

# **Non-Ventilation Conditions During Incubation at High Altitude Modify Embryonic Development and Hatchability of the Broiler Breeder's Eggs**

**Running title:** Hypercapnic incubation at high altitude of the broiler breeder's eggs

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## **Non-Ventilation Conditions During Incubation at High Altitude Modify Embryonic Development and Hatchability of the Broiler Breeder's Eggs**

### **Abstract**

This study explores the impact of gradually increasing high carbon dioxide (CO<sub>2</sub>) levels during the first 10 days of incubation at high altitude. Two ventilation conditions were compared. In the first, CO<sub>2</sub> concentration gradually accumulates through the embryo's metabolism in a non-ventilated (NV) incubator during the first 10 days of embryonic development (ED10). In the second condition, the incubator was normally ventilated (V). Both treatments received V conditions for the remainder of the incubation period. The CO<sub>2</sub> concentration in the V incubator remained at 0.13 % during the first ED10 days, whereas in the NV, the concentration gradually increased from 0.14 % to 0.9 %. Throughout the incubation, NV exhibited significantly lower ( $P < 0.05$ ) embryonic mortality compared to V. Remarkably, the hatchability of fertile eggs (HFE) was 10 % significantly higher ( $P < 0.05$ ) in NV conditions than V group. NV conditions at high altitude (2 230 m) produced embryos with heavier yolk-free body mass and a progressive trend of lighter yolk-sac weights from ED10 day until hatch. The weight of hatchlings in the NV treatment was 43.4 g, with a length of 17.5 cm, both of which were significantly higher ( $P < 0.05$ ) than hatched chicks from the V group (41.5 g and 17.2 cm respectively). The NV condition at high altitude positively influenced the quality of hatchling chicks. We conclude that the NV condition, with a gradual increase of CO<sub>2</sub> concentration during the first 10 days of incubation at high altitude, is preferable to the V conditions.

**Keywords:** Embryonic mortality; Egg mass loss; Hatchability of fertile eggs; Hypercapnia; Hatchling chick quality.

### **Study contribution**

The current study highlights the critical role of oxygen and carbon dioxide exchange during the first half of the domestic fowl embryonic development performed at high altitudes. The impact of non-ventilation conditions in the first 10 days of incubation on broiler breeders' fertile eggs was addressed. Restricting ventilation into the incubator allows CO<sub>2</sub> from the embryo's metabolism to build up. The results show a significant increase in hatchability due to lower embryo mortality and better quality of 1-day-old chicks in the non-ventilated group compared to standard ventilation. These findings suggest that controlled CO<sub>2</sub> increase during the early incubation stage can optimize hatchery outcomes. The current methodology is especially valuable for hatchery managers operating at high altitudes, where environmental factors such as oxygen levels and CO<sub>2</sub> concentrations directly influence embryonic development. Understanding these effects can lead to more effective incubation practices, ultimately improving hatchability and the overall health of newborn chicks.

### **Introduction**

Embryonic development (ED) in poultry is influenced by a combination of biotic and abiotic factors, such as genetic background and environmental conditions.<sup>(1)</sup>

The genetic background plays a crucial role in shaping the growth and development of poultry, as evidenced by the distinct characteristics observed in

broilers and layers.<sup>(2-4)</sup> Selective breeding over the years has resulted in significant differences between these two types of chickens, impacting their ED during incubation.<sup>(3, 5, 6)</sup> The abiotic factors that influence ED and its requirements for successful incubation include temperature, relative humidity (RH), air movement, air velocity, and the gaseous composition of the environment.<sup>(6, 7)</sup>

The gaseous environment is particularly important since it can influence the developmental trajectories of physiological regulatory systems in poultry.<sup>(6, 8-10)</sup> It has been proposed that altering the environment of a developing organism might change the developmental trajectories of some physiological regulatory systems. This phenomenon is known as heterokairy, a specific type of plasticity that describes environmentally driven, altered timing of development within a species.<sup>(11, 12)</sup> Plasticity in the timing of the onset of developmental events occurs at the individual level during their development.<sup>(12, 13)</sup>

To achieve optimal embryo development, adequate oxygen levels and the removal of sufficient carbon dioxide are needed.<sup>(14)</sup> Chronic hypoxia during critical phases of chick embryo development can have varying effects.<sup>(15)</sup> Chronic hypoxia during critical phases of chick ED can impact embryo survival, body weight, and cause developmental abnormalities.<sup>(13, 16-18)</sup> It is noteworthy that hypoxia has been observed to accelerate the developmental process of chick embryos, leading to earlier hatching attributed to the earlier maturation of the surfactant system.<sup>(13, 17, 19, 20)</sup> Hypoxia delays the onset of all cardiovascular responses during the development of certain vertebrate species,<sup>(21)</sup> and shifts the timing relative to the developmental program. This event illustrates what some researchers describe as the third form of heterokairy: where the onset of a

functional regulatory system is moved by altering both the onset of regulation and the length of the developmental program differentially.<sup>(8, 11–13)</sup>

In nature, during early avian embryo development, chronic hypercapnia, with concentrations up to 1 % CO<sub>2</sub>, is more prevalent than hypoxia.<sup>(9)</sup> The atmospheric air contains 0.03 to 0.04 % CO<sub>2</sub> and approximately 21 % O<sub>2</sub>. However, CO<sub>2</sub> concentrations can exceed 1 % under breeders during natural incubation, whereas the CO<sub>2</sub> concentration in ambient air remains at only 0.03 %. Although the supply of both O<sub>2</sub> and CO<sub>2</sub> during incubation is crucial for the development of chick embryos and their hatching process, it is also recognized that O<sub>2</sub> consumption and CO<sub>2</sub> production increase as the embryo develops.<sup>(22, 23)</sup> Research indicates that the role of CO<sub>2</sub> in embryonic development is complex, with studies focusing on factors such as O<sub>2</sub> exchange and pH levels rather than solely on elevated CO<sub>2</sub> levels at the beginning of the ED period.<sup>(6, 10)</sup>

The atmospheric pressure decreases at high altitude which affects, the partial pressure (Pa or mmHg) of individual gases such as O<sub>2</sub> and CO<sub>2</sub>.<sup>(24, 25)</sup> However, the percentage composition of these gases remains relatively constant, although blood parameters may not change significantly; the development of respiratory organs such as the heart and lungs could be influenced by the O<sub>2</sub> concentration in the incubator, especially for the altitude setting during incubation<sup>(16, 26–29)</sup>, oxygen and, consequently, CO<sub>2</sub> concentrations are known to influence embryo mortality and hatchability during incubation at high altitudes.<sup>(16, 23, 25, 26)</sup> To compensate for insufficient O<sub>2</sub>, hypertrophy is observed, leading to the development of abnormal and overgrown organs.<sup>(15, 16, 18, 26)</sup> Therefore, it is necessary to compensate for the oxygen content in the air

(23–25 %) or increase the atmospheric pressure in the setting at high altitude.<sup>(28, 30)</sup> However, both measures show technological challenges and dangerous risks. In addition, in high-altitude regions such as India, Pakistan, Iran, China, Turkey, South America, and Mexico (2 200–4 000 m), very poor hatchability rates have been reported.<sup>(22, 23, 25)</sup>

Traditionally, chicken hatching eggs are incubated in an environment containing 21 % oxygen and 0.5 % carbon dioxide.<sup>(22, 31)</sup> However, several reports suggest that higher CO<sub>2</sub> levels than those currently used (0.1–0.5 %) for artificial incubation of poultry eggs may be beneficial for embryo development and hatchability, depending on the time of application during incubation.<sup>(8, 22, 23, 27, 32)</sup>

The presence of higher CO<sub>2</sub> concentrations during incubation can influence hatchability, embryo weight, and the physiological development of chicken embryos, highlighting the significance of gas exchange conditions for optimal embryonic growth and development.<sup>(8–10, 32)</sup> Carbon dioxide plays a more significant role in the incubation process of chicken eggs than previously believed.<sup>(8, 10, 26)</sup> While it was traditionally thought that CO<sub>2</sub> is harmful to the developing embryo, this belief may have been due to the assumption that the embryo requires fresh air and a sufficient amount of oxygen for optimal chick development and hatchability.<sup>(9, 28, 30)</sup> Indeed, with atmospheric CO<sub>2</sub> levels typically around 0.03–0.04 %, and the average measured CO<sub>2</sub> under the hen at around 0.4 %, it is evident that hens incubate their eggs to maintain a certain level of CO<sub>2</sub> above what is found in the ambient environment. Higher CO<sub>2</sub> levels during

the incubation process have been shown to stimulate ED during the beginning of the incubation period.<sup>(8, 23, 26, 32, 33)</sup>

Modifying the ventilation to allow natural increase of CO<sub>2</sub> during the first 10 d of the ED in the incubator has been shown to result in higher absolute and relative body weight (to the egg weight) from ED10 to ED18.<sup>(8, 23, 34)</sup> This suggests that higher CO<sub>2</sub> levels may accelerate growth and elevate levels of corticosterone and plasma thyroxine.<sup>(8, 10, 32–35)</sup> El-Hanoun et al.<sup>(35)</sup> found that duck breeder eggs incubated in a closed incubator with a carbon dioxide concentration of 1 % at the end of incubation exhibited higher HFE and ducklings' weights, indicating that the NV condition with circulation of CO<sub>2</sub> for the first 10 d of incubation in ducks is preferable to the V condition.

In a study, incubating Ross 308 young breeder eggs at high altitude in an air-tight incubator allowing the CO<sub>2</sub> level to rise to 1.2 % at ED10 followed by a normal incubation procedure, there was 7 % improvement in HFE compared to the control.<sup>(22)</sup> Recently, Fares et al.<sup>(32)</sup> discovered that high carbon dioxide levels (0.9 %) during early ED (0–9 d) contributed to earlier and narrower spread of hatch, higher HFE, and hatched chick weight compared to the control V group. While the effects of CO<sub>2</sub> levels on egg hatchability have been fairly documented in the literature, the assumption in the incubation industry that increased levels of CO<sub>2</sub> at the beginning of incubation at high altitude enhance hatchability and chick quality is not yet supported by enough scientific results.<sup>(22, 23, 26, 36)</sup>

The research goal was to investigate whether the manipulation of ventilation had any impact on the natural evolution of CO<sub>2</sub> levels in the incubator, embryo growth, viability, hatching events, and the quality of one-day-old broiler

chicks. The current study also evaluated whether allowing CO<sub>2</sub> concentration to naturally increase without ventilation during the first 10 days, followed by normal ventilated incubation until hatch, had clear beneficial effects on hatchability and embryo survival at an altitude of 2 230 meters.

## **Materials and methods**

### **Ethical statement**

The experimental procedures reported in this experiment were carried out according to the Internal Committee for the Care and Use of Animals (Comité Interno para el Cuidado y Uso de Animales, CICUA) of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Nacional Autónoma de México.

### *Broiler breeder eggs*

The current study was conducted using fertile hatching eggs from a flock of 37-week-old Ross 308 breeders. The breeder farm was located at 1 350 m of altitude in Jiutepec, Morelos, Mexico. A total of 420 hatching eggs were collected and stored with the pointed end down in a cool room (18 °C and 75 % RH) for 3–7 days before setting in the incubators. All egg trays were transferred to experimental incubation setting located in Mexico City at 2 230 m of altitude. Upon arrival, all eggs were randomly assigned to two groups of equal size, identified, weighed, and placed in ten forced-draft commercial incubators (Hova-Bator® Mod. #1583 GQF Inc. Savannah, Georgia, USA).



### *Experimental design*

The first group of hatching chick eggs was incubated in a normally ventilated incubator (V) throughout the incubation period. The second group of hatching eggs was incubated in a non-ventilated incubator (NV) during the first ten days of incubation. The air-tight condition was achieved by closing 8/12 bottom vent dampers and two top outlets of the commercial incubator with Polypropylene adhesive Tuk®. Both treatments were kept under the same V condition from the tenth day of embryonic development (ED10) to ED18. A total of 42 eggs were incubated in each treatment, with five replicates per treatment. For the first two days all eggs were incubated at 100.0 °F with dry bulb, from 3 to 7 days at 99.9 °F, from 8 to 10 days at 99.7 °F, from 11 to 13 days at 99.5 °F, from 14 to 15 days at 99.3 °F, 16 and 17 at 99.0 °F, and from 18 to 21.5 days at 98.4 °F. In V condition from ED1 to ED18 days, and in the NV conditions from ED10 to ED18 days wet bulb was kept at 84.5 °F.

Eggs were turned every hour from 1 to 18 days of incubation. At 444 h of incubation, the eggs were weighed and candled. Those with living embryos were transferred to fixed-hatching baskets. The eggs were hatched in the same incubators converted into hatchers. The wet bulb of hatcher from ED18 to hatch was kept at 90.0 °F. The incubators were monitored six times daily to ensure proper operation. To accurately track hatch time, the time when the eggs were set in every incubator was recorded as hour zero. Concentrations of CO<sub>2</sub> and O<sub>2</sub> within each machine were measured three times a day (morning, afternoon, and night) using an infrared (NDIR) sensor and a galvanic cell sensor, respectively (Analox®, Analox Inst. Ltd. The Vale, London W3 7QE, UK). Before every

measurement, the gas analyzers were calibrated in the same manner using atmospheric air and precision calibration gases.

#### *Hatching egg and embryonic mass weight*

Eggs were individually weighed at 0, 10 and 18 days of incubation. The percentages of egg weight loss (EWL) during different incubation intervals (0–10, 10–18 and 0–18 days) were calculated for each ventilation condition. Fifteen eggs from each incubator were randomly picked to calculate EWL using the following equation:

$$EWL = \left( \frac{\text{EW at every sample d ED} - \text{EW of same egg at ED0}}{\text{EW of same egg at ED0}} \right) \times 100$$

The ratio of yolk-free embryo body weight and yolk sac weight to egg weight was determined in 5 eggs randomly sampled per incubator at 10, 12, 14, 16, and 18 days of incubation, and 9 chicks per hatching basket. The wet and dry weights of the yolk-free embryo, yolk sac, heart, and liver of every sampled egg and newly hatched chick were measured according to the methodology previously described by Willemsen et al.<sup>(37)</sup> Briefly, after careful separation of the embryo and yolk sac, both were weighed (**Figure 1 at left**). Embryos were weighed after excessive fluid was dried off using absorbent paper (Versi-Dry® Thermo Scientific). For the purpose of drying the embryo, internal organs, skin, and extra-embryonic membranes were excluded, while the complete yolk sac was dried as one piece. The yolk-free embryo, yolk sac, heart, and liver were dried through desiccation by heating them in a dry oven set at 140 °F for approximately 72 h, until reaching a stable final weight (**Figure 1 at right**).



**Figure 1.** Yolk-free embryo mass and yolk sac were separated at 10, 12, 14, 16, and 18 days of incubation and in newly hatched chicks (left). Desiccation was performed by heating them in a dry oven (right).

#### *Hatching events*

Following transfer to the hatching baskets on day 18, eggs were individually monitored at 2-h intervals, starting at 468 h and continuing for 48 h. During this period, the internal pipping, external pipping, and hatching were recorded for each egg. Chick hatchlings that had fully emerged from their eggs, exhibiting healed navels and dryness around the head and neck, were removed. For each egg, the incubation duration was defined as the period between setting and hatching. At the end of incubation, the hatchability of fertile eggs (HFE) was calculated according to the following equation:

$$HFE = \left( \frac{\text{Total of hatchlings chicks}}{\text{Total fertile eggs}} \right) \times 100$$

The hatchability of total eggs set (HES) was also determined using the following equation:

$$HES = \left( \frac{\text{Total of hatchlings chicks}}{\text{total eggs set}} \right) \times 100$$

After each collection of hatched chicks, they were weighed to the nearest 0.1 g, measured for length, and received a quality score under random double-blind conditions. The hatch window was recorded as the time elapsed between the first and last chick hatched within each treatment group.

#### *Embryonic mortality*

Eggs that failed to hatch after 21.5 days of incubation were opened and examined macroscopically to estimate the infertility rate and assess the developmental stage reached before the embryo died. The time of embryonic death was estimated in days to the extent feasible. The embryonic mortality percentage, expressed as a percentage of fertile eggs set, was recorded and classified into different periods of the ED. Early embryonic mortality occurs during stage I (days 1 to 7 of ED), mid embryonic mortality during stage II (day 8 to 17 of ED), late embryonic mortality during stage III (days 18 to 21 of ED) and cases with pipped but unhatched eggs before 516 hours were classified as stage IV.

#### *Quality score grading newly hatched chicks*

All one-day-old chicks were individually pulled out and numbered after the entire batch of chicks had hatched. Every chick was examined macroscopically to identify traits associated with excellent, good, average, poor, and unacceptable

quality. This methodology assessed chick quality based on field observations of various physical conditions crucial for successful chick development. The scoring of newly hatched chick quality was based on thirteen comprehensive characteristics (**Table 1**). These traits included physical conditions, such as body dryness and cleanness, activity level, appearance of eyes, retracted yolk-sac, remaining yolk-sac, conformation of legs, tarsometatarsus and toes integrity, appearance of the navel, presence of remaining membranes and debris, appearance of the vent, hydration condition, body weight and chick length (**Table 1**).

The quantitative traits, body weight and chick length, were recorded for each hatched chick. Chicks were individually weighed in grams. To measure chick length, each chick was positioned face down on a flat surface, ensuring that the neck and right leg were fully extended to their maximum length. Chick length was defined as the distance from the tip of the beak to the point where the nail is attached on the third toe (**Figure 2**).<sup>(34, 38-40)</sup> All these methods are described in **Table 1**.

**Table 1.** Assessment of different characteristics to index newly hatched chicks quality

<b>Parameter</b>	<b>Assessment</b>
Down and appearance	The 1-day-old broiler chick was examined for dryness and cleanness. It was regarded as normal if it is dry and clean. If it is wet or dirty or both (which can be a source of contamination), then it is not a normal condition.
Activity	It was assessed by laying the chick hatchling on its back to determine how quickly it returned to its feet. A quick spring back onto its feet was regarded as good, but dragging back onto its feet or remaining on its back was assessed as weak or very weak.
Eyes	The newborn broiler chick was put in front of the observer. The condition of eye brightness and the wideness of the gape of the eyelids were determined.
Retracted yolk	The hatchling was put on its back obliquely on the palm until abdominal movement stopped. The height and consistency of to touch of its abdomen were estimated. If the height of the abdomen was estimated to be higher and harder to touch than normal, then retracted yolk was regarded as large and consistent.
Remaining yolk	Observation of the navel area allowed estimation of the size of any remaining yolk. The size of any remaining yolk-sac

was classified as large (clear detection), small (almost no detectable), or no yolk sac.

Legs	The broiler chick was put on its feet to determine if it remained upright well. If the chick remained upright with difficulty, articulations of the knees were examined to detect signs of inflammation, redness, or both.
Tarsometatarsus and toes	The broiler chick was put on its feet, and the tarsometatarsus and toes were examined for their integrity. The toes were analyzed for how straight or crooked they were.
Navel	The navel and its surrounding areas were examined for closure and coloration. If the color was different from the skin color, it was regarded as poor; if this had an appearance like a navel button or a leaky navel, it was very bad.
Remaining membrane	Observation of the navel area allowed estimation of the size of any remaining membrane or debris. If present, the size was classified as large, small, or no membrane and debris.
Vent appearance	The broiler chick's cloacal area was examined for cleanness grading. It was regarded as normal if it is clean. If it was wet, with adherence of chalky white material, dirty or vent pasting or both (which can be a source of contamination), then it was not good.

Dehydration	The assessment of the skin and vascular vessels of the neck, wing and leg allowed estimation of dehydration condition. It was regarded as none, mildly dry, or severely dry.
Weight	All one-day-old broiler chicks were individually weighed in grams.
Length	The one-day-old broiler chick was laid on its ventral side, with the neck and right leg extended to their maximum length. Chick length was defined as the length from the tip of the beak to the point where the nail is attached on the third toe.

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**Figure 2.** Newly hatched chick length. The chick was positioned face down, ensuring that the neck and right leg were fully extended to their maximum length.

The grading score assigned to every newly hatched chick was used to create an index of chick hatchling quality. Each parameter was scored based on the subsequent performance of the birds during their rearing at the farm, as described previously by Tona et al.<sup>(41)</sup> and Willemsen et al.<sup>(34, 42)</sup> Discrete traits were scored on a scale of 0 to 100 points, as outlined in **Table 2**.

**Table 2.** Assignment of scores to different quality traits observed in hatchling broiler chicks

Parameters	Characteristics	Scores
Down and appearance	Clean and dry	8
	Wet	4
	Dirty and wet	0
Activity	Good	8
	Weak	4
	Very weak, chick remained lying down	0
Eyes	Both opened and bright	8
	Opened and not bright	4
	One or both closed	0
Retracted yolk	A body with a normal swallowed yolk sac	8
	A body with a regular swallowed yolk sac	4
	A body with a large yolk sac, and rather than being hard to touch	0
Remaining yolk	No yolk sac	8
	Small yolk sac	4
	Large yolk sac	0
Legs	Normal legs	8
	One infected leg or swelling of the hock joint	4
	Two infected legs or swelling of both hock joints	0
Tarsometatarsus and toes	Normal toes	8
	Lighter twisted toes	4
	Twisted toes	0
Navel	Completely closed and clean	10
	Unhealed (< 1.5 mm) and not discolored	4
	Unhealed (> 1.5 mm) and discolored, black navel button or leaky navel	0

Remaining membrane and debris	No membrane or debris	8
	Small membrane	4
	Large membrane	0
Vent appearance	Clean	8
	Wet	4
	Dirty or vent pasting	0
Dehydration	None	8
	Skin dry and wrinkled	4
	Skin very dry and wrinkled	0
Length	≥ 18 cm	10
	36–45 week-old	15–18 cm
Broiler breeders	≤ 15 cm	0
Weight	≥ 40 g	8
	36–45 week-old	37–40 g
Broiler breeders	≤ 37 g	0

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The quality score rank assignment was as follows: 91–100 points = Excellent, 81–90 = Good; 71–80 = Average; 61–70 = Poor, and < 60 = Unacceptable. The final score allowed grading each percentage rank in each ventilation treatment (V and NV). All measurements were performed in a double-blind manner, meaning that each parameter was measured for all chicks taken in random order from the entire batch before the next parameter was assessed.

#### *Statistical analysis*

The data were collected and analyzed using an ANOVA with a general linear model (GLM) (SAS/STAT 9.2. SAS Institute Inc., Cary, NC, USA). The response

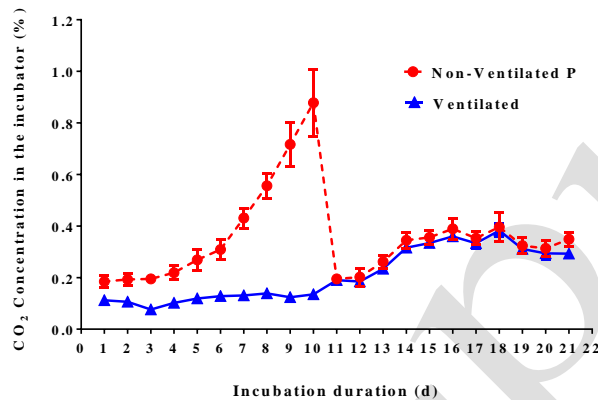
traits included egg weights at setting, the weight of the yolk sac-free embryo body, the weight of the embryo yolk sac, the weights of the heart and liver, and the weight of the newly hatched chicks in grams, as well as the length of the newly hatched chicks in centimeters. Before the statistical analysis, data from hatching events, chick hatchling quality, and egg weight losses underwent an arcsine square root transformation. All of this data was also analyzed using an ANOVA via the GLM. When significant differences were revealed between treatments, post hoc pairwise comparisons were conducted using Tukey's test ( $P < 0.05$ ) to discriminate specific differences among NV and V treatment groups. All values were expressed as mean  $\pm$  standard deviation (SD). Chi-square test of independence was employed for assessing embryonic mortality stages (I, II, III, and PUH), as well as quality scoring on one-day-old broiler chicks. Statements of statistical significance were based on a threshold of  $P < 0.05$ .

## Results

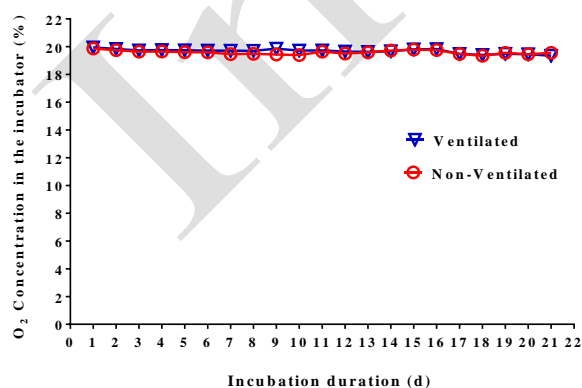
### *Incubation environment*

**Figure 3A** shows a lower (0.13 %) but steady CO<sub>2</sub> concentration in the V condition throughout the first 10 d of embryonic development, whereas the NV condition showed a gradual increase in CO<sub>2</sub> concentration from the outset of incubation until ED10, reaching a CO<sub>2</sub> concentration of 0.88 % at ED10 (**Figure 3**).

**A**



**B**



**Figure 3.** Dynamics of the CO<sub>2</sub> (A) and O<sub>2</sub> concentration, (B) into the air-tight and standard incubators (n = 5).

During the first 10 days of embryonic development, the V condition showed a higher O<sub>2</sub> concentration of 19.85 %, which was significantly greater (P < 0.05) than the 19.55 % of O<sub>2</sub> recorded in the NV treatment. From ED10 until hatching, the NV condition exhibited higher CO<sub>2</sub> concentration at 13, 14, and 21 d of embryonic development (**Figure 3A**), whereas both ventilation conditions maintained similar levels of O<sub>2</sub> during this period (**Figure 3B**). The hatchery room maintained an average temperature of 24.4 °C and an RH of 53.3 % throughout the experimental period. O<sub>2</sub> levels were measured at 19.88 %, with CO<sub>2</sub> concentration at 0.10 percentage.

#### *Egg-weight losses*

The mean egg weight at the outset of incubation was 61.3 ± 3.9 g in the V group, and 61.9 ± 3.5 g in the NV group, with no significant difference observed between the treatments. In the V condition, the EWL at ED10 was measured at 6.23 ± 1.16 %, significantly higher (P < 0.05) than the 5.47 ± 0.89 % recorded in the NV group (**Table 3**).

**Table 3.** Egg-weight loss of broiler breeder hatching eggs

<b>Egg weight variable</b>	<b>Ventilated</b>	<b>Non-ventilated <sup>1</sup></b>
Egg hatching weight at set (g)	61.34 ± 3.98	61.86 ± 3.50
Egg weight at day ED10 d (g)	58.27 ± 4.00	59.19 ± 3.51
Egg-weight loss at ED10 d (%)	6.23 ± 1.16 <sup>a</sup>	5.47 ± 0.89 <sup>b</sup>
Egg weight at day ED18 (g)	54.28 ± 3.77 <sup>b</sup>	55.41 ± 3.61 <sup>a</sup>
Egg-weight loss at ED18 d (%)	11.65 ± 1.71 <sup>a</sup>	10.47 ± 1.57 <sup>b</sup>
Egg-weight loss ED10-ED18 (%) <sup>ψ</sup>	4.92 ± 0.71	5.28 ± 0.75

<sup>1</sup> Non-ventilated air-tight incubator condition achieved through Polypropylene Tuk® tape (P) during the first 10 days of embryonic development.

<sup>a, b</sup> Means (± SD) in the same row with different superscript letter are significantly different (P < 0.05).

n = 75 Broiler eggs by group <sup>ψ</sup> Ventilated (n = 26) <sup>ψ</sup> Non-ventilated (n = 28)

When all eggs were transferred to baskets, the V group exhibited a mean EWL of 11.65 ± 1.71 %, which was significantly higher (P < 0.05) than the 10.47 ± 1.57 % observed in the NV group. At the same time, the mean egg weight in the V group was significantly lower (P < 0.05) compared to the mean egg weight recorded in the NV group. Egg-weight loss from ED10 to ED18 did not exhibit any difference between treatments (**Table 3**).

#### *Hatchings events*

In the V condition, the HFE was 56.71 %, which was significantly different (P < 0.05) from the 66.93 % of HFE observed in the NV group (**Table 4**). The

hatchability of all eggs set in the V condition was 53.18 %, which was significantly lower ( $P < 0.05$ ) compared to the 57.95 % recorded in the NV group (**Table 4**).

**Table 4.** Incubation variables and embryo mortality

<b>Incubation variables</b>	<b>Ventilated</b>	<b>Non-ventilated <sup>1</sup></b>
Fertility (%) <sup>ψ</sup>	92.26 ± 4.91	92.26 ± 7.87
Hatchability from fertile eggs (%) <sup>ψ</sup>	56.71 ± 10.19 <sup>b</sup>	66.93 ± 12.96 <sup>a</sup>
Hatchability of all eggs set (%) <sup>ψ</sup>	53.18 ± 10.01 <sup>b</sup>	57.95 ± 9.33 <sup>a</sup>
Early embryo mortality (%) <sup>φ</sup>	17.62 <sup>a</sup>	16.32 <sup>b</sup>
Middle embryo mortality (%) <sup>φ</sup>	17.09 <sup>a</sup>	9.33 <sup>b</sup>
Late embryo mortality (%) <sup>φ</sup>	17.35 <sup>a</sup>	7.43 <sup>b</sup>
Pipped but unhatched (%) <sup>φ</sup>	1.20 <sup>a</sup>	0.0 <sup>b</sup>
Total embryo mortality (%)	53.26	33.08

<sup>1</sup>Non-ventilated air-tight incubator condition achieved through Polypropylene Tuk® tape (P) during the first 10 days of embryonic development.

<sup>a, b</sup> <sup>ψ</sup>Means (± SD) in the same row with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>a, b</sup> <sup>φ</sup>Means in the same row with different superscript letters are significantly different ( $P < 0.05$ ).

n = 210 Broiler hatching eggs by group.



The embryos in the NV group started hatching 11.5 h earlier ( $P < 0.05$ ) than those in the V group. The hatch window was 49 h for chick hatchlings in the NV treatment and 41 h for the V treatment, with no significant difference observed between groups.

#### *Embryonic mortality*

During the first ED stage, no significant difference in embryo mortality was observed between the NV and V conditions. Results in **Table 4** revealed differences in embryo mortality between NV and V treatments from the middle stage onward. The NV treatment showed a middle embryo mortality rate of 9.33 %, which was significantly lower ( $P < 0.05$ ) compared to the 17.09 % observed in the V group (**Table 4**). Chick embryos that died in the late stage (18–21 days of ED) were predominantly observed in the V condition (17.35 %), and this was significantly higher ( $P < 0.05$ ) compared to the 7.43 % of embryo mortality observed in the NV group (**Table 4**). The percentage of eggs that pipped but were unhatched in the NV group was 0 %, significantly different from the 1.20 % observed in the V group. The higher total embryonic mortality was observed in group V (53.26 %), which was significantly higher than the 33.08 % observed in the NV group (**Table 4**).

#### *Embryonic development and newly hatched chicks*

The one-day-old broiler chick weight from the NV group, at  $43.41 \pm 1.84$  g, was significantly heavier ( $P < 0.05$ ) than the  $41.51 \pm 1.58$  g recorded in those chicks

from the control V group. However, there was no significant difference in the length of hatched broiler chicks between the two ventilation conditions (**Table 5**).

**Table 5.** Quality grade scoring and one-day-old broiler during the first half of incubation at high altitude

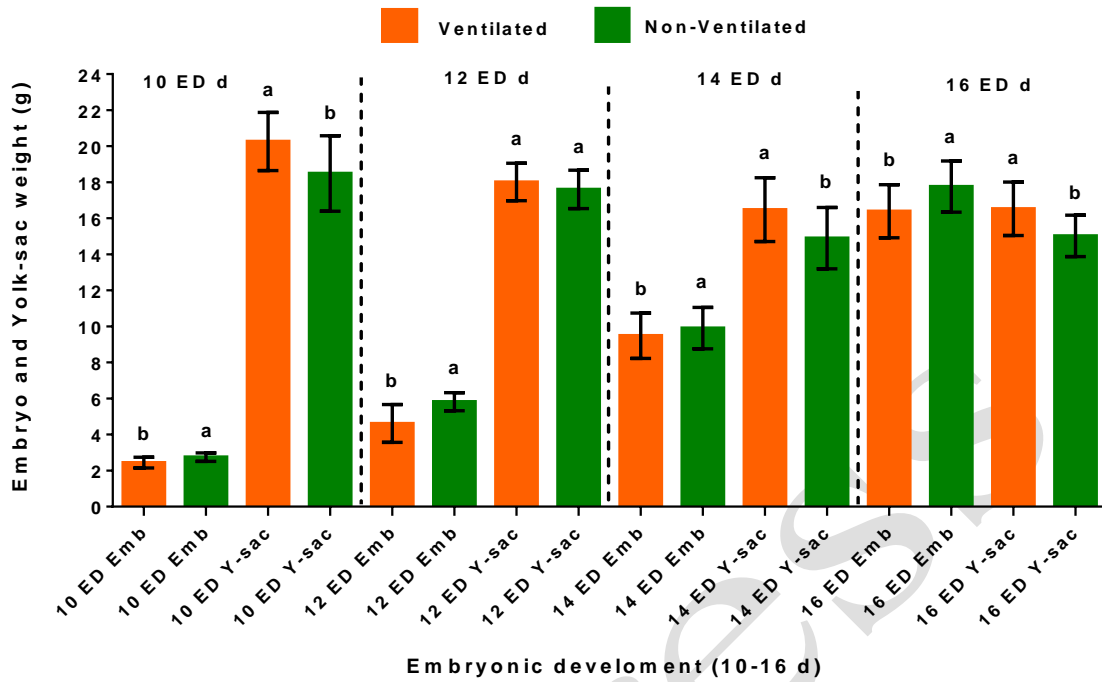
<b>Chick quality feature</b>	<b>Ventilated</b>	<b>Non-ventilated<sup>1</sup></b>
High quality (%)	0 ± 0	0 ± 0
Good quality (%)	24.72 ± 8.80 <sup>b</sup>	44.44 ± 11.11 <sup>a</sup>
Middle quality (%)	33.89 ± 10.46	31.11 ± 4.97
Poor quality (%)	25.28 ± 10.41	24.44 ± 9.29
Unclassified (%)	16.11 ± 6.92 <sup>a</sup>	0 ± 0 <sup>b</sup>
Chick hatchling mass (g)	41.51 ± 1.58 <sup>b</sup>	43.41 ± 1.84 <sup>a</sup>
Chick hatchling length (cm)	17.20 ± 0.60	17.48 ± 0.96

<sup>1</sup>Non-ventilated air-tight incubator condition achieved through Polypropylene Tuk® tape (P) during the first 10 days of embryonic development.

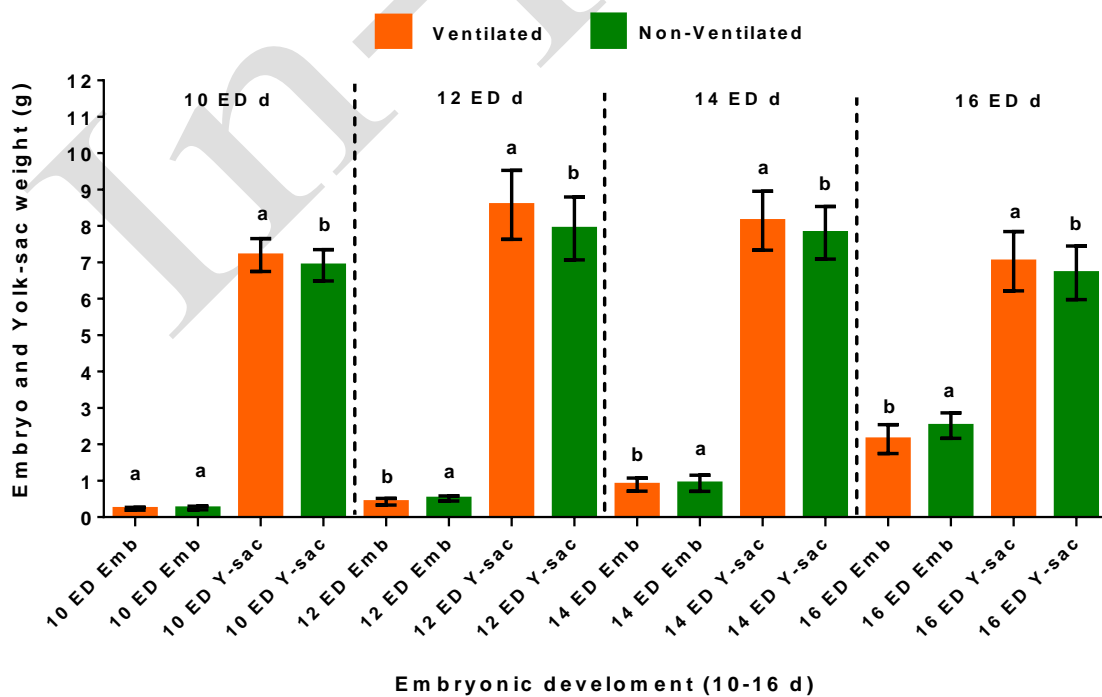
<sup>a, b</sup>Means (± SD) in the same row with different superscript letter are significantly different (P < 0.05).

n = 45 Chick hatchlings/group

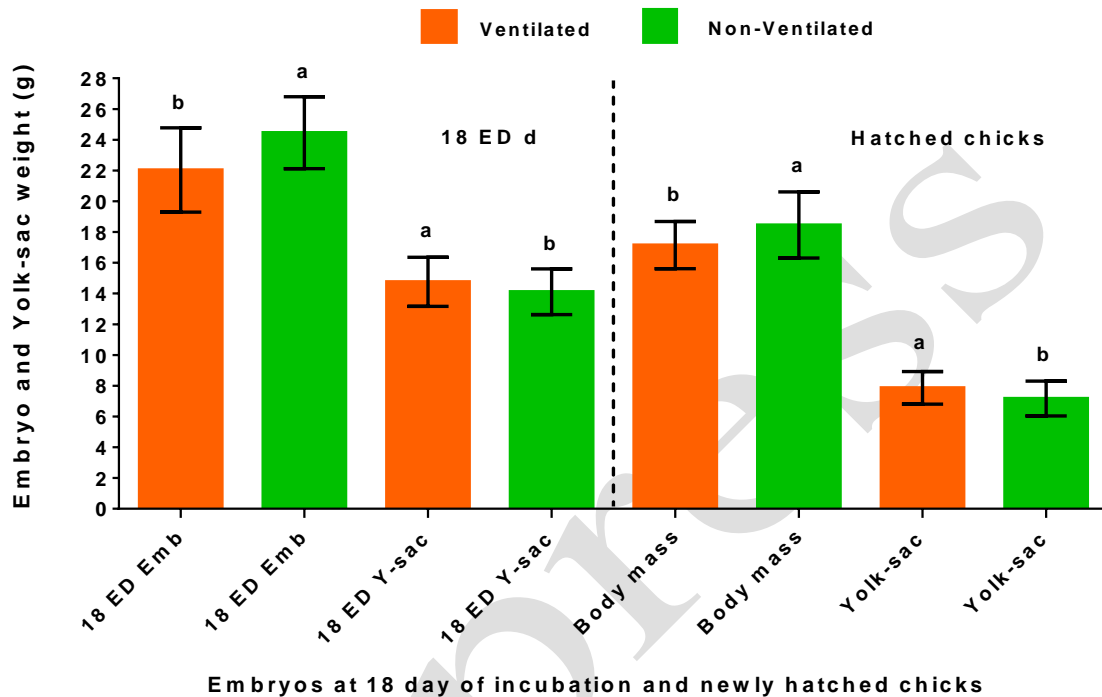
Yolk-free body mass from the NV treatment, whether fresh or dry, was consistently heavier compared to the control V group from ED10 to ED18, and this trend was maintained in the hatched broiler chicks (**Figures 4–7**).



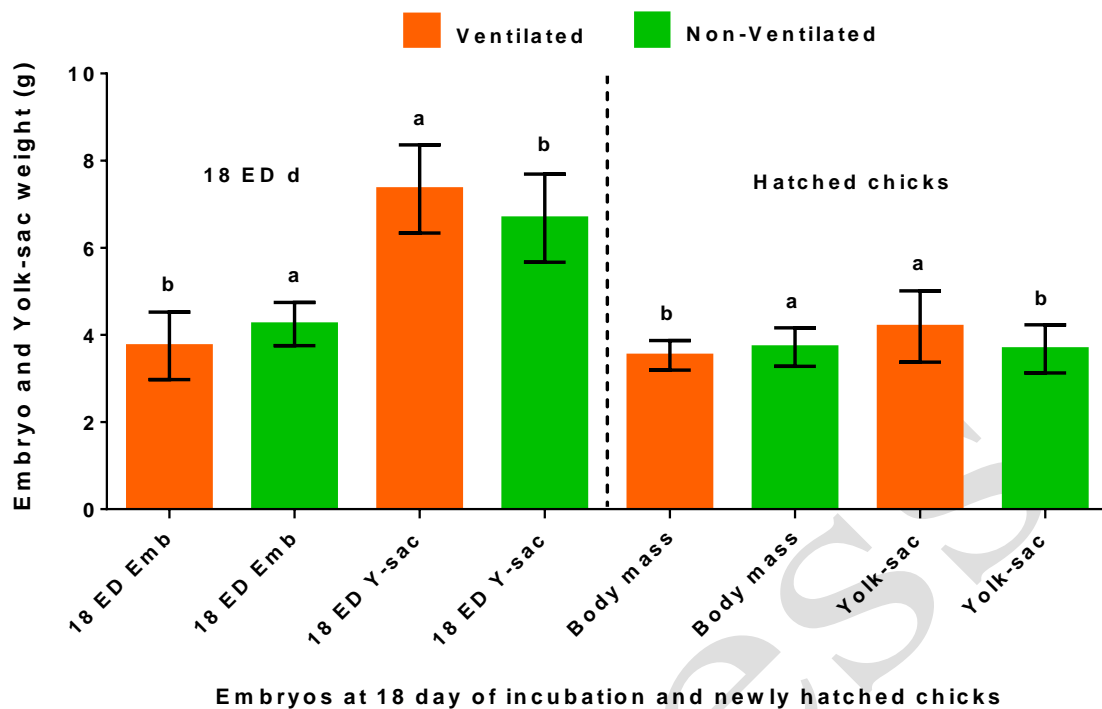
**Figure 4.** Effect of non-ventilation during the first ten days of incubation at high altitude on wet yolk-free body mass and wet yolk sac mass at 10, 12, 14 and 16 days of embryonic development (n = 25).



**Figure 5.** Effect of non-ventilation during the first ten days of incubation at high altitude on dry yolk-free body mass and dry yolk sac mass at 10, 12, 14 and 16 days of embryonic development (n = 25).



**Figure 6.** Effect of non-ventilation during the first ten days of incubation at high altitude on wet yolk-free body mass and wet yolk sac mass at 18 days of embryo development and hatching time (ED18 n = 25; one-day-old chicks n = 45).



**Figure 7.** Effect of non-ventilation during the first ten days of incubation at high altitude on dry yolk-free body mass and dry yolk sac mass at 18 days of embryo development and hatching time (ED18 n = 25; one-day-old chicks n = 45).

Otherwise, fresh and dry yolk-sac mass from the control V group was heavier ( $P < 0.05$ ) compared to that from NV group at 10, 14, 16, and 18 ED days, as well as in hatched broiler chickens (**Figures 4–7**). The fresh yolk-sac mass from the control V group did not show any difference at ED12 compared to the fresh yolk-sac mass from the NV group. Dry yolk-sac mass did not show any difference between groups, only at ED10 d (**Figure 5**). Hearts from embryos from ED12 to ED16 d were significantly heavier ( $P < 0.05$ ) in the NV group compared to those from the control V group, as well as in newly hatched chicks (**Table 6**). At ED18, there were no differences in heart weight between groups. However, the liver was significantly heavier ( $P < 0.05$ ) in the NV group compared to that from the control V group from ED12 to newly hatched chicks (**Table 6**).

**Table 6.** Heart and liver weight (g) of embryos and newly hatched chicks incubated at high altitude during the first ten days

Quality features	Ventilated	Non-ventilated <sup>1</sup>
Heart weight on ED12 d <sup>ψ</sup>	0.054 ± 0.01 <sup>b</sup>	0.067 ± 0.01 <sup>a</sup>
Liver weight on ED12 d <sup>ψ</sup>	0.089 ± 0.03 <sup>b</sup>	0.107 ± 0.05 <sup>a</sup>
Heart weight on ED14 d <sup>ψ</sup>	0.091 ± 0.02 <sup>b</sup>	0.125 ± 0.02 <sup>a</sup>
Liver weight on ED14 d <sup>ψ</sup>	0.194 ± 0.02 <sup>b</sup>	0.220 ± 0.04 <sup>a</sup>
Heart weight on ED16 d <sup>ψ</sup>	0.120 ± 0.02 <sup>b</sup>	0.140 ± 0.02 <sup>a</sup>
Liver weight on ED16 d <sup>ψ</sup>	0.300 ± 0.06 <sup>b</sup>	0.320 ± 0.05 <sup>a</sup>
Heart weight on ED18 d <sup>ψ</sup>	0.240 ± 0.02	0.240 ± 0.03
Liver weight on ED18 d <sup>ψ</sup>	0.420 ± 0.10 <sup>b</sup>	0.530 ± 0.11 <sup>a</sup>
Heart weight on hatchlings <sup>φ</sup>	0.320 ± 0.04 <sup>b</sup>	0.350 ± 0.04 <sup>a</sup>
Liver weight on hatchlings <sup>φ</sup>	0.900 ± 0.11 <sup>b</sup>	0.950 ± 0.09 <sup>a</sup>

<sup>1</sup>Non-ventilated air-tight incubator condition achieved through Polypropylene Tuk® tape (P) during the first ten days of embryo development.

<sup>a, b</sup>Means (± SD) in the same row with different superscript letters are significantly different (P < 0.05).

<sup>ψ</sup> n = 25 Embryos/group <sup>φ</sup> n = 45 Newly hatched chicks/group

#### *Hatchling chick quality*

None of the chickens received a high-quality grading score, and while there was a slight trend toward better quality in the NV-hatched chicks, ultimately, there was no significant difference between the groups (**Table 5**). Forty-four percent of newly hatched chicks from the NV condition received a good-quality score, a percentage significantly higher (P < 0.05) than the 24.7 % of hatched chicks from

the control V group receiving the same ranking score (**Table 5**). Otherwise, the control V group showed significantly higher proportion ( $P < 0.05$ ) of newly hatched chicks in the unclassified ranking score (16.1 %), compared to the NV group.

## **Discussion**

The lower hatchability observed in the standard V group, incubated at higher altitude, could be attributable to elevated rates of middle and late embryonic mortality, as well as pipped but unhatched chicks. In contrast, the NV group exhibited significantly reduced embryo mortality during the last two stages of embryonic development. The NV group displayed higher embryo weights from the 10<sup>th</sup> day of ED until hatch, suggesting a more robust ED than those observed in the control V group.

According to Willemsen et al.<sup>(34)</sup> embryos surviving the early NV condition exhibit enhanced morphophysiological conditions that enable them to accelerate their ED compared to embryos from the V condition. Several research groups have indicated that a gradual increase in CO<sub>2</sub> from 0.8 % to 1.5 % during the first half of chicken incubation accelerates ED, enhances embryo morphophysiological conditions, and contributes to increased hatchability and quality of hatched chicks.<sup>(8, 22, 23, 26, 32, 43)</sup> Bruggeman et al.<sup>(43)</sup> assessed hypercapnia conditions (NV) in the first half of incubation and found accelerated embryonic growth, increased embryo weight, faster hatching, and enhanced quality of the hatched chicks. These findings are similar to the results obtained in

our NV group. However, Bruggeman et al.<sup>(43)</sup> reported no significant improvement in hatchability or a decrease in the total embryonic mortality rate.

The precise reasons for the inconsistent improvement in hatchability parameters observed throughout all studied cases with increased of CO<sub>2</sub> during early incubation are not yet fully understood.<sup>(8, 22, 23, 27, 32, 35, 36, 43)</sup> Some studies have highlighted serious problems in the incubation of commercial broiler breeders, especially at high altitudes.<sup>(16, 22, 25, 27, 28, 30)</sup> For this reason, hatcheries for commercial breeder genotypes are generally established at low altitudes. Since local genotypes are adapted to high altitudes, there are not many problems with their incubation.<sup>(25, 44)</sup> However, this situation can be problematic in geographical areas with high altitudes and the need for commercial broiler chicks.<sup>(16, 22, 25, 27)</sup>

Leghorn chicks can oxygenate their hemoglobin fully at 2 500 to 3 000 m of altitude. However, broiler chicks showed low arterial blood oxygen even at low altitudes. Recently, Juárez-Estrada et al.<sup>(23)</sup> showed that high CO<sub>2</sub> during the first half of ED in Leghorn breeders' eggs produced positive results on major hatchability traits, but these were not as high as the outcomes observed here with Broiler breeders' eggs. Higher CO<sub>2</sub> during the first half of incubation could have more positive outcomes for high-altitude broiler breeder hatcheries than for other genotypes.

The beneficial effects of an early NV condition on hatchability seem to be attributed to several factors including genotype, breeding stock age, and even the altitude of the incubation setter, rather than solely the CO<sub>2</sub> concentration achieved during the first half of the incubation period.<sup>(4, 8, 22, 23, 26, 35)</sup> The 0.9 %



CO<sub>2</sub> concentration reached in our NV group during the first half of incubation at high altitude when compared to that in the control V group, resulted in a gradual increase of benefits, including accelerated ED, improved hatchability, and increased weight and quality of newly hatched chickens. This is aligned with the observations made by De Smit et al.,<sup>(8)</sup> who applied NV incubation on embryos from 60-week-old broiler breeders and achieved 1.0 % CO<sub>2</sub> concentration remarkably similar to CO<sub>2</sub> levels recorded in our NV incubators.

De Smit et al.<sup>(8)</sup> observed higher hatchability and increased embryo weights from ED10 to ED17 when compared to their control V group. In the NV condition, the weight of yolk-free body mass consistently exceeded that of embryos in the V group throughout the incubation period. Similarly, the weight of dry yolk-free body mass followed an identical pattern for almost the whole experiment. NV incubation resulted in accelerated embryonic growth and utilization of the primary energy reserves, particularly from the yolk sac. From the 10<sup>th</sup> d of ED onward, as the embryo's weight increased, the volume and weight of the yolk sac decreased. Yolk-free body mass serves as a more reliable indicator of post-hatch broiler development than embryo body weight, because it includes the weight of the residual, unmetabolized yolk.<sup>(40, 45)</sup>

According to Wolanski et al.,<sup>(45)</sup> there is an important correlation between wet and dry mass weight for the yolk sac compared to the embryo, a pattern that closely aligns with our findings. The NV group's accelerated embryo development suggests the embryo's ability to grow faster in conditions of hypercapnia and relative hypoxia throughout the first half of ED. The environmental conditions applied in the NV group could contribute to maximizing the growth and

functionality of the chorioallantoic membrane (CAM).<sup>(15, 17, 29, 43)</sup> After 10 d of incubation, early stimulation for enhanced CAM development and functionality might result in improved oxygen uptake during the exponential phase of ED (second half of ED). This could explain the beneficial effects of early hypercapnia and relative hypoxia in the later stages of ED observed in our NV group, perhaps enhancing the rate of energy supply from the yolk sac through biochemical mechanisms such as fatty acid beta-oxidation and gluconeogenesis.<sup>(17, 29, 45-47)</sup>

As ED progresses during natural incubation, the CO<sub>2</sub> concentration increases from 0.04 % to 0.5 %, while the O<sub>2</sub> levels decrease from 20.9 % to 20.3 %, as noted by Walsberg.<sup>(48)</sup> Similarly, our study found a similar trend of O<sub>2</sub> declining over the first ten days of ED. The NV group experienced a decrease from 19.8 % to 19.5 %. Conversely, the V group did not show any decrease in O<sub>2</sub> levels. Rahn et al.<sup>(44)</sup> discovered that gas levels in the nest do not remain constant throughout natural incubation and often change as ED progresses. Hens play a crucial role in maintaining the nest temperature by constantly monitoring the egg's temperature in nature. Once the embryos reach a stage when they can generate their heat autonomously, hens prefer to leave the nest more frequently to drink and feed, especially after the embryos' endothermic period (1-10 days of setting).<sup>(49)</sup> Hens improve ventilation by leaving the nest more frequently, which increases CO<sub>2</sub> removal and oxygen supply surrounding the nest eggs. This process continues progressively until hatching.<sup>(44, 48, 49)</sup>

The embryos in the NV group consistently weighed more than those in the V group. Willemsen et al.<sup>(34)</sup> observed that the weight of chicks at hatch had the highest predictive value for post-hatch performance, surpassing even the length

measurement of the chick when implemented as the sole predictive metric measurement. In the current study, the NV group showed the highest weights for one-day-old chicks. However, the chick length was found not to differ between the groups. Wolanski et al.<sup>(40, 50)</sup> found a correlation ( $r = 0.56$ ) between chick length and yolk-free body mass at hatch. However, several other authors have expressed doubts regarding the usefulness of chick length as a meaningful indicator of chick quality.<sup>(34, 51)</sup>

The NV treatment enabled the attainment of heavier yolk-free embryos along with a progressively lighter yolk-sac weight, a phenomenon previously reported by several research groups.<sup>(8, 22, 23, 40, 43)</sup> This may be attributed to the embryo's elevated early metabolic activity, particularly in tissue formation, and the fulfillment of energy requirements for its own development.<sup>(45, 46, 52, 53)</sup> The weight and volume of the remaining yolk-sac serve as measures of both the energy invested in the egg and the energy used by the embryo during its development.<sup>(45, 53–55)</sup> According to Moran<sup>(56)</sup> starting from the 12<sup>th</sup> d of ED, the gain in yolk-free body mass exhibits a negative correlation with yolk-sac weight. The embryos in the NV group exhibited a relatively smaller proportion of yolk-sac compared to the yolk-free body mass, suggesting that the chick hatchlings in this group may have matured earlier. In addition to increasing yolk-free body mass, surviving NV embryos exhibited higher heart and liver weights than the control V group. The observed increase in liver and muscle in NV embryos at 18 ED days suggests elevated storage of glycogen, indicating a more robust ED, as previously suggested by Druyan.<sup>(57)</sup> The glycogen storage in the liver and glycolytic muscles is needed to fuel the “pipping” and hatching processes and

serve as the main energy source until the newly hatched chick can access external food.<sup>(45, 46, 54, 55, 58)</sup>

Early incubation under NV not only generates optimal CO<sub>2</sub> and O<sub>2</sub> profiles but also promotes uniformity in eggshell temperature (EST). These combined effects promote the achievement of optimal ED and post-hatch growth.<sup>(37, 52, 53, 59)</sup> Post-hatch growth and organ function can be impaired if growth rates during ED deviate from the optimal EST.<sup>(6, 52, 59, 60-62)</sup> NV incubation helps to mitigate temperature oscillations caused by the forced exchange of fresh air from outside, which is commonly observed during conventional V incubation.<sup>(7, 22, 25, 60, 62)</sup> NV incubation ensures a balanced thermal environment over the first half of the incubation period.<sup>(22, 61)</sup> This ventilation approach is specifically applied during the most critical growth phase of the ED (1–10 d of ED), where keeping a warm and uniform EST is critical.<sup>(6, 7, 49, 52, 60, 61)</sup> Indeed, wet air transfers heat more efficiently than dry air.<sup>(7, 60)</sup> As a result, restricting ventilation during the first 10 d of ED promotes a uniform, humid and warm environment throughout the incubation period. This is particularly important at high altitudes, where the air is dry and cold.<sup>(7, 22, 23, 25)</sup>

A single exposure to a certain environment during early embryonic growth can have long-lasting consequences for the life of birds.<sup>(7, 11, 13, 33, 62)</sup> In the current study, the NV condition contributed to optimizing the subsequent development stages of embryos exposed to hypercapnia and relative hypoxia at an early stage.<sup>(10, 61)</sup> Some research groups have proposed that the high CO<sub>2</sub> content during the early stages of ED may exert a specific influence on the pH of the albumin.<sup>(36, 43, 47, 63, 64)</sup> Albumin pH is affected by an early upregulation in the

expression of pH-dependent enzymes, such as carbonic anhydrase, which plays a critical role in the early stages of ED.<sup>(29, 47)</sup> Early hypercapnia enhances the rupture of the chalaziferous membranes, causing a rapid loss of hardness in both the dense and aqueous phases of the albumen.<sup>(65)</sup> Furthermore, early hypercapnia has been shown to improve the formation of sub-embryonic fluid during the early stages of ED.<sup>(66)</sup> This phenomenon could potentially contribute to improved early ED and subsequently enhance hatchability outcomes.<sup>(8, 22)</sup>

Moreover, genotype, breeder flock age, and egg storage time have been shown to influence albumin pH, chalaziferous membrane breakdown, and the optimal production of sub-embryonic fluid.<sup>(65, 66)</sup> These interactions may explain the disparities in the effects of early hypercapnia observed throughout different studies, perhaps leading to contradictory conclusions.<sup>(10, 23, 26, 32, 33, 36)</sup> Further research will hopefully reveal more precisely the exact mechanisms by which CO<sub>2</sub> modifies early ED. Meanwhile, the development of novel incubation programs that consider optimal concentrations of O<sub>2</sub> and CO<sub>2</sub> during specific stages of ED for poultry breeding of different ages and genotypes is essential.

## **Conclusions**

The gaseous environment, specifically elevated carbon dioxide concentrations during critical incubation periods at high altitude, improves embryonic development, optimizes quality, and increases body weight in one-day-old broiler chicks. Incubating fertile eggs with increased CO<sub>2</sub> levels at high altitudes for the first ten days resulted in 10 % higher hatchability than the standard ventilated group. This method offers an effective strategy for increasing hatchability and the

quality of newly hatched broiler chicks, particularly in high-altitude commercial breeder hatcheries.

### **Data availability**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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### **Conflicts of interest**

The authors have no conflict of interest to declare concerning this publication

## **Author contributions**

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