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Increased CO_2 Levels during the First Half of Incubation at High Altitude Modifies Embryonic Development of Fertile Leghorn Breeder Eggs

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Abstract

The exchange of oxygen (O_2) and carbon dioxide (CO_2) within an incubator has a significant impact on embryonic development (ED) and hatching processes. This study examines the influence of non-ventilation (NV) conditions during the first ten days of incubation at high altitudes on Leghorn hens hatching eggs. Five hundred four hatching eggs were equally divided into three treatment groups and placed in twelve incubators (R = 4). The first group was subjected to standard ventilated conditions (V) during the setting phase. The ventilation inlet holes of the remaining incubators in the NV treatments were closed with either micropore (M) or polypropylene (P) tape, referred to as NVM and NVP groups, respectively. These two different airtight settings were intended to allow for a gradual rise in CO_2 naturally generated by the embryos. Results indicate that carbon dioxide concentration gradually increased during the first half of incubation, reaching 1.42% in the NVM group and 1.20% in the NVP group, while the V condition group remained at 0.15%. From 10 days of incubation onwards, normal V conditions were restored in all incubators. The highest hatchability of fertile eggs (HFE) was shown by the NVP group (55.7%), followed by the V (52.6%) and NVM (38.6%) groups. The NVP group showed a greater yolk-free body mass (YFBM) from 10 days of incubation until the hatch basket transfer. NV conditions during the first 10 days of incubation at high altitude produced higher YFBM with gradually decreasing yolk sac mass. In comparison to the NVM and V conditions, the particular NVP condition showed a beneficial impact on the quality of hatched chicks. Sustaining NVP condition (1.2% of CO_2) throughout the first half of incuba-

tion at high altitude generated the optimal environment in the incubator ensuring the best hatchability results. This study highlights how important it is for hatchery managers to recognize the influence of low O_2 and high levels of CO_2 on the development trajectories of Leghorn embryos during early incubation at high altitudes.

Keywords

Non-Ventilation, Hypercapnia, Hypoxia, Egg Mass Loss, Hatchability, Embryonic Mortality, Hatchling Chick Quality

1. Introduction

Specific management during artificial incubation (AI) can impact the welfare and health of hatchlings, thereby modifying their post-hatching development and performance [1]-[3]. Embryonic development (ED) is a remarkable process influenced by the genetic background of the embryo and the environmental condition in which it develops [4]. The chicken hatching egg is a self-contained life-support pack for the developing embryo. Artificial incubation requires a delicate balance of several factors to optimize the hatchability of fertile eggs (HFE) and the quality of hatchlings (QH). Initial factors to control include pre-incubation conditions tailored to the specific features of each chicken hatching egg. Key factors such as parental stock age, egg storage conditions, egg weight (EW) and eggshell conductance (G) collectively play a significant role in achieving successful ED in larger quantity of hatching eggs. Abiotic factors include environmental conditions such as temperature, relative humidity (RH), air circulation, gas concentrations, barometric pressure of the hatchery setting and biosecurity level, each exerting a specific effect on every hatching egg stock [2] [5]-[9]. Gas exchange during incubation is critical for optimal ED, influencing incubation results and the QH [3]. It is crucial to keep adequate oxygen (O_2) levels and eliminate excess carbon dioxide (CO_2) to achieve optimal ED [3]. Inappropriate ventilation rates, inadequate air circulation and a lack of fresh air replacement might result in serious hypoxic and hypercapnic issues during AI [10] [11]. Appropriate O_2 levels during the incubation of hatching eggs are critical for the metabolic processes that sustain the embryonic development. Insufficient ventilation rates lead to hypoxia, impairing the embryo's energy production, essential for cell division, differentiation, and growth. Hypoxia could inhibit embryo growth and produce abnormalities, which increases overall embryo mortality. Inappropriate ventilation avoids the removal of excess carbon dioxide, which might produce respiratory acidosis, disrupt the embryo's acid-base balance, and potentially lead to ED issues and higher embryo mortality. Planned hypercapnia could improve ED triggering specific physiological responses. For example, modest rises in CO_2 throughout early incubation can improve vascularization and general embryo robustness [5] [10] [12] [13]. Atmospheric air usually contains ~0.03% to

~0.04% carbon dioxide and 21% oxygen [2] [14]. Nevertheless, throughout natural incubation, the CO_2 content in the air within the nest approaches to 1% [15]. In multi-stage AI, chicken hatching eggs are incubated with CO_2 levels ranging from ~0.05% to 0.4% - 0.5%. In contrast, oxygen levels remain constant at 21% during the incubation [16] [17]. Alternatively, in single-stage AI, the damper can be closed (non-ventilation), mimicking higher natural CO_2 concentration during the first half of AI, like what occurs in nature [2] [3] [15] [18]. As altitude increases, the partial pressure of O_2 diminishes, implying a higher risk of hypoxia (low O_2), which in turn affects the gas exchange of the embryo [19]. Early prenatal hypoxia alters the endocrine functions of chick embryos, affecting both the duration of incubation and HFE [3] [7] [20] [21]. Eggs laid at sea level and subsequently incubated at high altitudes (1200 - 3700 m) may experience hypoxia, hypocapnia, and excessive water loss. This condition can lead to delayed ED and result in poor HFE rates [7] [19] [20]. Inappropriate concentrations of O_2 and CO_2 are known to increase embryonic mortality rates [22] [23]. To overcome these drawbacks, supplementary systems that increase O_2 levels from 21% to 23% - 25% (hyperoxia) in the setter and hatchery are now recommended [7] [21] [24]. However, the main obstacles to using supplemental O_2 are the economic cost and some critical safety management issues. Additionally, higher CO_2 levels (hypercapnia) throughout incubation have been found to have different effects on incubation, embryo morphology and physiology, and post-hatch performance, depending on factors such as exposure concentration, time, and duration [1]-[3] [6] [8] [12] [22] [25]-[29]. Previous research has shown that increasing the usual CO_2 level from 0.5% to 1.5% during the first ten days of chicken incubation in an airtight incubator gradually improves embryo growth and favors early hatching, eventually increasing the hatchability and quality of newborn chicks [1]-[3] [9] [12] [18] [26]. Relative hypoxia, hyperoxia and hypercapnia throughout incubation have been found to improve ED, with effects varying based on the extent of exposure and the specific stage of the ED [2] [22] [25]. However, such effects have been shown to differ amongst ages of breeder stocks, breeds, hybrid lines, and varieties of chickens [8] [9] [26] [30], turkeys [31], and ducks [32]. Although current results are promising, hatchery managers must understand the impact of low O_2, and high CO_2 concentrations on embryo growth trajectory during incubation at high altitudes to fully utilize this new knowledge. Chicken embryos appear to evolve epigenetic mechanisms that modify certain morphological and physiological traits to compensate for adverse conditions, such as early hypoxia. During artificial incubation, the atmospheric pressure interacts with the embryo's endocrine systems and the development of its chorioallantoic membrane blood vessels, heart, lung, and liver, resulting in different hatching outputs depending on the altitude of the incubation hatchery [2] [8] [9] [11] [12] [20] [21] [33] [34]. There have been no studies on the physiological implications that different high CO_2 levels throughout the first ten days show on Leghorn chicken embryos incubated at high altitudes (2000 - 3000 meters). The goal of this study was to find out how different CO_2

concentrations applied during the early stages of ED at high altitudes influence hatchability, embryonic trajectory development, average embryo mortality, and quality of hatched Leghorn chickens.

2. Materials and Methods

2.1. Fertile Eggs for Hatching

Fertile hatching eggs from a 36-week-old Bovans white breeder hen flock were utilized in this research. The Leghorn breeder flock was raised on the floor under standard commercial husbandry conditions. The breeder farm was located at an elevation of 1600 meters in Tehuacán, Puebla, Mexico. A total of 504 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 the experimental incubation setting in Mexico City at 2230 meters of altitude [2]. Before arrival, all eggs were randomly assigned to three equal-sized groups, identified, weighed, and placed in twelve forced-draft commercial incubators (Hova-Bator® Mod. #1583 G.Q.F. Inc. Savannah, GA, USA).

2.2. Experimental Design

The first group of hatching chick eggs was incubated in a one-stage standard ventilated incubator (V) throughout the entire incubation period. Hatching fertile eggs of the second experimental group were incubated in a non-ventilated (NV) condition during the first ten days. In order to establish an airtight setting, the incubator's top outlets and bottom vent dampers were closed with Micropore® tape (NVM). The third group of hatching eggs was incubated in an NV incubator throughout the first ten days. The airtight environment was achieved by covering the bottom vent dampers and top outputs with Polypropylene Tuk® tape (NVP). From the tenth day of ED (ED10) to ED18, both NV treatments were switched to standard V condition. Every treatment included 42 eggs, with four distinct repetitions for each one. During the first half of the incubation, all hatching eggs were kept at a dry bulb temperature of 100.0˚F. In the standard V condition, from ED1 to ED18 day, the wet bulb temperature was maintained at 85.0˚F and from ED10 to ED18 day in both NV treatments. The hatching eggs were rotated 24 times daily until day 18 of incubation. On the 18th day (432 h) of incubation, the eggs were weighed and candled. Those with living embryos were then transferred to fixed-hatching baskets. The eggs were hatched in the same incubators converted into hatchers (Hova-Bator® Mod. #1583 G.Q.F. Inc. Savannah, Georgia, USA). The hatchers maintained a dry bulb temperature of 98.9˚F at ED18, which gradually decreased to 98.2˚F at hatch time. The wet bulb temperature of the hatcher was maintained at 89.5˚F from ED18 to hatch. The machines were monitored 6 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_2 and O_2 within each machine were measured four times a day (morning, noon, 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 the experiment, the gas analyzers were calibrated identically using atmospheric air and precision calibration gases.

2.3. Hatching Egg Weight and Embryonic

Eggs were individually weighed in grams when set in every incubator (0 d), and at 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 selected to calculate EWL using the following equation:

EWL = EW at every sample d ED − EW of the same egg at ED0/EW of same egg at ED0 × 100.

Yolk-free body mass and yolk sac weight were determined in five eggs randomly sampled per incubator at 10, 12, 14, 16, and 18 days of incubation, and nine chicks per hatching basket. The wet and dry weights of the yolk-free body mass, yolk sac, heart, and liver of every embryonated egg and newborn chick were measured according to the methodology described by Wolanski *et al*. [35] and Willemsen *et al*. [36]. Briefly, after carefully separating the embryo and yolk sac, both were weighed. Embryos were weighed after excessive fluid was dried using absorbent paper (Versi-Dry® Thermo Scientific). To dry the fresh embryo, internal organs, skin, and extra-embryonic membranes were excluded, while the complete yolk sac was dried as one piece. The yolk-free body mass, 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.

2.4. Hatching Events

Following transfer to the hatching baskets on day 18, eggs were individually monitored at 2-hour intervals, starting at 468 hours and continuing for 48 hours. During this period, the occurrence of internal pipping (IP), external pipping (EP), and hatching were recorded for each egg. Chick hatchlings that had fully emerged from their eggs, exhibiting healed navels and dryness about 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:

Total of hatchlings chicks/total of fertile eggs × 100.

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

Total of hatchlings chicks/total eggs set × 100.

After each hatched chick collection, it was weighed to the nearest 0.1 g, measured for length, and given 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.

2.5. Embryonic Mortality

Eggs that failed to hatch after the full period of incubation were opened and examined macroscopically to estimate infertility rate and assess the developmental stage reached before the embryo died. The time of embryonic death was calculated 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 death (EED) occurs during Stage I (days 1 to 7 of ED), mid embryonic death (MED) during Stage II (days 8 to 17 of ED), late embryonic death (LED) during Stage III (days 18 to 21 of ED) and cases with pipped eggs that did not hatch before 520 hours (PNH) were classified as Stage IV.

2.6. Quality Score Grading Chicks

All one-day-old chicks were individually pull-out and numbered after the entire batch of chicks had hatched. Every chick was examined macroscopically to identify a comprehensive range of traits associated with excellent, good, average, or poor quality. This methodology assessed chick quality based on field observations of various physical conditions crucial for successful chick development, including initial weight and body length, activity level, feather condition, eye health, comb appearance, leg strength, dehydration level, condition of the navel area, vent integrity, and remaining yolk sac. The scoring of newborn chick quality was based on thirteen comprehensive characteristics. These traits included physical conditions, such as body dryness and cleanness, activity level, the 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, dehydration condition, body weight and chick length. The quantitative traits, body weight (measured in grams) and chick length (measured in centimeters) 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 to the third toe [35]-[39]. All these methods are stated in **Table 1**.

The grading score assigned to every newborn chick was used to create an index of chick hatchling quality (CHQ). Every parameter was scored based on the birds' subsequent performance during their rearing at the farm, as described previously by Willemsen *et al*. [39] and Tona *et al*. [40]. Discrete traits were scored on a scale of 0 to 100 points, as outlined in **Table 2**.

The quality score rank assignment was as follows: 90 - 100 points = Excellent, 80 - 89 = Good; 70 - 79 = Average; 60 - 69 = Poor and <59 = Unacceptable. The final score allowed grading of each percentage rank in each NV treatment. All

Table 1. Assessment of different characteristics to index Leghorn chick hatchling quality.

Parameter	Assessment*
Down and appearance	The 1-d-olf Leghorn 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 good.
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 new-born chick was put on front of the observer. The condition of eyes brightness and wideness of the gape of the eyelids were determined.
Retracte yolk	The hatchling was put on its back obliquely on the hand palm until abdominal movement totally stopped. The height and consistency to touch of its abdomen were estimated. If the height of abdomen was estimated to be higher and harder to touch than normal, then yolk retracted 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, small, or no yolk sac.
Legs	The 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 or redness or both.
Tarsometatarsus and toes	The 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	Navel and its surrounding areas were examined for closure of the navel and its coloration. If the color was different from the skin color, then it was regarded as no good, if this had appearance like navel button or leaky navel it was very bad.
Remaining membranes	Observation of the navel area allowed estimation of the size of any remaining membrane or debris. If there was, the size was classified as large, small or no membrane and debris.
Vent appearance	The chick cloaca 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), it was not good.
Dehydration	The appreciation of the skin and vascular vessels of the neck, wing and leg allowed estimate dehydration condition. It was regarded as none, skin dry and skin very dry.
Weight	All one-day-old Leghorn chicks were individually weighted in grams.
Length	The one-day-old 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 implantation of the nail on the third toe.

Note: *Measurements were performed using a double-blind procedure.

Table 2. Assessment of scores to different quality traits observed in hatclings Leghorn chicks.

Parameter	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

Continued

Eyes	Both opened and bright	8
	Opened and not bright	4
	One or both closed	0
Retracted yolk	Body with normal swallowed yolk sac	8
	Body with regular swallowed yolk sac	4
	Body with large yolk sac and rather 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 joint	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
Weight 36 - 45 week-old Leghorn breeders	>40 g	10
	37 - 40 g	4
	<37 g	0
Length 36 - 45 week-old Leghorn breeders	>18 cm	8
	15 - 18 cm	4
	<15 cm	0

Note: *Measurements were performed using a double-blind procedure.

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.

2.7. Statistical Analysis

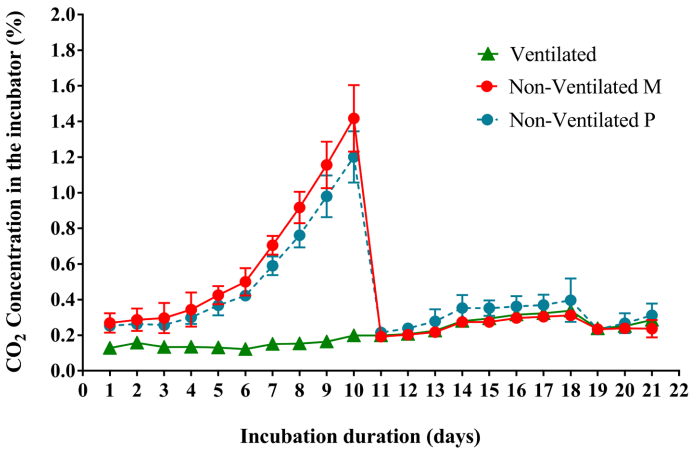
Data were gathered and analyzed using ANOVA with a general linear model (GLM) (SAS/STAT 9.2. SAS Institute Inc., Cary, NC, USA). The parametric traits include egg weights at setting, the weight of the yolk sac-free embryo body, the weight of the embryo yolk sac, the weights of the heart and liver, and the length and weight of the newborn chicks. Before the statistical analysis, data from hatching events 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 discern specific differences among treatment groups. All values were expressed as mean ± standard deviation (SD). Chi-square analysis assessed embryonic mortality stages (I, II, III and PNH), and quality scoring on 1-day-old Leghorn chicks. Statements of statistical significance were based on a threshold of $P < 0.05$.

3. Results

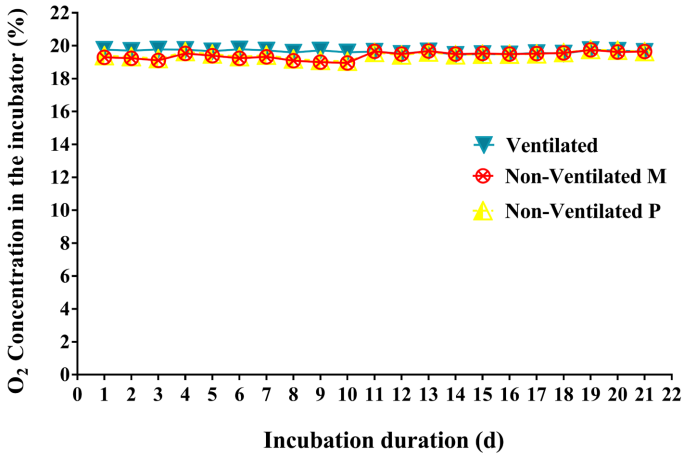
3.1. Incubation Environment

Oxygen concentration and discharged carbon dioxide into V and NV incubators were measured at regular intervals throughout the entire embryonic development period until hatch. **Figure 1(a)** illustrates a low (0.15%) but steady CO_2 concentration in the V condition during the first 10 days of ED, while the CO_2 rose gradually increased since the outset of incubation until ED10 day in both NV treatments. The NVP incubators exhibited a CO_2 concentration of 1.2%, while the NVM incubators showed a slightly higher concentration at 1.42% (**Figure 1(a)**).

During the initial ED10 days, the O_2 concentrations in NVM, NVP, and V incubators were measured at 19.0%, 19.3%, and 19.7%, respectively. From the ED10 day until hatching, all ventilation conditions showed similar levels of O_2 and CO_2 (**Figure 1(b)**). The experimental hatchery unit maintained an average



(a)



(b)

Figure 1. Kinetic of the CO_2 (a) and O_2 concentration (b) into the non-ventilated airtight and standard incubators (n = 4).

temperature of 24.0˚C and a relative humidity of 46% during the entire experiment. CO_2 levels were of 0.12%, and average concentration of O_2 was kept at 19.9%.

3.2. Egg-Weight Losses

The mean egg hatching weight at the outset of incubation was 57.01 ± 1.39 g, 56.44 ± 1.50 g and 56.50 ± 1.47 g in the V, NVM and NVP groups, respectively. No significant difference was observed between treatments. In the V condition, the early EWL at ED 10 day was measured at 5.77 ± 1.10%, significantly higher ($P < 0.05$) than the EWL observed in the NVM (3.99 ± 0.49%) and NVP conditions (3.99 ± 0.49%). However, the results observed in **Table 3** failed to demonstrate any significant impact of the initial ventilation conditions on EWL from ED10 to ED18. On day ED18, when all hatching eggs were transferred to baskets, the V group exhibited a mean EWL of 11.13 ± 2.22%, which was not significantly different from the EWL recorded in the NVM (10.66 ± 1.45%) or NVP conditions (10.55 ± 1.36%) (**Table 3**).

Table 3. Egg-weight loss at 10 and 18 days of incubation in ventilated and non-ventilated conditions of Leghorn hatching eggs.

Egg weight features	Ventilated	Non-ventilated M*	Non-ventilated P*
Egg hatching weight at set (g)	57.01 ± 1.39^{A} **	56.44 ± 1.50^{A} **	56.50 ± 1.47^{A} **
Egg weight at day ED10 day (g)	53.83 ± 1.50^{A}	54.31 ± 1.44^{A}	53.85 ± 1.64^{A}
Egg-weight loss at ED10 day (%)	5.77 ± 1.10^{A}	3.99 ± 0.49^{B}	4.46 ± 0.56^{B}
Egg weight at day ED18 (g)	50.60 ± 1.83^{A}	50.36 ± 1.53^{A}	50.56 ± 1.63^{A}
Egg-weight loss at ED18 day (%)	11.13 ± 2.22^{A}	10.66 ± 1.45^{A}	10.55 ± 1.36^{A}
Egg-weight loss ED10-ED18 (%)	5.36 ± 1.38^{A}	6.67 ± 0.61^{A}	6.10 ± 1.03^{A}

Note: *Non-ventilated airtight incubator condition through Micropore® membrane (M) or Polypropylene Tuk® tape (P) during the first 10 days of embryonic development. **Means (±SD) in the same row with no common superscript letter are significantly different ($P < 0.05$). n = 60 Leghorn hatching eggs per group.

3.3. Hatchings Events

In the NVP condition, the HFE was 55.7%, which was not significantly different from the HFE recorded in the V condition (52.6%). However, the NVM group exhibited an HFE of 38.5%, significantly lower ($P < 0.05$) than the former groups (Table 4). The hatchability of all eggs set in the NVP condition was 53.8%, compared to 50.9% in the V group and 36.2% in the NVM group (Table 4). The hatching eggs in the NVP group (53.8% of hatchlings) and V group (50.9% of hatchlings) started hatching 9 hours earlier ($P < 0.05$) than those in the NVM group (36.2% of hatchlings). The hatch window was 37 hours for chick hatchlings in the NVP treatment, 36.2 hours for the V treatment and 27.8 hours for the NVM condition.

3.4. Embryonic Mortality Analysis

Embryo mortality rates did not differ between the NV and V conditions during the first ED stage. However, results in Table 4 revealed differences in embryo mortality percentages between NV and V treatments from the middle stage onward. The NVM treatment showed a higher middle embryo mortality rate (21.28%), which was significantly different ($P < 0.05\%$) from embryo mortality observed in the NVP group (13.2%) or the V group (11.7%). Chick embryos that died in the late stage (18 - 21 days of ED) were predominantly observed in the V group (10.82%) which was higher compared to the NVP group (5.8%) and the NVM group (5.7%) (Table 4). The percentage of pipped eggs that remained unhatched in the NVP and V conditions was quite similar (12.7%). However, the rate of PNH in the NVM condition was higher (21.5%), showing a significant difference ($P < 0.05$) compared to the former groups (Table 4). A higher total embryonic mortality was observed in the NVM group (61.3%) (Table 4).

Table 4. Incubation traits and mortality of Leghorn embryos according to ventilated and non-ventilated conditions.

Parameters of incubations	Ventilated	Non-ventilated M*	Non-ventilated P*
Fertility (%)$^{\Psi}$	97.02 ± 1.19^{A}	95.24 ± 2.75^{A}	93.45 ± 3.58^{A}
Hatchability from fertile eggs (%)$^{\Psi}$	52.63 ± 8.63^{A}	38.65 ± 15.93^{B}	55.74 ± 11.86^{A}
Hatchability of all eggs set (%)$^{\Psi}$	50.88 ± 8.17^{A}	36.18 ± 16.26^{B}	53.82 ± 11.23^{A}
Early embryo mortality (%)$^{\phi}$	12.09^{A}	12.85^{A}	12.43^{A}
Middle embryo mortality (%)$^{\phi}$	11.67^{C}	21.28^{A}	13.20^{B}
Late embryo mortality (%)$^{\phi}$	10.82^{A}	5.75^{B}	5.87^{B}
Pipped dead (%)$^{\phi}$	12.78^{B}	21.46^{A}	12.76^{B}
Total of embryo mortality (%)	47.36	61.34	44.26

Note: *Non-ventilated airtight incubator condition through Micropore® membrane (M) or Polypropylene Tuk® tape (P) during the first 10 days of embryonic development. $^{\Psi}$Means (±SD) within the same line with no common superscript letter are significantly different ($P < 0.05$). $^{\phi}$Means within the same line with no common superscript letter are significantly different ($P < 0.05$). n = 168 Leghorn hatching eggs per group.

3.4. Embryonic Growth

The wet and dry yolk-free body mass showed significant differences between the NV and V conditions. Embryos from the NVP treatment, whether wet or dry, were consistently heavier ($P < 0.05$) compared to those from the NVM or V groups throughout almost the entire incubation period (Figure 2 and Figure 3). However, at hatch, both NV treatments did not show any difference compared to the control V group (Figure 3). On the other hand, there were also significant differences in wet and dry yolk sac mass between the NV and V conditions (Figure 4 and Figure 5).

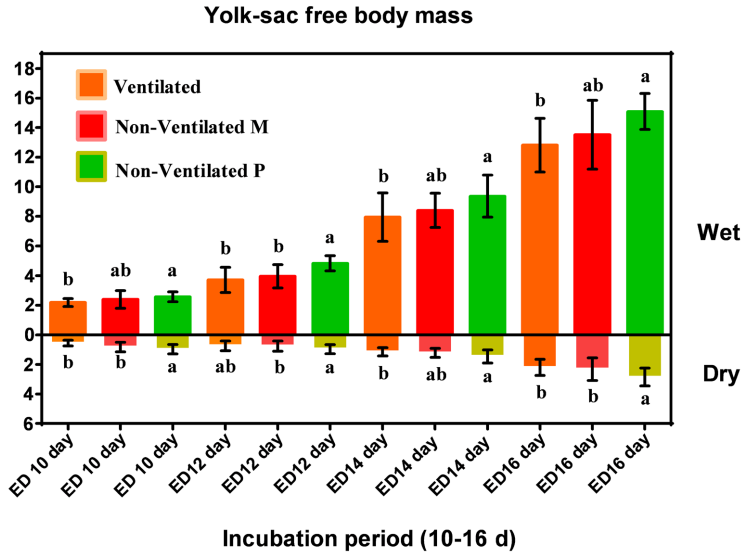


Figure 2. Effect of different ventilation conditions during early embryonic development on yolk-free body mass (g) at 10, 12, 14 and 16 days of embryo development (n = 20).

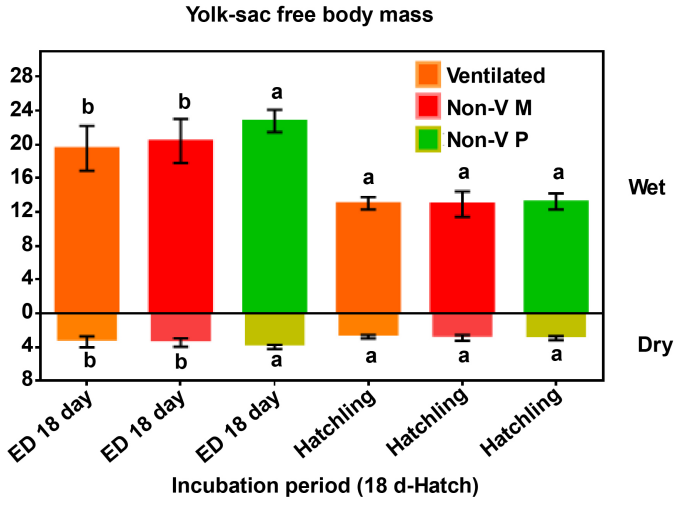


Figure 3. Effect of different ventilation conditions during early embryonic development on yolk-free body mass (g) at 18 days of embryo development (n = 20), and hatch (n = 36).

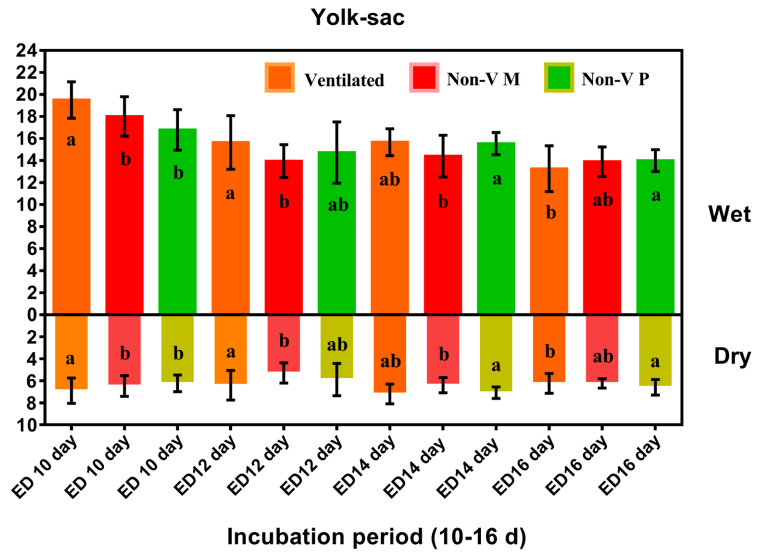


Figure 4. Effect of different ventilation conditions during early embryonic development on yolk sac weight (g) at 10, 12, 14 and 16 days of embryo development (n = 20).

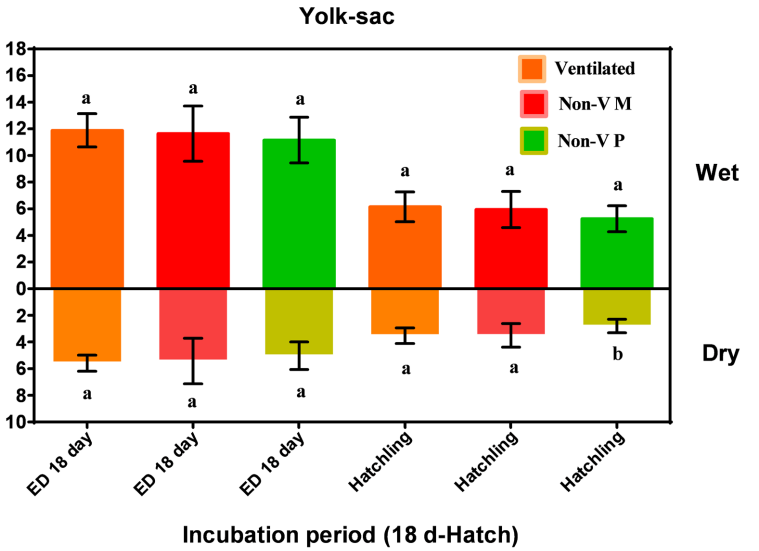


Figure 5. Effect of different ventilation conditions during early embryonic development on yolk sac weight (g) at 18 days of embryo development (n = 20) and hatch (n = 36).

Yolk sac and dry yolk sac masses from the NVP and NVM treatments were lighter ($P < 0.05$) than those from the ventilated group at 10 and 12 ED days. However, wet and dry yolk sac masses from the NVP group were heavier ($P < 0.05$) than NVM and ventilated groups at 14 and 16 ED days, respectively (Figure 4). The wet yolk sac mass from the NVP group at 18 ED days or hatch did not differ from the NVM or V groups (Figure 5).

However, the dry yolk sac mass from the NVP group at hatch was significantly lower ($P < 0.05$) than those of the NVM or V groups (Figure 5).

The hearts and livers in the NVP group of embryos at 14 ED days were significantly heavier ($P < 0.05$) than those in the control V group (Table 5).

Table 5. Measuring of heart and liver weights from embryos and newborn Leghorn chicks incubated according to ventilated and non-ventilated conditions during the first ten days of incubation.

Periods for organs weighing	Ventilated (g)	Non-ventilated M* (g)	Non-ventilated P* (g)
Heart weight on ED14 day$^{\Psi}$	0.06 ± 0.01^{B} **	0.09 ± 0.01^{AB} **	0.12 ± 0.01^{A} **
Liver weight on ED14 day$^{\Psi}$	0.11 ± 0.03^{B}	0.16 ± 0.04^{A}	0.17 ± 0.05^{A}
Heart weight on ED16 day$^{\Psi}$	0.14 ± 0.02^{A}	0.14 ± 0.03^{A}	0.14 ± 0.02^{A}
Liver weight on ED16 day$^{\Psi}$	0.33 ± 0.09^{AB}	0.35 ± 0.08^{A}	0.30 ± 0.05^{B}
Heart weight on ED18 day$^{\Psi}$	0.18 ± 0.02^{B}	0.21 ± 0.03^{A}	0.21 ± 0.02^{A}
Liver weight on ED18 day$^{\Psi}$	0.38 ± 0.09^{B}	0.46 ± 0.11^{A}	0.49 ± 0.10^{A}
Heart weight on Hatchlings$^{\Phi}$	0.24 ± 0.05^{B}	0.27 ± 0.04^{A}	0.25 ± 0.05^{AB}
Liver weight on Hatchlings$^{\Phi}$	0.75 ± 0.05^{A}	0.78 ± 0.07^{A}	0.76 ± 0.05^{A}

Note: *Non-ventilated airtight incubator condition through Micropore® membrane (M) or Polypropylene Tuk® tape (P) during the first 10 days of embryonic development. **Means (±SD) within the same line with no common superscript letter are significantly different ($P < 0.05$). $^{\Psi}$n = 20 embryos/group Φ n = 36 chick hatchlings/group.

At 16 ED days, there were no differences in heart weight between groups. However, the liver was significantly heavier ($P < 0.05$) in the NVM group compared to the NVP group, while the liver weight of the V group did not differ from any NV treatment (Table 5). Hearts in both NV groups of embryos at 18 ED days (NVM = 0.21 ± 0.03 g, NVP = 0.21 ± 0.02 g) were heavier ($P < 0.05$) than the heart weight recorded in embryos from the V condition (0.18 ± 0.02 g), and liver weight showed a similar trend (Table 5). The hearts of NVM hatchlings had a mean weight of 0.27 ± 0.04 g, which was heavier ($P < 0.05$) than the average heart weight recorded in newborn chicks from the V condition (0.24 ± 0.05 g). At hatch, one-day-old chicks from both NV and V groups did not show a difference in liver mean weight between them (Table 5).

3.4. Hatchling Chick Quality Index

Under the current experimental conditions, we conducted a comprehensive evaluation of QH. No chick received a high-quality rating, indicating the need for further investigation. Although the ranks for good quality between hatched chicks from each ventilation condition varied slightly, ventilation treatment had no significant effect (Table 6). Fifty percent of hatchlings from the NVP condition received a middle-quality score, a percentage significantly higher than the 33.9% of chicks from the NVM condition and the 36.9% from the control V group receiving the same ranking score (Table 6). The NVM condition showed a significantly higher quantity ($P < 0.05$) of hatchlings with poor quality (39.3%). Group V was the only one with 4.16% of newborn chicks receiving an unclassified rating. There was no significant effect of ventilation conditions on the body weight of hatched chicks (Table 6). However, the average length of one-day-old chicks in the NVM group, at 16.32 ± 0.5 cm, was shorter ($P < 0.05$) than the 16.65 ± 0.3 cm recorded in the NVP group. The V condition showed a length of 16.5 ± 0.3 cm, which was no different from NV treatments (Table 6).

Table 6. Quality grade scoring and quantitative traits in one-day-old Leghorn chickens from ventilated and non-ventilated conditions during the first half of incubation at high altitude.

Quality features	Ventilated	Non-ventilated M*	Non-ventilated P*
High quality (%)	0 ± 0 **	0 ± 0 **	0 ± 0 **
Good quality (%)	21.78 ± 14.88^{A}	26.78 ± 20.51^{A}	30.20 ± 9.23^{A}
Middle quality (%)	37.20 ± 19.42^{B}	33.92 ± 22.86^{B}	50.0 ± 13.60^{A}
Poor quality (%)	36.90 ± 15.74^{AB}	39.28 ± 11.45^{A}	19.79 ± 15.22^{B}
Unclassified (%)	4.16 ± 2.71^{A}	0 ± 0^{B}	0 ± 0^{B}
Chick hatchling mass (g)	39.67 ±1.72^{A}	39.15 ± 1.27^{A}	39.83 ± 1.81^{A}
Chick hatchling length (cm)	16.54 ± 0.33^{AB}	16.32 ± 0.48^{B}	16.65 ± 0.33^{A}

Note: *Non-ventilated airtight incubator condition through Micropore® membrane (M) or Polypropylene Tuk® tape (P) during the first 10 days of embryonic development. **Means (±SD) within the same row with no common superscript letter are significantly different ($P < 0.05$). n = 36 Newborn Leghorn chicks per group.

4. Discussion

During the first half of embryonic development, the NVM condition showed slightly higher levels of CO_2 (0.2%) than the NVP condition. Despite this slight shift, it was enough to explain the reduction in overall hatchability noticed in the NVM condition. Micropore (M) tape sealing generated a strict airtight environment, resulting in a significant rise in CO_2 (1.4%) and an eventual reduction in O_2 levels. The lower HFE noticed in the NVM group could be due to higher rates of early and middle embryonic mortality, and a greater proportion of pipped but unhatched chicks. Extremely high CO_2 levels (e.g. 1.4% in the NVM group) can negatively impact in viability of some embryos that showed a threshold of susceptibility toward toxic effects of high CO_2 during early incubation. At the finish of the study, it was found that the NVM group showed a higher total embryonic mortality than both the NVP and V groups. Despite the NVP group displaying higher middle embryonic mortality than the V group, this mortality declined significantly throughout the last two stages of embryonic development. Indeed, both NV groups showed better embryo weight from the 10th day of ED until hatch, and they also had lower embryonic mortality in the final stage of ED. According to Willemsen *et al.* [39], embryos surviving early NV conditions exhibit improved physiological conditions that enable them to accelerate their ED. NVP embryos that survived the middle stage of ED might have attained a more advanced morpho-physiological stage than NVM and V embryos. While the early recorded CO_2 levels in the NVP group may have mitigated some adverse effects at the onset of the middle stage, they were not as detrimental as in the NVM group. This suggests a physiological tolerance in NVP embryos to a subsequent higher increase in CO_2 during the later stages of ED [22] [41] [42]. The physiological mechanisms behind this time-dependent tolerance are not yet understood. Still, they are hypothesized to be related to the increasing buffering capacity of the embryo with age, which helps counteract acidosis caused by high levels

of dissolved CO_2 [22] [41] [42]. Several studies have indicated that modifying ventilation rates during the first half of poultry incubation increases gradual CO_2 (0.8% to 1.5%), and these concentrations accelerate the ED, enhance the morpho-physiological condition of embryos, and contribute to increased HFE and the QH [1]-[3] [6] [8] [9] [12] [26] [41]. Bruggeman *et al*. [41] investigated hypercapnia (NV) in the first half of incubation by injecting CO_2 directly into the incubator. They detected accelerated embryonic growth, higher embryo weight, faster hatching, and better-quality hatched chicks. These findings are similar to those gathered by our NVP group. Nevertheless, results from Bruggeman *et al*. [41] showed no significant improvement in hatchability or decrease of embryonic mortality rate. The exact reasons for the inconsistent improvement in hatchability parameters observed across all studied cases with increased CO_2 during early incubation are not yet fully understood [1]-[3] [8] [13] [26] [29] [41]. The positive effects of NV condition incubation observed on hatchability likely are attributed to various factors such as genotype, breeding stock age, and even the altitude of the incubation setter, rather than solely the CO_2 concentration reached during the first half of the incubation period [1] [3] [8] [9] [12] [13] [26] [32] [43]. Indeed, genotype and the age of the parent stock are critical factors influencing the metabolic rate of the embryo and, consequently, its response to rising CO_2 levels during the early stages of ED [1] [2] [8] [13] [43]. The concentration of 1.2% CO_2 reached in the NVP group during the first half of incubation resulted in a gradual increase of benefits, including accelerated ED, improved hatchability, and enhanced quality of hatched chicks, compared to the NVM group. This aligns with the research results of De Smit *et al*. [1], who carried out NV incubation on embryos from 60-week-old broiler breeders and reached a CO_2 concentration (1.0%) comparable to our NVP group. De Smit *et al*. [1] reported greater hatchability, increased embryo weight, and a faster and narrower spread of hatch than its standard V group. In contrast, when De Smit *et al*. [1] applied NV incubation conditions to embryos from 45-week-old broiler breeders, they reported greater CO_2 concentrations (1.5%), which were similar to our NVM group (1.4%). Otherwise, they found no changes between their experimental NV and control V groups, however, we detected significant differences between our NVM and V groups. While an early high concentration of CO_2 in broiler embryos may not be as harmful, the same early CO_2 concentration exhibited non-beneficial effects in Leghorn embryos [1] [2] [26] [43]. The essential mechanisms explaining Leghorn embryos' limited tolerance to increased CO_2 require further research. Through the incubation period, the NVP condition showed regularly greater yolk-free body mass than embryos from the standard V group. Similarly, dry yolk-free body mass weight showed a consistent trend throughout the course of the research. NVP incubation resulted in accelerated embryonic growth and efficient utilization of primary energy stores, particularly from the yolk sac. From the 10^{th} day of ED onward, as the weight of the embryo increased, there was a corresponding decrease in the weight of the yolk sac. According to Wolanski *et al*. [35], there is a

notable correlation between wet and dry weight for the yolk sac compared to the embryo, a pattern that coincides accurately with our findings in the NVP group. The NVP group's accelerated ED suggests the embryo's ability to grow much faster under hypercapnia and relative hypoxia during the first ten days of incubation. The moderate early hypercapnia and relative hypoxia observed in the NVP group can contribute to improving the growth and functionality of the chorioallantoic membrane (CAM) [8] [11] [12] [41] [44]. Early stimulation for enhanced development and functionality of the CAM could lead to improved oxygen uptake during the exponential phase of ED after ten days of incubation. This could account for the favorable effects of early hypercapnia and relative hypoxia in the later stages of ED, which could enhance the rate of energy supply from the yolk sac through processes such as fatty acid oxidation and gluconeogenesis [12] [44]-[46]. According to Walsberg [15], as ED advances during natural incubation, the CO_2 level increases from 0.04% to 0.5%, while the O_2 level declines from 20.9% to 20.3%. Similarly, our study revealed an analogous pattern of O_2 drop throughout the first ten days of ED. The oxygen concentration in the NVM group declined from 19.8% to 18.8%, whereas in the NVP group, it decreased from 19.8% to 19.0%. Conversely, the V group experienced only a 0.2% decrease. Rahn *et al*. [47] observed that gas levels are not fixed during natural incubation in the nest and typically vary as ED progresses. During natural incubation, breeder hens play a crucial role in regulating the nest temperature, frequently monitoring the eggs's temperature. Once the embryos reach a stage where they can generate their heat autonomously, breeder hens tend to leave the nest more regularly to drink and feed, especially after the endothermic phase of the embryos (1 - 10 days of setting) [48]. Breeder hens enable progressive ventilation by leaving the nest more frequently, which improves CO_2 removal and increases O_2 supply to the nest eggs. This process continues in phases until hatching [15] [47] [48]. The embryos in the NVP group were consistently heavier than those in the control V group, and this difference persisted until the transfer to the hatch baskets. Willemsen *et al*. [39] demonstrated that the weight of hatched chicks from a group with NV conditions exhibited the most significant predictive value for post-hatch performance, surpassing the chick length trait as predictive measure. The NVP group in the present study recorded the greatest measure of chick length. The studies conducted by Wolanski *et al*. [35] [49] indicated that chick length had a correlation with yolk-free body mass at hatch ($r = 0.56$). However, other authors doubt the usefulness of chick length as a meaningful indicator of chick quality [39] [50]. Whereas some investigators believe that this approach offers actual commercial applications, they must come up with more rigorous evidence that backs up these claims. Several research teams discovered that the NV method produced heavier yolk-free embryos and gradually lighter yolk sacs [1] [2] [35] [39] [41] [51]. This is linked to the embryo's effective early metabolic activity, especially in the growth of tissues, as well as the capacity to satisfy its energy requirements [45] [52]-[54]. The weight of the residual yolk sac measures both the

energy invested in the egg and the energy utilized by the embryo during its own development [53]-[55]. According to Moran [56], starting from the 12th day of ED, the gain in yolk-free body mass negatively correlates with yolk sac weight. The NVP embryos exhibited a relatively small proportion of yolk sac compared to the yolk-free body mass, indicating that the chicks hatched in this group may have matured earlier. In addition to exhibiting an increasing weight trend, the surviving NV embryos demonstrated greater weight of the heart and liver than those in the V group. According to Druyan [57], this suggests a more robust embryo development. The observed increase in liver and muscle in NPV embryos at 18 d ED suggests a greater glycogen storage. This glycogen stored in the liver and 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 [45] [54] [55] [58]. Early incubation under NV promotes suitable CO_2 and O_2 profiles and uniformity in eggshell temperature (EST). This combined effect contributes to optimal ED and post-hatch growth. Maintaining a warm and steady environment during the early stages of incubation is essential for uniform ED. Inconsistent EST can lead to developmental delays and greater mortality rates [27] [52] [53] [59]. Post-hatch growth and organ function can be affected if growth rates throughout ED deviate from the optimum [27] [45] [52] [59]. NV conditions during the first half of incubation mitigate temperature oscillations caused by the forced exchange of fresh air from outside, commonly observed in standard V incubation at high altitudes [2] [19]. Inconsistent temperatures can delay ED and increase mortality. NV settings guarantee controlled warmth throughout the first half of the ED. This ventilation approach was primarily implemented during the embryo's most critical growth time frame, the endothermic phase (1 - 10 days of ED), in which keeping a constant and warm EST is critical [27] [48] [52] [59]. Appropriate ventilation helps to maintain humidity levels, preventing the eggs from losing too much moisture, which tends to rise with altitude [2] [19] [47]. Wet air transfers heat more efficiently than dry air. Therefore, restricting ventilation during the first ten days of ED helps maintain a stable and warm temperature. This is especially important in high-altitude environments because, in this setting usually, the air is cold and dry [2] [19] [47] [52]. Precise ventilation at high altitudes guarantees that the eggshell maintains a proper temperature and moisture equilibrium, supporting gas exchange but without affecting the developing embryo's hydration [2] [9] [11] [19] [47]. A single exposure to a specific environment during early embryonic growth can have long-term consequences on the life of birds. In the current study, the NVP condition contributed to optimizing the subsequent development stages of embryos that were early exposed to hypercapnia and relative hypoxia. The increase in CO_2 concentration appears to function as a switch, triggering specific effects on certain physiological traits related to early ED. These effects are likely to persist throughout the entire incubation period, influencing the process of epigenesis [1]-[3] [8] [39]. Ventilation affects the pH of the albumen (egg white).

Some research groups have suggested that the high concentration of CO_2 during the early stages of ED may have a specific action on the pH of the albumen [22] [29] [41]-[43] [60] [61]. Changes in pH can influence the early activity of pH-dependent enzymes, such as carbonic anhydrase, crucial for various metabolic processes during the early phases of ED [29] [42] [61]. Early hypercapnia plays a significant role in accelerating the rupture of the chalaziferous membranes and results in a rapid loss of hardness in both dense and aqueous phases of the albumen [62]. Proper ventilation promotes the production of subembryonic fluid (SEF), which is required for nutrition delivery and embryo hydration. It has been noticed that early hypercapnia enhances the output of SEF in the early stages of embryo development [63]. This may contribute to improved ED and hatchability results. Genotype, breeder flock age, and length of egg storage time also influence the albumin's pH, chalaziferous membranes breakdown and optimal SEF formation [41] [44] [46] [60]. This explains the variability in the effects of early hypercapnia reported in different studies, which could lead to explain the contradictory results observed between them. Therefore, further studies on the precise physiological mechanisms displayed by the embryos during early incubation with hypercapnia and relative hypoxia are required. It is necessary to develop novel ventilation programs throughout incubation, considering optimal gas concentrations at different stages of embryo development for breeding hens of other species, ages, breeds and hybrid lines.

5. Conclusion

Implementing single-stage incubation with moderate levels of hypercapnia favored by non-ventilation conditions throughout the first half of embryonic development might serve as an effective strategy for high-altitude hatcheries aiming to improve the hatchability and quality of hatched chicks.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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