Does preincubational in ovo injection of buffers or antioxidants improve the quality and hatchability in long-term stored eggs?

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ABSTRACT A hypothesis was tested that providing buffer solutions or antioxidants during egg storage may help embryos in combating the harmful effect of longer holding periods. Hatching eggs were obtained from a breeder flock (35 wk) and stored for 13 d before setting. In experiment 1, the eggs were injected (d 4) with bicarbonate buffer solution (BBS) or PBS. For experiment 2, L-carnitine (LC), vitamin E (VE), and vitamin C (VC) were injected (d 7) at 3 different doses. The egg internal quality characteristics were evaluated at 2-d intervals after injection and the remaining eggs were incubated for 21 d under standard conditions. At 21 d, hatchability was recorded and unhatched eggs were broken open to assess the fertility and stage of embryonic mortality. No differences were noted in albumen pH due to using buffer solutions or antioxidants except for a decreased pH at 2 d postinjection of the high dose of VC (75 mg). In ovo injection of BBS increased the albumen index and Haugh unit at d 6 postinjection; however, the response to PBS was not different from that in the control group. In ovo injection of antioxidants did not influence the albumen index, Haugh unit, and yolk index; however, the yolk percentage was partly affected. Irrespective of the dosage, hatchability was greatly decreased following in ovo injection of buffers or antioxidants (as low as 4.3 vs. 87.5% in control), with the highest mortality percentage recorded at early embryonic stages (d 0 to 6). Data suggested that, despite improvement in certain egg internal qualities, preincubational in ovo injection of BBS, PBS, LC, VE, or VC was associated with a profoundly decreased hatchability for which the underlying mechanism(s) remain(s) to be clarified.

Key words: hatchability, in ovo injection, egg storage, buffer, antioxidant

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INTRODUCTION

Storing hatching eggs for extended periods is occasionally an inevitable practice in the broiler breeder industry due to uncoordinated demand for hatchlings in the market. On the other hand, it is beneficial to decrease the number of individual incubations in hatcheries for full exploitation of incubators. There is, however, a great body of literature suggesting a negative association between prolonged storage time and hatchability (Brake et al., 1997; Fasenko, 2007). Attempts have been made to decrease the detrimental effects of long-term storage on hatchability, including prestorage incubation (Fasenko, 2007), treating the eggshell with oil (Hill and Hall, 1980), storing the eggs in the small-end-up position or increased turning frequency during the subsequent incubation (Elibol and Brake, 2008), and storing the eggs in carbon dioxide (Walsh et al., 1995) or in water (van den Brand et al., 2008). Nonetheless, the decreased hatchability rate still appears to be a challenge during the long-term storage of eggs.

The quality of albumen, specifically the pH, is said to be the major contributor to embryonic metabolism and survival during long-term storage (Brake et al., 1997). The buffering capacity of albumen is weakest between the pH values of 7.5 and 8.5 (Cotterill et al., 1959), and long-term storage has been reported to reduce buffering capacity (Stern, 1991), associated with lower hatchability (Fasenko, 2007). Additionally, the presence of unsaturated fatty acids makes the eggs susceptible to peroxidative degradation, which can alter cellular functions and lead to the lysis of oxidized cell membranes (Cherian and Sim, 1997). Because of their antioxidizing effects, L-carnitine (**LC**), vitamin E (**VE**), and vitamin C (**VC**) have been broadly used in the poultry industry for a variety of purposes. Eggs possess low concentrations of LC (Chiodi et al., 1994) and chicken embryos have a limited capacity to synthesize it during incubation (Casillas and Newburgh, 1969). Furthermore, LC

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has been reported to have anti-apoptotic effects (Golzar Adabi et al., 2011), making it appropriate for testing to improve hatchability rates, as the rate of apoptosis seems to be higher in long-term stored eggs (Hamidu et al., 2011). Chickens can synthesize VC, but it is not transferred into the egg (Franchini et al., 2002). Hence, the levels of these antioxidants may not be sufficient to impart protection to the embryo.

To our knowledge, the effects of preincubational in ovo injection of buffer solutions and antioxidants on hatchability of long-term stored eggs have not been investigated. Therefore, 2 experiments were conducted aimed to improve the hatchability rate of long-term stored eggs by providing a more suitable microenvironment for the embryo during the storage period. In experiment 1, the hypothesis was tested that in ovo injection of bicarbonate buffer solution (**BBS**; as the principal extracellular buffering system) or PBS (as the principal intracellular buffering system) during the storage period may reinforce the albumen buffering capacity and enable the embryos to better withstand the adverse effects of the extended holding periods. In experiment two, 3 levels of LC, VE, or VC were injected in ovo during the storage period to provide supplementary sources of antioxidants, thereby helping the embryos to better tolerate the peroxidative stress during the holding period and possibly thereafter. To provide a better interpretation of the hatchability data, egg internal quality characteristics were also evaluated at certain times postinjection.

MATERIALS AND METHODS

Eggs and Storage Conditions

Hatching eggs of approximately similar weights (65 ± 2 g) were obtained from a breeder flock (Cobb 500) at 35 wk of age (n = 944). The eggs were numbered, fumigated with formaldehyde gas (20 min), and stored for a 13-d period (14°C and 75% RH) at the same height from the floor to minimize possible effects of different height-born humidity levels associated with different heights from the floor. A pilot study was conducted to determine the proper site (the yolk or albumen), appropriate depth, and suitable time of injection as well as specifying the effective dosing levels of buffers to decrease albumen pH. As a decrease in albumen pH was intended, the buffer solutions were injected into the albumen. Pricking the vitelline membrane with a needle to directly inject antioxidants into the yolk resulted in the leakage of yolk content to the albumen, which might be detrimental to the embryo; therefore, the albumen was regarded as the most suitable site for injecting the antioxidants. The same study showed a depth of 12 mm as adequate to inject the solutions at the closest proximity to the blastoderm without damaging the yolk membrane. Because the literature suggests that the greatest increase in albumen pH occurred during the first 4 d of storage (Lapão et al., 1999), d 4 of the storage period was selected for injecting the buffer solutions. For antioxidant solutions, however, d 7 of the storage period was selected for injection, because storing the eggs longer than 7 d negatively influences hatchability (Fasenko, 2007).

Solutions

Two (bicarbonate and phosphate) buffer solutions (experiment 1) and 3 antioxidants (LC, VE, and VC; experiment 2) were used. The solutions were prepared through dissolving in sterilized distilled water (**DW**) under a biological hood. The chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO) and utilized regarding sanitary considerations. The buffer and LC solutions as well as all utensils and containers were autoclaved for 20 min (120°C) to prevent any contamination. The corresponding time for VE was 15 min (50°C). The BBS provided an ionic strength of 0.0847 mol·dm^{-3}, a buffering capacity (β) of 6.976, and a molarity of 0.08. The respective values for PBS were 0.237 mol·dm^{-3}, 3.115, and 0.08. The pH of the buffers was adjusted at 8.5 for a working temperature of 18°C, using Buffer Maker 1.0.1.55 Software (2008–2009, BPP Marcin Borkowski Co., Marki, Poland). The LC stock solution (100 mg/mL) was diluted to 2 (**LC_2**), 8 (**LC_8**), and 12 (**LC_{12}**) mg of LC per final injection volume (0.75 mL), autoclaved, and stored away from sunlight until injected. A water-soluble derivative of VE, Trolox, was used to prepare solutions containing 0.25 (**$VE_{0.25}$**), 0.50 (**$VE_{0.50}$**), and 0.75 (**$VE_{0.75}$**) IU of VE, immediately before injection. A stock solution (100 mg/mL) of L-ascorbic acid (75 mg per 0.75 mL) was used to provide 3 doses of 25 (**VC_{25}**), 50 (**VC_{50}**), and 75 (**VC_{75}**) mg of VC per injection volume (0.75 mL). The VC solutions were prepared shortly before injection and sterilized using syringe filters (0.2 μm).

Treatments and Incubation

In experiment one, 248 eggs were stored for 13 d, out of which 200 eggs (5 replicates of 10 eggs per treatment) were used to determine hatchability and the remaining eggs for evaluating egg internal quality. On d 4, the eggs were injected with DW, BBS, or PBS, and an equal number of intact eggs served as the control. In experiment 2, a total of 696 eggs were stored for 13 d (5 replicates of 10 eggs per treatment for hatchability data and the remaining eggs for evaluating the egg internal quality). On d 7 of storage, the eggs were divided into 12 groups, control (intact), needle pricked (**N**), injected with the vehicle only (DW), LC_2, LC_8, LC_{12}, VC_{25}, VC_{50}, VC_{75}, $VE_{0.25}$, $VE_{0.50}$, or $VE_{0.75}$. Initially, the egg was cleaned with ethyl alcohol (70%) and the blunt end was punched using a micro-needle, taking care not to damage the outer egg membrane. The penetration site was then cleaned again with ethyl alcohol and the solutions were injected (0.75 mL/egg) using another disposable syringe equipped with a 27-gauge needle. The

eggs in group N were just pricked without injecting any solution (dry punch). The eggs were then sealed with melted wax and stored until incubation with their blunt ends up. For evaluating the egg internal quality, 4 eggs per treatment group were randomly selected on d 2, 4, and 6 (experiment 1), or on d 2 and 4 (experiment 2) postinjection during the storage period. After weighing, the eggs were opened to determine the albumen index (100 × albumen height divided by average of thick and thin albumen diameters), albumen pH, yolk index (100 × yolk height divided by yolk diameter), yolk percentage (100 × yolk weight divided by egg weight), and Haugh unit (**HU**) according to the equation (Haugh, 1937):

$$HU = 100 \times \log (H - 1.7 \times W^{0.37} + 7.57),$$

in which H = albumen height (mm) and W = egg weight (g).

The experimental egg trays, containing an equal number of replicates per treatment, were allotted to trolleys to ensure minimal positional effect. On d 14, the remaining eggs (n = 200 and 600 for experiments 1 and 2, respectively) were fumigated, preincubated at 24°C (8 h; 65% RH), and incubated at 37.8°C (60% RH). After 18 d, the eggs were transferred into the hatcher baskets such that each basket received an equal number of replicates per treatment and that eggs in each replicate were placed under an individual pedigree basket. After emergence at 21 d, the number of hatchlings was determined to calculate the hatchability of fertile eggs. All unhatched eggs were broken open to determine the age at death (Hamburger and Hamilton, 1951), presented as early (0 to 6 d), mid (7 to 17 d), or late (18 to 21 d plus pipped) embryonic mortality.

Statistical Analysis

The data were tested for equality of variances and normality using Levene's and the Kolmogorov-Smirnov tests, respectively. Each replicate of eggs served as the experimental unit. The data were arc-sine transformed where appropriate. Data on the egg internal quality indices were subjected to 2-way ANOVA for which the effects of solutions, time, and their interaction were included in the model. The hatchability data were analyzed by 1-way ANOVA in which the reweighted least-squares procedure was used with the number of fertile eggs per replicate as the weight. Where significant differences were found by the PROC GLM (SAS, 2002), the least squares means were compared, using the least significant difference procedure (SAS, 2002). Statements of statistical significance were based upon $P \leq 0.05$ unless otherwise indicated.

RESULTS

Experiment 1

Table 1 shows the effects of BBS and PBS on albumen index, albumen pH, HU, yolk index, and yolk percentage at 2, 4, and 6 d postinjection. The egg internal characteristics were not affected by treatment on d 2 or 4 postinjection. On d 6, however, the albumen index, HU, and yolk index were influenced by treatment. The highest albumen index value was recorded for BBS (76.3) and the lowest one for the DW (47.0) group, which was not different from the control eggs (55.8). In ovo injection of BBS increased the HU values compared with the control on d 6 postinjection (80.5 and 66.3, respectively). The main effect of time did not affect the egg internal characteristics ($P > 0.05$). In ovo injection of buffer solutions resulted in a profound decrease in the hatchability of fertile eggs ($P = 0.0001$), where the mean values of 32.0 ± 0.02 and 8.3 ± 0.04% were recorded for BBS and PBS groups, respectively. The hatchabilities of fertile eggs in control and DW groups were 87.5 ± 0.02 and 91.3 ± 0.04%, respectively. The highest proportion of embryonic mortality in the buffer-injected groups occurred during 0 to 6 d of incubation period (82.3 and 90.9% for BBS and PBS, respectively; data not tabulated).

Experiment 2

The effects of in ovo injection of LC, VE, or VC on egg quality measures and hatchability of fertile eggs at d 2 and 4 postinjection are presented in Table 2. Differences were observed for albumen pH ($P = 0.021$) and yolk percentage ($P = 0.039$) on d 2. The highest value for albumen pH was recorded for DW (9.53) and $VE_{0.50}$ (9.52) groups and the lowest one was found in VC_{75} (9.20). The albumen index, HU, and yolk index were not affected by solution × time interaction. Furthermore, the main effects of solution and time did not influence the egg internal characteristics ($P > 0.05$). In ovo injection of antioxidants profoundly decreased the hatchability of fertile eggs ($P = 0.0001$). In comparison with the control (87.5%), the values for LC ranged from 54.1 to 37.5% and those for VE varied between 33.3 and 4.3%. The corresponding values for the eggs injected with VC varied from 45.8 to 9.5%. The highest proportion of embryonic mortality was recorded at early stage, except for $E_{0.75}$ noted during mid-incubation (d 7 to 17). The proportions of embryonic mortality for LC_2, LC_8, LC_{12}, $VE_{0.25}$, $VE_{0.50}$, $VE_{0.75}$, VC_{25}, VC_{50}, and VC_{75} were 73.3, 85.7, 81.8, 73.3, 62.5, 86.4, 69.2, 73.7, and 68.4%, respectively (detailed data not shown).

DISCUSSION

The present study aimed to implement the in ovo injection of buffer solutions and antioxidants during the storage period to improve the hatchability in long-term stored eggs wherein the egg internal quality indices were also evaluated. In spite of our expectations, a profound decrease in hatchability was found in eggs receiving the buffer solutions or antioxidants in ovo, although an improvement in a certain number of egg quality attributes was noted.

Table 1. Effect of preincubational in ovo injection of bicarbonate buffer solution (BBS) or PBS and time (days postinjection, DPI) interaction on internal characteristics of long-term stored eggs[1]

	2 DPI					4 DPI					6 DPI				
Trait[2]	Control	DW	BBS	PBS	SEM	Control	DW	BBS	PBS	SEM	Control	DW	BBS	PBS	SEM
Albumen index	66.4	75.0	65.7	58.4	4.12	65.7	69.9	58.3	56.7	2.73	55.8^{bc}	47.0^{c}	76.3^{a}	59.6^{b}	2.97
Albumen pH	9.47	9.43	9.48	9.14	0.03	9.45	9.54	9.48	9.55	0.04	9.52	9.53	9.49	9.34	0.09
Haugh unit	73.7	82.7	71.1	69.0	4.48	72.6	74.5	67.5	70.2	3.32	66.3^{b}	68.0^{ab}	80.5^{a}	72.5^{ab}	2.38
Yolk index	42.0	37.5	46.2	39.2	6.74	41.7	42.4	41.6	41.2	5.05	40.2^{ab}	36.7^{b}	43.1^{ab}	45.1^{a}	2.93
Yolk (%)	33.9	33.7	34.3	32.0	1.25	33.6	32.8	32.1	34.9	1.27	34.3	34.1	32.5	35.1	1.47

[a–c]In each row, least squares means with no common superscript(s) differ significantly at $P \leq 0.05$.

[1]Eggs (n = 248) were stored for 13 d (14°C and 75% RH) and injected (d 4) with distilled water (DW), BBS, or PBS, or were not injected (control).

[2]The *P*-values for treatment × time (DPI) interaction effect were 0.007, 0.010, and 0.041 for albumen index, Haugh unit, and yolk index, respectively. Other effects were not significant ($P > 0.05$).

Table 2. Effect of preincubational in ovo injection of L-carnitine (LC), vitamin E (VE), or vitamin C (VC) solutions and time (days postinjection, DPI) interaction on internal characteristics and hatchability of long-term stored eggs[1]

Trait[2]	DPI	Control	DW	N	LC_2	LC_8	LC_{12}	$VE_{0.25}$	$VE_{0.50}$	$VE_{0.75}$	VC_{25}	VC_{50}	VC_{75}	SEM
Albumen index	2	66.7	66.3	65.7	60.1	57.5	58.6	60.9	58.4	62.5	61.5	57.4	58.9	3.94
	4	61.1	67.1	61.4	59.6	54.5	55.5	68.0	64.3	63.2	59.0	55.7	56.7	7.10
Albumen pH	2	9.45^{ab}	9.53^{a}	9.41^{ab}	9.40^{ab}	9.38^{ab}	9.48^{ab}	9.41^{ab}	9.52^{a}	9.48^{ab}	9.45^{ab}	9.32^{bc}	9.20^{c}	0.03
	4	9.52	9.55	9.49	9.35	9.43	9.61	9.40	9.57	9.52	9.52	9.47	9.40	0.07
Haugh unit	2	73.3	73.0	73.6	69.7	68.8	68.9	70.4	68.8	73.8	70.7	68.8	67.4	2.31
	4	71.1	63.7	71.0	76.9	69.7	65.6	74.7	75.5	73.1	70.4	69.2	70.5	4.41
Yolk index	2	41.7	40.4	39.4	40.7	40.2	40.0	40.2	40.2	39.1	39.8	39.8	39.1	1.82
	4	39.6	40.4	40.8	37.6	39.8	38.8	35.4	40.7	40.3	42.1	42.0	41.7	1.33
Yolk (%)	2	33.6^{ab}	35.0^{ab}	33.8^{ab}	35.0^{ab}	35.0^{ab}	34.8^{ab}	32.8^{b}	35.8^{ab}	32.5^{b}	36.5^{a}	36.5^{a}	35.6^{ab}	0.99
	4	34.9	33.6	34.4	33.4	33.8	35.0	33.0	34.0	34.3	35.8	36.0	34.1	1.15
Hatch of fertile[3] (%)		87.5^{a}	87.5^{a}	92.0^{a}	37.5^{bc}	39.1^{bc}	54.1^{b}	31.8^{cd}	33.3^{cd}	4.3^{e}	45.8^{bc}	20.8^{d}	9.5^{e}	0.034

[a–e]In each row, least squares means with no common superscript(s) differ significantly at $P \leq 0.05$.

[1]Eggs (n = 696) were stored for 13 d (14°C and 75% RH) and injected (d 7) with distilled water (DW), LC (2, 8, or 12 mg/egg), VC (25, 50, or 75 mg/egg), or VE (0.25, 0.50, or 0.75 IU/egg), or were not injected (intact: control; or pricked with a needle: N).

[2]The *P*-values for treatment × time (DPI) interaction effect were 0.021 and 0.039 for albumen pH and yolk percentage, respectively. Other effects were not significant ($P > 0.05$).

[3]Number of hatched chicks divided by the number of fertile eggs set.

In experiment 1, the albumen index and HU were increased by the BBS treatment. Decreases in albumen index found in the DW group could simply be due to the diluting effect of water injected. Compared with the DW group, in ovo injection of PBS resulted in a concurrent increase in albumen index and yolk index at 6 d postinjection. Regarding the formula used to calculate the albumen index, an increase in albumen index might be due either to an increase in albumen height or to a decrease in albumen diameter or both. Similarly, increased yolk height, decreased yolk diameter, or both might have involved in increased yolk index. Separate analysis of data on the height and diameter of albumen and yolk revealed that the PBS decreased the albumen diameter, but not the albumen height or HU. In the same group, the yolk height was increased, but the yolk diameter was not influenced by PBS. Contrary to the findings in experiment 1, in ovo injection of antioxidants (experiment 2) did not influence the albumen index, HU, and yolk index; however, the yolk percentage was affected to some extent.

No differences were found in albumen pH due to injection of BBS or PBS in the current work, suggesting the inefficiency of the buffer solutions in maintaining the pH values within narrow ranges. A pilot study aimed to determine the appropriate dosing level showed that an injection volume of 20 mL of BBS or PBS (5 *M*) was needed to decrease the albumen pH from 9 to 8.4, which could potentially be detrimental to the early embryo. Therefore, a lower volume (0.75 mL) and molarity (0.08 *M*) were used, but at a closer injection site to the yolk membrane so that the higher albumen pH could not negatively affect the blastoderm. Inability to lower the albumen pH might be due to such a low molarity. It seems that decreasing the albumen pH is hard to achieve via injecting buffer solutions, unless a high volume of a buffer with a high molarity is used. Regarding the injection time of the buffer solutions, the greatest increase in albumen pH, which occurs during the first 4 d of storage period (Lapão et al., 1999), led us to consider d 4 of storage an appropriate time for injection. However, it seems that injecting the buffers before d 4 of the storage period (before the early increase in albumen pH) might be an alternative. In experiment 2, the highest level of VC (VC_{75}) decreased the albumen pH compared with the control (9.20 and 9.45, respectively); however, the difference disappeared 2 d later. Being an acid, VC might slightly lower the pH, but could not probably compensate for the CO_2 outflow from the albumen thereafter.

Unexpectedly, in ovo injection of buffer solutions or antioxidants greatly decreased the hatchability of fertile eggs. Because the dry puncture by the needle did not influence hatchability, the conclusion is justified that the active ingredients injected disturbed the environment for the embryo detrimentally. The major proportion of embryonic mortality occurred during early incubation (d 0 to 6), which included those taking place during the storage period and those at early incubation. Given this, the determining factor(s) contributing to the lower hatchability rates might have acted during the storage period, early incubation, or both. Alternatively, early embryonic mortality might be due to a carry-over effect of the injections that predisposed the embryos to death at early incubation, but not during the storage period. Further analysis of the age of embryos at death (Hamburger and Hamilton, 1951) revealed that more than 96% of the overall early embryonic mortality occurred between d 3 to 6 of the incubation period. In other words, this group of embryos could withstand the storage conditions (for 13 d) and start to further development following the setting, but could not survive beyond d 6 of the incubation period. Therefore, it seems that the buffer solutions or antioxidants acted in a lethal way that inhibited the further development of embryos toward mid- or late-incubation.

The high pH values recorded in all experimental groups through 1 to 13 d of storage would be inappropriate for embryos, as the optimum albumen pH for embryonic development has been estimated to be between 7.9 and 8.4 (Reijrink, 2010). However, the current data give the albumen pH a minimal contribution to the decreased hatchability in the experimental groups because the hatchability of the control eggs (intact, N, or DW groups) remained high despite an increased albumen pH, which was in line with those recorded in the eggs receiving buffer solutions or antioxidants. A more crucial contribution has been attributed to albumen pH during early incubation compared with that of the storage period (Reijrink, 2010). In the present study, the egg internal characteristics were not evaluated during the incubation period; therefore, the probable differences in albumen pH among the treatment groups during the early incubation could not be determined. However, factor(s) other than the albumen pH might be sought to explain the decreased hatchability rate in buffer- or antioxidant-injected eggs.

The hatchability of fertile eggs was greatly decreased following the in ovo injection of antioxidants, where the lowest values of 4.3 and 9.5% were recorded for the highest doses of vitamins E ($VE_{0.75}$) and C (VC_{75}), respectively. The yolk content of unsaturated fatty acids is susceptible to free radicals (Cherian and Sim, 1997). Apart from being an anti-oxidizing agent, LC is said to have anti-apoptotic properties (Golzar Adabi et al., 2011). In addition, from unaffected hatchability rates following in ovo injection of up to 8 mg of LC per 18-d-old embryo, Keralapurath et al. (2010) proposed to study higher levels of LC. Therefore, we used the low, medium, and high levels of LC, VE, and VC, which were injected in ovo on d 7; yet, the hatchability was profoundly decreased in all the treatment groups. This might be attributed to pro-oxidizing, rather than anti-oxidizing effect of these compounds on the blastoderm, as was reported to be the case for vitamins C (McGregor and Biesalski, 2006) and E (Chen et al., 1998). Evaluating the activity of enzymes contributing to scavenging the reactive oxygen species may provide

more insight into the mechanism(s) involved. If this is true, then the lower dosing levels of these antioxidants would be suggested to be investigated for in related studies.

The egg internal characteristics, forming the surrounding micro-environment of the embryo, change during the storage period. These changes may have consequences for the protection of the embryo against microorganisms and availability of nutrients to the embryo during incubation, thereby affecting cell death, embryonic viability, and hatchability (Reijrink, 2010). Among a variety of factors critical to the embryonic viability is the ionic integrity (Buttgereit and Brand, 1995). The ability of early chick blastoderm in unidirectional transport of Na^+, K^+, H^+, and Cl^- ions as well as water (Stern, 1991) implies its capability in active modification of its microenvironment (Brake et al., 1997). Further, the endodermal cells facing the yolk have been reported to be involved in the transport of water and Na^+ ions from albumen to the yolk sac (Bakst and Holm, 2003). Failure of adequate transfer of water to the yolk sac between 3 and 7 d of incubation might be detrimental to the embryo (Deeming, 1989). It is speculated that the ingredients injected in ovo in the present study might have disturbed the ionic balance of the egg internal environment. Regarding experiment 1, we tried to prepare a buffer solution with ionic strength as low as possible to make sure that the egg ionic balance was minimally affected. However, it might be inferred from the poor hatchability rates that there is a fragile ionic balance in the egg that cannot be disturbed without losing embryonic survival. It seems that the pH or osmolarity of the buffer solutions or both might have adversely affected the surrounding microenvironment of the early embryo in the present work, which resulted in a higher embryonic death wherein the highest mortality rate was recorded during early stages (82.3 and 90.9% for BBS and PBS, respectively).

Increased albumen pH, which naturally occurs during storage and early incubation, does not correspond to the optimal pH for the embryo (Reijrink et al., 2008). The vitelline membrane and chalaziferous layer constitute a boundary between the embryo and the albumen, which protects the embryo against the alkaline albumen during the storage and the first few days of incubation when the amnion is not yet developed (Sadler, 1955). The maintenance of an effective barrier may result in a depletion of energy reserves available to the embryo and in embryonic mortality (Walsh et al., 1995). On the other hand, the embryonic cells have a limited energy budget (Reijrink, 2010) that might be used to recover the biological balance of the cells being exposed to strange ingredients. It might be assumed that the active ingredients injected in ovo in the present work may result in more expenditure of energy resources to reconstitute cell homeostasis. This, in turn, might adversely influence the efficacy of barrier against the alkaline pH, viability of cells, or both (Walsh et al., 1995). Given that the major proportion of the early embryonic mortality in the current study comprised the 3- to 6-d-old embryos, it could be stated that the negative effects of the injections became more evident during early incubation, although a decrease in the number of viable embryonic cells may have happened previously during the storage period.

During early incubation, metabolic activity increases as does the CO_2 production of the embryonic cells. An embryo with a higher number of viable cells may better form an effective barrier between the inside of the embryo and its exterior environment. Further, it may better produce a sufficient amount of CO_2 than an embryo with a lower number of viable cells. A higher CO_2 production may reduce the pH in the embryo's own surrounding microenvironment from a pH around 9.0 to a pH around 8.0 (Reijrink et al., 2008). Further studies, however, are needed to specify the contribution of these speculative mechanisms to the lower hatchability rates found in the present study. It can be hypothesized, as did Reijrink et al. (2010), that there is a particular stage of differentiation requiring a minimum number of viable cells to continue the embryonic development successfully. Otherwise, early embryonic mortality would happen due to the inability of the embryo to compensate for the cell death before morphological changes continued.

In the current work, the hatchability of the control eggs was not adversely influenced by 13-d-long storage period (87.5%), possibly due to the age of the flock from which the eggs were obtained (35 wk). It was demonstrated that the higher albumen quality in young flocks might be associated with a lower hatchability rate, probably due to higher albumen viscosity (Lapão et al., 1999). Regarding the buffer solutions, an improved albumen index and HU at the late storage period (d 10) might worsen the situation because albumen liquefaction is critical to provide the embryo with sufficient resources of nutrients and with an efficient gaseous diffusion (Brake et al., 1997). Therefore, exploiting the buffer solutions for the eggs from the older flocks might be a proposition. The higher yolk index in the buffer-treated eggs might result in a thinning along with a reduced stability of yolk membrane (Jones and Musgrove, 2005). This, in turn, may increase the leakage of nutrients into the albumen and diminish its antimicrobial potency (Gast and Holt, 2000), which could be associated with a decreased hatchability. In summary, the decreased hatchability rates in the eggs receiving buffer solutions or antioxidants in the present work might be due to 1) a disturbance in the ionic balance of the egg internal environment, 2) an inefficacy of the chalaziferous layer and yolk membrane in protecting the embryo against the alkaline albumen pH, 3) a decreased number of viable embryonic cells, 4) a decreased antimicrobial potency of albumen due to the leakage of nutrients from yolk, or 5) a combination of these mechanisms.

Overall, the data suggest that despite improvement in certain egg internal quality attributes, preincuba-

tional in ovo injection of BBS, PBS, LC, VE, or VC was associated with profoundly decreased hatchability rates. The pH of the albumen seems not to be the determining factor in hatchability rates in the present study. Generally, it appears that the homeostasis of the embryo might be readily disturbed during the storage period compared with that of the incubation period, suggesting a fragile biological balance may be present within the egg that cannot be disturbed without damaging embryonic survival. However, the question of why the treatments detrimentally affected the embryo is still an interesting one, as the answer to that would lead to new approaches to overcome this adverse effect. Perhaps a combination of biological buffers, including Tris and HEPES, would provide an optimal alternative for an efficient maintaining of the pH, especially at early incubation. Because the egg internal characteristics were not negatively affected by injecting the antioxidants, lower concentrations might be beneficial to achieve higher hatchability rates during the long-term storage of eggs.

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