Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2023 / v.25 / n.2 / 001-012

http://dx.doi.org/10.1590/1806-9061-2022-1728

Original Article

# Beneficial Impact of Hypercapnic Conditions During Early Incubation on Broiler Hatchability. Embryo Mortality and Postnatal Performance

■Author(s)

Kroetz Neto FL[I,II] https://orcid.org/0000-0001-9370-2228
Gonzales E[III] https://orcid.org/0000-0002-6962-3105
Novaes GA[II] https://orcid.org/0000-0001-6449-7253
Pereira RJG[II] https://orcid.org/0000-0003-3908-1007

[I] Aviagen America Latina Ltda.
[II] Study Group for Avian Multiplication – GEMA, Department of Animal Reproduction. College of Veterinary Medicine and Animal Sciences, University of São Paulo, São Paulo, Brazil.
[III] Department of Animal Science, Animal Physiology and Embryology, College of Veterinary Medicine and Animal Sciences, Paulista State University, Botucatu, São Paulo, Brazil.

■Mail Address

Corresponding author e-mail address
Dr Felipe Lino Kroetz Neto
Department of Animal Reproduction, College of Veterinary and Animal Sciences, University of São Paulo, São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508-900, São Paulo, SP, Brazil.
Phone: 5519 99625-1795
Email: felipekroetzneto@gmail.com

■Keywords

$CO_2$ concentration; chick quality; embryonic development; hatch window.



Submitted: 15/September/2022
Approved: 13/February/2023

## ABSTRACT

We assessed the extent to which $CO_2$ levels altered different hatching and chick parameters. In Experiments 1 and 2, a total of 16,184 eggs from Cobb 500 breeders were incubated in single stage incubators under three different conditions: (a) standard ventilated incubator (CON, Exp.1 and 2); (b) increasing $CO_2$ levels during the first 10 days of incubation until 0.7% (V7000, Exp. 1) and (c) until 0.8% (V8000, Exp. 2). High levels of $CO_2$ improved hatchability, possibly due to lower embryo mortality from ED18 to ED21. Internal and external pipping in experiment V8000 started later than in CON; nevertheless, the hatch still occurred before in V8000 as a result of the shorter durations of external pipping and hatch. In Experiment 3, a total of 12,138 eggs from Cobb 500 were incubated in single stage incubators under three different conditions: (a) standard ventilated incubator (CON); (b) increasing $CO_2$ levels until 1.0% with ventilation (V10000); and (c) increasing $CO_2$ levels until 1.0% without ventilation (NV10000). Hypercapnic conditions led to better hatchability and lower embryo mortality from ED18 to ED21. Internal pipping started earlier in NV10000, but only V10000 differed from CON in terms of the average time for hatch. Hypercapnic groups also showed shorter durations of external pipping and hatch when compared to CON. Post-hatch analysis revealed no differences among incubation conditions in terms of body weight gain, feed conversion ratio, mortality by sudden death syndrome, and production factor. Nevertheless, V10000 showed a lower mortality by ascites and a better viability when compared to CON, while NV10000 presented a higher mortality by other causes. Altogether, our findings indicate that in addition to not being detrimental to embryo survival, high $CO_2$ levels reduce embryonic mortality at 18-21 days of incubation and increase hatchability.

## INTRODUCTION

Due to intense genetic progress in broiler production, individuals from modern fast-growing lines nowadays take only 35 days to reach their slaughter weight, which means that more than a third of these birds' life consists of embryo development (Ismail *et al.*, 2016; Tallentire *et al.*, 2018) chick quality, secondary sex ratio, and some blood biochemical parameters at hatch and after thermal challenge at 60 days of age. A total number of 1200 suitable hatching eggs were taken from Mamoura strain laying hens, Eggs were randomly divided into equal 4 treated groups( 300 eggs each. Therefore, it is undeniable that hatcheries are increasingly becoming a strategic division of the poultry industry. Evidence of this can be found in several reports demonstrating that pre-incubation and incubation changes can improve hatching parameters, chick quality, and post-hatch performance, emphasizing the relevance of the embryonic environment to achieve the bird's full

genetic potential (De Smit *et al.*, 2006; Onagbesan *et al.*, 2007; Bergoug *et al.*, 2013; Ismail *et al.*, 2016).

Previous studies in chicken embryos have shown that, depending on timing and duration, increased $CO_2$ levels (above 0.1–0.5%) can optimize hatchability and chick performance (De Smit *et al.*, 2006; Bruggeman *et al.*, 2007; Tona *et al.*, 2007; Willemsen *et al.*, 2008). Although the physiological mechanisms of this process remain to be fully elucidated, Verhoelst *et al.* (2011) and Druyan *et al.* (2012) confirmed that hypercapnia or hypoxia during the first 10 days of incubation promote angiogenesis in the chorioallantoic membrane, and there are those who believe that other related organs may be positively affected (ensuring a better blood supply during prenatal and postnatal growth) (Decuypere *et al.*, 2006; Fernandes *et al.*, 2017). Initial investigations on $CO_2$ manipulation revealed that levels above 1% at different incubation periods increased embryo mortality and reduced hatchability (Taylor *et al.*, 1956; Taylor & Kreutziger, 1965, 1966; Everaert *et al.*, 2010). However, subsequent data showed that whenever $CO_2$ levels were gradually increased up to 1-1.5% at embryonic day (ED) 10, hatchability and other hatching parameters were positively affected (De Smit *et al.*, 2006; Bruggeman *et al.*, 2007; Tona *et al.*, 2007; Willemsen *et al.*, 2008). Some of these researches indicated that the developing embryo acquires greater $CO_2$ tolerance over the course of incubation, possibly through its increasing capacity to mitigate the effects of acidosis caused by gas saturation (Bergoug *et al.*, 2013).

In light of the above, this study determined the influence of early prenatal hypercapnic incubation (increasing $CO_2$ levels up to 0.7%. 0.8% and 1.0% at ED10) on embryonic growth, incubation and hatching parameters (eggshell temperature, egg weight loss, hatchability, time of internal pipping, external pipping and hatching, and embryo mortalities), and hatchling characteristics (body weight, chick length, residual yolk weight, yolk-free body weight, chick quality and blood parameters). Additionally, in Experiment 3 (when $CO_2$ levels reached 1.0%) we monitored post-hatch characteristics from birth to slaughter (body weight gain, feed conversion ratio, blood parameters, mortality, ascites and sudden death incidence, and carcass traits). Although previous studies have addressed hypercapnic conditions on early incubation, we felt the need to test $CO_2$ manipulation in larger sample sizes (above 4,000 eggs per group), collecting a vast number of parameters (as listed above) from the same experimental groups in order to provide better overview of the impact of controlled levels of $CO_2$ during the first half of incubation.

# MATERIAL AND METHODS

## Experimental Design

Experimental procedures complied with the current regulations established by the Institutional Animal Care and Use Committee at the Faculdade de Ciências Agrárias e Veterinárias – Universidade Estadual Paulista (FCAVJ – UNESP). Three experiments were set up using eggs from the same breeding flock (Cobb 500) housed by Perdigão Agroindustrial S/A (Castro, Paraná, Brazil). Incubations were performed using single-stage incubators (Petersime SR 84 for 8400 eggs with 150-eggs trays, adapted to automatically monitor temperature, relative humidity, ventilation, and egg turning using a CASP E Line system) located at FCVAJ – UNESP. Eggs (8,092 units) collected at 36 weeks of age were randomly divided into two equal-sized treatments during Experiment 1: (a) standard ventilated incubator (control – CON; i.e. without $CO_2$ pumping); and (b) ventilated incubator with a gradual injection of $CO_2$ from ED1 to ED10 until the level of 0.7% was reached (V7000). The same number of eggs (collected at 40 weeks of age) and treatment allocation were adopted in Experiment 2, however $CO_2$ levels were increased until 0.8% at ED10 (V8000). In Experiment 3, 12,138 eggs were collected from the same flock at 48 week of age and randomly assigned into three equal-sized groups: (a) standard ventilated incubator (CON); (b) ventilated incubator with a gradual injection of $CO_2$ until 1.0% from ED1 to ED10 (V10000); and (c) non-ventilated incubator with a gradual injection of $CO_2$ until 1.0% from ED1 to ED10 (NV10000). In all hypercapnic groups, $CO_2$ pumping was terminated after 240 h of incubation, and incubation conditions thereafter were the same as in the controls. In all experiments, $CO_2$ levels in both treated and control groups were registered every 2 h using a computerized system with a $CO_2$ sensor (Vaisala GMM221, Waarloos, Belgium). These $CO_2$ level increase patterns are depicted in Fig. 1.

## Egg Storage, Processing and Incubation

Eggs used in this study were stored for 3 days at the breeders' farm at a temperature of 21°C (69,8°F) and later transported in specially designed egg trucks at temperatures between 23-25°C (73,4-77°F). Shortly after arrival, eggs were removed from their boxes and accommodated in incubation trays and trolleys, with 360 eggs from each treatment group being individually weighed and numbered in order to assess eggshell temperature, egg weight loss, hatching parameters, and hatchling characteristics. The total number of eggs within each treatment group was used to estimate

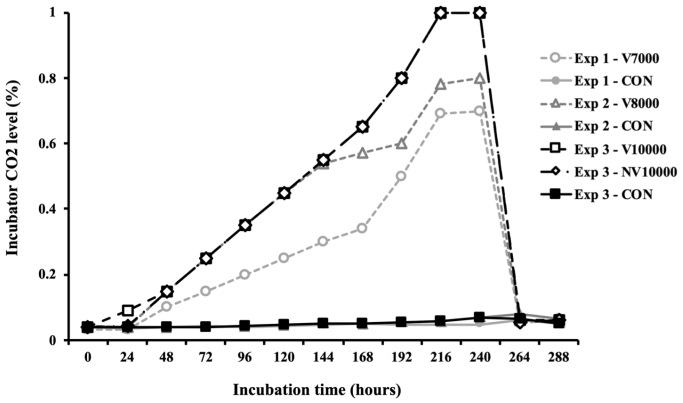


**Figure 1** – Changes in $CO_2$ levels of the incubators during the first 12 days of incubation. Control (ventilated incubators without $CO_2$ pumping, CON) and hypercapnic groups are represented by solid and dotted lines, respectively. V7000, V8000, and V10000 are ventilated incubators with a gradual increase of $CO_2$ until 0.7%. 0.8% and 1.0% at E10, respectively. NV10000 represents the non-ventilated incubator with a gradual injection of $CO_2$ until levels reached 1.0% at ED10.

embryonic mortality and hatchability. Additionally, we measured the egg specific gravity of 1,176 eggs from the same batches to verify whether general eggshell quality was between 1,075 and 1,085, but these eggs were not included in our study.

In all experiments, eggs were pre-heated during 5 h with a gradual increase in temperature from 26.5°C to 37.8°C (79,7 to 100°F), and incubators were set to execute the same temperature and relative humidity programs (from 37.89°C to 37.28°C or 100,2 to 99,1°F and from 56.24% to 41.72%, respectively). These settings were established based on previous tests to guarantee that eggshell temperature was around 37.7°C (99,9°F) throughout incubation. Egg turning was performed on an hourly basis until ED18. An automated monitoring system was also used to register all incubation parameters (temperature, relative humidity, ventilation, and egg turning) every 30 minutes for greater precision. Incubators remained closed until ED10, and were opened to allow for the removal of accumulated gases from the interior. After 456 hours of incubation, these incubators were configured for the hatcher function at 36.9°C (98,4°F) and relative humidity of 55% for the first 24 hours, and 36,7°C (98°F) and relative humidity of 61% for the remaining 24 hours. The number of eggs in each hatching tray was standardized to avoid temperature differences that could affect the hatching process. At 504 h, chicks were removed from the hatcher, sexed, and individually weighed.

## Eggshell Temperature and Embryonic Parameters

Between ED10 and ED18, eggshell temperatures were determined inside each incubator on a daily basis using an infrared thermometer (Braun 270, Germany). Over the same period, 30 eggs per day were randomly removed from each incubator to evaluate the yolk-free embryonic body weight using a semi-analytical scale with an accuracy of 1 milligram (Adventure ARD110, Ohaus, USA).

## Hatching Events, Hatchability and Embryonic Mortalities

From 444 to 504 h of incubation, 360 transferred eggs per treatment group were individually checked every 2 h for internal pipping (IP), external pipping (EP), and hatching. In addition to IP, EP and hatching, we calculated the duration of IP, EP and Hatch (i.e., time intervals between IP-EP, EP-Hatch and IP-Hatch, respectively). Hatched chicks were subsequently counted and tagged with leg bands (corresponding to the number of the incubated egg). Unhatched eggs were opened and macroscopically categorized into the following groups: unfertile; embryonic mortality at 0-4 days; embryonic mortality at 5-10 days; embryonic mortality at 11-17 days; embryonic mortality at 18-21 days; pipped eggs; and contaminated eggs. Hatchability (%) was calculated as the number of hatched chicks divided by fertile eggs and multiplied by 100.

## Hatchling Assessment

Hatchlings from marked eggs were weighed, and lengths from the tip of the beak to the tip of the middle-toe were recorded by the same person. Later on, chick quality was evaluated according to criteria established by Tona *et al.* (2003), with some minor modifications (Table 1). After quality scoring, animals were euthanized by cervical dislocation and their yolk sac was removed and weighed. Additionally, heparinized blood samples from another 30 chicks per treatment group per period were analyzed for hematocrit (HCT), red blood cell count (RBC), mean corpuscular volume (MCV), and hemoglobin concentration (Hb) (Automated Cell Counter CELM 550, Germany).

## Post-hatch Performance

During Experiment 3, 3,000 hatchlings were shipped to an experimental farm (Perdigão Agroindustrial S/A, Videira – SC, Brazil), where they were randomly assigned to 60 floor pens (20 pens per treatment group, each containing 25 males and 25 females). Environmental conditions were the same for all groups. Chicks were raised for 42 days with water and feed *ad libitum* using manual feeding systems and nipple drinkers. The feeding program formulated with corn and soybean meal was divided into three stages: (a)

**Table 1 –** Description of the parameters used to determine chick quality.

| Parameter | Definition | Classification | Score |
|---|---|---|---|
| Activity | Assessed by laying the chick on its back to determine how quickly it returned to its feet | Fast<br>Slow<br>No return | 16<br>8<br>0 |
| Down | Normal appearance was considered dry and clean | Clean and dry<br>Clean and wet<br>Dirty and wet | 12<br>6<br>0 |
| Eyes | The chick was put on the legs, and its eyes were observed. The state of brightness and wideness of the gape of the eyelids were estimated. | Open and bright<br>Open and dull<br>Closed | 10<br>5<br>0 |
| Navel area | Navel and surrounding areas were examined for closure of the navel and its color | Closed and clean<br>Partially closed and normal color<br>Unclosed and abnormal color | 12<br>6<br>0 |
| Remaining membrane | Estimation of the size of any remaining membrane in the navel area | No membrane<br>Small<br>Large | 12<br>6<br>0 |
| Abdomen | Visual inspection of the abdomen. It was considered bad if swollen | Normal<br>Slightly swollen<br>Swollen | 12<br>6<br>0 |
| Legs | The chick was put on its feet to examine if it remained properly upright | Normal legs. knees and feet<br>One leg. knee or foot abnormal<br>Both legs. knees or foot abnormal | 10<br>5<br>0 |
| Shin | Shins were examined in terms of brightness and color. Normal shin must be bright and slightly reddish | Bright and reddish<br>Bright and pale<br>Dull and pale | 16<br>8<br>0 |

starter diet (1-18 days, 3100 kcal of ME/kg and 21.0% CP); (b) grower diet (19-35 days, 3200 kcal of ME/kg and 20.0% CP); and (c) finisher diet (36-42 days, 3250 kcal of ME/Kg and 19.0% CP). Feed consumption and mortality were daily recorded, and individual weights were collected on a weekly basis. These data were later used to calculate body weight gain (g), feed conversion ratio (g feed/ g gain), viability (100 – mortality in percent), and production factor ((viability % x daily weight gain in grams) / feed conversion ratio x 10)). Apart from total mortality, we also registered mortality caused by ascites (ASC) and sudden death syndrome (SDS) following necropsy of all dead individuals. Ascites diagnosis was based on the appearance of the ascitic fluid in the abdominal cavity or in the cardiac sac (with or without ventricle enlargement), whereas SDS diagnosis was made according to the macroscopic criteria proposed by Gonzales. At 40 days of age, blood samples were collected from 30 chicken per treatment group (15 males and 15 females), which were later analyzed for HCT, RBC, MCV, and Hb. At 42 days of age, 100 birds per treatment group (5 birds per floor pen with weighs close to the mean body weight (±10%)) were euthanized in the slaughter house located at the Experimental farm (a total of 300 birds). These individuals were used to estimate both carcass and part yields, including breast, drumsticks plus thighs, and giblets (heart, liver and lungs). The remaining animals were weighed for calculations of body weight gain, feed conversion ratio, viability, and production factor, and were later transported to a commercial slaughter house.

## Statistical Analysis

We had only two incubators available for Experiments 1 and 2, and therefore the different hypercapnic conditions (0.7% and 0.8%) were only compared to control conditions. Data were analyzed using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Firstly, the variables were evaluated for the normality of the residues and homogeneity of the variances using a SAS package (Guided Data Analysis). Parametric and non-parametric analyzes were used for the variables that did and did not follow the statistical premises, respectively. In Experiments 1 and 2, in order to compare the observed variables (egg weight loss, eggshell temperature, hatchability, hatching events, embryonic mortality, and chick parameters) from each hypercapnic condition (V7000 and V8000) to the control conditions (CON), we used Student's T test (PROC TTEST) and Wilcoxon Signed Rank Test (PROC NPAR1WAY WILCOXON) for parametric and non-parametric variables, respectively. In Experiment 3, to compare the three groups (CON, V10000 and NV10000), we used the ANOVA test (PROC GLM – ANOVA) for parametric variables and the Kruskal-Wallis test (PROC NPAR1WAY) for non-parametric variables. Additionally, we analyzed post-hatch performance (e.g. body weight, feed

conversion ratio and mortality) in Experiment 3 using repeated measure ANOVA (PROC MIXED). Tukey HSD and Dunn's were used as post hoc tests for parametric and nonparametric variables, respectively. All data are expressed as mean ± SD and there were considered to be mean differences when $p<0.05$.

## RESULTS

Egg weight did not differ between E10 and E18 in Experiments 1 and 2, while it was higher in hypercapnic groups only at ED18 in Experiment 3 (Tables 2 and 3). On several occasions, embryo weights under hypercapnic conditions (V7000, V8000, and V10000) were higher than those in the controls. In Experiment 3, embryos from NV10000 were heavier than CON the entire time, except for ED18. With regard to eggshell temperature, most values in Experiments 1 and 2 were higher in hypercapnic incubations than in the controls (Fig. 2). Nevertheless, the same pattern was only observed in Experiment 3 for V10000, with NV10000 exhibiting equal or lower values than CON for most of the time. Both incubators in Experiment 2 had problems to adjust eggshell temperature in days 14, 15 and 17 of incubation, possibly because of the high temperatures of the room during mid-summer. However, these short-period oscillations (which varied from 37.4 to 37.7ºC or 99,3 to 99,9ºF) did not seem to impact hatchability or embryonic mortality when compared to group averages from the other two experiments described here.

All hypercapnic conditions showed better hatchability than the controls (Tables 4 and 5). Although V7000 did not differ in egg weight loss from CON during Experiment 1, eggs from V8000 apparently lost more weight than CON in Experiment 2. Curiously, both hypercapnic conditions in Experiment 3 displayed different outcomes, with NV10000 and V10000 losing more and less egg weight than CON, respectively. Embryonic mortality at 11-17 days was only lower than the controls in V7000 during Experiment 1, while all hypercapnic treatments exhibited lower embryonic

**Table 2 –** Mean ± SEM of egg weight, embryo weight, and eggshell temperature per treatment group for Experiments 1 and 2.

| Day[1] | Egg weight | | | | Embryo weight[1] | | | |
|---|---|---|---|---|---|---|---|---|
| | Experiment 1 | | Experiment 2 | | Experiment 1 | | Experiment 2 | |
| | CON | V7000 | CON | V8000 | CON | V7000 | CON | V8000 |
| 10 | 65.72 ± 0.93 | 66.03 ± 0.69 | 63.90 ± 0.98 | 64.19 ± 0.84 | 3.13 ± 0.03 | 3.27 ± 0.04** | 3.15 ± 0.04 | 3.34 ± 0.04** |
| 11 | 65.01 ± 0.78 | 65.87 ± 0.77 | 64.44 ± 0.78 | 65.08 ± 0.99 | 4.95 ± 0.07 | 4.96 ± 0.07 | 4.68 ± 0.05 | 4.89 ± 0.05** |
| 12 | 67.14 ± 0.86 | 66.66 ± 0.89 | 62.72 ± 0.86 | 62.03 ± 0.69 | 7.37 ± 0.09 | 7.63 ± 0.09* | 7.06 ± 0.09 | 7.48 ± 0.08*** |
| 13 | 66.00 ± 0.67 | 64.63 ± 0.81 | 64.66 ± 0.74 | 63.49 ± 0.81 | 10.41 ± 0.12 | 10.97 ± 0.13** | 10.25 ± 0.15 | 10.90 ± 0.13*** |
| 14 | 64.89 ± 0.85 | 65.09 ± 0.80 | 63.77 ± 0.78 | 63.29 ± 0.79 | 14.43 ± 0.19 | 15.18 ± 0.19** | 14.11 ± 0.15 | 15.29 ± 0.19*** |
| 15 | 63.93 ± 0.73 | 65.69 ± 0.87 | — | — | 18.87 ± 0.19 | 18.99 ± 0.21 | — | — |
| 16 | 63.78 ± 0.81 | 63.60 ± 0.79 | — | — | 22.89 ± 0.24 | 23.95 ± 0.30** | — | — |
| 17 | 64.54 ± 0.86 | 63.55 ± 0.96 | — | — | 27.77 ± 0.37 | 27.86 ± 0.37 | — | — |
| 18 | 63.14 ± 0.83 | 62.63 ± 0.90 | — | — | 31.58 ± 0.44 | 32.06 ± 0.31 | — | — |

[1]Day of incubation. [2]Yolk-free embryo weight. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ between control and its respective treatment.

CON = ventilated without $CO_2$ pumping (control incubators); V7000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10; V8000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10.

**Table 3 –** Mean ± SEM of egg weight and embryo weight per treatment group for Experiment 3.

| Day[1] | Egg weight | | | Embryo weight[1] | | |
|---|---|---|---|---|---|---|
| | CON | V10000 | NV10000 | CON | V10000 | NV10000 |
| 10 | 64.94 ± 0.91 | 66.02 ± 0.75 | 66.05 ± 0.67 | 3.22 ± 0.04[b] | 3.49 ± 0.04[a] | 3.52 ± 0.05[a] |
| 11 | 64.77 ± 0.69 | 66.00 ± 0.69 | 64.99 ± 0.91 | 4.89 ± 0.05[c] | 5.07 ± 0.07[b] | 5.47 ± 0.06[a] |
| 12 | 64.62 ± 0.84 | 64.99 ± 0.71 | 64.26 ± 0.86 | 6.93 ± 0.09[b] | 7.55 ± 0.09[a] | 7.77 ± 0.07[a] |
| 13 | 63.93 ± 0.75 | 64.51 ± 0.87 | 64.42 ± 1.07 | 10.80 ± 0.16[b] | 11.43 ± 0.10[a] | 11.49 ± 0.20[a] |
| 14 | 63.56 ± 0.89 | 64.21 ± 0.68 | 61.79 ± 0.72 | 14.67 ± 0.18[b] | 15.20 ± 0.12[a] | 15.43 ± 0.21[a] |
| 15 | 63.97 ± 0.84 | 63.26 ± 0.76 | 62.42 ± 0.74 | 19.23 ± 0.18[b] | 20.34 ± 0.21[a] | 20.29 ± 0.18[a] |
| 16 | 62.56 ± 0.72 | 61.70 ± 0.91 | 62.75 ± 0.79 | 23.95 ± 0.22[b] | 24.45 ± 0.22[ab] | 24.85 ± 0.29[a] |
| 17 | 60.38 ± 0.81 | 62.08 ± 0.95 | 59.97 ± 0.87 | 28.47 ± 0.41[b] | 29.88 ± 0.29[a] | 29.30 ± 0.36[a] |
| 18 | 58.64 ± 1.02[b] | 62.18 ± 0.79[a] | 61.60 ± 0.93[a] | 34.38 ± 0.51 | 34.30 ± 0.36 | 34.21 ± 0.32 |

[1]Day of incubation. [2]Yolk-free embryo weight. Columns with different superscripts differ significantly ($p<0.05$)

CON = ventilated without $CO_2$ pumping (control incubators); V10000 = ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10; NV10000 = non-ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10.

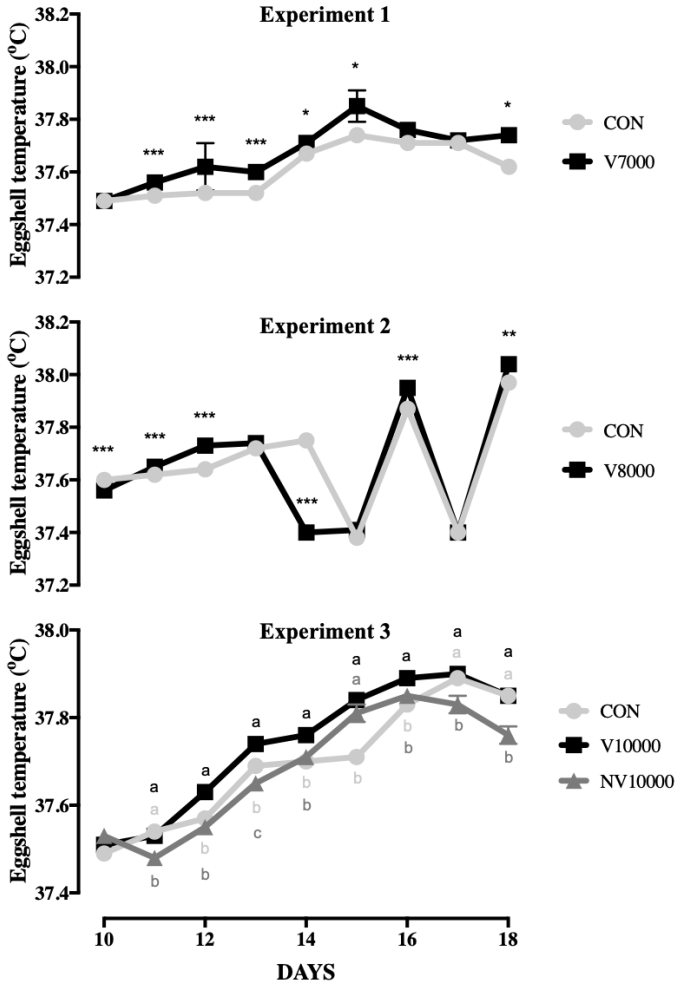


**Figure 2** – Changes in eggshell temperature between 10 and 18 days of incubation in all three experiments of this study. In Experiments 1 and 2, asterisks (*, ** and ***) indicate significant differences between treatment groups at a given time point during incubation ($p$<0.05, $p$<0.01 and $p$<0.001, respectively). In Experiment 3, data sharing no common letters are significantly different ($p$<0.05).

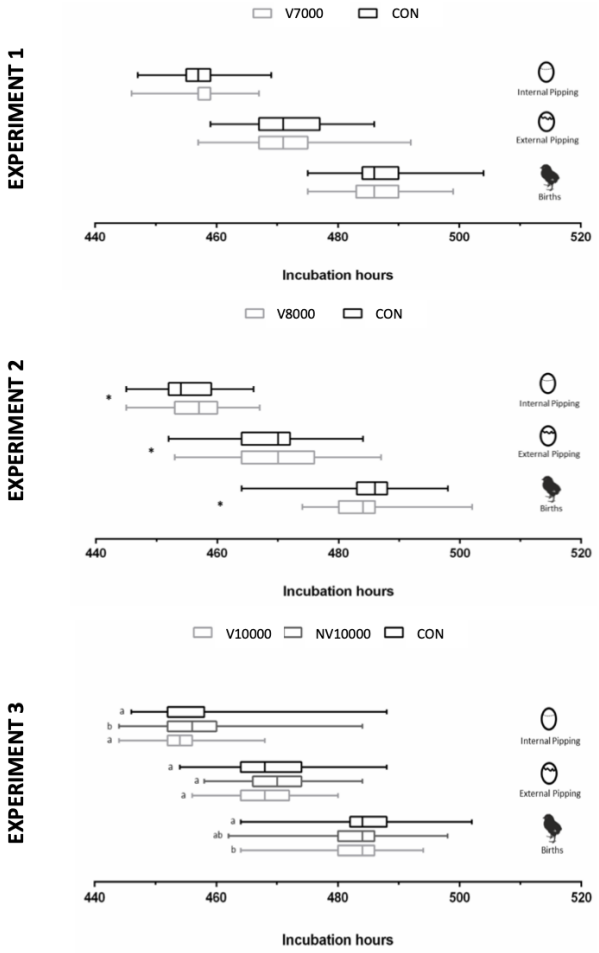


**Figure 3** – Representation of the hatch windows of eggs subjected to 0.7% (Exp. 1), 0.8% (Exp. 2), and 1% (Exp. 3) of $CO_2$ gradually in the first ten days of incubation. It is possible to observe the beginning of internal and external pipping and births, as well as the duration of each of these events. In Experiments 1 and 2, asterisks (*, ** and ***) indicate significant differences between treatment groups at a given time point during incubation ($p$< 0.05, $p$< 0.01 and $p$<0.001, respectively). In Experiment 3, data sharing no common letters are significantly different ($p$<0.05).

**Table 4 –** Mean ± SEM of hatching parameters per treatment group for Experiments 1 and 2.

| Parameters | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | CON | V7000 | CON | V8000 |
| Fertility (%) | 92.87 ± 0.52 | 93.19 ± 0.46 | 94.86 ± 0.31 | 96.06 ± 0.27** |
| Hatchability of fertile eggs (%) | 89.92 ± 0.52 | 91.87 ± 0.64* | 90.17 ± 0.63 | 91.70 ± 0.38* |
| Egg weight loss | 11.73 ± 0.13 | 11.42 ± 0.12 | 10.05 ± 0.09 | 11.22 ± 0.11*** |
| Embryonic mortality % (0-4 days) | 2.67 ± 0.24 | 2.92 ± 0.31 | 2.34 ± 0.27 | 2.22 ± 0.22 |
| Embryonic mortality % (5-10 days) | 1.00 ± 0.15 | 0.71 ± 0.17 | 0.84 ± 0.18 | 1.29 ± 0.21 |
| Embryonic mortality % (11-17 days) | 2.18 ± 0.25 | 1.41 ± 0.20* | 1.39 ± 0.27 | 1.05 ± 0.14 |
| Embryonic mortality % (18-21 days) | 2.47 ± 0.34 | 1.34 ± 0.23* | 5.00 ± 0.94 | 2.39 ± 0.27* |
| Pipped eggs (%) | 0.92 ± 0.16 | 0.63 ± 0.17 | 0.96 ± 0.16 | 0.77 ± 0.14 |
| Contaminated eggs (%) | 0.92 ± 0.16 | 0.63 ± 0.17 | 0.96 ± 0.16 | 0.77 ± 0.14 |
| Average time for IP (h) | 457.03 ± 0.23 | 457.21 ± 0.21 | 455.01 ± 0.25 | 456.99 ± 0.29* |
| Average time for EP (h) | 471.50 ± 0.36 | 471.83 ± 0.39 | 468.52 ± 0.31 | 469.96 ± 0.44* |
| Average time for hatch (h) | 486.65 ± 0.31 | 487.11 ± 0.30 | 485.55 ± 0.33 | 483.51 ± 0.35* |
| Duration of IP (h) | 14.36 ± 0.39 | 14.41 ± 0.39 | 11.72 ± 0.82 | 13.23 ± 0.39 |
| Duration of EP (h) | 15.37 ± 0.33 | 15.47 ± 0.29 | 16.69 ± 0.83 | 12.92 ± 0.32*** |
| Duration of hatch (h) | 29.73 ± 0.32 | 29.97 ± 0.31 | 27.95 ± 0.77 | 26.15 ± 0.31* |

* $p$<0.05; ** $p$<0.01; *** $p$<0.001 between control and its respective treatment. CON = ventilated without $CO_2$ pumping (control incubators); V7000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10; V8000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10.

**Table 5 –** Mean ± SEM of hatching parameters per treatment group for Experiment 3.

| Parameters | CON | V10000 | NV10000 |
|---|---|---|---|
| Fertility (%) | 94.13 ± 0.36 | 94.94 ± 0.38 | 94.77 ± 0.37 |
| Hatchability of fertile eggs (%) | 91.02 ± 0.54[b] | 94.00 ± 0.39[a] | 93.11 ± 0.42[a] |
| Egg weight loss | 11.59 ± 0.09[b] | 11.08 ± 0.11[c] | 11.95 ± 0.11[a] |
| Embryonic mortality % (0-4 days) | 3.00 ± 0.28 | 2.07 ± 0.23 | 2.41 ± 0.21 |
| Embryonic mortality % (5-10 days) | 1.03 ± 0.15 | 0.91 ± 0.18 | 0.92 ± 0.17 |
| Embryonic mortality % (11-17 days) | 1.40 ± .24 | 0.99 ± 0.16 | 1.39 ± 0.21 |
| Embryonic mortality % (18-21 days) | 2.75 ± 0.25[b] | 1.44 ± 0.14[a] | 1.48 ± 0.23[a] |
| Pipped eggs (%) | 0.11 ± 0.07 | 0.11 ± 0.05 | 0.20 ± 0.09 |
| Contaminated eggs (%) | 0.11 ± 0.07 | 0.15 ± 0.07 | 0.20 ± 0.09 |
| Average time for IP (h) | 454.65 ± 0.31[a] | 454.41 ± 0.26[a] | 455.77 ± 0.34[b] |
| Average time for EP (h) | 469.06 ± 0.33 | 468.74 ± 0.29 | 469.51 ± 0.34 |
| Average time for hatch (h) | 484.86 ± 0.34[a] | 483.47 ± 0.27[b] | 484.04 ± 0.30[ab] |
| Duration of IP (h) | 14.54 ± 0.34 | 14.33 ± 0.31 | 13.74 ± 0.32 |
| Duration of EP (h) | 15.86 ± 0.28[a] | 14.72 ± 0.27[b] | 14.53 ± 0.26[b] |
| Duration of hatch (h) | 30.33 ± 0.34[a] | 29.13 ± 0.29[b] | 28.43 ± 0.30[b] |

Columns with different superscripts differ significantly ($p<0.05$)

CON = ventilated without $CO_2$ pumping (control incubators); V10000 = ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10; NV10000 = non-ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10.

mortality at 18-21 days when compared to controls. In Experiment 2, internal and external pipping started later in V8000 than in CON but, in spite of that, the hatch occurred before as a result of shorter durations of external pipping and hatch in V8000 (Table 4 and Fig. 3). Internal pipping in Experiment 3 started earlier in NV10000 than in CON, but only V10000 differed from CON in terms of the average time for hatch. Both hypercapnic groups exhibited shorter durations of external pipping and hatch when compared to CON.

Tables 6 and 7 summarize chick variables observed in Experiments 1, 2 and 3. None of the hypercapnic conditions differed from controls for chick weight, yield, and quality. Likewise, blood parameters were not positively or negatively influenced by the levels of $CO_2$ used here. Nevertheless, V7000 (Exp1) and NV10000 (Exp3) presented lower yolk-free hatchling weights and higher yolk sac weights than the controls.

Weekly measurements of body weight, feed conversion ratio, and mortality in Experiment 3 indicated no differences among groups whenever results from males and females were jointly assessed (Table 8). However, females from V10000 differed from CON in body weight during the 2nd week (0.522 ± 0.003 and 0.507 ± 0.004, respectively – $p<0.05$) and in feed conversion in the 4th week (1.56 ± 0.01 and 1.49 ± 0.02, respectively – $p<0.05$). Yet, overall analysis (i.e., from 1 to 42 days) revealed no differences among incubation conditions in relation to body weight gain, feed conversion ratio, mortality by sudden death syndrome, and production factor. On the other hand, V10000 showed lower mortality by ascites and better

**Table 6 –** Mean ± SEM of chick parameters per treatment group for Experiments 1 and 2.

| Parameters | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | CON | V7000 | CON | V8000 |
| Chick weight (g) | 51.67 ± 0.11 | 51.83 ± 0.11 | 49.42 ± 0.08 | 49.23 ± 0.09 |
| Chick Length (cm) | 18.81 ± 0.03 | 18.59 ± 0.03* | 18.48 ± 0.03 | 18.61 ± 0.03* |
| Chick yield (%) | 71.01 ± 0.13 | 71.05 ± 0.14 | 71.22 ± 0.12 | 70.89 ± 0.12 |
| Yolk-free hatchling weight (g) | 46.07 ± 0.17 | 45.50 ± 0.15* | 43.34 ± 0.12 | 43.31 ± 0.12 |
| Yolk sac weight (g) | 5.58 ± 0.13 | 6.15 ± 0.10* | 6.06 ± 0.09 | 5.89 ± 0.08 |
| High quality chicks % (score ≥ 80) | 96.39 ± 0.72 | 96.62 ± 1.25 | 92.78 ± 0.90 | 93.41 ± 1.07 |
| Low quality chicks % (score < 80) | 3.60 ± 0.72 | 3.38 ± 1.25 | 7.22 ± 0.90 | 6.59 ± 1.07 |
| HCT (%) | 21.30 ± 0.85 | 21.89 ± 0.98 | 18.23 ± 1.09 | 18.75 ± 1.11 |
| RBC ($10^6$/ $mm^3$) | 2.47 ± 0.10 | 2.51 ± 0.12 | 1.98 ± 0.13 | 2.06 ± 0.13 |
| MCV ($\mu m^3$) | 87.68 ± 0.47 | 87.84 ± 0.44 | 94.62 ± 0.70 | 93.20 ± 0.54 |
| Hb (g/dL) | 13.52 ± 0.44 | 14.22 ± 0.52 | 11.59 ± 0.80 | 11.75 ± 0.70 |

* $p<0.05$; ** $p<0.01$; *** $p<0.001$ between control and its respective treatment.

CON = ventilated without $CO_2$ pumping (control incubators); V7000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10; V8000 = ventilated incubator with a gradual injection of $CO_2$ until 0.7% at ED10.

HCT – hematocrit; RBC – red blood cell count; MCV – mean corpuscular volume; Hb – hemoglobin concentration.

**Table 7 –** Mean ± SEM of chick parameters per treatment group for Experiments 1 and 2.

| Parameters | CON | V10000 | NV10000 |
|---|---|---|---|
| Chick weight (g) | 49.17 ± 0.09 | 49.30 ± 0.09 | 49.30± 0.09 |
| Chick length (cm) | 18.93 ± 0.03[a] | 18.97 ± 0.02[a] | 18.81 ± 0.03[b] |
| Chick yield (%) | 69.84 ± 0.12 | 70.04 ± 0.12 | 69.73 ± 0.12 |
| Yolk-free hatchling weight (g) | 43.53 ± 0.13[a] | 43.67 ± 0.15[a] | 42.51 ± 0.12[b] |
| Yolk sac weight (g) | 5.63 ± 0.09[a] | 5.60 ± 0.15[a] | 6.55 ± 0.09[b] |
| High quality chicks % (score ≥ 80) | 86.16 ± 1.79 | 89.67 ± 1.16 | 87.68 ± 2.74 |
| Low quality chicks % (score < 80) | 13.84 ± 1.79 | 10.33 ± 1.16 | 12.31 ± 2.74 |
| HCT (%) | 39.02 ± 2.41 | 39.67 ± 1.32 | 38.10 ± 1.60 |
| RBC ($10^6$/ $mm^3$) | 3.04 ± 0.22 | 3.65 ± 0.18 | 3.12 ± 0.19 |
| MCV ($\mu m^3$) | 129.38 ± 2.97 | 112.29 ± 1.12 | 118.12 ± 0.66 |
| Hb (g/dL) | 19.24 ± 0.52 | 22.82 ± 0.64 | 20.11 ± 0.64 |

Columns with different superscripts differ significantly ($p$<0.05)

CON = ventilated without $CO_2$ pumping (control incubators); V10000 = ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10; NV10000 = non-ventilated incubator with a gradual injection of $CO_2$ until 1.0% at ED10.

HCT – hematocrit; RBC – red blood cell count; MCV – mean corpuscular volume; Hb – hemoglobin concentration.

**Table 8 –** Mean ± SEM of body weight, feed conversion ratio, mortalities, viability, and production factor for Experiment 3.

| Week | Parameters | CON | V10000 | NV10000 |
|---|---|---|---|---|
| 1st | Body weight (kg) | 0.20 ± 0.001 | 0.20 ± 0.001 | 0.20 ± 0.001 |
| | Feed conversion ratio (g feed/ g gain) | 1.06 ± 0.004 | 1.06 ± 0.003 | 1.06 ± 0.005 |
| | Mortality (%) | 0.30 ± 0.16 | 0.10 ± 0.10 | 0.52 ± 0.25 |
| 2nd | Body weight (kg) | 0.53 ± 0.005 | 0.54 ± 0.004 | 0.53 ± 0.005 |
| | Feed conversion ratio (g feed/ g gain) | 1.30 ± 0.005 | 1.29 ± 0.004 | 1.29 ± 0.004 |
| | Mortality (%) | 2.40 ± 0.40 | 2.67 ± 0.67 | 3.09 ± 0.49 |
| 3rd | Body weight (kg) | 1.02 ± 0.01 | 1.04 ± 0.009 | 1.03 ± 0.01 |
| | Feed conversion ratio (g feed/ g gain) | 1.41 ± 0.006 | 1.41 ± 0.007 | 1.42 ± 0.006 |
| | Mortality (%) | 1.90 ± 0.49 | 1.20 ± 0.37 | 2.69 ± 0.53 |
| 4th | Body weight (kg) | 1.66 ± 0.03 | 1.68 ± 0.03 | 1.68 ± 0.02 |
| | Feed conversion ratio (g feed/ g gain) | 1.45 ± 0.02 | 1.49 ± 0.02 | 1.47 ± 0.01 |
| | Mortality (%) | 4.10 ± 0.90 | 2.00 ± 0.48 | 4.16 ± 0.66 |
| 5th | Body weight (kg) | 2.28 ± 0.04 | 2.30 ± 0.04 | 2.30 ± 0.04 |
| | Feed conversion ratio (g feed/ g gain) | 1.81 ± 0.02 | 1.82 ± 0.02 | 1.82 ± 0.01 |
| | Mortality (%) | 6.20 ± 1.25 | 3.20 ± 0.76 | 5.86 ± 0.76 |
| 6th | Body weight (kg) | 2.89 ± 0.05 | 2.91 ± 0.05 | 2.90 ± 0.04 |
| | Feed conversion ratio (g feed/ g gain) | 2.07 ± 0.03 | 2.09 ± 0.03 | 2.12 ± 0.02 |
| | Mortality (% | 8.00 ± 1.43 | 5.76 ± 0.98 | 8.25 ± 0.95 |
| Overall[1] | Body weight gain (kg) | 2.84 ± 0.05 | 2.87 ± 0.05 | 2.86 ± 0.04 |
| | Feed conversion ratio (g feed/ g gain) | 1.84 ± 0.02 | 1.89 ± 0.02 | 1.86 ± 0.02 |
| | Total Mortality (%) | 8.10 ± 1.45 | 4.80 ± 0.93 | 7.40 ± 0.95 |
| | Mortality by ASC (%) | 6.50 ± 0.71[b] | 3.10 ± 0.66[a] | 5.40 ± 0.68[b] |
| | Mortality by SDS (%) | 1.20 ± 0.39 | 1.20 ± 0.44 | 1.20 ± 0.39 |
| | Mortality by other causes (%) | 0.21 ± 0.14[b] | 0.11 ± 0.11[b] | 0.80 ± 0.22[a] |
| | Viability (%) | 92.00 ± 1.43[b] | 95.10 ± 0.96[a] | 92.16 ± 0.99[ab] |
| | Production factor | 338.00 ± 8.02 | 344.00 ± 7.78 | 336.0 ± 6.53 |

[1]Outcomes from 1 to 42 days of age. Columns with different superscripts differ significantly ($p$<0.05). ASC – ascites; SDS – sudden death syndrome. Viability = 100 – mortality in percent; Production factor = (viability % x daily weight gain in grams)/ feed conversion ratio x 10.

viability when compared to CON, while NV10000 presented higher mortality by other causes. At the slaughter house, females from NV10000 had better body weights than those in CON (Table 9), but in spite of that, only males from V10000 exhibited higher carcass weight. There were no differences among groups for part yields.

## DISCUSSION

This study aimed to understand better about the influence of increased $CO_2$ levels during the first half of incubation on embryonic development, hatching parameters, and postnatal performance. Our findings seem compatible with previous observations, since they

**Table 9 –** Mean ± SEM of carcass yield and part yields of broilers euthanized at 42 days of age during Experiment 3.

| Parameters | MALES | | | FEMALES | | | MALES + FEMALES | | |
|---|---|---|---|---|---|---|---|---|---|
| | V1000 | NV10000 | CONTROL | V1000 | NV10000 | CONTROL | V1000 | NV10000 | CONTROL |
| Body weight (kg) | 3.07 ± 0.01 | 3.02 ± 0.01 | 3.03 ± 0.01 | 2.61 ± 0.01$^{ab}$ | 2.62 ± 0.01$^{a}$ | 2.57 ± 0.01$^{b}$ | 2.83 ± 0.02 | 2.82 ± 0.02 | 2.80 ± 0.02 |
| Carcass weight (kg) | 2.38 ± 0.01$^{a}$ | 2.34 ± 0.01$^{b}$ | 2.34 ± 0.01$^{b}$ | 2.04 ± 0.01 | 2.04 ± 0.01 | 2.01 ± 0.01 | 2.20 ± 0.02 | 2.19 ± 0.02 | 2.17 ± 0.02 |
| Carcass yield (%) | 77.51 ± 0.25 | 77.58 ± 0.22 | 77.01 ± 0.22 | 78.12 ± 0.19 | 77.71 ± 0.23 | 78.02 ± 0.18 | 77.81 ± 0.16 | 77.65 ± 0.16 | 77.52 ± 0.15 |
| Breast yield (%) | 37.72 ± 0.23 | 37.68 ± 0.24 | 38.20 ± 0.24 | 38.79 ± 0.27 | 38.62 ± 0.24 | 39.09 ± 0.27 | 38.25 ± 0.18 | 38.15 ± 0.18 | 38.65 ± 0.18 |
| Thigh yield (%) | 29.31 ± 0.22 | 29.33 ± 0.21 | 29.47 ± 0.21 | 28.03 ± 0.21 | 28.25 ± 0.21 | 27.99 ± 0.22 | 28.67 ± 0.16 | 28.79 ± 0.16 | 28.73 ± 0.17 |
| Heart yield (%) | 0.60 ± 0.01 | 0.60 ± 0.01 | 0.60 ± 0.01 | 0.56 ± 0.01 | 0.58 ± 0.01 | 0.59 ± 0.01 | 0.58 ± 0.01 | 0.59 ± 0.01 | 0.59 ± 0.01 |
| Liver yield (%) | 2.29 ± 0.04 | 2.36 ± 0.04 | 2.34 ± 0.04 | 2.33 ± 0.03 | 2.42 ± 0.04 | 2.40 ± 0.03 | 2.31 ± 0.02 | 2.39 ± 0.03 | 2.37 ± 0.03 |
| Lung yield (%) | 0.74 ± 0.02 | 0.72 ± 0.02 | 0.73 ± 0.02 | 0.62 ± 0.02 | 0.63 ± 0.02 | 0.61 ± 0.02 | 0.68 ± 0.01 | 0.67 ± 0.01 | 0.67 ± 0.01 |

Columns with different superscripts differ significantly ($p<0.05$).

indicated that gradual increases in $CO_2$ levels (0.7% - 1.0%) accelerated embryo growth and improved hatchability, apart from reducing external pipping and hatch intervals (0.8% and 1.0%) (Bruggeman *et al.*, 2007; Decuypere *et al.*, 2007; Peacock *et al.*, 1990). Similar to the reports by De Smit *et al.* (2006), we noticed that yolk-free embryonic weights at ED18 did not differ among groups, despite embryo growth being boosted by hypercapnic conditions from ED10 to ED17. This lack of $CO_2$ effect on embryo weights during egg transfer and in hatchling weights was also described by Decuypere *et al.* (2006) and Bruggeman *et al.* (2007). Some authors attribute the faster embryonic development under hypercapnic conditions to a more rapid acidification of the albumen, which triggers changes in different physiological pathways of the embryo. According to Bruggeman *et al.* (2007), the albumen acts as a compensatory system to the acidosis caused by high $CO_2$ levels, absorbing and hydrolyzing carbon dioxide into bicarbonate that is diffused through the embryonic circulation and regulates blood alkalinity. This and other signaling mechanisms seem to activate the expression of pH-dependent enzymes such as carbonic anhydrase, components that are directly implicated in the formation of sub-embryonic fluid (SEF), which in turn is essential for early development (Deeming, 1989). Besides, previous data reveal that systemic acidosis induced by early prenatal hypercapnia leads to greater chorioallantoic membrane (CAM) vascularization through the expression of vascular endothelial and basic fibroblast growth factors (VEGF and bFGF, respectively), which ensures better circulatory support for the developing embryo (Everaert *et al.*, 2008; Verhoelst *et al.*, 2011; Fernandes *et al.*, 2017).

Hypercapnic conditions in all experiments improved hatchability and reduced embryo mortality from ED18 to ED21. Our results coincide with previous studies in which gradual increases in $CO_2$ during early incubation led to higher hatchability as compared to standard conditions (De Smit *et al.*, 2006; Tona *et al.*, 2007; Willemsen *et al.*, 2008; El-Hanoun *et al.*, 2019). However, data on this matter are not consistent, since other researchers found negative or no effects of environmental hypercapnia up to ED10 on hatchability (Bruggeman *et al.*, 2007; Willemsen *et al.*, 2008; Reijrink *et al.*, 2010; Fernandes *et al.*, 2017; Okur, 2019; Özlü *et al.*, 2019). According to Özlü *et al.* (2019), the influence of hypercapnic conditions on embryonic development and survival may depend on the level and timing of $CO_2$ exposure. Chicken embryos seem to acquire an increasing tolerance to higher $CO_2$ levels from ED4 onwards, and therefore gradual instead of sudden rises in $CO_2$ culminate in beneficial effects on embryonic growth and hatching parameters (Bruggeman *et al.*, 2007). Druyan *et al.* (2012) speculated that the best time for higher $CO_2$ levels to enhance embryo growth and hatching is between ED5 and ED11, the interval that comprises CAM formation. Furthermore, factors such as strain and breeder age also appear to interfere in the hypercapnic impact on hatchability (De Smit *et al.*, 2006). Altogether, these aspects somehow explain the variability of results seen in literature regarding the effects of higher $CO_2$ levels on hatchability.

In this study, embryos submitted to increasing $CO_2$ levels up to 0.8% and 1.0% at ED10 in ventilated incubators exhibited shorter average times for hatch (approximately 2 hours) as a result of shorter durations of external pipping and hatch. Similar findings were observed by De Smit *et al.* (2006) and Bruggeman *et al.* (2007) in broiler eggs, and by Tona *et al.* (2013) in layer eggs. It is known that at the end of internal pipping, chick embryos begin lung ventilation. Shortly after, high $CO_2$ and low $O_2$ levels in the air chamber induce the hatching process, a phenomenon accompanied by and dependent on the rise of thyroid hormones and corticosteroids (De Smit *et al.*, 2006; Decuypere *et al.*, 1990). Earlier studies have shown that embryos

incubated under hypercapnic conditions during the first 10 days had higher $T_3$ and corticosteroids plasma concentrations as a consequence of higher $CO_2$ levels in the air chamber (De Smit *et al.*, 2006; Tona *et al.*, 2013). Such hormonal stimulus combined with faster growth of embryos submitted to hypercapnia may explain the shorter hatch windows observed in V8000 and V10000 but not in V7000.

Despite the known effects of hypercapnia on angiogenesis in the CAM during development, as well as the stimulus that $CO_2$ saturation has on blood parameters of developing embryos (Tazawa *et al.*, 2012), none of the blood parameters were positively or negatively affected by 0.7%, 0.8%, or 1.0% of $CO_2$. These results appear to contradict those obtained by El-Hanoun *et al.* (2019), whereby non-ventilated conditions induced increases in hemoglobin concentration (Hb), packed cell volume (PCV) and red blood cells (RBCs) in hatchlings and ducklings. Perhaps these divergences occurred due to distinct $CO_2$ pumping curves or exposure intervals of eggs to the plateau level (1%) during early incubation. Hypoxia from E5 through E12 also elevated hematocrit values on E13, E14 and at hatch without affecting hemoglobin concentrations (Druyan *et al.*, 2012). Nevertheless, the lack of studies investigating the impact of incubator $CO_2$ levels on blood parameters of hatchlings along with conflicting data on this matter makes it difficult to reach more definitive conclusions.

Overall, weekly measurements of body weight, feed conversion ratio, and mortality showed no differences between hypercapnic and standard incubations, but when sexes were assessed separately, females from V10000 differed from CON in body weight and feed conversion in the 2$^{nd}$ and 4$^{th}$ week, respectively. Likewise, Fernandes *et al.* (2014) observed no effects of high $CO_2$ levels during incubation on broiler performance or heart and liver relative weights. Conversely, De Smit *et al.* (2006) reported that increased $CO_2$ during the first 10 days improved the post-hatch body weight of broilers. Even more interestingly, these authors registered a more pronounced impact on the body weight of females, an outcome that to some extent resembles ours regarding performance. In addition, El-Hanoun *et al.* (2019) found lower body weight, and body weight gain, and higher feed consumption, and feed conversion ratio in ducks subjected to higher $CO_2$ during early incubation. Once again, the causes of such divergence among studies in the post-hatch performance are very difficult to determine considering that variations among protocols may have occurred not only in the level and timing of $CO_2$ exposure during incubation, but also in the housing, management, and nutrition of the chicks.

Several authors believe that hypoxia or hypercapnia throughout incubation may exert a positive influence on ascites susceptibility due to cardiocirculatory, pulmonary, and endocrine changes during embryonic development (Decuypere *et al.*, 2000a; Hassanzadeh *et al.*, 2004; De Smit *et al.*, 2006; Hassanzadeh *et al.*, 2008; Bahadoran *et al.*, 2010). Nevertheless, the length and severity of the hypoxia or hypercapnia must be considered during incubation, otherwise it may lead to profound structural and functional cardiovascular abnormalities instead of being beneficial, as previously described by Rouwet *et al.* (2002). In our study, broilers incubated at the highest $CO_2$ level (1%) with ventilation had lower incidence of ascites, corroborating previous results from Buys *et al.* (1998) and Hassanzadeh *et al.* (2002). In contrast, animals from the non-ventilated incubator with increasing $CO_2$ levels up to 1.0% showed no decrease in their mortality by ascites, which perhaps relates to an exacerbated decline in $O_2$ levels for a prolonged period of time during incubation. Yet, in spite of this difference, increasing $CO_2$ levels until 1.0% produced similar results in both ventilated and non-ventilated groups. This information is extremely pertinent for modern fast-growing broiler lines, in which accelerated growth and metabolism demand highly effective oxygen transport and favors the emergence of diseases such as right heart failure caused by ascites, and sudden death (Peacock *et al.*, 1990; Decuypere *et al.*, 2000).

As mentioned above, the success of induced hypercapnia during chicken embryogenesis depends on the gas level, moment, and time window in which it is performed. As stated by Sadler *et al.* (1954), embryos are extremely sensitive to $CO_2$ rises in the first 4 days, but as incubation progresses, they become gradually resistant to hypercapnia, coping with the effects of acidosis caused by gas saturation. This can explain the wide range of results found in literature concerning embryonic mortality (Taylor *et al.*, 1956; Taylor & Kreutziger, 1965; De Smit *et al.*, 2006; Tona *et al.*, 2007; Willemsen *et al.*, 2008; Everaert *et al.*, 2011).

Overall, our data demonstrated that, regardless of the $CO_2$ level (0.7%, 0.8% or 1.0%), hypercapnic conditions during early incubation did not negatively affect embryo survival and, as a matter of fact, reduced embryonic mortality at 18-21 days of incubation and increased hatchability. Thus, from the findings

presented here, it can be concluded that $CO_2$ pumping under the proposed conditions may be an important tool for broiler egg incubation, with the potential of accelerating embryonic growth, increasing hatchability, advancing hatch, reducing late embryo mortality, and decreasing ascites susceptibility.

## ACKNOWLEDGMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors would like to acknowledge CASP for its technical assistance with incubator updating and BRF for kindly providing eggs for the experiments. Many thanks go to all professors, staff, and graduate students from the Departments of Animal Science, Animal Physiology and Embryology of the Faculdade de Ciências Agrárias e Veterinárias – Universidade Estadual Paulista (FCAVJ – UNESP).

## AUTHOR CONTRIBUTIONS

F.L.K.N.: Acquisition of data, drafting the manuscript, revising the manuscript. E.G.: Conception and design of the study, acquisition of data. G.A.N.: statistical analysis, drafting the manuscript, revising the manuscript. R.J.G.P.: statistical analysis, acquisition of data, drafting the manuscript, revising the manuscript. All authors have approved the version to be published and agreed to be accountable for all aspects of the work.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## FUNDING

Master and PhD scholarships were provided by the Coordination for the Improvement of Higher Education Personnel (CAPES)

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [FLKN or RJGP], upon reasonable request.

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