocyte progenitor cells during the second week of embryonic life which proliferate and form follicles (Paramithiotis and Ratcliffe, 1994). Thus, the immunosuppressive or immunostimulatory effects on the acquired immune response can also be exerted via cell proliferation rate. Our data show significant dose-specific effect of AA on cell proliferation in bursal follicles. Intra egg injection of 2% AA increased cell proliferation in bursal follicles, however decreases with higher injection doses.

The bird cellular defense system consists of monocytes, heterophils, basophils, eosinophils, thrombocytes and lymphocytes (Morgulis, 2002), whereas the first five act in innate immune defense and last in acquired immune defense. Stress may depress or stimulate the immune defense system, quantitatively altering the presence of cells in the blood (Borges *et al*., 2003) which makes granulocytes and heterophils and the usual H/L ratio indicators of response to stress in birds (Gross and Siegel, 1983; Macari and Luquetti, 2002; Laganá and Ribeiro, 2007). Bird immune response can also be modulated by vitamin agents such as vitamin C which improve response to heat stress (Pardue and Thaxton, 1984; Pardue *et al*., 1985; Campo and Dávila, 2002; Furlan and Macari, 2002). In this study, the leucogram data were used to determine whether intra egg injection of Ascorbic Acid (AA) before incubation at thermoneutral and hot temperatures alter the potential of the innate immune response in chicks. According to our data, there were no changes in total leukocyte count or amounts of cell types of the immune defense system-basophils, eosinophils, heterophils, thrombocytes and monocytes-and in the H/L ratio, related to hot temperature incubation, suggesting that the innate immune defense system may be adapted to changes in temperature during intra egg development which remain at conditions within the limit of thermal tolerance.

The present study shows the effect of immunomodulating dose and temperature-dependent ascorbic acid on development of the bursa of Fabricius during incubation and that 4 and 6% AA injection induces leukocytosis. Although, from a practical standpoint, intra egg injection of AA during pre-incubation appears to be a feasible expedient in producing chicks with higher potential for immune defense at hatching, further research on this potential action of the ascorbic acid is required.

ACKNOWLEDGMENTS

The authors are grateful to the Foundation for Research Support of the State of São Paulo (FAPESP) for financial support for the project (Proc. No. 2010/15280-0) and the Ph.D. scholarship granted to S.S. (Proc. No. 2010/01923-7).

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[1]Detection kit, Starr Trek Universal HRP, Biocare Medical, USA.

[2]PCNA Clone PC10, Biocare Medical, USA, origin: mouse monoclonal anti-body.