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NON-LINEAR EGG WEIGHT LOSS IN LEGHORN BREEDER EGGS INCUBATED AT HIGH ALTITUDE IMPROVES HATCHLING PHYSICAL QUALITY TRAITS

Marco Antonio Juarez-Estrada[1a]*, Erick Iraim Lopez-Ruiz[1b], Sonia Lopez-Cordova[2], Victor Manuel Petrone-Garcia[3], and Omar Francisco Prado-Rebolledo[4]

[1a] Departamento de Medicina y Zootecnia de Aves, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Cd. Universitaria, Ciudad de México 04510, Mexico
https://orcid.org/0000-0003-4376-0651

[1b] Departamento de Medicina y Zootecnia de Aves, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Cd. Universitaria, Ciudad de México 04510, Mexico
https://orcid.org/0009-0006-0276-4727

[2] Dirección General de Salud Animal-Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, Centro Nacional de Servicios de Diagnóstico en Salud Animal, Tecámac, Edo. de México 55740, Mexico
https://orcid.org/0009-0008-3609-3188

[3] Departamento de Ciencias Pecuarias, Facultad de Estudios Superiores de Cuautitlán (FESC), Universidad Nacional Autónoma de México, Cuautitlán 54714, Mexico
https://orcid.org/0000-0001-6109-6366

[4] Facultad de Medicina Veterinaria y Zootecnia, Universidad de Colima, Tecomán, Colima 28100, Mexico
https://orcid.org/0000-0001-8802-0177

* Corresponding author: britoco@unam.mx

ABSTRACT

Artificial incubation (AI) is essential for producing healthy, high-quality one-day-old chicks; however, continuous optimization of incubation conditions, particularly at high altitudes, remains necessary. Among these conditions, relative humidity (RH) is a crucial factor. Egg weight loss (EWL) during incubation plays a key role in embryonic development and the hatching process. This study evaluated the effects of non-linear and standard EWL patterns during incubation at high altitude. Hatching eggs from 36- and 41-week-old Leghorn breeders (n=672) were evaluated. An airtight experimental incubation system was designed to allow a gradual increase of CO_2 concentration and RH, generating differential conditions between treatments during the early stage of incubation (days 0-10). Results revealed that the non-linear EWL protocol applied during AI at high altitude did not cause any significant increase in embryonic mortality. Hatchability of fertile eggs (HFE) was slightly higher (>3-5%) than that observed under standard EWL incubation conditions. The hatchling physical quality index under the non-linear EWL approach, from both breeder hen age groups, was significantly increased (>9%) compared with the respective standard EWL groups. Therefore, non-linear EWL management during incubation at high altitude has the potential to improve HFE, hatchling physical quality score, and thus post-hatch performance in the field. However, before the non-linear EWL method can be adopted as a standard protocol in AI at high altitude, further adjustments should be investigated, as these may vary according to each hatchery and individual egg batch.

Keywords: Air chamber, chicks, embryo mortality, hatchability, hypercapnic incubation, physical quality, relative humidity.

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INTRODUCTION

Environment management procedures during artificial incubation (AI) of poultry have been shown to play a critical role in the health and welfare of hatchlings, thereby influencing their post-hatch field performance (De Smit et al., 2006; Ipek et al., 2014; Vieira et al., 2025). When environmental conditions in setters and hatchers meet the demands of the embryo, conversion of egg content into embryo and hence the development of the embryo will be optimal (Mueller et al., 2022; Juárez-Estrada et al., 2024).

Successful hatchability depends largely on the environmental conditions created throughout AI smarter protocols (Tona et al., 2022; Peebles, 2023; Fares et al., 2023). Therefore, AI requires a delicate balance of several factors to optimize the hatchability and the quality of one-day-old chicks (Boleli et al., 2016; Araújo et al., 2016; Juarez-Estrada et al., 2025). Among the initial factors to be controlled are pre-incubation conditions, which should be tailored to the specific features of each batch of hatching eggs (Reijrink et al., 2008; Ar and Deeming, 2009).

Embryonic development largely depends on the embryo's genetic background and the environmental conditions under which it develops (Mueller et al., 2022; Vieira et al., 2025). Factors such as parental stock age, egg storage conditions, egg weight, and eggshell conductance collectively play a significant role in achieving successful embryonic development in hatching eggs (Ar and Rhan, 1980; Visschedijk, 1980; Peebles et al., 2001; Ar and Deeming, 2009; Araújo et al., 2017).

Abiotic factors, including environmental conditions such as temperature, relative humidity (RH), air circulation, gas concentrations, barometric pressure of the hatchery setting, and biosecurity level, each exert a specific effect on every batch of hatching egg (Yilmaz-Dikmen et al., 2014; Boleli et al. 2016; Juarez-Estrada et al., 2025; Vieira et al., 2025). The AI procedures should consider all these interacting variables to maximize hatchability and chick quality, by monitoring and controlling temperature, egg turning, humidity, and respiratory gas levels, in accordance with the stage of embryonic development (Tona et al., 2022; Vieira et al., 2025).

In addition to adequate egg turning, the primary priority during incubation is the precise regulation of embryo's internal temperature, typically measured through eggshell temperature measurements (Lourens et al., 2005, 2007; Mueller et al., 2022). The second priority is the control of RH (Bruzual et al., 2000). Humidity within the setter influences egg heat loss through evaporation, with lower RH leading to increased egg weight loss (EWL) (Bruzual et al., 2000; Araújo et al. 2016; Peebles, 2023). An appropriate EWL between 10.4 and 12% relative to the initial egg weight to transfer time can contribute to positive hatching results (Vieira et al., 2025). However, due to the wide variation in eggshell conductance, the ideal humidity level inside the setters and hatcher can also vary throughout AI (40–60%) (Ar and Rhan, 1980; Bruzual et al., 2000; Araújo et al. 2016, Araújo et al., 2017; Peebles, 2023). Hence, achieving optimal environmental conditions throughout the AI process to optimize EWL is critical (Peebles et al., 1987; Banwell, 2008; Ar and Deeming, 2009).

Most of the weight lost through the eggshell is water vapor (Ar and Rhan, 1980). Before the chick starts to break the eggshell, the egg chamber must already be formed; this helps the chick to adapt its lungs quickly to the external atmosphere before hatching (Mueller et al., 2022). In poultry, the air cell is only formed if the hatched eggs lose between 10.4 and 12% of their weight upon transfer to the hatcher baskets (Ar and Rhan, 1980; Ar and Deeming, 2009; Peebles et al., 2023).

Achieving optimal EWL is a challenge during the AI process (Ar and Rhan, 1980). It is known that hatchability and chick quality decrease with increasing altitude, depending on the level of O_2 in the air (Boleli et al., 2016; Okur, 2019). In high-altitude regions, such as parts of India, China, and South America (2700-4000 m), low hatchability rates of approximately 20-45% have been reported under standard conditions (Lan et al., 2012; Ahmed et al., 2013).

EWL becomes particularly critical at high altitude due to the increase in kinetic energy of the water vapor molecules, which increases gaseous diffusion through the eggshell; this results in gas exchange dynamics that differed from those observed at sea level due to higher barometric pressure (Visschedijk, 1985; Lan et al., 2012; Ahmed et al., 2013; Yilmaz-Dikmen et al., 2014; Okur et al., 2022).

During standard incubation, EWL regularly follows a linear trajectory; each incubated egg gradually loses water, and this loss continues on a linear basis throughout the incubation process (Peebles et al., 2023; Juarez-Estrada et al., 2025). However, some studies have reported improved incubation outputs when a non-linear EWL protocol is implemented; this non-linear EWL approach promotes higher levels of early fluids during the first half of embryonic development (Banwell, 2008; Peebles, 2023). The purpose for retaining more fluids during the first half of embryonic development is to cushion a potential adverse effect on embryo metabolism due to an early water imbalance caused by a high rate of premature metabolic water loss (>6.5% at 10 days of incubation) (Lourens et al., 2005; Banwell, 2008; Peebles et al., 2021; Mueller et al., 2022; Fares et al., 2023; Peebles, 2023). A non-linear EWL protocol has been evaluated in several previous trials, obtaining a significant improvement in hatchability of broiler chicks (Banwell, 2008; García et al., 2013; Juárez-Estrada et al., 2024). However, assays linking this novel non-linear weight loss dynamic with a hypercapnic incubation system during the first half of the avian Leghorn ED are lacking (García et al., 2013; Vieira et al., 2022; Okur et al., 2022; Juarez-Estrada et al., 2025).

The rate of diffusion of gases and fluids can be regulated through careful control of the environment surrounding the eggs (Ar and Rhan, 1980; Walsberg, 1980). Major hatching features obtained in a non-linear EWL system during incubation at high altitude are yet unknown, particularly given the critical role of the environmental conditions surrounding the egg to achieve optimal hatchability and chick quality (Visschedijk, 1985; Yilmaz-Dikmen et al., 2014; Araújo et al., 2017; Okur, 2019; Okur et al., 2022; Juárez-Estrada et al., 2025).

The objective of the study was to evaluate the effects of a non-linear egg mass loss management strategy during the first half of the embryonic development at high altitude on hatchability and physical quality traits of hatchlings, and to determine whether these effects differ according to the age of young or prime-age breeder Leghorn hens.

MATERIAL AND METHODS

Egg collection and storage conditions

Fertile hatching eggs were collected from Bovans White breeder hen flocks aged 36 and 41 weeks. Both flocks were reared on deep-litter floors under standard husbandry conditions. Both Leghorn breeder flocks were located close to each other at an altitude of 1,600 m in Puebla state, Mexico. Hatching eggs were stored with the pointed end down in a cool room (18°C and 75% RH) for 3-7 days before being set in the incubators. All egg trays were transferred to the experimental incubation setting located in Mexico City at 2,230 m of altitude. Upon arrival, all eggs of from each breeder age group (36 and 41 weeks) were randomly assigned to two equal-sized groups, individually identified, weighed, and distributed among eight forced-draft commercial incubators (Hova-Bator® Mod. #1583 G.Q.F. Inc. Savannah, Georgia, USA), according to breeder age.

Experimental Design and incubation

The first group of hatching eggs was incubated under standard EWL conditions in a single-stage ventilated incubator throughout the AI period. The second group was incubated in an airtight incubator during the first 10 embryonic days (ED). Airtight conditions were achieved by sealing the bottom dampers and top outlets of the incubator with polypropylene adhesive tape to gradually increase the CO_2 concentration and HR. From ED1 to ED3, all incubators contained the same water content in the floor pan.

At the end of ED3, all water was removed from each airtight incubator to implement the non-linear EWL strategy. From ED10 to ED18, the non-linear EWL treatment was transitioned to a standard ventilated condition by adding water in the same manner as in the standard incubators. A total of 42 eggs were incubated per treatment, with four replicates per treatment. All eggs were incubated at dry bulb temperature of 100.0°F during the setter phase. Under standard EWL conditions, wet-bulb temperature was maintained at 85.0 ºF from ED1 to ED18, and under non-linear EWL conditions from ED10 to ED18. Eggs were turned 24 times per day until ED18 day. On ED18 (432 h), all eggs were weighed and candled, and those with viable embryos were transferred to fixed hatching baskets. Eggs were hatched into hatchers (Hova-Bator® Mod. #1583 G.Q.F. Inc. Savannah, Georgia, USA). The hatchers were maintained a dry-bulb temperature of 98.9 ºF at ED18, which was gradually reduced to 98.2 ºF at hatch. The wet-bulb temperature in all hatchers was maintained at 89.5 ºF from ED18 until hatch. All machines were monitored six times daily to ensure proper operation. To accurately determine hatch time, the time when eggs were set in each incubator was defined as hour zero. Concentrations of CO_2 and O_2 in each incubator and hatcher were measured four times daily (morning, noon, afternoon, and night) using an infrared non-dispersive (NDIR) sensor and a galvanic cell sensor, respectively (Analox®, Analox Inst. Ltd. The Vale, London W3 7QE, UK). Prior to each measurement, the gas analyzers were calibrated consistently using atmospheric air and precision calibration gases.

Hatching egg and embryo weight

Eggs were individually weighed (g) at set (ED0), and at ED10 and ED18 in each incubator. The percentage of EWL during different intervals (ED 0-10, 10-18, and 0-18) was calculated for each treatment. Fifteen eggs per incubator were randomly selected to calculate EWL using the following equation:

EWL = EW at every sample ED - EW of same egg at ED0/ EW of same egg at ED0 × 100

According to the methodology described by Willemsen et al. (2011), yolk sac-free body weight and yolk sac weight were measured at hatch in five chicks per hatching basket from 36-week-old breeder hens, and in nine chicks per basket in the 41-week flock. Afterward, the yolk sac-free body mass was carefully dissected, and the liver and heart from each chick were weighed separately.

Hatchability traits

Following transfer to the hatching baskets at ED18, eggs were individually monitored at 2-hour intervals, starting at 460 h and continuing for 68 h. During this period, the occurrence of internal pipping (IP), external pipping (EP), and hatching for each embryo was recorded. Newly hatched chicks that had fully emerged from the eggs and exhibited healed navels, as well as dry plumage around the head and neck, were selected removed.

For each egg, the incubation duration was defined as the interval between setting and hatching.

At the end of incubation, hatchability of fertile eggs (HFE) was calculated according to the following equation:

Total of hatchling chicks/total of fertile eggs × 100

Hatchability of total eggs set (H) was also determined using the following equation:

Total of hatchlings chicks/total eggs set × 100

After collection, each chick was weighed to the nearest 0.1 g, measured for length, and assigned a quality score under randomized double-blind conditions, as previously described by Juárez-Estrada et al. (2024). The hatch window for each treatment group was defined as the time elapsed between the first and last chick hatched.

Embryonic mortality

All unhatched eggs at the end of the incubation period were opened and examined macroscopically by a single experienced evaluator to determine infertility and assess the developmental stage at which embryonic death occurred. The timing of embryonic death was estimated in days where feasible.

Embryonic mortality (EM) was expressed as a percentage of the total number of fertile eggs set. The EM diagnosis was classified into different intervals of embryonic development. Early embryonic death occurs during stage I (ED1 to ED7), middle embryonic death (stage II; ED8 to ED17), and late embryonic death (stage III; ED18 to ED21). Pipped but unhatched chicks prior to 528 h (PNH) were classified as stage IV (Juárez-Estrada et al., 2024).

Physical quality score for hatchlings

All one-day-old chicks were pulled out from hatching baskets once they were hatched. Every newly hatched chick was examined macroscopically to identify physical traits associated with high, good, middle, poor, and unclassified quality grades according to the methodology described by Juárez-Estrada et al. (2024). Briefly, this methodology assessed hatchling physical quality based on previous field observations of major physical conditions crucial for successful post-hatch performance (Willemsen et al., 2008). The scoring of chick hatchling quality was based on thirteen comprehensive characteristics, including body dryness and cleanness, activity level, eye appearance, retracted yolk sac, residual yolk sac, conformation of legs, tarsometatarsus integrity, toes integrity, navel appearance, presence of residual membranes and debris, vent appearance, dehydration status, body weight, and chick length. Quantitative traits, body weight (g) and chick length (cm), were recorded for each hatched chick. All assessment parameters are summarized in Table 1.

The grading score assigned to each newly hatched chick was used to generate a hatchling quality index. Each parameter was weighted according to its association with subsequent performance during rearing at the farm, as previously described by Willemsen et al (2008, 2011), and Tona et al (2022). Discrete traits were scored on a scale of 0 to 100, as outlined in Table 2.

Quality score categories were defined as follows: 90 - 100 points = high, 80 - 89 = good; 70 - 79 = moderate; 60 - 69 = poor, and <59 = unclassified. The physical quality scores assigned to each newly hatched chick were used to generate a hatchling quality index for each treatment.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) within a general linear model (GLM) (SAS/STAT 9.2, SAS Institute Inc., Cary, NC, USA). For parametric traits, including egg weight at setting, yolk-sac free embryo weight, yolk sac weight, heart and liver weights, and newborn chick weight and length, the experimental unit was the hatching egg. For hatching events, the experimental unit was the single-stage setter/

Table 1. Parameters used to assess newborn chick quality.

Parameter	Assessment*
Down and appearance	Considered normal when dry and clean. Wet and/or dirty (potential sources of contamination) were classified as poor.
Activity	Chicks were placed on their back to determine righting ability. A rapid return to standing position was considered good, whereas delayed or failed righting was classified as weak or very weak.
Eyes	Eye condition was evaluated based on brightness and degree of eyelid opening.
Retracted yolk	If abdomen height was estimated to be higher and harder than normal by palpation, then yolk retracted was regarded as large and consistent.
Residual yolk	The size of the residual yolk sac at navel area was classified as large, small, or absent.
Legs	Posture and ability to remain upright were evaluated. Kee joints were examined to detect signs of inflammation or redness.
Tarsometatarsus and toes	Integrity of the tarsometatarsus and toes was assessed. Toes were analyzed for straightness and deformities.
Navel	A navel matching the surrounding skin was regarded as normal. Redness, discoloration, a “black button” or leakage were classified as poor or very poor.
Residual membrane	Presence of membranes or debris at navel area were classified as large, small or absent.
Vent appearance	The cloaca area was regarded as normal when clean. Wetness, presence of chalky white material, dirty, or vent pasting were classified as poor.
Dehydration	Hydration status was assessed based on skin and a vascular appearance in the neck, leg, and wing, as classified as normal, mid-dehydrated (skin dry), and severely dehydrated (very dry skin).
Body weight	All one-day-old Leghorn chicks were individually weighted (g).
Chick length	Newly hatched chicks were placed on ventral side position, with the neck and right leg extended to their maximum length. Length was measured from the tip of the beak to the implantation of the nail on the third toe.

*Measurements were performed using a double-blind procedure.

hatcher, while the observational unit was the individual egg.

Prior to analysis, proportional data (hatching events and egg weight loss) were subjected to arcsine square root transformation. These data were subsequently analyzed using ANOVA with the GLM procedure. When significant treatment effects were detected, differences among means were assessed using Tukey’s post-hoc test. Results were expressed as mean ± standard deviation (SD). A chi-square test of independence was performed to assess differences in embryonic mortality stages (I, II, III, and PNH), and chick quality scoring scale on one-day-old Leghorn chicks. Statements of statistical significance were based on a threshold of $P < 0.05$.

RESULTS

Environment during incubation

During the initial 10 ED, the O_2 concentrations in the non-linear EWL and standard EWL incubators of eggs of 36-week-old breeders were measured at 19.3 and 19.7%, respectively. Non-linear EWL exhibited a carbon dioxide concentration of 1.2% at ED10, while standard incubators showed a CO_2 concentration of 0.1%. From ED10 until the start of external pipping, all incubators showed similar levels of O_2 and CO_2. At ED20, the non-linear EWL group showed 0.27% of CO_2 and 19.72% of O_2, and the standard group exhibited 0.29% of CO_2 and 19.64% of O_2. Standard EWL incubation of eggs from 41-week-old breeders at ED10 showed low

Table 2. Scoring criteria for evaluating newborn chick traits.

Parameters	Characteristics*	Scores
Down and appearance	Clean and dry	8
	Wet	4
	Dirty and wet	0
Activity	Normal	8
	Weak	4
	Very weak, chick remained lying down	0
Eyes	Both well open 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
Residual 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 button or leaky	0
Residual 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
	Dry and wrinkled skin	4
	Very dry and wrinkled skin	0
Length	> 18 cm	10
36–45 week-old	15-18 cm	4
Leghorn breeders	< 15 cm	0
Weight	> 40 g	8
36–45 week-old	37-40 g	4
Leghorn breeders	< 37 g	0

*Measurements were performed using a double-blind procedure.

carbon dioxide levels (0.08%), while under non-linear EWL conditions, CO_2 concentration rose gradually from the onset of incubation, reaching 0.72% CO_2 by ED10. At ED20, there were no differences between the non-linear EWL group (0.33% CO_2; 20.72% O_2), and the standard EWL conditions (0.34% CO_2, 20.63% O_2). The hatchery room maintained an average temperature of 24.0 °C and 46% of RH throughout the experimental period, while the mean level of O_2 was 19.9%, and 0.12% of CO_2.

Egg-weight loss

At the onset of incubation, the mean egg weight of 41-week-old breeders exceeded that of 36-week-old breeders by 3.5 g. No significant difference was observed between treatments. The non-linear EWL applied on eggs from 36-week-old breeders at ED 10 was measured at 4.46 ± 0.56%, significantly less ($P<0.05$) than the EWL observed in the standard group (5.77 ± 1.10%). However, the HR management in the non-linear EWL group from ED10 to ED18 was significantly improved; therefore, at ED18, when all hatching eggs were transferred to baskets, the non-linear EWL group exhibited a mean EWL of 10.55 ± 1.36%, which was not significantly different from the 11.13 ± 2.22% recorded in the standard EWL group, both groups showed recommended EWL at ED18 (Table 3). In the standard EWL condition of eggs from 41-week-old breeders at ED 10, EWL was measured at 6.48 ± 1.15%, being significantly higher ($P<0.05$) than the EWL observed in the non-linear EWL condition (3.04 ± 1.06%). Table 3 showed that HR management in this latter group was not enough to reach the optimal EWL at ED18. The standard EWL group at ED18 reached 10.83±1.97%, which was higher ($P<0.05$) than the 7.67±1.88 % of EWL shown by the non-linear EWL group.

Hatchability

HFE and H did not differ between non-linear and standard EWL conditions during incubation of eggs from 36-week-old breeders, with biological differences between treatments of approximately 3% (Table 4). In eggs from 41-week-old breeders, the HFE under non-linear EWL was 57.4%, which statistically was not different from the 52.6% observed under the standard EWL group (Table 4). Similarly, H was 55.3% under the non-linear, and 49.2% under standard conditions, with no significant differences between treatments (Table 4).

Embryonic mortality

Embryonic mortality rates during the early and mid-stages did not differ between the non-linear and standard EWL conditions in eggs from 36-week-old breeders. However, as shown in Table 4, late embryonic mortality was 5.87% in the non-linear EWL group, which was lower ($P<0.05$) than the 10.82% observed in the standard group. The proportion of chicks that pipped but failed to hatch did no differ between the two groups (Table 4). In eggs from 41-week-old breeders, non-linear EWL management showed 7.35% early embryonic mortality, which was lower ($P<0.05$) than the 13.42% exhibited by the standard group (Table 4). Embryonic mortality rates did not differ between incubation treatments during the mid- and late stages (Table 4). In eggs from 36-week-old breeders, non-linear and standard EWL conditions did not differ in internal pipping time or onset of hatch (Table 5). The hatch window was 37 h for under non-linear EWL, and 36 h under standard conditions. In eggs from 41-week breeders, the internal pipping of hatchlings under non-linear EWL occurred earlier (465 h) ($P<0.05$) than hatchlings under standard conditions (471 h). Similarly, external pipping occurred earlier

Table 3. Egg weight loss at 10 and 18 embryonic days (ED) under non-linear and standard egg weight loss (EWL) conditions applied to Leghorn hatching eggs from breeders aged 36 and 41weeks.

Egg mass features	Non-linear EWL 36 s	Standard EWL 36s	Non-linear EWL 41 s	Standard EWL 41 s
Egg hatching weight at set (g)	$56.50 \pm 1.47^{a*}$	$57.01 \pm 1.39^{a*}$	$60.06 \pm 4.38^{A**}$	$60.45 \pm 5.91^{A**}$
Egg weight at ED10 day(g)	53.85 ± 1.64^{a}	53.83 ± 1.50^{a}	58.23 ± 1.95^{A}	56.53 ± 4.06^{B}
Egg weight loss at ED10 day (%)	4.46 ± 0.56^{b}	5.77 ± 1.10^{a}	3.04 ± 1.06^{B}	6.48 ± 1.15^{A}
Egg weight at ED18 day(g)	50.56 ± 1.63^{a}	50.60 ± 1.83^{a}	55.45 ± 2.82^{A}	53.90 ± 3.39^{B}
Egg weight loss at ED18 day (%)	10.55 ± 1.36^{a}	11.13 ± 2.22^{a}	7.67 ± 1.88^{B}	10.83 ± 1.97^{A}
Egg weight loss ED10-ED18 (%)	6.10 ± 1.03^{a}	5.36 ± 1.38^{a}	4.63 ± 0.77^{A}	4.35 ± 1.57^{A}

*Means (±SD) within the same row that do not share a common superscript lowercase letter are significantly different ($P<0.05$).

**Means (±SD) within the same row that do not share a common superscript uppercase letter are significantly different ($P<0.05$). *n*= 42 hatching eggs per group.

Table 4. Incubation outcomes and embryonic mortality rates of Leghorn embryos incubated at high altitude under non-linear or standard egg weight loss (EWL) management.

Incubation traits	Non-linear EWL 36 s	Standard EWL 36s	Non-linear EWL 41 s	Standard EWL 41s
Fertility (%)$^{\psi}$	93.45 ± 3.58^{a}	97.02 ± 1.19^{a}	96.81 ± 1.37^{A}	93.64 ± 3.63^{A}
Hatchability of fertile eggs (%)$^{\psi}$	55.74 ± 11.86^{a}	52.63 ± 8.63^{a}	57.40 ± 5.39^{A}	52.60 ± 3.80^{A}
Hatchability of total eggs (%)$^{\psi}$	53.82 ± 11.23^{a}	50.88 ± 8.17^{a}	55.53 ± 4.94^{A}	49.17 ± 2.71^{A}
Early embryonic mortality (%)$^{\phi}$	12.43^{a}	12.09^{a}	7.35^{B}	13.42^{A}
Mid-stage embryonic mortality (%)$^{\phi}$	13.20^{a}	11.67^{a}	10.06^{A}	8.99^{A}
Late embryonic mortality (%)$^{\phi}$	5.87^{b}	10.82^{a}	22.94^{A}	22.55^{A}
Pipped, dead (%)$^{\phi}$	12.76^{a}	12.78^{a}	2.25^{A}	2.44^{A}
Total embryonic mortality (%)	44.26	47.37	42.60	47.40

$^{\psi}$ Means (±SD) within the same row and within the same breeder eggs age that do not share a common superscript letter are significantly different (P<0.05).

$^{\phi}$Means within the same row and between breeder ages that do not share a common superscript letter are significantly different (P<0.05). *n*= 42 Leghorn hatching eggs per group.

(P<0.05) under non-linear EWL (480 h) compared with the standard group (486 h) (Table 5). The hatch window was shorter under non-linear EWL than under standard conditions, reaching 30 h and 37 h, respectively (Table 5).

Quality hatchling score

Hatchlings from both non-linear and standard EWL groups of the 36-week-old breeders flock did not reach the high-quality category. The proportion of good-quality chicks did not differ between groups. However, the non-linear EWL group showed a higher proportion of medium-quality chicks (50%) than the standard group (37.2%) (P<0.05). Conversely, the standard group exhibited higher proportions of poor-quality and unclassified chicks than the non-linear EWL group (Table 6). In the 41-week-old breeders flock, hatchlings under non-linear EWL showed higher proportions of high-, good-, and medium-quality categories compared with those from the standard EWL group (P<0.05) (Table 6). In contrast, the standard group exhibited a higher proportion of unclassified chicks than the non-linear EWL group (Table 6).

In eggs from 36-week-old breeders, yolk sac-free body weight did not differ between non-linear and standard EWL conditions. However, the yolk sac mean weight was slightly higher (P<0.05) in the standard EWL group (6.02 g) than in the non-linear EWL group (5.56 g) (P<0.05) (Table 7). Heart and liver weights did not differ between groups (Table 7). In eggs from 41-week-old breeders, yolk-sac free body weight was higher (P<0.05) under non-linear EWL (15.38 g) than under standard EWL (14.07 g) (Table 7). Yolk sac and heart weights did not differ between treatments. However, liver weight was slightly higher (P<0.05) in chicks from the standard EWL group than in those from the non-linear EWL group (Table 7).

DISCUSSION

The results in previous trials using hatching eggs from breeders of different ages have suggested that HR management exerts a significant effect on non-linear EWL patterns between ED0 and ED18 (Peebles et al., 2001; Banwell, 2008). In the present research, the EWL observed in hatching eggs from 41-week-old breeders was insufficient to reach the required rate by ED18. However, the EWL in eggs from 36-week-old breeders more closely approached the EWL recommended values at this stage. Despite this, when compared to the other groups, the 41-week group showed the highest HFE, decreased early embryonic mortality, a narrower hatch window, and improved physical quality traits in the newly hatched chicks. Although these favorable outcomes may be partially attributed to early hypercapnic conditions previously described in the specialized literature (Okur, 2019; Fares et al., 2023; Juarez-Estrada et al., 2025), the non-linear EWL dynamics observed in eggs from 41-week-old breeders appeared to have improved overall performance.

Flock age is a crucial factor influencing genotype-environment interactions throughout the AI process of fertile eggs (Suarez et al., 1997; Peebles et al., 2001; Araújo et al., 2016). Banwell (2008) pointed out that beneficial effects from a non-linear EWL management are more pronounced in broiler breeder flocks aged 46 to 54 weeks (>1.43% increase in HFE) than in younger flocks aged 28 to 36 weeks (>0.46% increase in

Table 5. Internal and external pipping and hatch window of Leghorn eggs incubated under non-linear and standard egg weight loss (EWL) management.

Hatching features	Non-linear[1] EWL 36s	Standard[1] EWL 36s	Non-linear[2] EWL 41s	Standard[2] EWL 41s
Internal pipping (h)	467^{a*}	466^{a*}	465^{B**}	471^{A**}
External pipping (h)	481^{a}	479^{a}	480^{B}	486^{A}
Last hatching (h)	518^{a}	515^{a}	510^{B}	523^{A}
Hatch window (h)	37^{a}	36^{a}	30^{B}	37^{A}

*Means (±SD) within the same row that do not share a common superscript lowercase letter are significantly different (P<0.05).

**Means (±SD) within the same row that do not share a common superscript uppercase letter are significantly different (P<0.05).

Table 6. Quality scores and quantitative traits of one-day-old Leghorn chicks from eggs of 36- and 41-week-old breeders incubated under non-linear and standard egg weight loss (EWL) conditions.

Chick quality traits	Non-linear EWL[1] 36 s	Standard EWL 36 s	Non-linear EWL[1] 41 s	Standard EWL 41 s
High quality (%)	0 ± 0	0 ± 0	47.22 ± 8.8^{A}	38.00 ± 6.9^{B}
Good quality (%)	30.20 ± 9.23^{a}	21.78 ± 14.88^{a}	39.33 ± 7.41^{A}	31.29 ± 8.06^{B}
Medium quality (%)	50.0 ± 13.60^{a}	37.20 ± 19.42^{b}	10.04 ± 2.06^{A}	6.61 ± 1.15^{B}
Poor quality (%)	19.79 ± 15.22^{b}	36.90 ± 15.74^{a}	1.28 ± 0.82^{A}	2.90 ± 2.37^{A}
Unclassified (%)	0 ± 0^{b}	4.16 ± 2.71^{a}	2.13 ± 1.81^{B}	21.20 ± 7.39^{A}
Chick hatchling weight (g)	39.83 ± 1.81^{a}	39.67 ± 1.72^{a}	40.07 ± 1.09^{A}	38.80 ± 1.03^{B}
Chick hatchling length (cm)	16.65 ± 0.33^{a}	16.54 ± 0.33^{a}	16.61 ± 0.23^{A}	16.22 ± 0.49^{B}

[1] Non-linear egg weight loss was achieved by maintaining an air-tight conditions during the first 10 days of embryonic development.

^{a,b,A,B}Means (±SD) within the same row and within each breeder age that do not share common superscript letters differ significantly (P<0.05); lowercase letters refer to 36-week-old breeders, and uppercase letters refer to 41-week-old breeders. n= 62 Chick hatchlings per group.

Table 7. Yolk sac-free body weight, and yolk sac, heart, and liver weights of newborn Leghorn chicks from eggs of 36- and 41-week-old breeders incubated under non-linear and standard egg weight loss (EWL) conditions at high altitude.

Embryonic mass features	Non-linear[1] EWL 36s	Standard[1] EWL 36s	Non-linear[2] EWL 41s	Standard[2] EWL 41s
Yolk sac free body mass (g)	13.16 ± 0.99^{a*}	13.01 ± 0.74^{a*}	15.38 ± 3.19^{A**}	14.07 ± 2.28^{B**}
Yolk sac (g)	5.56 ± 1.17^{b}	6.02 ± 1.24^{a}	6.48 ± 1.33^{A}	6.51 ± 1.06^{A}
Heart weight (g)	0.25 ± 0.05^{a}	0.24 ± 0.05^{a}	0.32 ± 0.05^{A}	0.32 ± 0.03^{A}
Liver weight (g)	0.76 ± 0.05^{a}	0.75 ± 0.05^{a}	0.92 ± 0.10^{B}	1.01 ± 0.10^{A}

*Means (±SD) within the same row that do not share a common superscript lowercase letter are significantly different (P<0.05).

**Means (±SD) within the same row that do not share a common superscript uppercase letter are significantly different (P<0.05). [1] n= 20 [2] *n*= 36 hatchlings per group.

HFE), a period in which successful hatching becomes difficult (Suarez et al., 1997; Bruzual et al., 2000; Peebles et al., 2001; Celen et al., 2009).

De Smit et al. (2006) observed a beneficial hatchability effect using hypercapnic incubation in older breeder hens (60-week-old) compared to intermediate-age-old breeder hens (45-week-old). The authors describe an epigenetic effect after the hypercapnic incubation was performed during the first half of embryonic development. In the present study, it is feasible that non-linear EWL management applied to the 41-week group may have induced epigenetic effects; however, further research is needed to test this hypothesis. At ED18, De Smit et al. (2006) reported a mean EWL of 8.72%, in hatching eggs from 60-week-old breeder hens incubated under airtight conditions, compared with 10.06% in the standard group. Notably, the EWL observed under airtight conditions at ED18 was closer to that recorded in the present study for the 41-week non-linear EWL group (7.67%), indicating that a similar non-linear EWL effect may have occurred in this airtight treatment. Although the EWL of the standard group observed by De Smit et al (2006) in 60-week breeder eggs was closer to the recommended EWL at ED18, this group exhibited lower hatchability (<3.9%) compared to the airtight condition; these results partially agree with the finding of the present study regarding the 41-week-old non-linear EWL group when compared with the standard group. Although the airtight conditions applied to hatching eggs from to 41-week-old breeders significantly increased CO_2 concentrations at ED10, early embryonic mortality was reduced, resulting in improved overall hatchability in this group.

The effect of breeder age on optimal hatchability traits has become the subject of several investigations (Celen et al., 2009; Grochowska et al., 2019). Recently, there has been increasing interest in the physical quality of chicks at hatch, and several studies suggest the optimal hatchability traits are associated with breeders of intermediate-age-old (Suarez et al., 1997; Celen et al., 2009; Araújo et al., 2016; Grochowska et al., 2019). Araújo et al. (2016) found an interaction between breeder age and incubator type (single-stage or multi-stage) only on hatchling physical quality. The authors concluded that the physical quality of chicks derived from eggs from intermediate-age-old breeder hens was superior when eggs were incubated in single-stage incubators. In the present study, the non-linear EWL protocol applied to eggs from 41-week-old breeder hens incubated in this type of incubators resulted in nearly half of the hatched chicks being classified in the highest physical quality grade, in contrast with the standard EWL group, in which fewer chicks achieved the same classification. This finding may be directly related to the breeder age, as 36-week-old breeder hens failed to produce any hatchlings classified within the highest quality grade, irrespective of the EWL protocol used.

The physical quality score was the most important hatchability variable, as shown by intermediate-aged breeders with a non-linear EWL incubation approach. When eggs are incubated at high altitudes, increased EWL, slower embryonic developmet, prolonged incubation periods, and reduced hatchability are generally expected (Visschedijk, 1985; Lan et al., 2012; Ahmed et al., 2013; Tullet, 2013; Yilmaz-Dikmen et al., 2014; Okur et al., 2022).

Due to the limitations described before, under standard incubation conditions above 1,000 m above sea level, the manufacturer (Hova-Bator®) recommends full opening of dampers to enhance O_2 intake. However, this practice may have contributed to the high early embryonic mortality observed in the hatching eggs from the 41-wek flock under standard EWL protocol. this effect may be attributed to early temperature and RH variations within standard incubators, while the airtight conditions applied in the non-linear EWL treatment likely mitigated these environmental oscillations during the most critical period of the embryonic development (ED1-ED10) (Lourens et al., 2005; Lan et al., 2012; Juarez-Estrada et al., 2025). Araújo et al (2016), reported higher early embryonic mortality (0-4 days) in embryos from young breeders (<30 weeks), regardless of incubator type (single-stage or multi-stage), while Okur (2019) observed increased early mortality in lighter eggs compared with heavier eggs.

Our results from 36-week-old breeder hens support these findings, as higher mortality rates were observed in both 36-week groups when compared to the non-linear EWL group from 41-week-old breeders. A critical factor that influenced the poor HFE and physical quality of hatchlings in the standard groups was the high altitude of the hatchery setter (Ahmed et al., 2013; Okur et al., 2022). Although both standard groups showed an apparently normal incubation trajectory (e.g., appropriate EWL at ED10 and ED18), evidence suggests that, during the final stage of embryonic development, limitations in O_2 exchange capacity and maintenance of embryonic thermoregulatory systems (eggshell temperature) adversely affected outcomes (Lourens et al., 2007). This effect was particularly pronounced in the standard EWL group from 36-week old breeders, which showed nearly double late embryonic mortality compared with the non-linear EWL group (10.8 vs 5.8%, respectively). In contrast, eggs from 41-week-old breeder hens did not show any difference between EWL groups in late or pipped-but-unhatched embryonic mortality. However, the narrower hatch window in the non-linear EWL group from 41-week-old breeders contributed to achieving a quick withdrawal of a larger quantity of high-quality chicks, reducing the risk of early dehydration in the hatcher (Fares et al., 2023).

All gases are subject to the inverse relationship between gas conductance and barometric pressure (Ar and Deeming, 2009). Accordingly, although the partial pressure of oxygen in the ambient air decreases with increasing altitude, its diffusion into the egg is facilitated, whereas CO_2 and H_2O vapor escape more easily from the egg as the altitude increases (Visschedijk, 1980). However, the poor O_2 concentration in the gas exchange rate in the EWL standard group from 36-week-old breeders might have been associated with the lower temperature and RH recorded throughout the incubation period. This effect, combined with the reduced partial pressure of O_2 typically observed at 2,230 m of altitude (~16.38 kPa) (Visschedijk, 1985; Celen et al., 2009; Lan et al., 2012)), likelycontributed to high mortality in all groups, but with greater impact on both standard incubation treatments

Although standard groups showed normal EWL values at ED18, a methodological limitation related to the recording of hatch times may have contributed to the high embryonic mortality observed at the late and pipped-but-unhatched stages across all groups and breeder ages. Incubators were opened every 2 h after internal pipping to observe hatching events. However, under commercial incubation conditions, it is strongly recommended to avoid opening incubators or hatchers during operation, as this could disrupts the internal environment, leading to great oscillation in temperature and RH, which can have negative consequences on the survival of the weaker embryos (Lourens et al., 2007). While this methodological factor could have affected all groups equally, the uncertainty factor, and consequently the lower HFE could have been magnified compared to the optimal HFE to obtain at high altitudes (Visschedijk,1985; Lan et al., 2012; Ahmed et al., 2013; Juarez-Estrada et al., 2025).

After ED10, the non-linear EWL management should be adjusted through RH control to promote adequate weight loss, such that by ED18 it approximates the levels typically recommended under standard sea level incubation conditions (10.4 -12%). In this context, Banwell (2008) found that a non-linear EWL group lost 10.8% at ED18 in a non-linear treatment, with higher hatchability than in the standard EWL. More recently, Juárez-Estrada et al. (2025) obtained similar results in this type of non-linear EWL group incubated under an early hypercapnia regimen at high altitude. The authors also determined a positive effect of the interaction of high $>CO_2$ and $>H_2O$ during the first half of embryonic development on HFE and physical quality hatchling traits. According to Peebles (2023), a substantial proportion of early embryonic mortality may be attributed to variations in embryonic gas exchange capacity, while most mortality occurring after ED14 is associated with EWL imbalances, resulting from either insufficient or excessive water loss during the early stage of incubation. Inappropriate EWL can hamper proper air cell formation at the time of internal pipping (Ar and Deeming, 2009). In larger eggs, Lourens et al. (2005) reported that the thermoregulation stress of the embryo might easily occur due to the increase in egg water loss. One of the most important aspects of performing a non-linear EWL approach during AI is to adjust RH during the last stages of embryonic development. To ensure an appropriate EWL during incubation and achieve the optimal EWL at 18 ED, some commercial incubator manufacturers (e.g. BioStream™) have incorporated certain RH management systems into equipment, such as Dynamic Weight Loss System™ (Petersime). This management approach promotes an optimal air cell development to optimize pulmonary respiration, leading to healthier chicks. Such dynamic EWL systems contribute to ensuring an optimal weight loss trajectory with a non-linear EWL profile that closely resembles the natural incubation conditions in which hens incubate their own eggs within the nest (Walsberg, 1980). This specific RH management continuously weighs the eggs at short intervals and dynamically adjusts the incubator RH to maintain the desired EWL profile.

Juarez-Estrada et al. (2025) found that a hypercapnic incubation at high altitude of hatching eggs from broiler breeders hens contributed to improved early fluid retention during the first 18 days of incubation, in a comparable manner to the non-linear EWL approach evaluated in the present study. Hypercapnic conditions have also been shown to increase yolk-free embryonic mass while reducing yolk sac weight through embryonic development (De Smit et al., 2006; Fares et al., 2023; Juarez -Estrada et al., 2025).

In addition to these similarities, the non-linear EWL condition applied in Leghorn breeder hatching eggs in the present study also favored increased chick length at hatch and overall physical chick quality. As noted by Bruzual et al.

(2000), hatchability and yolk-free embryo mass are parameters that can be effectively controlled through careful monitoring of EWL, and chick quality can significantly be improved when this factor is optimal managed. Accordingly, EWL should be constantly monitored throughout incubation, with particular attention to eggshell conductance to water vapor, which is intrinsically linked to breeder age and nutritional status, and it tends to increase as breeder hens age (Ar and Deeming, 2009; Araújo et al., 2017; Grochowska et al., 2019).

During EWL monitoring, not only should daily weight loss be assessed and RH adjusted accordingly, but the eggshell temperature must also be carefully monitored for adjust incubation conditions to specific requirements for each batch of incubated eggs; hatchability is primarily influenced by temperature and ambient gas tensions, particularly O_2, CO_2 and H_2O vapor (Visschedijk, 1980; Lourens et al., 2005 and 2007; Ar and Deeming, 2009; Mueller et al., 2022).

Hatchability at high altitude depends on intrinsically eggshell conductance and O_2 supply; however, not only must the partial pressures of O_2 and H_2O in the ambient be considered at high altitude, but CO_2 levels must also be regulated under these conditions (García et al., 2013; Fares et al., 2023; Juarez-Estrada et al., 2025; Vieira et al., 2025). As hatchability appears to be correlated to embryonic metabolic rate under reduced partial pressure of O_2 in the ambient air, further studies are necessary to evaluate the combined effects of barometric pressure and abnormal EWL rates throughout AI period. In particular, studies should focus on O_2 consumption and CO_2 production, and their influence on HFE and the physical quality of newly hatched chicks.

At present, the most practical approach to optimizing non-linear EWL management while allowing a controlled increase in CO_2 during the early stage of embryonic development or at internal pipping is the use of single-stage incubators, which have shown clear advantages in recent studies (Araújo et al., 2016; Grochowska et al., 2019; Juarez-Estrada et al., 2025). Hatchery efficiency is mainly based on hatchability and the proportion of salable, high-quality chicks. In this context, the incorporation of novel methodologies, such as non-linear EWL, into modern single-stage incubation systems may contribute substantially to reaching these production goals.

CONCLUSIONS

Non-linear management of egg weight loss (EWL) at day 10 of embryonic development of Leghorn breeder eggs incubated at high altitude, followed by an accelerated weight loss phase to achieve the recommended egg weight loss at 18 day of embryonic development, indicates that non-linear EWL management resulted in a significant improvement of the physical quality traits of hatchlings compared with the standard EWL pattern.

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Author contributions

All authors contributed actively to the bibliographic review, methodology, data interpretation, and manuscript preparation, and reviewed and approved of the final version of the article.

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