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Effects of Intra-Egg Injection of Vitamin C on the Eggshell Mineral Absorption, Embryo Mortality and Hematological Variables in Chicks at Hot Incubation Temperature

Sarah Sgavioli[1], Vitor Rosa de Almeida[1], Maria Fernanda F.M. Praes[1], Tamiris Iara Vicentini[1], João Batista Matos Júnior[1], Viviane de Souza Morita[1], Euclides Braga Malheiros[2] and Isabel Cristina Boleli[1]
[1]Department of Animal Morphology and Physiology, [2]Department of Exact Sciences,
Faculty of Agricultural and Veterinary Sciences, São Paulo State University, UNESP, Brazil

Abstract: This study examined whether preincubation injection of vitamin C intra-eggs influences the eggshell mineral absorption, embryo mortality and hematological characteristics of chicks from eggs incubated at usual or hot temperatures. Five hundred fertile eggs from broiler breeder (Cobb®) were used in an experiment consisting of a 5 x 2 factorial arrangement (five treatments: no injection or injection of 0%, 2, 4, or 6% vitamin C/100 µL water; two incubation temperatures: 37.5°C and 39.0°C). The percentages of vitamin C injected into the eggs did not influence the hematological characteristics of the chicks when compared to non-injected controls. However, chicks from eggs injected with 4% vitamin C had a higher total number of Red Blood Cells (RBCs) and levels of Hematocrit (HCT) and Hemoglobin (HGB) when incubation occurred at hot temperatures. The lowest percentage of Phosphorus (P) from the eggshells incubated at hot temperatures was observed with 2% vitamin C and the highest with 6% vitamin C. Embryo mortality was higher in eggs injected with 2 and 4% of vitamin C than in eggs not injected with vitamin C. The data indicate that intra-eggs injected vitamin C influences the phosphorus absorption from the shell and injection of 4% vitamin C improves potential gas transport in the blood of chicks from eggs incubated at hot temperatures but increases the embryo mortality.

Key words: Ascorbic acid, erythrogram, heat stress, blood pH

INTRODUCTION

Heat stress decreases hatching rate and chick quality, influencing chick performance post-hatching (Molenaar *et al*., 2011). At the same time, however, during embryonic stages, heat stress can imprint a higher thermotolerance to temperature deviations during the chick's development, expanding its thermal tolerance zone (Tzschentke and Plagemann, 2006; Tzschentke, 2007).

High temperatures accelerate fetus development which requires increased metabolism and thereby higher oxygen (O_2) levels and elimination of carbon dioxide (CO_2), for which a cardiovascular and/or hematologic adjustment is necessary. Respiratory, cardiovascular and metabolic potential can be analyzed by respiratory and biochemical characteristics in the blood which are particularly sensitive to temperature changes, making them important indicators of physiological responses of the bird to different stressors. Heat stress can cause changes in Hematocrit (HCT) levels, the number of Red Blood Cells (RBCs) and Hemoglobin (HGB) levels in erythrocytes (Borges *et al*., 2003b). Increases in respiration rate can result in a significant loss of CO_2, causing a decrease in the partial pressure of CO_2 (pCO_2) and leading to drops in the concentrations of carbonic acid (H_2CO_3) and hydrogen (H^+) and an increase in blood pH. In response, the kidneys increase the excretion of bicarbonate (HCO_3^-) and reduce the excretion of H^+ in an attempt to maintain the acid-base balance of the bird (Rondon *et al*., 2000; Borges *et al*., 2003b; Mushtaq *et al*., 2005), known as respiratory alkalosis. Sodium (Na^+), potassium (K^+), chloride (Cl^-) and phosphate (PO_4^{---}) are essential ions in maintaining the osmotic pressure and acid-base balance of bodily fluids. K^+ is the main cation of the intracellular fluid, while Na^+ and Cl^- are the major ions of the extracellular fluid (Rondon *et al*., 2000). Phosphorus has important participation in the energy utilization, storage and transference by ATP, ADP and ATP, several enzymatic processes and structure of nucleic acids (Lenhninger *et al*., 2002). In the skeleton, the phosphate ion is encountered under the form of calcium phosphate and provides bone rigidity. The concentrations of K^+ and Na^+ decrease (Borges *et al*., 2002), whereas Cl^- (Belay and Teeter, 1993) and P^{+++} concentrations increase as the temperature rises. Consequently, changes in the homeostasis of this ions affect cellular functions (Borges *et al*., 2003b; Mushtaq *et al*., 2005).

Corresponding Author: Isabel Cristina Boleli, Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellani s/n, 14884-900 Jaboticabal-SP, Brasil

Data from the literature have shown that vitamin C can prevent or minimize the effects of heat stress (Mahmoud *et al*., 2004), as well as improve the development of birds in a thermoneutral environment (Pardue and Thaxton, 1986). This vitamin enhances the absorption of iron, consequently increasing both the number of RBCs and HGB levels (Moura and Pedroso, 2003) and improving the respiratory potential of the animal. The developing embryo nutritionally depends on the composition of the egg which may therefore influence hatching rate and chick quality both during and after hatching (Finkler *et al*., 1998). Thus, intra-egg administration of nutrients may be an alternative method for manipulating chick quality and performance after hatching. If vitamin C is an anti-stressor and enhances the performance of the bird (Pardue and Thaxton, 1986; Mahmoud *et al*., 2004), it is possible that intra-egg injection of vitamin C may be beneficial to embryos under thermoneutral or heat-stress conditions. Although reports in the literature have described the effects of vitamin C on the development of birds (Zakaria and Al-Anezi, 1996; Ghonim *et al*., 2009; Mohammed *et al*., 2011; Nowaczewski *et al*., 2012), these studies refer to intra-egg injection of vitamin C during the later stages of embryonic development. There are no data in the literature on the effects of vitamin C injected into the egg preincubation on the hematologic characteristics of chicks hatched from eggs incubated under thermoneutral and heat-stress conditions. This study examined whether preincubation intra-egg injection of vitamin C influences the eggshell mineral absorption, embryo mortality and hematological characteristics of chicks from eggs incubated under thermoneutral and heat-stress conditions.

MATERIALS AND METHODS

Experimental design: The experimental protocol of this study was approved by the Ethics Committee on Animal Use (protocol number 7377/10), from the Faculty of Agricultural and Veterinary Sciences, São Paulo State University-UNESP. Fertile eggs from broiler breeder (Cobb) 47-week-old were weighed and used in a completely randomized 5 x 2 factorial arrangement with five treatments (uninjected or intra-egg injection of 0, 2, 4, or 6% vitamin C in 100 mL water) and two incubation temperatures (thermoneutral: 37.5°C and hot: 39.0°C). Two incubators were used to house 40 fertile eggs per treatment at each temperature and the average weight of the eggs per treatment and repetition was 67±2 g. Incubators (model IP-200, Premium Ecologic, Belo Horizonte, MG, Brazil), with automatic temperature control and rotation of the eggs every 2 h, were maintained at 60% relative humidity until day 18 of incubation, from which 70% relative humidity was used and the egg rotation was stopped. The injection of an aqueous solution of vitamin C was performed preincubation with the eggs kept in a horizontal position. After local disinfection of the shell with 100% ethanol, a solution of vitamin C (Sigma) was injected into the albumen approximately 1 cm from the tapered end of the egg and at a depth of 5 mm of depth using a sterile needle (model 0.38 x 13, 27.5 G1/2", Injex, Ourinhos, SP, Brazil). After injection, the hole was sealed with an identification label that noted the treatment and replicate. The vitamin C solution was prepared shortly before application with Milli-Q water, autoclaved and kept in a dark environment due to its photosensitivity. From day 18 of incubation, the eggs were individually maintained into bags of tulle, isolating chicks from different treatments, well as avoiding the lost of eggshell particles.

Blood characteristics: At hatching, eight male chicks per treatment were used in the analysis the following blood variables: RBCs (cells/mm^3) HCT (% PCV) HGB (g/dL), pH, pCO_2 (mm Hg), pressure of oxygen (pO_2) (mm Hg) base excess (BEecf) (mM), total of carbon dioxide (TCO_2) (mM), hemoglobin oxygen saturation (SO_2) (%), Na^+ (mM), K^+ (mM), ionized Ca (iCa) (mM), HCO_3^- (mM) and glucose (mg/dL). Blood amounts were collected from the jugular vein using a syringe containing sodic heparin (15 µl heparin/1 mL blood; Glistab, cat. 29th, Labtest Diagnostica) and immediately analyzed using a portable clinical analyzer (I-STAT Co., Abbott Laboratories, USA, Cg8+cartridge). For RBC and leucocyte counts, blood collected from the jugular vein was kept in plastic tubes and maintained on ice for analyses. The counts were carried out in a Neubauer chamber, using diluted blood samples (1:100) in a Natt and Henrick (1952) solution. The pH, pCO_2 and pO_2 were adjusted to the average surface temperature according to Richard *et al*. (1971).

Percentage, thickness, ash and minerals from the eggshell: The percentage, thickness and mineral composition of the eggshells at the end of the incubation period were obtained from an analysis of 10 eggs per treatment after removal of the inner and outer membranes and cuticle following the method of Rahn *et al*. (1981), keeping the shell fragments in a boiling aqueous solution of 0.5% NaOH. Following that procedure, the shells were washed in distilled water and dried at room temperature for 72 hours for subsequent analyses. The weight of the eggshells is provided as a percentage relative to the weight of the egg preincubation. Shell thickness was obtained from a measurement of fragments from the equatorial region of the eggshells, using a digital micrometer (model MDC-Lite, 0.001-mm resolution, Mitutoyo). The mineral composition (percentage of calcium, phosphorus) and ash of the eggshells was performed according to methods described by Silva and Queiroz (2002).

Statistical analysis: Data were subjected to analysis of variance by the General Linear Model (GLM) using the SAS program (SAS, 2002). Data were analyzed for the presence of outliers (Box-and-Whisker plot), normal distribution of studentized errors (Cramer-Von-Mises test) and homogeneity of variances (Brown-Forsythe) (Littell *et al*., 2002). In cases of significance, a comparison of means among vitamin C levels was performed by 5% probability polynomial orthogonal contrasts: contrast 1: comparison between the treatment versus the control average of treatments with 0, 2, 4, or 6% vitamin C; contrasts 2, 3 and 4: comparisons using three regression models: linear, quadratic and cubic (Robbins *et al*., 1979), with the purpose of verifying the effects of vitamin C levels.

RESULTS

Hematologic respiratory characteristics: The effects of different percentages of intra-egg injection of vitamin C on the respiratory characteristics of chicks hatched from eggs incubated at 37.5 and 39°C (thermoneutral and hot temperatures, respectively) are shown in Table 1. There was a significant interaction (P<0.05) between the percentages of vitamin C and incubation temperatures for the three erythrocyte characteristics analyzed: RBC count and HGB and HCT levels. According to Table 2, RBC values were higher (P<0.05) after incubation at 39 than at 37.5°C in chicks from eggs injected with 4% vitamin C. Furthermore, there was no significant difference (P>0.05) between RBC values in chicks from eggs injected with different percentages of vitamin C and those not injected, regardless of incubation temperature. Additionally, there was a quadratic effect of the inoculation levels of vitamin C on RBC values after incubation at the thermoneutral temperature (P<0.05) and a cubic effect of the inoculation levels of vitamin C at the hot temperature (P<0.05; Fig. 1). The injection of water alone (0% vitamin C) increased the RBC count at the thermoneutral temperature, whereas a higher RBC count was observed after injection of 4% vitamin C at the hot temperature (Fig. 1). Tables 3 and 4 show the percentage effect of the interaction between vitamin C and incubation temperature on HGB and HCT. There were no significant differences (P>0.05) in the HGB and HCT values between chicks from uninjected eggs and those injected with different percentages of vitamin C at any of the temperatures studied. Additionally, there were neither significant differences (P>0.05) between control treatment and vitamin C percentages nor significant regression (P>0.05) of the percentages of vitamin C and the HGB and HCT values. However, the HGB and HCT values of chicks from eggs injected with 4% vitamin C were higher (P<0.05) after incubation at 39°C than at 37.5°C.

Biochemical characteristics of blood: The values reported for p H, pCO_2, pO_2, BEecf, TCO_2, SO_2, HCO_3^-, Na^+, K^+, ionized calcium and glucose are shown in Table 5. None of these variables were influenced by the different percentages of vitamin C injected into the eggs or by the temperature of incubation (P>0.05). However,

Table 1: Erythrocytic values of newly-hatched broiler chicks from eggs injected with different vitamin C percentages and incubated at 37.5°C and 39°C

	RBC[1] (cels/mm^3)	HCT (%PCV)	HGB (g/dL)
Treatments (T)			
Control	352.920 (12.58)	15.36	5.24
Vitamin C-0%	373.511 (12.63)	14.00	4.75
Vitamin C-2%	194.400 (12.05)	14.17	4.80
Vitamin C-4%	642.300 (12.59)	15.67	5.33
Vitamin C-6%	431.000 (12.73)	13.77	4.68
Temperatures (TP)			
37.5°C	298.527 (12.20)	14.90	5.06
39°C	491.721(12.81)	14.41	4.90
Probability			
T	0.2746	0.8123	0.7972
TP	0.0089*	0.8441	0.8703
T x TP	0.0154*	0.0465*	0.0466*
CV (%)	6.64	26.15	26.31

CV: Coefficient of variation, *Significant at P<0.05

[1]Comparison from data transformed by log (values between brackets). RBCs: Total number of red blood cells, HCT: Levels of hematocrit and HGB: Hemoglobin

Table 2: Interaction between intra-eggs injected vitamin C levels and incubation temperature on number of red blood cells (RBC[1], cels/mm^3) of newly-hatched broiler chicks

	Temperatures		
Treatments	37.5°C	39°C	Probability
Control	293.533 (12.35)	442.000 (12.93)	0.2891
Vitamin C-0%	474.800 (12.95)	292.480 (12.38)	0.3153
Vitamin C-2%	129.600 (11.59)	226.800 (12.28)	0.2469
Vitamin C-4%	279.040 (11.54)b	1098.400 (13.90)a	0.0002*
Vitamin C-6%	280.800 (12.50)	581.200 (12.95)	0.4505
Polynomial		Probability	
Control x vitamin C levels		0.6848	0.8721
Linear for vitamin C levels		0.5606	0.0127*
Quadratic for vitamin C levels		0.0426*	0.1242
Cubic for vitamin C levels		0.9017	0.0001*

a-b: Means in the same row with distinct superscripts are different at p<0.05. *Significant at p<0.05.

[1]Comparison from data transformed by log (values between brackets)

Table 3: Interaction between intra-eggs injected vitamin C levels and incubation temperature on hematocrit[1] (HCT, %PCV) of newly-hatched broiler chicks

	Temperatures		
Treatments	37.5°C	39°C	Probability
Control	17.50 (17.40)	14.50 (13.67)	0.1171
Vitamin C-0%	11.00 (14.50)	10.00 (13.50)	0.7957
Vitamin C-2%	13.00 (14.25)	14.00 (14.00)	0.9404
Vitamin C-4%	10.00 (12.00)b	24.00 (18.60)a	0.0150*
Vitamin C-6%	16.50 (15.33)	11.00 (12.43)	0.1822
Polynomial		Probability	
Control x vitamin C levels		0.1401	0.5874
Linear for vitamin C levels		0.9815	0.8778
Quadratic for vitamin C levels		0.4376	0.1171
Cubic for vitamin C levels		0.4350	0.1240

a-b: Means in the same row with distinct superscripts are different at p<0.05.

[1]Comparasion from data transformed by log (values between brackets)

Table 4: Interaction between intra-eggs injected vitamin C levels and incubation temperatures on hemoglobin[1] values (HGB, g/dL) of newly-hatched broiler chicks

	Temperatures (T)		
Treatments	37.5°C	39°C	Probability
Control	5.95 (5.92)	4.95 (4.67)	0.1238
Vitamin C-0%	3.70 (4.90)	3.40 (4.60)	0.8203
Vitamin C-2%	4.40 (4.83)	4.75 (4.75)	0.9477
Vitamin C-4%	3.40 (4.07)b	8.20 (6.34)a	0.0146*
Vitamin C-6%	5.65 (5.21)	3.75 (4.21)	0.1784
Polynomial		Probability	
Control x vitamin C levels		0.1381	0.6093
Linear for vitamin C levels		0.9569	0.8878
Quadratic for vitamin C levels		0.4423	0.1164
Cubic for vitamin C levels		0.4412	0.1177

a-b: means in the same row with distinct superscripts are different at P<0.05.

[1]Comparison from data transformed by log (values between brackets)

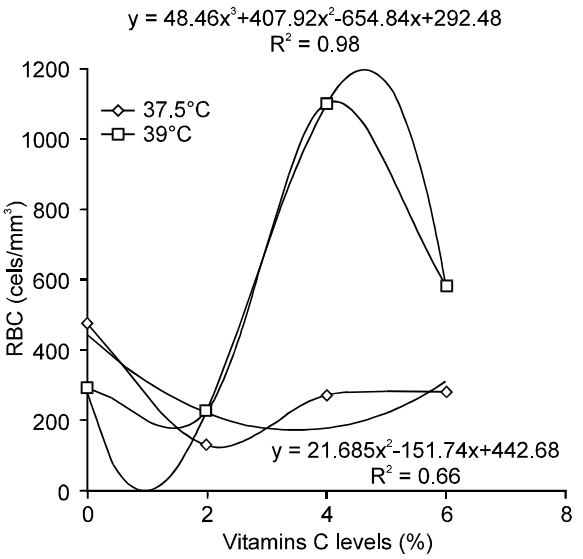

Fig. 1: Number of red blood cells (RBC, cels/mm^3) in newly-hatched chicks from eggs incubated at 37.5°C or 39°C temperature as a function of intra-eggs injected vitamin C levels

there were significant interactions (P<0.05) between the percentages of vitamin C and temperatures that affect blood pH, in which the data show that chicks from eggs injected with 6% vitamin C had a higher pH after incubation at the hot temperature than at the thermoneutral temperature (Table 6). Moreover, there was a significant quadratic effect (P<0.05) of the percentages of vitamin C on the blood pH of chicks from eggs incubated at 39°C (Fig. 2), in which the lowest values were recorded for chicks from eggs injected with 2% vitamin C and the highest values were observed in chicks from eggs injected with 6% vitamin C. Additionally, there were no significant differences (P>0.05) in blood pH between chicks from uninjected eggs and those injected with vitamin C. With respect to

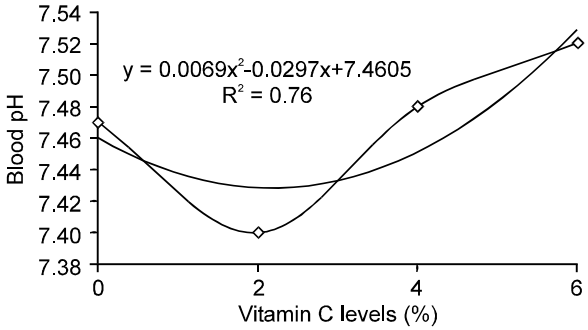

Fig. 2: Blood pH of newly-hatched broiler chicks from eggs incubated at 39°C as a function of intra-eggs injected vitamin C levels

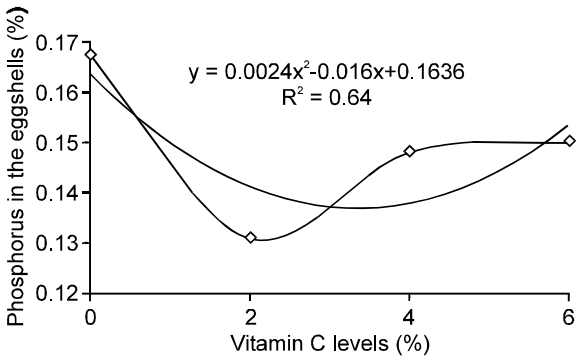

Fig. 3: Phosphorus percentages in the eggshells incubated at 39°C as a function of intra-eggs injected vitamin C levels

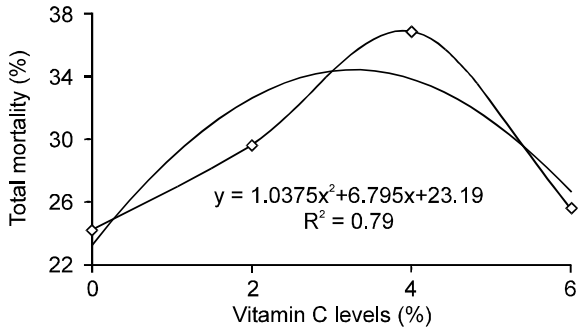

Fig. 4: Total mortality percentages a function of intra-eggs injected vitamin C levels

the concentrations of Na^+, K^+, ionized calcium and glucose, no significant effects were recorded (P>0.05) between the percentages of vitamin C and incubation temperatures and no significant interactions between these two factors regarding these blood variables were observed.

Percentage, thickness and eggshell minerals: As shown in Table 7, the percentage, thickness and

Table 5: Blood biochemical characteristics of newly-hatched broiler chicks from eggs injected with different vitamin C percentages and incubated at 37.5°C or 39°C

	pH	pCO_2 (mmHg)	pO_2 (mmHg)	BEecf (mmol/l)	TCO_2 (mmol/l)	sO_2 (%)	HCO_3^- (mmol/l)	Na^+ (mmol/l)	K^+ (mmol/l)	iCa (mmol/l)	Glucose (mg/dL)
Treatments (T)											
Control	7.43	20.97	19.00	-10.47	15.93	50.20	15.07	130.00	4.53	0.595	156.87
Vitamin C-0%	7.46	19.11	19.60	-10.71	15.14	59.00	14.53	129.75	3.63	0.320	136.11
Vitamin C-2%	7.43	20.27	17.87	-10.71	15.50	46.79	14.70	129.31	4.32	0.486	156.50
Vitamin C-4%	7.47	18.74	18.08	-10.15	15.54	53.00	14.77	130.92	4.04	0.540	149.50
Vitamin C-6%	7.45	19.19	18.78	-11.20	15.13	53.00	14.39	128.17	4.91	0.360	141.11
Temperatures (TP)											
37.5°C	7.44	20.74	19.03	-10.45	15.93	52.14	15.19	129.41	4.17	0.483	156.53
39°C	7.46	18.92	18.33	-10.83	15.11	51.23	14.31	129.56	4.55	0.434	143.95
Probability											
T	0.6424	0.2136	0.8337	0.9645	0.8548	0.5482	0.9060	0.1362	0.2648	0.3577	0.3054
TP	0.4346	0.0891	0.7184	0.8522	0.5107	0.6821	0.4470	0.7539	0.2816	0.4384	0.1919
T x TP	0.0071*	0.0939	0.0925	0.0618	0.0748	0.1518	0.0736	0.7025	0.8730	0.1602	0.4487
CV (%)	0.96	16.74	20.62	-36.32	20.61	27.13	21.33	2.28	32.59	40.55	18.51

CV: Coefficient of variation. *Significant at $p<0.05$. pCO_2: Pressure of carbon dioxide, pO_2: Pressure of oxygen, BEecf: base excess (mM), TCO_2: Total of carbon dioxide, SO_2: hemoglobin oxygen saturation, H_2CO_3: Concentrations of carbonic acid, Na^+: Sodium, K^+: Potassium, iCa: Ionized Ca

amount of ash and calcium from the eggshell at the end of the incubation period were not affected significantly ($P>0.05$) by either the percentage of vitamin C or by incubation temperature but there was a significant interaction between these factors regarding the percentage of P in the eggshell. In accordance with the unfolding of this interaction (Table 8), there were differences in the percentage of P between eggshell incubated at 37.5 and 39°C but only in eggs injected with 0 or 2% vitamin C ($P<0.05$). The percentage of P was greater after incubation at 39°C than at 37.5°C in eggs injected with water (0% vitamin C) and lesser in eggs that received an injection of 2% vitamin C. Moreover, there was a significant quadratic effect ($P<0.05$) of the percentages of vitamin C on the percentage of P in the eggshell at the end of the incubation period (Table 8) at the hot temperature, with the lowest percentage of P in eggshells at the end of the incubation period for eggs injected with water (0% vitamin C) (Fig. 3).

Mortality: According to Table 8, the percentages of precoces, intermediate and latter mortality were not influenced significantly ($P>0.05$) by either the percentage of vitamin C or by incubation temperature but occurred significant effect of the vitamin C on total percentage of mortality which was higher in eggs injected with 4% of vitamin C (Fig. 4).

DISCUSSION

The second half of the incubation period of bird eggs is characterized by intense fetal growth, involving increased metabolism (Tullet, 1990; French, 1997; Meijerhof, 1999; Tazawa and Whittow, 2000); therefore, increased demand for gas exchange (O_2 uptake and CO_2 removal) becomes larger after incubation at hot temperatures. Considering that vitamin C is related to an increase in RBC number and HGB and HCT levels (Moura and Pedroso, 2003), the present study examined whether intra-egg injection of vitamin C influences such hematological respiratory and biochemical variables related to respiratory metabolism (pH, pCO_2, pO_2, BEecf, TCO_2, SO_2, HCO_3^-, Na^+, K^+, iCa and glucose), that minimize the effects of increased metabolic demand and provide the chicks from eggs incubated under thermoneutral and hot conditions with a greater respiratory potential.

Chicks from eggs injected with 4% vitamin C had higher numbers of RBC and greater HGB and HCT levels when the incubation was performed at hot temperatures, indicating that an improvement in the gas exchange potential of the blood in those chicks and therefore an increase in hematopoietic process and respiratory potential occurred. Despite that, a 26% increase in the embryo mortality of these eggs was reported in the present study. Increased values of HGB and HCT may be related to dehydration (Campbell, 1994). However, although weight loss is higher for eggs after incubation at 39°C, there were no effects of this temperature on the relative weight of the chicks during hatching or evidence of chicks with characteristics of dehydration (Sgavioli *et al*., submitted). One cannot overlook that higher numbers of RBC, as well as greater HGB and HCT levels, grant a greater potential for gas exchange to chicks. However, it is possible that chicks from eggs injected with 4% vitamin C and incubated at hot temperature had presented a increased blood viscosity and cardiac demand that induced higher mortality rate. Ghonim *et al*. (2009) and Pires *et al*. (2011) did not report effects of vitamin C injection on erythrocyte characteristics of broiler chicks; however, the authors only looked at the effects of vitamin A in eggs incubated under thermoneutral conditions.

According to the data for blood pH, there was a dose-dependent effect of vitamin C in eggs incubated at hot temperature. Increased blood pH in chickens under conditions of heat stress is due to a reduction in pCO_2, resulting from increased respiratory rate and acid-base imbalance (Furlan and Macari, 2002) which may be

Table 6: Interaction between intra-eggs injected vitamin C levels and incubation temperatures on blood pH of newly-hatched broiler chicks

	Temperatures (T)		
Treatments	37.5°C	39°C	Probability
Control	7.45	7.42	0.4167
Vitamin C-0%	7.44	7.47	0.5279
Vitamin C-2%	7.47	7.40	0.0694
Vitamin C-4%	7.46	7.48	0.7212
Vitamin C-6%	7.39b	7.52a	0.0012*
Polynomial		---------- Probability ----------	
Control x vitamin C levels		0.7378	0.1044
Linear for vitamin C levels		0.3720	0.1168
Quadratic for vitamin C levels		0.1515	0.0429*
Cubic for vitamin C levels		0.8186	0.1015

a-bMeans in the same row with distinct superscripts are different at p<0.05. *Significant at p<0.05

Table 7: Shell characteristics at the end of incubation period of eggs injected with different vitamin C percentages and incubated at 37.5°C and 39°C

	Weight[1] (%)	Thickness (mm)	Ash (%)	Phosphates (%)	Calcium (%)
Treatments (T)					
Control	8.01	0.534	96.89	0.154	38.23
Vitamin C-0%	7.98	0.542	96.92	0.156	38.16
Vitamin C-2%	8.02	0.540	96.95	0.146	38.12
Vitamin C-4%	8.32	0.555	96.87	0.147	38.17
Vitamin C-6%	8.29	0.555	97.18	0.146	38.30
Temperatures (TP)					
37.5°C	8.20	0.544	96.99	0.150	38.21
39°C	8.04	0.546	96.95	0.150	38.19
Probability					
T	0.4925	0.3928	0.1109	0.4852	0.3025
TP	0.3518	0.7417	0.4364	0.9253	0.6187
T x TP	0.1001	0.1385	0.8181	0.0197*	0.0923
CV (%)	7.96	6.07	0.34	13.33	0.59

CV: Coefficient of variation. [1]Calculated in relation to egg weight before incubation. *Significant at p<0.05

Table 8: Interaction between intra-eggs injected vitamin C percentages and incubation temperatures on phosphates percentage in eggshell at the end of incubation period

	Temperatures		
Treatments	Usual	Hot	Probability
Control	0.156	0.153	0.7899
Vitamin C-0%	0.144b	0.167a	0.0364
Vitamin C-2%	0.160a	0.131b	0.0096
Vitamin C-4%	0.147	0.148	0.9418
Vitamin C-6%	0.140	0.150	0.3460
Polynomial		---------- Probability ----------	
Control x vitamin C levels		0.3941	0.6394
Linear for vitamin C levels		0.4985	0.2540
Quadratic for vitamin C levels		0.1769	0.0130*
Cubic for vitamin C levels		0.3531	0.0570

a-b: Means in the same row with distinct superscripts are different at p<0.05. *Significant at p<0.05

accompanied by a reduction in blood levels of Ca (Macari *et al*., 2002). In the present study, however, no changes were detected in the blood values of the variables analyzed (i.e., pCO_2, pO_2, BEecf, TCO_2, SO_2, HCO_{3-}, Na^+, K^+ and iCa) that could be correlated with increased blood pH. To maintain blood pH, the body uses buffers as well as respiratory and renal regulatory

Table 9: Mortality in broiler breeder eggs injected with different vitamin C percentages before incubation at 37.5°C and 39°C

	Mortality (%) days			
	0-7	7-14	14-21	Total
Treatments (T)				
Control	2.23	0.00	11.05	13.28b
Vitamin C-0%	8.18	5.79	10.23	24.20ab
Vitamin C-2%	6.72	4.80	13.81	29.60a
Vitamin C-4%	14.65	5.42	16.73	36.80a
Vitamin C-6%	6.07	1.98	16.27	25.60ab
Temperatures (TP)				
37.5°C	4.42	4.63	13.12	24.30
39°C	9.67	2.92	13.95	26.96
Probability				
T	0.9247	0.0799	0.6584	0.0028
TP	0.1398	0.2739	0.7971	0.3583
T x TP	0.9274	0.0655	0.2182	0.5617
CV (%)	108.92	102.45	56.77	26.54
Polynomial		----------	Probability	----------
Control x vitamin C levels		-	-	0.0007
Linear for vitamin C levels		-	-	0.4941
Quadratic for vitamin C levels		-	-	0.0260
Cubic for vitamin C levels		-	-	0.2230

CV: Coefficient of variation. a-b: Means in the same row with distinct superscripts are different at p<0.05

mechanisms. The buffer systems involved are the following: carbonic acid/bicarbonate, phosphate and HGB (Margotto, 2004). According to the results from this study, the quadratic model showed that the absorption of P from eggshells increased with increasing percentages of vitamin C injected into the eggs incubated at the hot temperature. Thus, we must consider that the increase in blood pH is related to changes in P levels. According to Borges (2001), trace elements have the ability to work with electrolytes which can affect acid-base and electrolytic balance in birds. This finding is interesting because it draws attention to the importance of P uptake from the shell to maintain blood pH via the phosphate buffer system.

According to results, it is concluded that pre-incubation injection of 4% vitamin C into eggs incubated under hot temperature increases the phosphorus absorption from eggshell, the values of hematological respiratory variables (RBC, HCT, HGB) in chicks and the embryo mortality. Better understanding of vitamin C's role on these erythrocytic variables are necessary to understanding of its effect on gas exchange modulation under hot temperature.

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