Effects of pre-incubation storage duration and nonventilation incubation procedure on embryonic physiology and post-hatch chick performance

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ABSTRACT This study investigated the effects and possible interactions of storage and nonventilation during incubation for eggs from Sasso broiler breeder flock on pre- and post-hatch incubation results.

A total of 1,260 Sasso eggs from a 58-wk-old broiler breeder flock were individually numbered, weighed and stored for 7 d or for 18 d in a climate-controlled room (16°C, 75% RH). After storage, eggs were weighed, and randomly assigned equally into 2 incubators. One of the incubators was ventilated (**V**) for the entire incubation and the second was nonventilated (**NV**) for the first 12 d. At d 18, the eggs were weighed, candled, and fertile eggs were transferred from the turning trays to hatching baskets. During the last 3 d of incubation, hatching eggs were checked individually every 3 h for hatching events and hatchability of fertile eggs. After pull out at d 21.5, post-hatch performances was determined until 1 wk of age.

Results showed that, embryo weights from eggs in NV incubator was significantly higher ($P < 0.05$) in both stored eggs compared to those from eggs in ventilated incubator, but embryos from eggs stored for 18 d were smaller ($P < 0.05$) than those from eggs stored for 7 d. Hatchability was higher ($P < 0.0001$) in NV incubator compared to V incubator in both 7 d and 18 d stored eggs and an interaction was found between incubation ventilation and storage duration on both hatchability and embryonic mortality ($P < 0.0001$). Chick weights from NV incubator at 7 d post-hatch was greater ($P = 0.0009$) than those from V incubator. Serum Tri-iodothyronine ($\mathbf{T_3}$) and Thyroxin ($\mathbf{T_4}$) concentrations were significantly higher ($P < 0.05$) in NV compare to V group.

It was concluded that the effect of long-term pre-incubation storage on embryonic physiology and post-hatch growth interacted significantly with incubation ventilation and that nonventilation can compensate for the negative effects of storage on some hatching and post-hatch performances.

Key words: nonventilation, storage, thyroid hormone, embryo physiology, Sasso

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INTRODUCTION

Hatchery operation is one of the most important integral components of poultry production which requires serious attention to achieve high post-hatch performances. Optimal hatchability and chick quality from chickens' eggs require a fine balance between various environmental factors, including gas exchange, which is crucial for the development of embryos (Onagbesan et al., 2007). The health of the hatchlings and their post-hatch growth performances depends on the incubation management (Fernandes et al., 2017). Thus, it is very important to ensure appropriate incubation conditions by adjusting several factors in order to achieve an optimum hatchability and chick quality.

Pre-incubation storage duration is well known as an important factor that affects egg quality, which in turn plays an important role in embryonic development, hatchability, and hatching time. Different studies have shown that hatchability of eggs decreases quickly after 7 d of storage time for ducks (Onbaşılar et al., 2007) and 4 d of storage length for guinea fowl eggs (Moreki and Ditshupo, 2012). Kustra et al. (2020) observed the significantly decrease of hatchability of golden pheasants after

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3 to 4 d of storage. For chickens, it was suggested that pre-storage incubation had no effect on hatchability, when storage duration was shorter than 8 d and can be detrimental when storage duration was prolonged (Reijrink et al., 2009). In fact, Whitehead et al. (2002) pointed out that the optimum storage duration for chicken hatching egg is 7 d and every extension of this duration decreases hatchability by increasing embryonic mortality. Furthermore, every day exceeding 7 d of post-ovipositional storage results in extending the average hatching time for one hour, due to retarded pre-hatch development.

Increasing storage duration is tantamount to increasing loss of CO_2 through the eggshell, resulting in an increased albumen pH which may have a negative effect on the initiation of embryonic development (Tona et al., 2003). Kouame et al. (2019) demonstrated that guinea fowl egg albumen pH increased with storage duration and therefore retarded embryonic development. Based on this knowledge, it is possible that the negative impact on incubation and post-hatch growth of prolonged storage duration is caused by excessive loss of CO_2.

In these conditions, high carbon dioxide concentrations in the incubator may help to optimize the pH level in the micro environment of the embryo as it is known that additional CO_2 in the incubator causes a faster decrease in albumen pH. Bruggeman et al. (2007) investigated the changes in acid-base balance that were caused by nonventilation condition and showed a clear acidifying effect of high CO_2 levels during the first 10 d of incubation on the albumen; in contrast with alkalization of the albumen caused by loss of CO_2 during pre-incubation storage of breeder eggs; and ultimately resulted in delayed embryonic development, prolonged incubation time, and low hatchability. Other studies had reported that nonventilation during the first half of incubation accelerated embryonic growth, hatching time and improved hatchability and post-hatch growth (De Smit et al., 2008; Tona et al., 2013; El-Hanoun et al., 2019).

The accelerated hatching time by nonventilation is known to be related to embryo physiological changes. Indeed, narrower spread of hatch due to high incubator CO_2 level was associated with higher corticosterone, tri-iodothyronine ($\mathbf{T_3}$), and tetra-iodothyronine ($\mathbf{T_4}$) levels (Tona et al., 2013, El-Hanoun et al., 2019). This information suggests that embryonic growth and total incubation duration depend on thyroid hormones, as it is well established that these hormones play critical roles in the hatching process of chick embryo.

Based on this knowledge on the effect of pre-incubation storage and nonventilation during incubation on pre- and post-hatch chick development, we hypothesized that the negative impact of long-term storage can be compensated by increasing CO_2 level in the incubator during incubation. Thus, this study investigated the impact of nonventilation during the first 12 d of incubation on embryo development, thyroid hormones, and juvenile growth of chicks from 7 d and 18 d stored hatching eggs.

MATERIALS AND METHODS

Experimental Design

The present study was conducted on eggs from a 58-wk-old parental stock of Sasso chicken broiler line (Hendrix Genetics, France). It was approved by the Institutional Animal Ethics Committee guidelines of the Regional Center of Excellence on Poultry Sciences, University of Lome (**CERSA-UL**). Sasso broiler breeders were reared in separate floor pens under standard husbandry conditions.

A total of 1,260 hatching eggs were individually numbered, weighed, and stored for 7 d or for 18 d in a climate-controlled room (16°C, 75% RH). Experiment were carefully planned such that egg collection and storage were timed to the exact setting period. Eggs of 18 d storage were collected 11 d prior to the collection of eggs of 7 d storage.

After storage, eggs were reweighed, and randomly assigned equally into 2 incubators with a capacity of 600 each (PasReform, Zeddam, the Netherlands, SmartPro-Combi model); only experimental hatching eggs were incubated in these incubators (single-stage incubators). One of the incubators (Control) was ventilated (**V**) for the entire incubation and the second was nonventilated (**NV**) for the first 12 d. Half of the eggs of each group (4 replicates of 75 eggs/group) (ventilated group) were set for incubation for 18 d in a forced-draft incubator at a temperature of 37.7°C, humidity of 55 % and were turned once every hour at 90° angle. The other half (4 replicates of 75 eggs/group) were incubated in a forced-draft incubator (temperature of 37.7°C, humidity of 55%) where the dampers were closed (nonventilated) during the first 12 d of incubation. In this machine, the relative humidity increase about 65 % and the CO_2 level in both incubators was recorded on the screen every 5 h daily for the first 12 d of incubation. After 12 d of incubation, the dampers were opened for the rest of incubation and the incubation was continued in the same way as the ventilated incubation.

At d 18 of incubation, the eggs were reweighed, candled, and fertile eggs were transferred from the turning trays to the same hatching baskets. During the last 3 d of incubation, hatching eggs were checked individually every 3 h for hatching events and hatchability of fertile eggs.

Post-hatch Growth

After pull out at d 21.5, a total of 420-day-old chicks for all experimental groups were individually weighed and then transferred into randomly assigned floor pens in groups of ≈105-day-old chicks per pen according to treatments, with 4 replicates (≈26 birds/replicates) for each treatment. The chicks were raised to 7-day-old of age on a starter diet containing 21% crude protein (**CP**) and 2,950 kcal metabolizable energy (**ME**). Feed and water were offered *ad libitum* during the experimental periods. Chicks body weight (g) at 7 d post-hatch were

recorded according to experimental groups. Average body weight and average body weight gain (g) were then calculated at the end of 7 d post-hatch.

Albumen pH Measurement During Storage

Thick albumen pH of 15 eggs/group was measured before the commencement of storage and incubation. For each broken egg, the pH of the thick albumen was measured with a pH meter after calibration of the electrode with buffered solutions of pH 7 and 10. Between 2 consecutive measurements, the probe was cleaned with distilled water. Thick albumen pH was measured with an oxythermometer VWR - pH 110 and the accuracy was 0.01.

Egg, Albumen, and Embryo Weights

Before the start of storage, incubation and at 18 d of incubation, egg weight (**EW**) was recorded and were used to determine the egg weight loss (**EWL**) during storage and at d 18 of incubation using the formula:

$$\text{Relative egg weight loss}(EDx)[\%] = \frac{EW(ED0) - EW(EDx)}{EW(ED0)} \times 100 \quad (1)$$

Where x = d 18 and 0 = start of incubation

From embryonic day 12 (**ED12**) to the internal pipping stage (ED19.5), 15 eggs/treatments were opened at each embryonic day to record embryo weights. The remaining albumen from the same eggs was also recorded.

Pipping, Hatching Events, Hatchability, and Chicks Quality Determination

Between 456 and 510 h of incubation, the eggs transferred into the hatchers were checked individually every 3 h for hatching events. Eggs in which the beak of embryo penetrates the inner shell membrane (internal pipping, **IP**) where transferred to a new basket and checked individually every 3 h for eggs in which the shell over the air cell is then cracked (external pipping, **EP**). The external pipped eggs were put in separate baskets to determine individual hatching time. The hatched chicks were left in the incubator until the machine was stopped. All individual times of IP, EP and hatching were recorded to determine average time and duration of IP, EP and hatch. At IP, EP, or hatching stages, incubation duration was defined as the time between setting and the occurrences of these events for each egg. Then, the timing of the occurrence of hatching events was used to calculate their durations as follows:

IP duration (dIP) = duration between IP and EP

EP duration (dEP) = duration between EP and hatching, and

Hatching duration (dHatch) = duration between IP and hatching

The total incubation duration was defined as the duration between setting and hatching.

On the day of hatch, the numbers of the hatched chicks were recorded according to treatments to determine fertile hatchability. Eggs that failed to hatch were counted, opened, and visually evaluated to determine embryonic mortality (**EM**). Day old chicks were then weighed according to treatments to determine average 1-day-old chick weight.

Chick's quality was determined using Tona scoring method (Tona et al., 2003). According to this method, physical parameters including reflex, down and appearance, eyes, conformation of legs, navel area, yolk sac, remaining membranes, and yolk were scored. The chick quality score was defined as the sum of the scores assigned to each quality parameter.

$$\text{Fertile Hatchability}(\%) = \frac{\text{total number of hatched chicks at the end of incubation}}{\text{number of fertile eggs transfered to hatching baskets at ED18}} \times 100 \quad (2)$$

$$EM(\%) = \frac{\text{total number of unhatched but fertile eggs at the end of incubation}}{\text{number of fertile eggs transfered to hatching baskets at ED18}} \times 100 \quad (3)$$

Blood Sampling and Hormone Analysis

At IP, hatch and 7 d post-hatch, blood sample was taken from the neck vein (*vena jugularis*) of 15 embryos and chicks according to treatments with a 27-G needle and 1 mL syringe. The blood samples were centrifuged at 3,000 rpm for 15 min, and the serum obtained were stored in a freezer at −20 ℃ until analyzed. A volume of 100 μL of serum was used for tetra-iodothyronine (T_4) and tri-iodothyronine (T_3) concentrations determination in an automated VIDAS systems, which is an enzyme-linked fluorescent assay (**ELFA**) technique. Antibody anti-T_3 and anti-T_4 of mutton, provided by VIDAS were used in the assay for the determination of the concentrations of tri-iodothyronine (T_3) and thyroxin (T_4), respectively. For each serum hormone, all the samples were run in the same assay in order to avoid interassay variability.

Statistical Analysis

The data were processed with the SAS statistical software package (SAS Version 6.124).

*t*Test was used to analyze the effect of storage duration on albumen pH and egg weight loss during storage. The Generalised Linear Model Procedure was used to analyze egg weight, embryo weights, serum T_3, T_4 concentrations, egg weight loss, incubation duration, body weight, and weight gain. Data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement of treatments. When the treatment effects

of the general model were statistically significant the means were further compared using Tukey's post-test. For all analyses, *P*value of 0.05 was used as the level of significance. The model was:

$$Yijk = \mu + \alpha i + \tau j + (\alpha\tau)ij + eijk$$

where Yijk = incubation duration, embryonic weight, T_3, T_4 concentrations, body weight, weight gain of chicken k from incubator ventilation i and storage duration j according to incubation stage; μ = the overall mean; αi = the main effect of incubator ventilation; τj = the main effect of storage duration; $(\alpha\tau)ij$ = the interaction between incubator ventilation and storage duration; and eijk = the random error term.

Data of hatching percentage were analyzed using a logistic regression model.

RESULTS

Incubator Conditions

The measured patterns of incubator CO_2 levels are shown in Figure 1. CO_2 concentration increased curvilinearly between ED3 and ED12 of incubation from 0.05 to 0.25% in nonventilated incubator and remained constantly at about 0.03% during the first 12 d of incubation in the ventilated incubator.

Initial Egg Weight, Egg Weight Loss, and Albumen pH During Storage Period, and Egg Weight at Setting

The initial egg weight, egg weight loss, and albumen pH during storage period are shown in Table 1.

The average egg weight (g) before storage was 59.88 ± 0.68 and 60.10 ± 0.76, respectively for 7 d and 18 d stored eggs. No differences were found in the initial egg weight before their storage ($P = 0.8298$). Nevertheless, significant differences were found in egg weight loss during the storage period as a function of its length ($P = 0.0002$). Egg weight loss increased with storage duration before incubation; the eggs stored for 18 d showing the higher weight loss. Egg albumen pH before storage did not differ ($P = 0.7156$) between 7 d and 18 d stored eggs. However, at start of incubation (end of storage), albumen pH of 7 d stored eggs was significantly lower ($P = 0.0068$) than that of 18 d stored eggs.

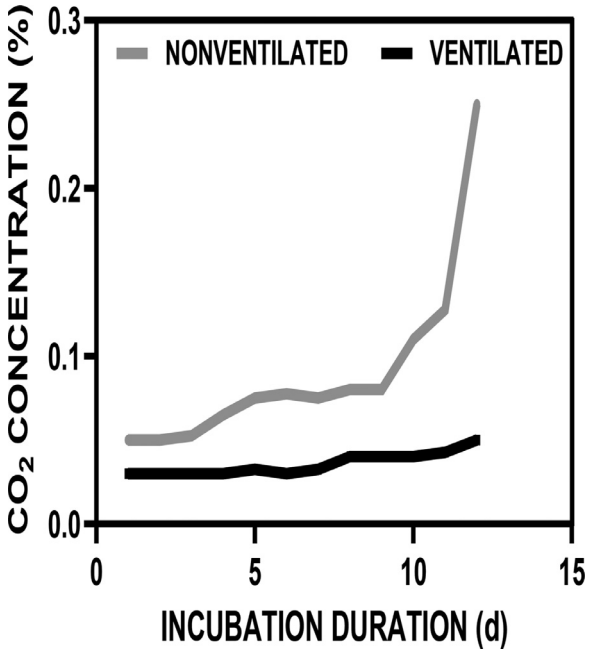

Figure 1. Changes of CO_2 concentration (%) in the ventilated and nonventilated incubator during the first 12 d of incubation.

Table 1. Egg weight loss (%) during storage, albumen, and yolk pH according to storage duration.

Items	Storage duration		*P* value
	d 7	d 18	
Egg weight loss after storage (%)	1.36 ± 0.11[b]	2.36 ± 0.13[a]	0.0002
Albumen pH before storage	8.24 ± 0.14	8.18 ± 0.06	0.7156
Albumen pH at setting	9.18 ± 0.04[b]	9.36 ± 0.03[a]	0.0068

[a, b]For each row, data sharing no common letter are significantly different ($P < 0.05$).

The average egg weight at setting was significantly higher ($P = 0.0101$) in 7 d stored eggs compared to 18 d stored eggs in both ventilated and nonventilated incubator (Figure 2A); whereas, egg weight at setting did not differ ($P = 0.9727$) between nonventilated and ventilated incubator for eggs stored for the same duration.

Egg Weight Loss, Embryo Weight, and Albumen Weight up to ED18 of Incubation

Egg weight loss (**EWL**) at ED18 according to incubation treatment and storage duration was shown on Figure 2B. The effect of storage duration was obvious in ventilated incubator where EWL from eggs that were stored for 7 d was significantly higher ($P < 0.0001$) than those that were stored for 18 d. Nonventilation did not impact EWL in 18 d stored eggs, whereas, EWL was significantly higher ($P = 0.0004$) in ventilated incubator compared to nonventilated incubator in 7 d stored eggs group, suggesting a significant interaction between storage duration and incubation ventilation ($P = 0.0151$).

The embryo weights increased with incubation duration in both nonventilated and ventilated incubators. Nonventilation and storage duration significantly affected the embryo weight (Figure 3A). The relative embryo weights from 7 d stored eggs was significantly higher ($P < 0.05$) compared to those from 18 d stored eggs. However, the impact of nonventilation was only obvious at ED14 ($P = 0.0112$) in 18 d stored eggs, ED12 ($P = 0.0025$) and at internal pipping stage ($P = 0.0022$) where embryo weights from nonventilated incubator was significantly higher than those from ventilated incubator in both 7 d and 18 d stored eggs.

The albumen weight was significantly lower in nonventilated incubator ($P = 0.0191$) compared to ventilated incubator in both groups; and in both nonventilated and ventilated groups; albumen weight from 7 d stored eggs was significantly lower

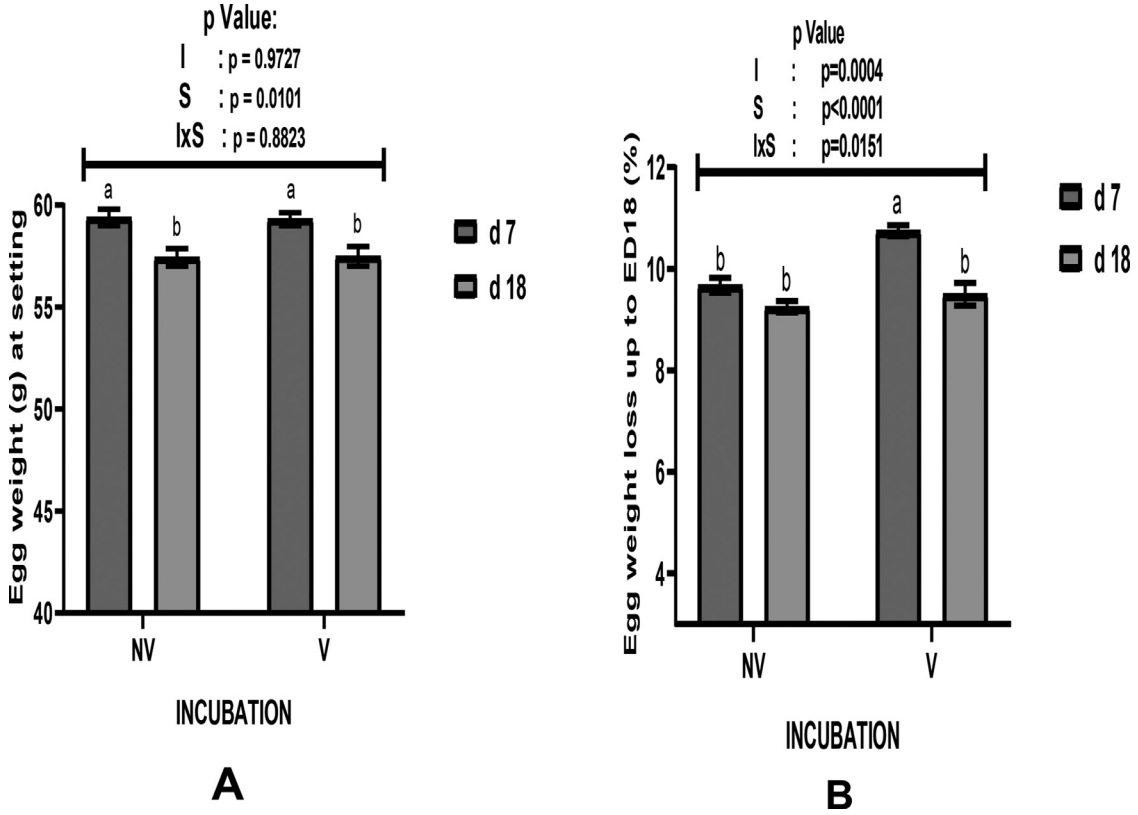

Figure 2. Egg weight (g) at setting (A) and egg weight loss (%) up to ED18 (B) according to storage duration and incubation ventilation. Data sharing no common letter are significantly different ($P < 0.05$).

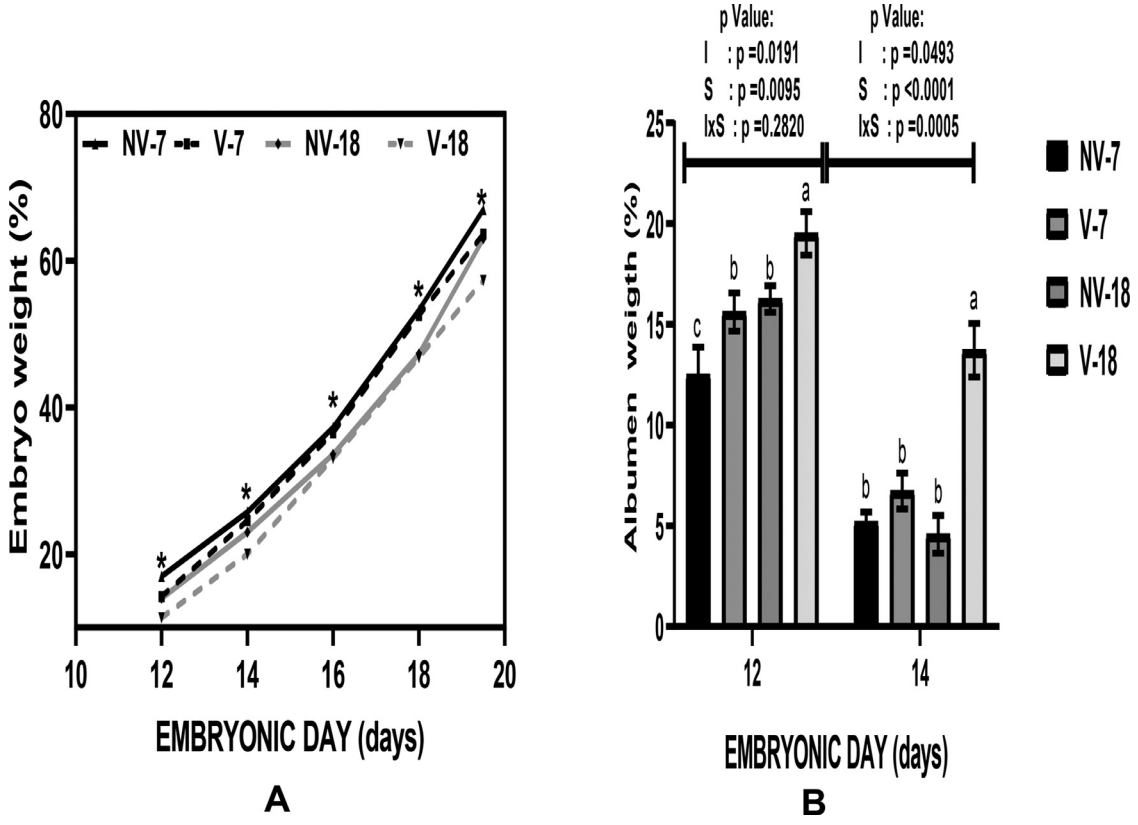

Figure 3. Embryo weight (%) (A) and albumen weight (%) (B) according to storage duration and incubation ventilation. * indicate a significant difference between treatments and data sharing no common letter are significantly different ($P < 0.05$). Abbreviations: I, incubation ventilation; S, storage duration.

Table 2. Incubation duration (h) up to internal pipping (IP), external pipping (EP), and hatching (Hatch) according to storage duration and incubation group[1].

Affecting factors		IP (h)	EP (h)	Hatch (h)
Incubation	V	462.6 ± 3.52[a]	472.4 ± 4.95[a]	487.7 ± 4.00[a]
	NV	457.7 ± 2.42[b]	467.1 ± 4.00[b]	481.3 ± 4.07[b]
Storage	7 d	457.2 ± 1,88[b]	465.3 ± 2.15[b]	480.2 ± 3.12[b]
	18 d	463.1 ± 2.97[a]	474.2 ± 3.10[a]	488.3 ± 3.05[a]
Incubation × Storage	V-7 d	459.0 ± 0.48[c]	467.8 ± 0.60[c]	483.7 ± 0.48[c]
	NV-7 d	455.6 ± 0.40[d]	463.2 ± 0.49[d]	477.2 ± 0.39[d]
	V-18 d	466.2 ± 0.77[a]	477.6 ± 0.66[a]	491.7 ± 0.40[a]
	NV-18 d	460.3 ± 0.63[b]	471.2 ± 0.74[b]	485.5 ± 0.58[b]
P value				
Incubation		<0.0001	<0.0001	<0.0001
Storage		<0.0001	<0.0001	<0.0001
Incubation × Storage		0.0034	0.0216	0.8032

[abcd]For each column, data sharing no common letter are significantly different ($P < 0.05$).
[1]NV, nonventilated; V, ventilated.

($P = 0.0009$) than 18 d stored eggs at ED12 (Figure 3B). At ED14, albumen weight did not differ between NV-7 and V-7, but V-18 showed higher ($P = 0.0493$) albumen weight compared to NV-18. NV-18 and NV-7 showed similar albumen weight but V-7 presented lower ($P < 0.0001$) albumen weight compared to V-18, indicating a significant interaction ($P = 0.0005$) between incubation ventilation and storage duration.

Hatching Process

The average time of the different hatching events was dependent on the storage duration (Table 2). Eggs stored for 7 d attained the different hatching events at significantly ($P < 0.0001$) earlier time points compared to 18 d stored eggs in both ventilated and nonventilated incubator. Additionally, the average time of occurrence of IP, EP and hatching was shorter ($P < 0.0001$) in nonventilated incubator compared to ventilated incubator in both stored eggs. An interaction was found between incubation ventilation and storage duration for average time of IP ($P = 0.0034$) and for average time of EP ($P = 0.0216$).

The duration between internal and external pipping (**dIP**) was significantly lower ($P = 0.0017$) for 7 d stored eggs compared to 18 d stored eggs in both groups (Table 3). IP duration was significantly affected by incubation ventilation. However, IP duration did not differ between nonventilated and ventilated incubator in 7 d stored eggs group; nevertheless, IP duration was shorter ($P = 0.005$) in nonventilated incubator compared to ventilated incubator in 18 d stored eggs groups. The duration between external pipping and hatching (**dEP**) and between internal pipping and hatching (**dHatch**) were significantly affected by both incubation ventilation and storage duration. Despite, dEP and dHatch were not affected by incubation ventilation in 18 d stored eggs, duration was significantly reduced in nonventilated incubator for 7 d stored eggs. The impact of storage duration on external pipping duration was obvious in ventilated incubator where dEP of 7 d stored eggs was shorter ($P = 0.047$) than 18 d stored eggs, indicating a significant interaction ($P = 0.0314$) between incubation ventilation and storage duration. However, the impact of storage duration on dHatch was obvious in nonventilated incubator where dHatch of 7 d stored eggs was shorter ($P = 0.0009$) compared to 18 d stored eggs, suggesting a significant interaction ($P = 0.0056$) between incubation ventilation and storage duration.

Figure 4 shows the spread of hatch according to incubation ventilation and storage duration treatments. The chicks of the NV-7 group commenced to hatch 6 h earlier than those of NV-18 group; but chicks of V-7 group start to hatch 9 h earlier than those of V-18 group. The first chicks was recorded at 467 h in NV-7 group, 3 h earlier

Table 3. Duration (h) of internal pipping (dIP), external pipping (dEP), and the total hatching process (dHatch) according to storage and incubation group[1].

Affecting factors		dIP (h)	dEP (h)	dHatch (h)	Chick quality scores
Incubation	V	9.80 ± 1.40[a]	14.75 ± 0.70[a]	24.80 ± 0.45[a]	90.00 ± 2.13[a]
	NV	8.87 ± 1.57[b]	14.08 ± 0.07[b]	23.20 ± 1.90[b]	91.30 ± 2.30[a]
Storage	7 d	7.85 ± 0.55[b]	14.73 ± 0.72[a]	22.83 ± 1.52[b]	92.87 ± 0.73[a]
	18 d	10.83 ± 0.37[a]	14.10 ± 0.05[b]	25.18 ± 0.07[a]	88.43 ± 0.57[b]
Incubation × Storage	V-7 d	8.40 ± 0.40[c]	15.45 ± 0.45[a]	24.35 ± 0.35[a]	93.60 ± 0.76[a]
	NV-7 d	7.30 ± 0.48[c]	14.00 ± 0.01[b]	21.30 ± 0.30[b]	92.13 ± 0.57[a]
	V-18 d	11.74 ± 0.48[a]	14.05 ± 0.05[b]	25.25 ± 0.25[a]	89.00 ± 0.91[b]
	NV-18 d	10.09 ± 0.57[b]	14.15 ± 0.15[b]	25.10 ± 0.10[a]	87.87 ± 1.16[b]
P value					
Incubation		0.005	0.0473	0.0039	0.1439
Storage		0.0017	0.0470	0.0009	<0.0001
Incubation × Storage		0.5145	0.0314	0.0056	0.8499

[abc]For each column, data sharing no common letter are significantly different ($P < 0.05$).
[1]NV, nonventilated; V, ventilated.

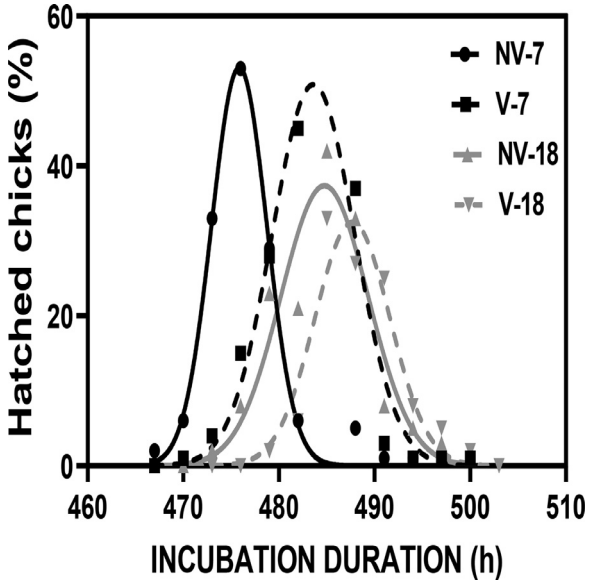

Figure 4. Spread of hatch of hatching time expressed as percentage of hatched chicks according to incubation ventilation and storage duration treatments.

compared to V-7 group; however, the first chick in NV-18 was recorded at 473 h, 6 h earlier compared to V-18 group.

Hatchability, Embryonic Mortality, Day-Old-Chick Weight, and Chick Score

Figure 5A shows the result of hatchability of the eggs set for the different groups. Overall, hatchability was lower ($P < 0.0001$) in the ventilated incubator compare to nonventilated incubator in both storage groups. A higher hatchability was recorded for eggs stored for 7 d in both incubators ($P < 0.0001$).

Figure 5B shows the embryonic mortality levels recorded for the groups. The lowest embryo mortality was recorded in 7 d-stored eggs and in the nonventilated incubator ($P < 0.0001$). An interaction between incubation ventilation and storage duration was observed on hatchability and embryo mortality ($P < 0.0001$).

Day-old chick weights according to incubation ventilation and storage duration are shown in Figure 7A. For both incubation schedules, average weight was significantly higher ($P < 0.0001$) in chicks from eggs stored for 7 d compared to 18 d storage. However, for the same storage duration, incubation ventilation had no effect on chick hatch weight ($P = 0.0606$).

Nonventilation had no impact on average chick score ($P = 0.1439$: Table 3) in both 7 d- and 18 d-stored eggs. Chicks from eggs that were stored for 7 d showed higher score ($P < 0.0001$) than those from eggs stored for 18 d in both ventilated and nonventilated groups.

Serum Levels of Tri-iodothyronine (T_3; Pmol/L), Thyroxin (T_4; Pmol/L), and the Ratio of T_3:T_4

There was a significant effects of storage duration and incubation ventilation on serum T_3, T_4 hormones and on T_3/T_4 ratio. The highest T_3 concentration was recorded in nonventilated incubator ($P < 0.0001$) and in 7 d stored eggs ($P < 0.0001$) at internal pipping stage (Figure 6A). At hatch and at 7-d post-hatch,

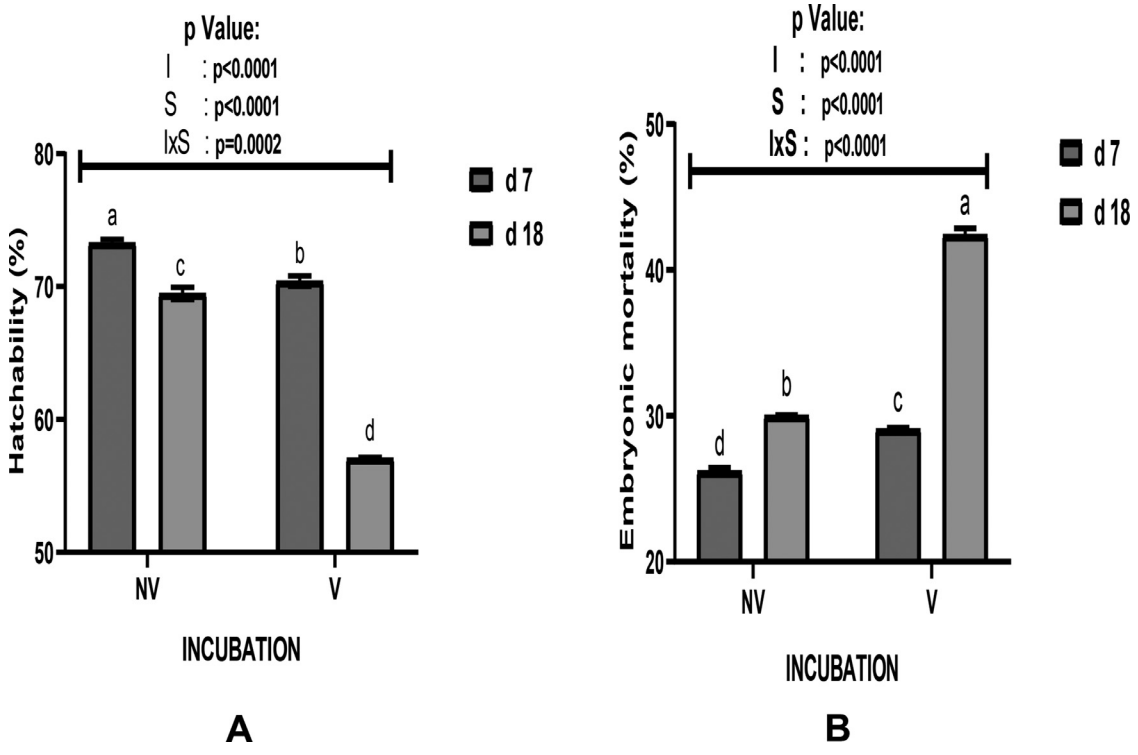

Figure 5. Hatchability (%) (A) and embryo mortality (%) (B) according to storage duration and incubation ventilation. Data sharing no common letter are significantly different ($P < 0.05$).

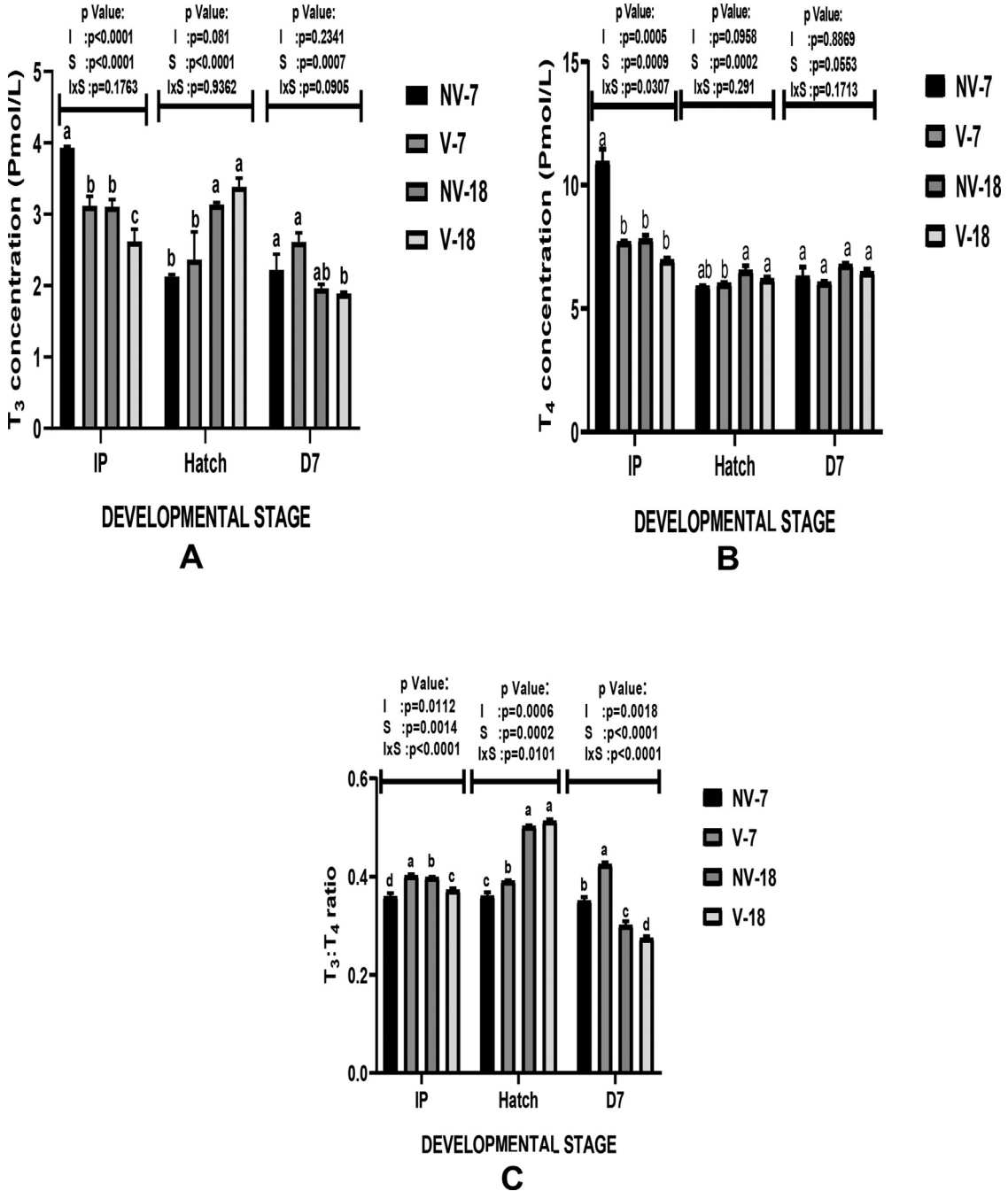

Figure 6. Serum tri-iodotyronine (T_3), Thyroxin (T_4) levels and T_3:T_4 ratio according to incubation ventilation and storage duration. Data sharing no common letter are significantly different ($P < 0.05$).

nonventilation did not impact serum T_3 level in both stored eggs; but 18 d stored eggs had higher ($P < 0.0001$) (at hatch) and lower ($P = 0.0007$) (at 7 d post-hatch) T_3 level compared to 7 d stored eggs.

Nonventilation did not impact T_4 level at hatch ($P = 0.0958$) and at 7 d post-hatch ($P = 0.8869$) in both groups; the nonventilation effect on T_4 level was only obvious at internal pipping stage in 7 d stored eggs where NV-7 embryo showed higher ($P = 0.0005$) thyroxin level than V-7 embryos, indicating a significant interaction ($P = 0.0307$) between incubation ventilation and storage duration (Figure 6B). At internal pipping, 7 d stored eggs had higher ($P = 0.0009$) thyroxin level than 18 d stored eggs in nonventilated incubator; however at hatch, the impact of storage duration on thyroxin level was visible only in ventilated incubator where V-18 chicks had higher ($P = 0.0002$) T_4 level compare to V-7 chicks.

The ratio was significantly affected by incubation ventilation and storage duration at IP, at hatch and at 7 d post-hatch (Figure 6C). The ratio was significantly higher in ventilated incubator compared to nonventilated incubator in 7 d stored eggs whereas the reverse was observed in 18 d stored eggs at both IP, hatch and 7-d post-hatch. The ratio was significantly higher ($P < 0.0001$) in 7 d stored eggs at 7 d post-hatch; but the reverse was observed at hatch where d 18 stored eggs had higher ($P = 0.0002$) T_3/T_4 ratio. A significant interaction was found between incubation ventilation and storage duration at IP ($P < 0.0001$), hatch ($P = 0.0101$) and 7-d post-hatch ($P < 0.0001$).

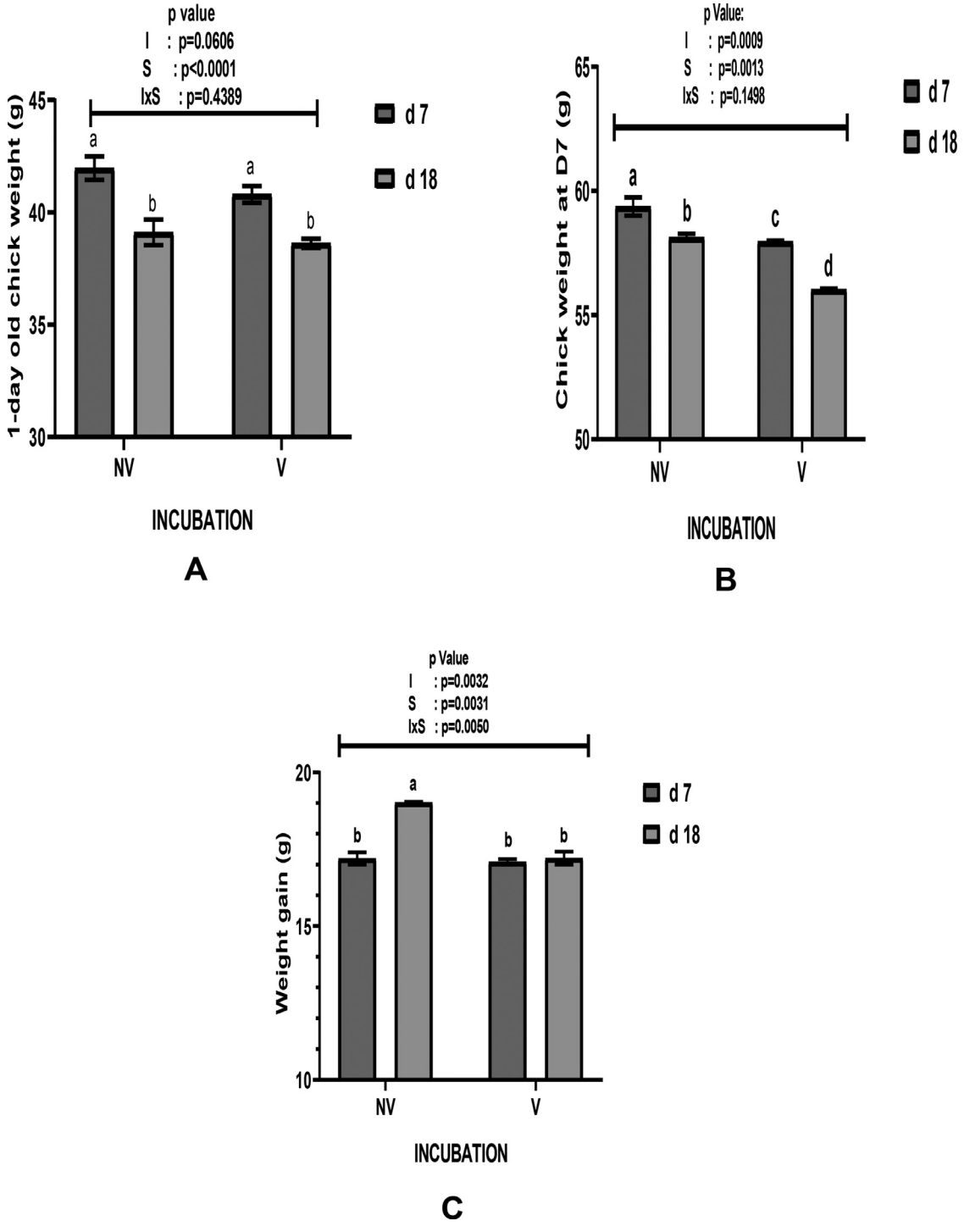

Figure 7. One-day-old chicks weight (A), average chicks weight at d 7 (B) and average weekly chick weight gain (C) according to incubation ventilation and storage duration. Data sharing no common letter are significantly different ($P < 0.05$).

Post-hatch Performances

Chicks from nonventilated incubator were significantly heavier ($P = 0.0009$) than those from ventilated incubator in both 7 d stored eggs and 18 d stored eggs (Figure 7B). Storage duration significantly influenced the chick's weights up to 7 d post-hatch. The weight of chicks from eggs that were stored for 7 d was higher ($P = 0.0013$) compared to those from eggs that were stored for 18 d.

The average chick weight gain did not differ between 7 d and 18 d stored eggs in ventilated incubator but the weight gain was significantly higher ($P = 0.0031$) in the chicks of 18 d stored eggs compared to those of 7 d stored eggs in nonventilated incubator (Figure 7C).

DISCUSSION

This study investigated the influence of nonventilation during incubation on eggs of different pre-incubation storage duration. This study clearly showed that embryonic and post-hatch development of broiler

chickens were affected by extended pre-incubation storage and nonventilation during the first 12 d of incubation.

The difference in egg weights between 7 d and 18 d stored eggs at setting in both ventilated and nonventilated incubator was a result of water loss during storage which was higher in 18 d stored eggs compared to 7 d stored eggs, since there was no significant difference in initial egg weights of the groups at collection. This result corroborates the recent report of Kouame et al. (2019) who showed that egg weight loss increased with storage duration. It is well known that during storage, eggs lose water, and CO_2 from the albumen. Thus, the increase in the albumen pH with storage duration can be attributed to the excessive diffusion of carbon dioxide from inside to the outside of the egg, which in turn provoke a decrease of protons in the albumen. In fact, diffusional loss of CO_2 causes a shift in the Henderson - Hasselbalch equation ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) to the left, resulting in a more alkaline pH of the albumen (Onagbesan et al., 2007).

Alkalinization of the albumen pH destabilizes the lysozyme-ovomucine complex and results in increasing liquidity of the albumen which negatively affect the egg quality. Day-old chick quality is related to egg characteristic such as egg quality (Tona et al., 2005). Therefore, the lower chick quality score for chicks from 18 d stored eggs can be attributed to the deterioration of the egg quality. The result of the present study is in accordance to the findings of Tona et al. (2003) who reported higher chick quality from eggs stored for 3 d compared to 18 d stored eggs.

The lower albumen weight and the higher embryo weight observed in nonventilated incubator compared to ventilated incubator in both stored eggs at ED 12 and at internal pipping stage could be the result of the inverse relationship between embryo weight and residual albumen weight (Peebles et al., 2000; Tona et al., 2005). Indeed, decrease in albumen weight during the second half of incubation is a result of albumen resorption by the embryo which begins at about ED 12 but becomes particularly active after ED 14 (Romanoff, 1960). Presents results showed that it is very likely that nonventilation during early incubation is associated with an acceleration in embryonic growth and hence, albumen resorption.

Meanwhile, embryos, that were stored for 18 d before start of incubation, weighed significantly less than those of 7 d stored eggs in both incubators. This result could be explain by the fact that embryonic development from eggs of long storage duration did not immediately initiate in response to incubation temperatures and these embryos proceeded at a slower rate during the first phase of incubation (Fasenko et al., 2002). It is known that embryos from long storage duration eggs not only lag behind in development, but their metabolism proceeds at a slower rate than embryos from eggs of short storage duration (Segura et al., 2006). This corresponds to the thyroid hormones levels in this study. Indeed, the lower T_3 concentration observed for embryos from 18 d stored eggs compared to embryo from 7 d stored eggs indicates a slower metabolic rate as it is generally assumed that T_3 is the calorigenically active form of thyroid hormones (Tona et al., 2013). Thus, the delay in the initiation of internal pipping, external pipping and hatching for chicks from 18 d stored eggs could be attributed to their lower metabolic rate due to the lower T_3 concentration.

It is well known that, nonventilation during the egg incubation process leads to an increase in pCO_2 which is accompanied by an increase in the production of corticosterone which has been implicated in the conversion of T_4 into T_3 leading to the initiation of hatching (De Smit et al., 2006). Therefore, the higher T_3 concentration observed in nonventilated group could be attributed to a higher pCO_2 and a higher corticosterone level in nonventilated incubator. This higher T_3 concentration could explain the fact that chicks of the nonventilated group hatched earlier than those of the ventilated group in both stored eggs, similar to the reports of earlier studies (De Smit et al., 2008; Tona et al., 2013; El-Hanoun et al., 2019). This finding was in agreement with studies by El-Hanoun et al. (2019) who reported that duck embryos from nonventilated incubator had higher T_3, T_4, and corticosterone levels and hatched earlier than those from ventilated incubator. The interaction of the thyroid and adrenal axis also extends to the hypothalamo-pituitary axis as corticotropin releasing hormone (**CRH**) induces an elevation of glucocorticoids (corticosterone) but also of thyrotropin (**TSH**) and hence raises T_4 concentrations which are a substrate for T_3 production. These parameters are interrelated, as corticosterone has been implicated in the maturation of thyroid hormone metabolism, and glucocorticoids and thyroid hormones are involved in preparation for pipping and hatching (Decuypere et al., 1991). The length of time between ventilated and nonventilated group in 7 d stored eggs (6.5 h) tended to be higher than the time frame in 18 d stored eggs (6.2 h). This suggests that the margin for improvement due to nonventilation tended to be larger in the eggs that were stored for 18 d.

The early commencement of the different hatching events in the nonventilated chicks can be attributed to higher tri-iodothyronine concentration at internal pipping, as the hatching process is known to be stimulated by T_3 and corticosterone (El-Hanoun et al., 2019). Moreover, the shorter interval between internal pipping and hatching observed in the nonventilated chicks compared to ventilated chicks in 7 d stored eggs group can be linked with the higher value of their thyroxin level at IP, since the length of the interval between the start of pulmonary respiration and hatching is thyroxin-dependent and higher thyroid hormone levels have been linked to a shorter time span between IP and hatch (Decuypere et al., 1991).

Regardless of the incubation treatment, 1-d and 7-d post-hatch chicks of the eggs that were stored for 18 d before start of incubation weighed significantly less than those of 7 d stored eggs in the present study. It should be noted that weighing of chicks was always done at the

same time point, without considering the biological age of the chicks. So, the observed differences in chick weights might be due to a difference in biological age, rather than a difference in daily weight gain; since there was no difference in weight gain between weights of the chicks of the 2 pre-incubation storage durations under ventilated incubator and the higher weight gain of chicks from eggs that were stored for 18 d compared to chicks from 7 d stored eggs in nonventilated incubator did not compensate either for this difference of chicks weight at 7 d post-hatch.

At hatch, absolute chick weights did not differ between the 2 incubation conditions. This result corroborating with the results of Bruggeman et al. (2007) and De Smit et al. (2008). Witters et al. (2008) showed no significant effect of nonventilation on day-old chick weight from 58-wk-old broiler breeders and a significant effect for 32-wk-old broiler breeders; suggesting that the effect of nonventilation on day-old chick weight might be age-dependent. The positive effect of nonventilation on chick weight was observed at 7 d post-hatch. In the present study, the difference in chick weight between nonventilated and ventilated groups in 18 d stored eggs can be attributed to higher weight gain in nonventilated incubator. This result is partially similar to the report of Witters et al. (2008) who showed that nonventilation improved post-hatch growth of chicks from 17 d stored eggs. However, the difference in chick weights between nonventilated and ventilated group in 7 d stored eggs might be due to a difference in biological age as no difference was observed in their weight gain.

If this is so, the advantage of early nonventilation incubation can mainly be attributed to an early hatching time and increased hatchability. In fact, a gradual build-up of CO_2 by closing the incubator dampers increased the fertile hatchability in both stored eggs. This result corresponds to the lower embryonic mortality (late embryonic mortality) in nonventilated incubator compared to ventilated incubator. In this way, one may suggest that nonventilation during the first few days of incubation can serve as a tool to reduce the percentage of embryo death due to long-term storage, as the highest embryonic mortality was observed in eggs that were stored for 18 d and incubated in a ventilated incubator. Several studies showed that nonventilation during the first 10 d of incubation in an airtight incubator improved hatchability (Tiryaki and Yildiri, 2011; El-Hanoun et al., 2019).

The eggs of nonventilated incubator had a lower EWL than those of the ventilated incubator in 7 d stored eggs. This finding may be due to the saturation of external air with CO_2, covering the surface of the eggshell as a layer and obstructing the evaporation of water vapour and gases from inside the eggs (El-Hanoun et al., 2019). Possible other explanation was the higher relative humidity observed in the nonventilated incubator compared to ventilated incubator confirming the result of Özlü et al. (2018) who showed in his third experiment that, the eggs of nonventilated incubator had a lower EWL than those of the ventilated incubator and this difference was due to slightly higher relative humidity in the nonventilated machine than that in the ventilated machine. The higher egg weight loss for 7 d stored eggs compared to 18 d stored eggs is in line with the report of Tona et al. (2003) and Kouame et al. (2019) who demonstrated that egg weight loss during incubation increased with storage duration.

In conclusion, this study has shown a beneficial impact of nonventilation procedure on the hatching process and on post-hatch development of broilers. It was shown that the effect of long-term pre-incubation storage on embryonic development and post-hatch growth interacted significantly with incubation ventilation. The results of this study has established that early nonventilation can partially compensate for the negative effects of storage on hatchability, total incubation duration, and post-hatch growth.

Since nonventilation can affect the eggshell temperature (although this was not the case in this study), it would be of interest for further studies to take into account the interaction between eggshell temperature and nonventilation treatment. It would be also for great importance to take into account different breeder flock ages.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Bruggeman, V., A. Witters, L. De Smit, M. Debonne, N. Everaert, B. Kamers, O. M. Onagbesan, P. Degraeve, and E. Decuypere. 2007. Acid−base balance in chicken embryos (Gallus domesticus) incubated under high CO_2 concentrations during the first 10 days of incubation. J. Respir. Physiol. Neurobiol. 159:147–154.

De Smit, L., V. Bruggeman, M. Debonne, K. Tona, B. Kamers, N. Everaert, A. Witters, O. Onagbesan, L. Arckens, J. De Baerdemaeker, and E. Decuypere. 2008. The Effect of Nonventilation during early incubation on the embryonic development of chicks of two commercial broiler strains differing in ascites susceptibility. Poult. Sci. 87:551–560.

De Smit, L., V. Bruggeman, K. Tona, M. Debonne, O. Onagbesan, L. Arckens, J. De Baerdemaeker, and E. Decuypere. 2006. Embryonic developmental plasticity of the chick: Increased CO_2 during early stages of incubation changes the developmental trajectories during prenatal and postnatal growth. J. Comp. Biochem. Physiol. 145:166–175.

Edited By Decuypere, E., E. Dewil, and E. R. Kühn. 1991. The hatching process and the role of hormones. Pages 239−256 in Avian Incubation. S. G. Tullet, ed. Butterworths − Heinnemann, London, UK Edited By.

El-Hanoun, A., K. El-Sabrout, M. Abdella, and M. Eid. 2019. Effect of carbon dioxide during the early stage of duck egg incubation on hatching characteristics and duckling performance. Physiol. Behav. 208:112–582.

Fasenko, G. M., F. E. Robinson, J. C. Segura, J. J. R. Feddes, and C. A. Ouellette. 2002. Long term hatching egg storage alters the metabolism of broiler embryos. Poult. Sci. 80:62.

Fernandes, J. I. M., C. Bortoluzzi, J. M. Schmidt, L. B. Scapini, T. C. Santos, and A. E. Murakami. 2017. Single stage incubators and Hypercapnia during incubation affect the vascularization of the chorio-allantoique membrane in broiler embryos. Poult. Sci. 96:220–225.

Kouame, Y. E. A, D. Nideou, K. Kouakou, and K. Tona. 2019. Effect of guinea fowl egg storage duration on embryonic and physiological parameters, and keet juvenile growth. Poult. Sci. 98:6046–6052.

Kustra, K., M. Trela, B. Tombarkiewicz, S. Lapinski, K. Pawlak, and M. W. Lis. 2020. Selected factors that affect the results of artificial hatching of the golden pheasant (Chrysolophus pictus) in aviary breeding-a preliminary study. Eur. Poult. Sci. 84, doi:10.1399/eps.2020.313.

Moreki, J. C., and T. Ditshupo. 2012. Effect of storage time on hatchability of guinea fowl eggs. J. Anim. Sci. Adv. 2:631–636.

Onagbesan, O., V. Bruggeman, L. De Smit, M. Debonne, A. Witters, K. Tona, N. Everaert, and E. Decuypere. 2007. Gas exchange during storage and incubation of Avian eggs: effects on embryogenesis, hatchability, chick quality and post-hatch growth. J. Worlds Poult. Sci. 63:557–573.

Onbaşılar, E. E., Ö. Poyraz, and E. Erdem. 2007. Effects of egg storage period on hatching egg quality, hatchability, chick quality and relative growth in Pekin ducks. Arch. Geflügelk. 71:187–191.

Özlü, S., A. Uçar, R. Banwell, and O. Elibol. 2018. The effect of increased concentration of carbon dioxide during the first 3 days of incubation on albumen characteristics, embryonic mortality and hatchability of broiler hatching eggs. Poult. Sci. 98:771–776.

Peebles, E. D., C. W. Gardner, J. Brake, C. E. Benton, J. J. Bruzual, and P. D. Gerard. 2000. Albumen height and yolk and embryo compositions in broiler hatching eggs during incubation. Poult. Sci. 79:1373–1377.

Reijrink, I. A. M., R. Meijerhof, B. Kemp, E. A. M Graat, and H. Van den Brand. 2009. Influence of pre-storage incubation on embryonic development, hatchability, and chick quality. Poult. Sci. 88:2649–2660.

Romanoff, A. L. 1960. The Avian Embryo: Structural and Functional Development. The MacMillan Company, New York, NY.

Segura, J. C., C. Ouellette, J. J. R. Feddes, G. M. Fasenko, and M. J. Zuidhof. 2006. Development of a metabolic calorimeter system to measure heat production of domestic avian embryos during incubation. Can. Bios. Engin. 48:41–46.

Tiryaki, S., and I. Yildiri. 2011. The effects of non-ventilation environments in setters on hatching characteristics of broiler breeder eggs and progeny performance. Selçuk Tarım Gıda Bilimleri Dergisi 25:60–64.

Tona, K., F. Bamelis, B. De Ketelaere, V. Bruggeman, V. M. B. Moreas, J. Buyse, O. Onagbesan, and E. Decuypere. 2003. Effects of egg storage time on spread of hatch, chick quality, and chick juvenile growth. Poult. Sci. 82:736–741.

Tona, K., V. Bruggeman, O. Onagbesan, F. Bamelis, M. Gbeassor, K. Mertens, and E. Decuypere. 2005. Day-old chick quality: relationship to hatching egg quality, adequate incubation practice and prediction of broiler performance. Avian Poult. Biol. Rev. 16:109–119.

Tona, K., N. Everaert, H. Willemsen, M. Gbeassor, E. Decuypere, and J. Buyse. 2013. Effects of interaction of incubator CO_2 levels and mixing hatching eggs of different embryo growth trajectory on embryo physiological and hatching parameters. Br. Poult. Sci. 54:545–551.

Whitehead, C. C., M. H. Maxwell, R. A. Pearson, and K. M. Herron. 2002. Influence of egg storage on hatchability, embryonic development and vitamin status in hatching broiler chicks. Braz. J. Poult. Sci. 26:221–228.

Witters, A., M. Debonne, N. Everaert, H. Willemsen, L. De Smit, B. Kamers, P. Garain, D. Berckmans, E. Decuypere, and V. Bruggeman. 2008. Differential effects of high CO2 during the first half of incubation on embryonic chick development according to broiler breeder age and storage time. WPSA Working Group 6 (Reproduction): Meeting 2008. Avian Biol. Res. 1:129–130.