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Impact of late-stage hypoxic stimulation and layer breeder age on embryonic development, hatching and chick quality

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ABSTRACT

The present study examined the effects of breeder age and oxygen (O_2) concentrations during the late chorioallantoic membrane **(CAM)** growth stage on embryo development, hatching dynamics, chick quality, bone mineralization and hatchability. A total of 1200 eggs from 33- and 50-week-old ISA layer breeders, weighing 53.85 g and 60.42 g on average respectively, were incubated at 37.7°C and 56 % relative humidity. From embryonic day (ED) 13 to 15, experimental eggs were exposed to hypoxia (15 % or 17 % O_2 for 1 hr/day) while the control was at 21 % O_2. Results showed significant interactions ($p = 0.040$) between breeder age and oxygen level, with embryos exposed to 15 % and 17 % O_2 exhibiting slower growth by ED 17. However, embryo weight at internal pipping (IP) was unaffected ($p > 0.05$). At hatch, chick weights were higher in hypoxic groups due to increased yolk sac retention ($p = 0.024$), while yolk-free weights were influenced only by breeder age ($p < 0.001$). Hypoxia at 15 % O_2 reduced chick length, toe length, and tibia parameters ($p < 0.05$), likely due to impaired calcium and phosphorus absorption. Embryos exposed to 15 % O_2 had longer internal and external pipping events, delaying hatch time. Embryonic mortality was highest ($p < 0.001$) at 15 % O_2, contributing to the reduced hatch of fertile eggs. This research demonstrates that controlled hypoxic conditions can slow embryonic development, conserve yolk nutrients, improve organ maturation and chick weight across breeder ages.

Introduction

The developmental environment of avian embryos significantly influences their growth, survival, and overall quality at post-hatch. Gas exchange during incubation is essential for embryonic development, hatching performance, and chick quality. Adequate oxygen supply and carbon dioxide removal are crucial for normal embryo development (Ar and Deeming, 2009). This process is influenced by factors such as egg structure, environmental contaminants, and energy sources. The spherical shape of avian eggs promotes gas exchange, facilitated primarily by the chorioallantoic membrane **(CAM)** and eggshell porosity (Barta and Székely, 1997; Onagbesan et al., 2007). Oxygen is critical for metabolic processes throughout development (Marsico et al., 2023) with increasing O_2 consumption and CO_2 production as the embryo matures (Decuypere et al., 2001; Fernandes et al., 2014). Hypoxia, a condition characterized by reduced oxygen levels, is experienced at high altitudes. At low altitude, during incubation the quality of eggshell could influence the exchange of O_2 and CO_2 (Silva et al., 2017). A potential imbalance of these factors could lead to a hypoxic or hypercapnic environment for the developing embryo. Hypoxia can disrupt metabolism and cause developmental challenges in embryos, however, controlled hypoxic conditions may enhance cardiovascular and respiratory development, which may improve chick quality and post-hatch performance (Druyan et al., 2018; Ben-Gigi et al., 2021; Haron et al., 2022).

Pre-incubation and incubation factors significantly affect oxygen consumption and embryo development. Key factors include parental flock age, egg weight, storage conditions, eggshell conductance, temperature, humidity, and O_2/CO_2 concentrations, as well as environmental altitude (Bergoug et al., 2013; Kasielke, 2020; Narinç et al., 2021; Abd Abd El-Hack et al., 2022; Meijerhof, 2022; Tona et al., 2022; Tainika et al., 2024). At high altitudes, where oxygen levels are lower, Bahadoran et al. (2010) found that chicks incubated at these altitudes hatched earlier and had higher weights than those incubated at lower elevations. Tibetan chickens exhibit superior chick quality in such

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environments due to genetic, physiological, and microbial adaptations (Zhang et al., 2007; Li and Zhao, 2009; Wang et al., 2015; Zhou et al., 2016; Huang et al., 2019; Li et al., 2019).

Breeder age is known to significantly influence the development of embryos during incubation, affecting various parameters such as hatchability, chick quality, and overall embryonic development. Research indicates that as breeders age, the quality of the eggs they produce declines, primarily due to changes in eggshell characteristics and nutrient composition. Older breeders tend to lay eggs with thinner shells that have a higher number of pores, which can lead to increased moisture loss during incubation and higher embryo mortality rates (Araújo et al., 2016; Prado-Rebolledo et al., 2023). This decline in eggshell quality is critical as it affects gas exchange and moisture retention, both of which are essential for successful embryonic development (Araújo et al., 2016; Prado-Rebolledo et al., 2023). Moreover, the size and composition of eggs also change with breeder age. Studies have shown that eggs from older breeders are generally larger and contain more yolk and albumen, which can enhance the nutrient availability for the developing embryo (Alo, 2024; Avcılar, 2023). This increased yolk weight is particularly beneficial as it provides essential energy and nutrients, allowing embryos from older breeders to utilize these resources more effectively during the later stages of development (Machado et al., 2020; Souza, 2023).

Consequently, embryos from older breeders often exhibit greater tissue gain and higher overall weights compared to those from younger breeders (Machado et al., 2020; Souza, 2023). In terms of hatchability, eggs from younger breeders typically exhibit better hatch rates and lower embryonic mortality compared to those from older breeders (Alsobayel et al., 2012; Silva et al., 2017). This discrepancy can be linked to the overall quality of the eggs, as younger breeders produce eggs with thicker shells that provide a more stable environment for embryo development (Prado-Rebolledo et al., 2023; Alsobayel et al., 2012). However, it is also noted that while younger breeders may have higher initial hatchability, the chicks that do hatch from older breeders often show improved growth performance and robustness due to the richer nutrient profile of their eggs (Machado et al., 2020; Silva et al., 2017).

The physiological responses of embryos to incubation conditions can vary based on the age of the breeder. For example, embryos from older breeders have been shown to have better adaptability to temperature fluctuations during incubation, which can enhance their survival rates and overall development (Yalçın et al., 2012; Nangsuay et al., 2016). Similarly, oxygen levels within the incubator significantly impact the embryo’s physiological and metabolic processes. This adaptability is partly attributed to the higher lipid content in the yolk of eggs from older breeders, which plays a crucial role in thermoregulation and energy provision during critical developmental phases (Yalçın et al., 2012; Koppenol et al., 2015).

The CAM plays a critical role in calcium **(Ca)** and phosphorus **(P)** absorption from the eggshell and yolk. Mobilization of Ca from the eggshell occurs mainly between days 10 and 12 of incubation (Torres and Korver, 2018) and peaks around day 17 (Obara et al., 2022). Responsive to hypoxia, the CAM undergoes distinct developmental stages, with regression starting at embryonic day 13 (Harper et al., 2021). Chorioallantoic membrane development and vascularization differ across breeder ages under hypoxic conditions, with older breeders exhibiting greater CAM weight, vascular density, and fractal dimension when exposed to early hypoxia (Agbehadzi et al., 2024). Embryonic development exhibits stage-specific metabolic and morphological responses to hypoxia (Dzialowski et al., 2002; Molenaar et al., 2010) indicating that the effects of hypoxia on growth vary across developmental phases. The intensity and duration of hypoxia are critical factors in shaping embryonic growth outcomes (Chan and Burggren, 2005; Zhang and Burggren, 2012). In broilers, the interaction between breeder age and incubator oxygen levels has been shown to influence yolk-free embryo weight at embryonic day (ED) 18 and not at ED 14 (Nangsuay et al., 2021). The oxygen demand and hypoxic tolerance of the embryo are lowest in the first five days of incubation and increase over time (Taylor et al., 1971; Everaert et al., 2007).

Although prior research primarily focused on hypoxic effects on broiler egg incubation because of the high metabolic response during embryogenesis, literature, specifically on layer breeder eggs exposed to hypoxia during incubation remain scarce. Broilers and layers are different breeds of chickens that have different developmental trajectories during incubation (Tona et al., 2001; Hamidu et al., 2011) and therefore may respond differently and in terms of age to hypoxic stimulation. The present study therefore evaluates the effects of layer breeder age and late-CAM-stage (ED 13-15) hypoxia on embryonic traits, hatching dynamics, chick quality, calcium and phosphorus uptake in bones, embryonic mortality and hatchability of fertile eggs.

Materials and methods

Experimental site, ethics and facilities

The current study was conducted at the hatchery, research farm, and laboratory facilities of the Regional Center of Excellence for Poultry Sciences **(CERSA)** at the University of Lomé, Togo. All experimental protocols adhered to ethical standards and were approved by the Animal Ethics and Scientific Committee following the CERSA-UL guidelines (Approval No. 008/2021/BC-BPA/FDS-UL). The experimental site and incubators were located at a geographical position of 6°1′95″N latitude, 1°2′53″E longitude, and an elevation of 26 meters above sea level (Google, 2024).

Experimental design

A total of 1,200 hatching eggs were utilized in a 2 × 3 factorial experimental design, incorporating two breeder flock ages (33 and 50 weeks) and three oxygen concentration (O_2) levels. The experimental groups were maintained at 15 % and 17 % O_2, while the control group was set at 21 % O_2. Each age group received 600 eggs, divided equally across the three O_2 levels, with 200 eggs assigned to each concentration. Each O_2 group was subdivided into four replicates of 50 eggs, which were incubated on separate setter trays. To achieve the target O_2 concentrations of 15 % and 17 %, a controlled air-N_2 mixture was introduced into the experimental incubators (PasReform, Zeddam, Netherlands, SmartPro Combi model) for 1 h per day from embryonic day 13 to 15. oxygen (O_2) levels within the incubators were continuously monitored and maintained using an O_2 gas detector (Model: HFP-1201 BX, Xi’an Huafan Technology Co., Ltd., China) (Druyan et al., 2012; Zhang and Burggren, 2012).

Hatching Eggs, Storage and Incubation Conditions

Hatching eggs with average weights of 53.85 ± 2.40 g and 60.42 ± 2.02 g were collected from ISA Brown breeder flocks at 33 and 50 weeks of age, respectively. The eggs were stored for 4 days at a temperature of 18°C and a relative humidity of 75 %. Subsequently, the eggs were prewarmed at 24°C for 6 h, individually weighed, and numbered before incubation. Hatching eggs were maintained at an incubation temperature of 37.7°C and a relative humidity of 56 %, with automated turning at a 90° angle every hour until embryonic days **(ED)** 18 before transfer into the hatcher. Experimental eggs (15 and 17 % O_2 level) were subjected to an air-N_2 flushing to lower the oxygen concentration for 1 hr from ED 13 to 15. Embryos were returned to the normal incubation condition after the 1 hr exposure period. On day 18 of incubation, all eggs were candled and those showing signs of viable embryos were weighed and transferred from the turning trays to hatching baskets, where they remained under standard conditions until hatching on ED 21.

Data collection

Egg weight loss, embryo and embryonic characteristics measurements

After the exposure period, on ED 17 and during internal pipping, a total of 12 live embryos per treatment were used for embryo and embryonic development measurements which included egg weight loss, eggshell weight, embryo weight, embryo length, residual albumen weight and residual yolk weight. All absolute weight(s) including embryo and embryonic characteristics were measured using a sensitive weighing scale (Ohaus STX8200 Scout) and expressed as a percentage of egg weight using the formulas below (Biesiasa-Drzazga et al., 2022).

- Egg weight loss (%) = [(initial weight, ED 0 (g)) - final egg weight, (g)) / (initial egg weight, ED 0(g))] × 100;
- Embryo weight (%) = [(embryo weight (g)) / (egg weight (g))] × 100;
- Residual embryonic weight (%) = [(residual embryonic weight (g)) / (egg weight (g))] × 100;
- Residual albumen weight (%) = [(residual albumen weight (g)) / (egg weight (g))] × 100;
- Residual yolk-sac weight (%) = [(residual yolk weight (g)) / (egg weight (g))] × 100;
- Embryo length was measured with a compass from the tip of the beak to the tip of the middle toes and then placed on a meter rule to determine the length (Browne, 2006; Willemsen et al., 2011; Agyekum et al., 2022).

Hatching events

Between 445 and 508 h of incubation, eggs were screened for internal and external pipping **(IP and EP)** using light. Internal pipping was identified when the embryo's beak pierced the inner shell membrane, while external pipping was marked by a crack in the eggshell. IP eggs were monitored every 3 h for EP and moved to separate baskets for chick emergence **(CE)**. The times for IP, EP and CE were recorded to calculate their averages and estimate hatching durations. Incubation duration **(Dur)** for each stage was defined as the time between setting and the specific event, following Meteyake et al. (2023). The incubation and hatching durations were estimated as follows:

- IP Dur = EP Time - IP Time
- EP Dur = CE Time – EP Time
- Hatch Dur = CE Time - IP Time
- Hatch window = time of the last chick hatched when incubation was stopped – time of the first chick hatched.
- The spread of hatch was estimated in percentiles by considering the average of chicks hatched at 25 %, 50 %, 75 % and 100 % of hatched eggs (Tona et al., 2008)

Post-hatch chick quality assessment

The quality of day-old chicks hatched was assessed for each treatment according to chick weight, yolk-free chick weight **(YFCW)**, yolk sac weight, external quality measurements and Tona chick score (Tona et al., 2003). The yolk sac weight was expressed as a percentage of chick weight. The formulas used are below:

- Yolk sac weight (%) = [(yolk sac weight (g)) / (chick weight (g))] × 100.

A total of twelve (12) chicks per treatment were randomly selected for measurements of external qualities, including chick length, shank length, and toe length. Chick length was measured from the beak tip to the middle toe, while the shank length was measured from the tip of the shank to the midpoint between the feet using a compass and dimensions taken on a ruler (Hamidu et al., 2011; Willemsen et al., 2011; Agyekum et al., 2022). Chick quality was scored using the Tona scoring method which is based on physical parameters, including reflex or activity, down and appearance, eyes, leg conformation, navel area, yolk sac, remaining membrane and remaining yolk. The total score was estimated by the summation of all the scores observed from each parameter.

Tibia and femur morphometric measurement

The tibia and femur of 12 chicks were removed and air-dried for 72 h. The weight, length and diameter (width at the midpoint and endpoint) were measured using a highly sensitive scale (Ohaus STX8200 Scout) and digital Vernier caliper. The relative tibia or femur weight, Seedor index (SI), and robusticity index (RI) were calculated using the following formulas by Riesenfeld (1972) and Evaris et al. (2021):

Relative tibia or femur weight (%) = [(tibia or femur weight)/(yolk free chick weight)] × 100;

$$SI = \frac{weight\ of\ bone\ (g))}{length\ bone\ (cm)};\ RI = \frac{length\ of\ bone\ (mm)}{\sqrt[3]{weight\ of\ bone\ (g)}}$$

Determination of calcium and phosphorus in the tibia and femur bone of chicks at hatch

To determine the calcium **(Ca)** and phosphorus **(P)** content in the tibia and femur bones of day-old chicks, eight samples from each treatment group were cleaned with alcohol and benzene for 96 h and dried in an oven (Memmert Universal Oven U, Germany) at 105°C until a constant weight was achieved. The specimens were burned to ashes at a temperature of 550°C for 6 hr in a muffle furnace (Nabertherm GmbH, Bahnhofstr 20, 28865 Lilienthal/Bremen, Germany). The Ca content was determined by titration with $KMnO_4$ in a 0.02 N EDTA solution from a red to blue endpoint (Moss, 1961; Okalebo et al., 2002; Song et al., 2022). Calcium in samples was estimated as follows:

Ca (mg) = Titer value of EDTA x 0.4008

$$\text{Ca } (\%) = \frac{mg\ Ca}{\text{Sample wt x volume}}\text{x100}$$

The phosphorus concentrations were measured on the Spectronic 20 spectrophotometer to give absorbance measurements at a wavelength of 420 nm. The observed absorbance was used to determine the P content from the standard curve (Okalebo et al., 2002). The percentage of P was calculated as:

$$\text{P content (g) in 100 g sample (P \%)} = \frac{C\ x\ df\ x\ 100}{1\ 000\ 000} = \frac{C\ x\ 1000\ x\ 100}{1\ 000\ 000} = \frac{C}{10},$$

Where C = concentration of P (µg/ml) as read from the standard curve; *df* = dilution factor, which is 100 *10 = 1000.

Hatching performances and embryo mortality

At the end of incubation, the number of hatched chicks was recorded for each treatment group to determine hatching percentages based on the total number of eggs set, fertile eggs and hatched chicks. The hatch of fertile (**HOF**) eggs was quantified as the percentage of chicks hatched from fertile eggs. This metric was calculated separately for each combination of breeder age and oxygen (O_2) level across the treatment groups using the following formula:

- Hatch of fertile (HOF) = [(chicks hatched / fertile eggs) x 100]

Unhatched eggs were counted and visually examined and classified as embryonic mortality across distinct developmental stages. The classification criteria were adapted from Lourens et al. (2006) with minor modifications to align with the observed progression of chorioallantoic membrane (CAM) development in the present study. Embryonic mortality was categorized as follows: early stage (1–11th embryonic day [ED]), middle stage (12–15th ED), late stage (16–19th ED), and pipping stage (19th ED to hatch). Total mortality was added by summing all stages of embryonic mortality. Early-stage embryonic mortality was not considered although they counted to avoid error in mortality estimation, since reduced oxygen effects were only at the middle stage. The specific modifications to the original classification are illustrated in Fig. 1.

Statistical analysis

The experimental samples in the current research were eggs, embryos and chicks. In Minitab Statistical Software, version 21.2 (Minitab, LLC, NY, US, 2021), the data collected were arranged as completely randomized design with a 2 × 3 factorial arrangement of treatments and subjected to a two-way ANOVA using the model: $\mathbf{Yijk} = \boldsymbol{\mu} + \mathbf{Ai} + \mathbf{Oj} + \mathbf{AO_2ij} + \mathbf{eijk}$; where **Yijk** is the variable measured, **μ** is the general mean, **Ai** is the main effect of breeder age (i = 33- or 50wks), $\mathbf{O_2j}$ is the effect of oxygen concentration (j = 15 %, 17 % or 21 %), $\mathbf{AO_2ij}$ is the interaction term between breeder age and oxygen concentration in the incubator and **eijk** is the random residual error term. The resulting relative embryo weights and relative yolk weights were corrected by analysis of covariance with their initial egg weights. Analyses for data (expressed as percentages) were conducted after the square root of the arc sine transformation of the data. Mean comparison and separation were done using the Tukey Test at a significance of $P < 0.05$.

Results

Egg weight loss and embryonic characteristics

Tables 1 and 2 present the effects of breeder age and oxygen **(O_2)** concentration level on egg weight loss and embryonic characteristics at embryonic day (ED) 17 and internal pipping (IP). Following the exposure to air-N_2, the results in Table 1 showed a significant interaction between breeder age and O_2 levels on embryo length ($p = 0.042$), with embryos from 33-week-old breeders at 17 % and 21 % O_2 showing greater lengths compared to 50-week-old breeders at 15 % O_2. Relative embryo weight ($p = 0.040$) and absolute embryo weight ($p = 0.007$) also demonstrated interaction effects, with older breeders (50 weeks) showing higher egg weight loss ($p = 0.007$) and lower absolute ($p = 0.011$) and relative embryo weights ($p < 0.001$) compared to younger breeders (33 weeks). Embryos incubated under 15 % O_2 had the lowest absolute embryo weight ($p < 0.001$) and highest residual yolk sac and albumen weights ($p = 0.002$ and $p = 0.001$, respectively). In comparison, 21 % O_2 resulted in the highest egg weight loss ($p = 0.002$).

In Table 2, the egg weight loss and embryonic characteristics at internal pipping are displayed. Results showed no significant interaction between breeder age and O_2 levels on egg weight loss, embryo weight, or relative embryonic weight. A trend was observed for yolk sac weight, especially in older breeders at 15 % O_2, but it wasn't significant ($p = 0.525$). Oxygen levels, however, significantly influenced yolk sac weight relative to both embryo and egg weight, with 15 % and 17 % O_2 showing higher values than 21 % O_2 ($p = 0.003$ and $p = 0.002$, respectively).

Hatching events

Pipping Time and Duration

Table 3 shows the impact of breeder age and oxygen levels on pipping and hatching times. The interaction between these factors significantly affected external pipping **(EP)** time ($p = 0.001$), chick emergence **(CE)** time ($p = 0.030$), internal pipping **(IP)** duration ($p < 0.001$), and hatch time ($p = 0.009$). Breeders at 33 weeks of age incubated at 15 % O_2 had the longest EP (485.21 hr) and CE (495.45 hr) times, while 21 % O_2 resulted in the shortest times (459.20 hr and 468.11 hr). IP duration was also longest at 15 % O_2 (28.38 hr) and shortest at 21 % O_2 (6.83 hr) for 33-week-old breeders compared to those at 50 weeks. Breeder age influenced all hatching times and durations except EP duration ($p = 0.759$) and hatch window ($p = 0.479$). Older breeders (50 weeks) generally exhibited longer incubation times, including IP (460.81 hr vs. 454.66 hr, $p < 0.001$) and hatch time (33.55 hr vs. 27.19 hr, $p < 0.001$). Oxygen levels significantly affected EP time, CE time, IP duration, and hatch time (all $p < 0.001$), with lower O_2 levels leading to prolonged hatching. The 21 % O_2 level led to the shortest IP duration (11.22 hr) and hatch time (20.05 hr).

Internal and external pipping percentile of chicks

The IP and EP of the total embryo expressed in percentile are displayed in Table 4. Breeder age and oxygen level interaction significantly impacted internal pipping at the 100th percentile ($p = 0.017$), with 33-week breeders at 17 % O_2 showing the longest time (486.00 hrs) compared to 21 % O_2 (464.00 hrs). No significant interaction was found at the 25th, 50th, or 75th percentiles of internally pipped embryos. Breeder age significantly affected the 75th percentile ($p = 0.002$), with 50-week breeders taking longer (470.67 hrs). Oxygen level significantly influenced total internally pipped embryos at the 25th, 50th, 75th and

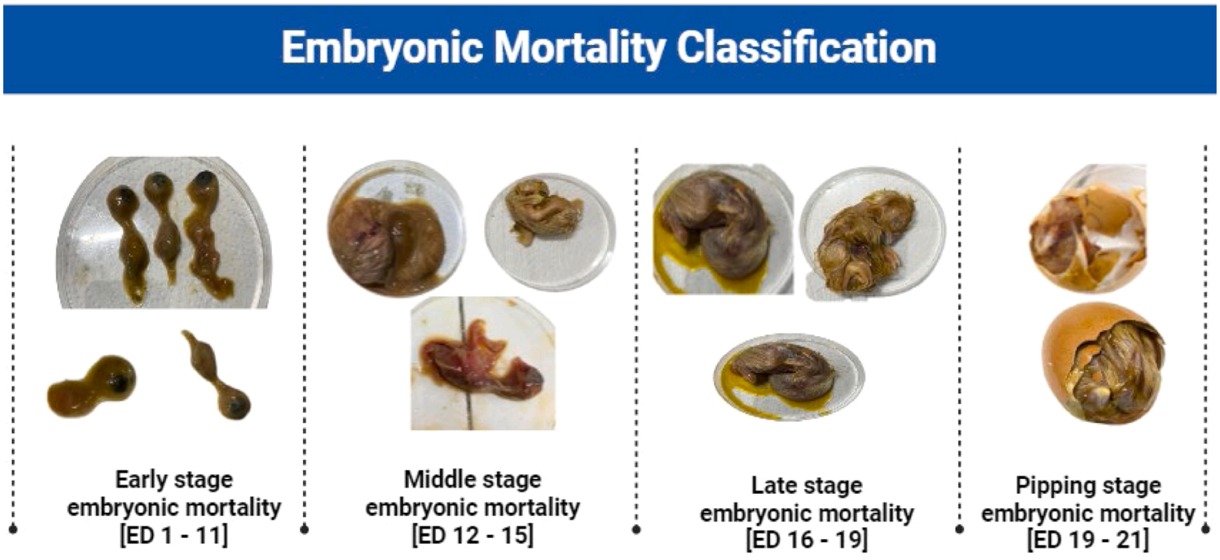

Figure 1. Stages of classification of embryonic mortality.

Table 1

Effect of layer breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on embryonic quality at embryonic day (ED) 17 after exposure.

Parameters	Egg weight loss (%)	Eggshell weight (%)	Embryo length (cm)	Absolute embryo weight (g)	Relative embryo weight[3] (%)	Residual embryonic weight (%)	Residual yolk sac weight (%)	Residual albumen weight (%)
Breeder age (A_b)								
33wks	9.37^{b}	11.82	10.39	15.31^{a}	32.09^{a}	88.22	18.58	3.77
50wks	10.40^{a}	11.89	10.33	14.35^{b}	28.62^{b}	88.85	18.83	4.13
SEM[1]	0.259	0.250	0.166	0.262	0.594	0.266	0.633	0.341
Oxygen level (O_2)								
15 %	8.98^{b}	12.06	9.52^{b}	13.51^{b}	27.34^{c}	88.56^{ab}	20.72^{a}	5.29^{a}
17 %	10.04^{ab}	11.65	10.91^{a}	15.37^{a}	30.33^{b}	89.29^{a}	18.90^{ab}	3.17^{b}
21 %	10.64^{a}	11.84	10.65^{a}	15.60^{a}	33.39^{a}	87.75^{b}	16.51^{b}	3.39^{b}
SEM[1]	0.318	0.306	0.203	0.321	0.728	0.326	0.775	0.417
Interaction (A_b * O_2)								
33wks * 15 %	8.64	12.32	9.54^{bc}	13.87	29.59^{b}	87.68^{bc}	20.27	5.08
33wks * 17 %	9.19	11.44	10.90^{a}	15.53	31.86^{ab}	88.67^{abc}	18.36	3.14
33wks * 21 %	10.28	11.70	10.73^{ab}	16.52	34.82^{a}	88.31^{abc}	17.12	3.08
50wks * 15 %	9.32	11.80	9.50^{c}	13.16	25.08^{c}	89.43^{ab}	21.16	5.50
50wks * 17 %	10.89	11.87	10.92^{a}	15.22	28.81^{bc}	89.92^{a}	19.44	3.20
50wks * 21 %	10.99	11.98	10.58^{abc}	14.68	31.96^{ab}	87.19^{c}	15.90	3.70
SEM[1]	0.449	0.433	0.288	0.454	1.030	0.462	1.100	0.590
***P-value*[2]**								
A_b	0.007	0.844	0.787	0.011	< 0.001	0.103	0.779	0.451
O_2	0.002	0.646	< 0.001	< 0.001	< 0.001	0.006	0.002	0.001
A_b * O_2	0.442	0.502	0.042	0.214	0.040	0.007	0.513	0.888

Abbreviations: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks.

[a-c] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability value.

[3] Expressed as a percentage of egg weight and the data were first transformed to arcsine before analysis.

Table 2

Effect of layer breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on embryo and embryonic quality at internal pipping.

Parameters	Egg weight loss (%)	Absolute embryo weight (g)	Relative embryo weight (%)	Embryonic weight (%)	Yolk sac weight[3] (%)	Yolk sac weight[4] (%)
Breeder age (A_b)						
33wks	11.67	26.81	58.83	79.63	34.55	20.78
50wks	12.06	27.62	58.32	80.62	36.19	21.15
SEM[1]	0.317	0.334	0.683	0.651	1.190	0.449
Oxygen level (O_2)						
15 %	11.27^{b}	26.83	58.44	79.53	37.97^{a}	22.44^{a}
17 %	11.72^{ab}	27.22	57.51	81.38	37.07^{a}	20.91^{ab}
21 %	12.60^{a}	27.58	59.78	79.47	31.08^{b}	19.54^{b}
SEM[1]	0.388	0.409	0.836	0.797	1.450	0.550
Interaction (A_b * O_2)						
33wks * 15 %	11.44	26.38	58.72	79.13	35.79	21.47
33wks * 17 %	11.52	27.40	57.70	80.36	36.86	20.90
33wks * 21 %	12.05	26.64	60.07	79.41	31.01	19.97
50wks * 15 %	11.10	27.28	58.15	79.92	40.14	23.41
50wks * 17 %	11.92	27.04	57.32	82.40	37.28	20.91
50wks * 21 %	13.15	28.52	59.48	79.53	31.15	19.12
SEM[1]	0.549	0.578	1.180	1.130	2.060	0.778
***P-value*[2]**						
A_b	0.390	0.094	0.598	0.290	0.335	0.562
O_2	0.050	0.431	0.167	0.167	0.003	0.002
A_b * O_2	0.430	0.164	0.995	0.688	0.525	0.194

Abbreviations: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks.

[a-b] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability value.

[3] Expressed as a percentage of embryo weight.

[4] Expressed as a percentage of egg weight at internal pipping.

100th percentiles.

The results for external pipping of embryos in Table 4 indicate no interaction between breeder age and O_2 level on external pipping percentiles. However, breeder age significantly influenced externally pipped embryos at the 50th, 75th, and 100th percentiles ($p < 0.05$), with 50-week-old breeders exhibiting prolonged times at the 75th percentile

Table 3

Effect of layer breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on pipping time, duration and hatch window.

Parameters	IP time (hr)	EP time (hr)	CE time (hr)	IP dur (hr)	EP dur (hr)	Hatch time (hr)	Hatch window (hr)
Breeder age (A_b)							
33wks	454.66^{b}	471.03^{b}	481.85^{b}	16.37^{b}	10.82	27.19^{b}	22.67
50wks	460.81^{a}	483.81^{a}	494.36^{a}	23.00^{a}	10.55	33.55^{a}	21.33
SEM[1]	0.864	0.946	1.040	0.725	0.616	0.761	1.290
Oxygen level (O_2)							
15 %	458.91	487.63^{a}	499.55^{a}	28.14^{a}	11.34^{ab}	40.06^{a}	19.50^{b}
17 %	458.02	477.13^{b}	488.44^{b}	19.69^{b}	11.89^{a}	31.00^{b}	25.50^{a}
21 %	456.28	467.49^{c}	476.32^{c}	11.22^{c}	8.83^{b}	20.05^{c}	21.00^{ab}
SEM[1]	1.060	1.160	1.270	0.888	0.754	0.932	1.580
Interaction (A_b * O_2)							
33wks * 15 %	456.83	485.21^{a}	495.45^{b}	28.38^{a}	10.24	38.62^{ab}	20.00
33wks * 17 %	454.79	468.69^{c}	482.00^{c}	13.90^{b}	13.31	27.21^{c}	26.00
33wks * 21 %	452.37	459.20^{d}	468.11^{d}	6.83^{c}	8.91	15.74^{d}	22.00
50wks * 15 %	460.98	490.05^{a}	503.65^{a}	27.90^{a}	12.43	41.50^{a}	19.00
50wks * 17 %	461.25	485.57^{a}	494.88^{b}	25.48^{a}	10.48	34.79^{b}	25.00
50wks * 21 %	460.18	475.79^{b}	484.54^{c}	15.61^{b}	8.75	24.36^{c}	20.00
SEM[1]	1.500	1.640	1.800	1.260	1.070	1.320	2.240
***P-value*[2]**							
A	< 0.001	< 0.001	< 0.001	< 0.001	0.759	< 0.001	0.479
O_2	0.213	< 0.001	< 0.001	< 0.001	0.014	< 0.001	0.050
A_b * O_2	0.472	0.001	0.030	< 0.001	0.073	0.009	0.967

Abbreviation: CAM, chorioallantoic membrane; IP, internal pipping; EP, external pipping; CE, chick emergence; dur., duration; hr, hour; ED, embryonic day; wks., weeks.

[a-d] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability value.

Table 4

Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on internal and external pipping time of total chicks hatched (expressed in percentiles).

Parameters	Internal pipping				External pipping			
	25th Percentile	50th Percentile	75th Percentile	100th Percentile	25th Percentile	50th Percentile	75th Percentile	100th Percentile
Breeder age (A_b)								
33wks	451.67	454.67	462.00^{b}	476.00	462.33	468.00^{b}	472.00^{b}	482.00^{b}
50wks	455.00	456.33	470.67^{a}	479.33	467.67	477.00^{a}	481.33^{a}	487.00^{a}
SEM[1]	1.430	1.130	1.580	1.870	4.000	2.900	2.820	1.560
Oxygen level (O_2)								
15 %	458.50^{a}	459.00^{a}	470.50^{a}	480.00^{a}	470.50	477.50	482.00	488.50^{a}
17 %	451.00^{b}	454.50^{ab}	466.00^{ab}	482.00^{a}	464.50	472.50	478.00	487.50^{a}
21 %	450.50^{b}	453.00^{b}	462.50^{b}	471.00^{b}	460.00	467.50	470.00	477.50^{b}
SEM[1]	1.760	1.380	1.940	2.290	4.900	3.550	3.450	1.910
Interaction (A_b * O_2)								
33wks * 15 %	457.00	459.00	469.00	478.00^{ab}	470.00	476.00	481.00	488.00
33wks * 17 %	449.00	453.00	461.00	486.00^{a}	461.00	468.00	474.00	486.00
33wks * 21 %	449.00	452.00	456.00	464.00^{b}	456.00	460.00	461.00	472.00
50wks * 15 %	460.00	459.00	472.00	482.00^{a}	471.00	479.00	483.00	489.00
50wks * 17 %	453.00	456.00	471.00	478.00^{ab}	468.00	477.00	482.00	489.00
50wks * 21 %	452.00	454.00	469.00	478.00^{ab}	464.00	475.00	479.00	483.00
SEM[1]	2.480	1.960	2.740	3.240	6.930	5.020	4.880	2.710
***P-value*[2]**								
A_b	0.126	0.318	0.002	0.232	0.364	0.048	0.037	0.043
O_2	0.012	0.025	0.039	0.012	0.347	0.180	0.080	0.003
A_b * O_2	0.973	0.743	0.214	0.017	0.863	0.509	0.291	0.191

Abbreviation: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks;.

[a-b] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments;.

[1] SEM, pooled standard error of means;.

[2] P, probability value.

(481.33 h, $p = 0.037$) compared to 33-week-old breeders. Oxygen (O_2) levels only affected externally pipped embryos at the 100th percentile ($p = 0.003$), where embryos incubated at 15 % O_2 showed the longest external pipping times (488.50 h) compared to the 21 % O_2 (477.50 h).

Hatching percentiles of chicks

The data in Table 5 shows the spread of hatching time (in percentiles) for chicks from 33- and 50-week-old breeders exposed to varying O_2 levels (15 %, 17 %, and 21 %). There was a significant interaction between breeder age and O_2 level at the 100th percentile ($p = 0.033$), with the 33- and 50-week-old breeders at 15 % O_2 showing the longest time (503, 507.00 hr), and those at 21 % O_2 the shortest (481, 489.00 hr). Breeder age significantly affected the spread of hatch at the 25th ($p = 0.001$) and 100th ($p = 0.028$) percentiles, with older breeders showing longer times compared to younger breeders. Oxygen levels significantly affected the 25th ($p = 0.022$) and 100th ($p < 0.001$) percentiles, where 15 % O_2 led to the longest times (505.00 hr).

Table 5

Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on spread of total chicks hatched (expressed in percentiles).

Parameters	25th Percentile	50th Percentile	75th Percentile	100th Percentile
Breeder age (A_b)				
33wks	474.33^{b}	480.33	484.33	494.22^{b}
50wks	486.33^{a}	488.56	492.33	499.00^{a}
SEM[1]	2.070	3.260	2.910	1.360
Oxygen level (O_2)				
15 %	485.50^{a}	491.50^{a}	494.00	505.00^{a}
17 %	481.50^{ab}	485.83^{ab}	490.00	499.83^{a}
21 %	474.00^{b}	476.00^{b}	481.00	485.00^{b}
SEM[1]	2.530	3.990	3.560	1.660
Interaction (A_b * O_2)				
33wks * 15 %	482.00	491.00	493.00	503.00^{a}
33wks * 17 %	477.00	482.00	486.00	498.67^{ab}
33wks * 21 %	464.00	468.00	474.00	481.00^{c}
50wks * 15 %	489.00	492.00	495.00	507.00^{a}
50wks * 17 %	486.00	489.67	494.00	501.00^{a}
50wks * 21 %	484.00	484.00	488.00	489.00^{bc}
SEM[1]	3.580	5.640	5.030	2.350
***P-value*[2]**				
A_b	0.001	0.100	0.075	0.028
O_2	0.022	0.051	0.063	0.000
A_b * O_2	0.191	0.437	0.511	0.033

Abbreviations: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks.

[a-c] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability value.

Table 6

Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on external chick quality assessment, chick weights, yolk-free chick weight and yolk sac weight.

Parameters	Chick length (cm)	Shank length (cm)	Toe length (cm)	Chick weight (g)	YFCW (g)	Yolk sac weight (%)
Breeder age (A_b)						
33wks	16.54	2.38	1.91^{b}	33.06^{b}	29.83^{b}	9.58
50wks	16.74	2.36	1.97^{a}	36.50^{a}	32.77^{a}	10.20
SEM[1]	0.149	0.035	0.021	0.371	0.392	0.236
Oxygen level (O_2)						
15 %	16.24^{b}	2.39	1.87^{b}	36.12^{a}	32.00	11.00^{a}
17 %	16.67^{ab}	2.34	1.94^{ab}	34.93^{a}	31.29	9.88^{b}
21 %	17.01^{a}	2.37	1.99^{a}	33.30^{b}	30.62	8.80^{c}
SEM[1]	0.182	0.043	0.025	0.454	0.48	0.289
Interaction (A_b * O_2)						
33wks * 15 %	16.02	2.44	1.82	35.44^{a}	31.28	10.96^{a}
33wks * 17 %	16.67	2.31	1.92	32.74^{b}	29.78	9.03^{b}
33wks * 21 %	16.93	2.38	1.98	31.00^{b}	28.42	8.77^{b}
50wks * 15 %	16.47	2.34	1.92	36.79^{a}	32.71	11.05^{a}
50wks * 17 %	16.68	2.37	1.97	37.11^{a}	32.80	10.73^{a}
50wks * 21 %	17.09	2.36	2.01	35.61^{a}	32.81	8.83^{b}
SEM[1]	0.258	0.061	0.036	0.642	0.679	0.408
***P-value*[2]**						
A_b	0.338	0.658	0.048	< 0.001	< 0.001	0.061
O_2	0.017	0.664	0.005	< 0.001	0.137	< 0.001
A_b * O_2	0.696	0.450	0.610	0.024	0.104	0.040

Abbreviations: CAM, chorioallantoic membrane; YFCW., yolk-free chick weight; ED, embryonic day; wks., weeks.

[a-b] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability value.

Post-hatch chick quality assessment

The effects of breeder age and oxygen (O_2) levels on chick weight, yolk-free chick weight (YFCW), yolk sac weight, chick length, shank length, and toe length were assessed, with results shown in Table 6. There was a significant interaction between breeder age and O_2 levels for chick weight ($p = 0.024$) and yolk sac weight ($p = 0.040$), with 50-week-old breeders under 15 % and 17 % O_2 producing heavier chicks than 33-week-old breeders. However, the interaction had no significant effect on YFCW, chick length, shank length, or toe length. Breeder age significantly influenced chick weight and YFCW, with older breeders (50 weeks) producing heavier chicks (36.50 g vs. 33.06 g, $p < 0.001$) and greater YFCW (32.77 g vs. 29.83 g, $p < 0.001$) compared to 33-week-old breeders. Older breeders also produced chicks with longer toe lengths ($p = 0.048$). Yolk sac weight was higher in older breeders but not significantly ($p = 0.061$). Oxygen concentration significantly affected chick length, toe length, chick weight, and yolk sac weight ($p < 0.05$). Chicks incubated at 21 % O_2 had longer chick lengths (17.01 cm vs. 16.24 cm, $p = 0.017$) and toe lengths (1.99 cm vs. 1.87 cm, $p = 0.005$). However, chicks under 15 % O_2 were heavier (36.12 g vs. 33.30 g, $p < 0.001$) and had higher yolk sac weights (10.95 % vs. 8.80 %, $p < 0.001$).

Tibia and femur morphometry

The effect of breeder age and O_2 levels during late CAM growth on chick tibia and femur morphometry at hatch is presented in Tables 7 and 8, respectively. Tibia morphometry (Table 7) demonstrated a significant interaction between breeder age and O_2 level on both tibia length ($p = 0.042$) and diameter ($p < 0.001$). Breeder age significantly influenced relative tibia weight ($p = 0.001$) and length ($p = 0.019$), with 33-week-old breeders producing longer tibias than 50-week-old breeders (23.95 mm vs. 22.94 mm). Oxygen level affected relative tibia weight ($p = 0.034$), length ($p = 0.003$) and diameter ($p = 0.005$), with 17 % and 21 % O_2 levels leading to longer and wider tibias compared to 15 % O_2 level. Tibia robusticity and Seedor index showed no significant differences across any interaction or main effects ($p > 0.05$).

The interaction between breeder age and O_2 levels significantly impacted absolute femur weight ($p = 0.006$), relative femur weight ($p = 0.032$), and Seedor index ($p = 0.003$) in hatchlings (Table 8). Breeder age independently influenced absolute femur weight ($p = 0.008$), relative femur weight ($p < 0.001$), femur length ($p = 0.004$), and Seedor index ($p = 0.014$), with the 33-week-old group showing higher values than the 50-week-old group. No significant O_2 effect was observed on femur morphometry.

Calcium and phosphorus levels in the tibia and femur bone of chicks at hatch

The results in Table 9 demonstrate the effects of breeder age and O_2 levels during incubation on calcium **(Ca)** and phosphorus **(P)** absorption in the tibia and femur bones at hatch. There was no significant interaction between breeder age and O_2 levels or any breeder age effect on Ca and P absorption. However, O_2 levels significantly influenced Ca and P levels ($p < 0.001$), with higher values in both the 33-week and 50-week age groups at 21 % O_2 compared to hypoxic conditions (15 % and 17 % O_2 levels).

Table 7

Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on tibia morphometry at hatch.

Parameters	Absolute weight (g)	Relative weight (%)	Length (mm)	Diameter (mm)	Robuscity (mm/g)	Seedor index (g/mm)
Breeder age (A_b)						
33wks	0.05	0.16^{a}	23.95^{a}	2.32	6.59	0.020
50wks	0.04	0.13^{b}	22.94^{b}	2.41	6.57	0.019
SEM[1]	0.002	0.006	0.294	0.036	0.076	0.001
Oxygen level (O_2)						
15 %	0.04	0.13^{b}	22.37^{b}	2.24^{b}	6.46	0.020
17 %	0.05	0.15^{ab}	23.99^{a}	2.41^{a}	6.66	0.020
21 %	0.05	0.16^{a}	23.97^{a}	2.44^{a}	6.63	0.019
SEM[1]	0.002	0.007	0.360	0.044	0.093	0.001
Interaction (A_b * O_2)						
33wks * 15 %	0.05	0.15	22.86^{bc}	2.20^{c}	6.42	0.020
33wks * 17 %	0.05	0.18	25.12^{a}	2.52^{ab}	6.68	0.021
33wks * 21 %	0.05	0.16	23.86^{abc}	2.25^{c}	6.67	0.019
50wks * 15 %	0.04	0.12	21.88^{c}	2.29^{bc}	6.50	0.018
50wks * 17 %	0.04	0.13	22.86^{bc}	2.31^{bc}	6.63	0.018
50wks * 21 %	0.05	0.15	24.07^{ab}	2.62^{a}	6.58	0.021
SEM[1]	0.003	0.010	0.510	0.062	0.131	0.001
P-value[2]						
A_b	0.067	0.001	0.019	0.099	0.848	0.148
O_2	0.110	0.034	0.003	0.005	0.261	0.524
A_b * O_2	0.073	0.195	0.042	< 0.001	0.798	0.164

Abbreviations: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks.

[a-c] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability values.

Table 8

Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on femur morphometry.

Parameters	Absolute weight (g)	Relative weight (%)	Length (mm)	Diameter (mm)	Robuscity (mm/g)	Seedor index (g/mm)
Breeder age (A_b)						
33wks	0.034^{a}	0.11^{a}	18.01^{a}	2.21	5.60	0.018^{a}
50wks	0.029^{b}	0.09^{b}	17.33^{b}	2.70	5.64	0.017^{b}
SEM[1]	0.001	0.004	0.159	0.216	0.061	0.001
Oxygen level (O_2)						
15 %	0.031	0.10	17.46	2.53	5.50	0.018
17 %	0.032	0.10	17.69	2.62	5.64	0.018
21 %	0.033	0.11	17.86	2.21	5.73	0.017
SEM[1]	0.002	0.005	0.194	0.264	0.074	0.001
Interaction (A_b * O_2)						
33wks * 15 %	0.036^{a}	0.11^{ab}	17.86	2.20	5.46	0.020^{a}
33wks * 17 %	0.036^{a}	0.12^{a}	18.23	2.31	5.57	0.020^{a}
33wks * 21 %	0.031^{ab}	0.11^{abc}	17.94	2.11	5.77	0.017^{ab}
50wks * 15 %	0.026^{b}	0.08^{c}	17.06	2.86	5.54	0.015^{b}
50wks * 17 %	0.028^{ab}	0.09^{bc}	17.14	2.93	5.71	0.016^{ab}
50wks * 21 %	0.034^{ab}	0.11^{abc}	17.79	2.32	5.68	0.019^{a}
SEM[1]	0.002	0.008	0.275	0.373	0.105	0.001
P-value[2]						
A_b	0.008	< 0.001	0.004	0.109	0.592	0.014
O_2	0.580	0.342	0.353	0.523	0.098	0.714
A_b * O_2	0.006	0.032	0.221	0.793	0.545	0.003

Abbreviations: CAM, chorioallantoic membrane; ED, embryonic day; wks., weeks.

[a-c]: Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.

[1] SEM, pooled standard error of means.

[2] P, probability values.

Hatching performances and embryonic mortality

The results in Table 10 summarize the effects of breeder age and oxygen exposure on embryonic mortality, hatch of fertile and chick quality score. A significant interaction between breeder age and O_2 levels was observed for mid ($p = 0.002$) and total embryonic mortality ($p = 0.045$), with 50-week-old breeders at 15 % O_2 showing the highest mortality. Breeder age had a significant effect on the hatch of fertile eggs ($p = 0.021$), mid ($p = 0.025$) and total mortality ($p = 0.023$). Oxygen levels significantly affected hatch of fertile ($p < 0.001$), mid ($p < 0.001$), total mortality ($p < 0.001$), and chick score ($p = 0.024$), with 17 % and 21 % O_2 producing higher chick scores than 15 % O_2 level.

Discussion

The present study which aimed to evaluate the effect of layer breeder age and reduced incubator oxygen concentration **(O_2)** level at the late phase of chorioallantoic membrane (CAM) maturation on embryo development, hatching events, hatching performances and chick quality showed that, following the hypoxic exposure, there was an immediate reduction in the growth rate of the embryo because of suppressed metabolic activity during the exposure period. The interaction effect as observed between breeder age and oxygen levels about egg weight also indicates an effect on other embryonic components aside from the embryo. While embryo weights from the 50-weeks-old (older) breeder flocks were notably lesser compared to the 33-weeks-old breeder flock

Table 9
Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on calcium and phosphorus level in the tibia and femur bone of chicks at hatch.

Parameters	Bone Ca (% DM)	Bone P (% DM)
Breeder age (A_b)		
33wks	12.00	9.82
50wks	11.52	9.28
SEM[1]	0.300	0.336
Oxygen level (O_2)		
15 %	10.56^{b}	7.96^{b}
17 %	11.27^{b}	8.96^{b}
21 %	13.45^{a}	11.74^{a}
SEM[1]	0.368	0.411
Interaction (A_b * O_2)		
33wks * 15 %	10.79	8.06
33wks * 17 %	12.10	9.51
33wks * 21 %	13.11	11.90
50wks * 15 %	10.32	7.87
50wks * 17 %	10.43	8.40
50wks * 21 %	13.80	11.57
SEM[1]	0.520	0.582
P-value[2]		
A_b	0.265	0.264
O_2	< 0.001	< 0.001
A_b * O_2	0.094	0.698

Abbreviations: CAM, chorioallantoic membrane; Ca., calcium; P., phosphorus; ED, embryonic day; wks., weeks.
[abc]: Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.
[1] SEM, pooled standard error of means.
[2] P, probability values.

(younger), the effect of, 15 and 17 % reduced O_2 level, was predominantly observed in the older breeder flock compared to the younger (33-weeks) breeders. Older hens, with more advanced reproductive physiology, lay larger, heavier eggs, characterized by increased albumen, yolk, eggshell mass and pore number. These larger eggs likely have greater oxygen demands, which were unmet in the hypoxic conditions used in this study, particularly during critical developmental stages (ED 13-15), resulting in significant embryo weight reduction compared to younger breeder eggs.

As embryonic development progresses, oxygen requirements rise due to increased demands on respiration and metabolism. Reduced metabolic activity under hypoxic conditions also led to a reduction in embryo length compared to control groups (21 % O_2 level). Additionally, yolk sac metabolism was slower, as evidenced by larger yolk sac and albumen weights in eggs incubated at 15 % O_2 compared to those at 17 % and 21 %. This suggests that more severe hypoxia slows yolk sac metabolism, consistent with findings by Molenaar et al. (2010) who reported increased yolk sac weight under hypoxic conditions. The reduced metabolic heat production under hypoxia appears to conserve yolk nutrients, supporting embryonic survival and growth. Similarly, Chan and Burggren (2005) observed reduced embryo growth after exposure to 15 % oxygen for six days. Rohlicek et al. (1998) proposed that decreased oxygen consumption during hypoxia may represent an adaptive response, prioritizing survival over growth functions like thermogenesis and tissue development, rather than a direct consequence of oxygen deprivation.

Hypoxia is mostly considered detrimental to embryo development. However, in addition to its role in inducing phenotypic adaptations and enhancing cardiovascular responses, hypoxia may offer a potential advantage by slowing embryonic development. This deceleration in development could be particularly beneficial in temperate regions, where external incubator temperatures are consistently elevated, thereby influencing internal incubator conditions and embryo metabolism. By slowing the developmental rate under hypoxic conditions, embryos might gain additional time for organ maturation and complete yolk sac absorption, provided that optimal early incubation conditions are maintained (Agbehadzi et al., 2024). Older breeder eggs, particularly from 50-week-old flocks, exhibited greater responses to both hypoxic and normoxic conditions due to increased egg weight loss compared to younger flocks. This observation aligns with the findings of Tullett and Board (1977) and Peebles and Brake (1987) who demonstrated that older breeders tend to produce eggs with thinner shells, leading to increased water loss during incubation. In contrast, eggs from younger flocks, with thicker shells, exhibited reduced moisture loss (Brake et al., 1997; Nasri et al., 2019).

In the present study, while hypoxic conditions of 15 % and 17 % oxygen resulted in lower egg weight loss and higher yolk sac weights compared to controls, no interaction between breeder age and oxygen

Table 10
Effect of breeder age and reduced incubator oxygen level (ED 13-15) during the late stage of CAM growth on embryonic mortality, hatchability profile and chick score.

Parameters (%)	Hatch of fertile	Mid mortality	Late mortality	Pipping mortality[3]	Total Mortality	Chick Score
Breeder age (A_b)						
33wks	72.42^{a}	18.08^{b}	4.68	3.32	27.58^{b}	96.00
50wks	66.45^{b}	23.27^{a}	6.25	1.72	33.85^{a}	95.33
SEM[1]	1.700	1.440	1.350	0.578	1.900	0.444
Oxygen level (O_2)						
15 %	32.91^{c}	53.41^{a}	7.07	3.75	67.21^{a}	94.44^{b}
17 %	83.63^{b}	6.70^{b}	6.14	1.93	16.37^{b}	96.22^{ab}
21 %	91.77^{a}	1.92^{b}	3.20	1.88	8.57^{b}	96.33^{a}
SEM[1]	2.080	1.760	1.660	0.708	2.340	0.544
Interaction (A_b * O_2)						
33wks * 15 %	39.93	44.28^{b}	9.17	5.35	60.07^{b}	94.67
33wks * 17 %	85.07	7.88^{c}	2.76	3.29	14.93^{c}	96.44
33wks * 21 %	92.26	2.07^{c}	2.13	1.33	7.74^{c}	96.89
50wks * 15 %	25.89	62.54^{a}	4.96	2.14	74.34^{a}	94.22
50wks * 17 %	82.18	5.51^{c}	9.53	0.57	17.82^{c}	96.00
50wks * 21 %	91.27	1.76^{c}	4.27	2.44	9.40^{c}	95.78
SEM[1]	2.940	2.490	2.350	1.000	3.295	0.769
P-value[2]						
A_b	0.021	0.025	0.429	0.073	0.023	0.290
O_2	< 0.001	< 0.001	0.266	0.148	< 0.001	0.024
A_b * O_2	0.075	0.002	0.103	0.102	0.045	0.882

Abbreviations: CAM, chorioallantoic membrane; Mid, middle; ED, embryonic day; wks., weeks.
[a-b] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.
[1] SEM, pooled standard error of means.
[2] P, probability value.
[3] Pipping mortality.

level was observed in embryo weight at internal pipping. Similar findings were reported by Nangsuay et al. (2021) who also observed no interaction between breeder age and oxygen level on yolk-free body weight at embryonic day 14 (ED 14) in broilers. However, Nangsuay et al. (2021) observed an interaction effect apparently at ED18. The difference between the results of Nangsuay et al. (2021) results when compared to the present finding at internal pipping and the present study at internal pipping highlights the critical role of the developmental stage in determining the embryo's response to hypoxia. Additionally, the potential for breed-specific differences such as those between layers and broilers may contribute to this variation, as oxygen requirements and developmental trajectories likely differ across breeds, particularly during the later stages of incubation. Contrary to these findings, Nangsuay et al. (2016) reported no differences in yolk-free body mass between broiler breeder flocks of 29 and 53 weeks of age. These discrepancies underscore the complex interplay between genetic factors, breeder age, and environmental conditions in shaping embryonic development under hypoxic conditions (Tona et al., 2004; Hamidu et al., 2007; Tona et al., 2010; Ho et al., 2011).

Druyan et al. (2012) demonstrated that daily exposure to a 17 % oxygen concentration during the development of the chorioallantoic membrane (CAM) resulted in embryo weights comparable to those of control embryos. This finding suggests the potential for a catch-up growth mechanism when embryos are returned to normoxic incubation conditions. The mechanisms underlying catch-up growth are physiological and molecular. In response to nutrients and oxygen availability, the insulin-like growth factor (IGF) signaling pathway plays a key role in regulating growth. Re-oxygenation promotes cell proliferation and growth in embryos by activating IGF signaling (Kamei et al., 2011). In addition, reactive oxygen species (ROS) generated during re-oxygenation are thought to facilitate catch-up growth (Zasu et al., 2022). According to Zhao et al. (2017), ROS play a crucial role in facilitating recovery processes such as angiogenesis and tissue repair following reoxygenation. The present study corroborates these outcomes, because no significant interaction or main effect of breeder age or oxygen level was observed on embryo weight at internal pipping after the exposure period. It is well-established that different stages of embryonic development exhibit variable responses to hypoxic conditions, both metabolically and morphologically (Dzialowski et al., 2002; Molenaar et al., 2010) underscoring the stage-specific effects of hypoxia on growth. The duration and severity of hypoxic exposure are key factors influencing its impact on embryonic development (Chan and Burggren, 2005; Zhang and Burggren, 2012). Generally, reduced oxygen availability hampers embryonic growth by limiting the metabolic pathways required for nutrient utilization, affecting embryos from both young and older breeder flocks (Nangsuay et al., 2021).

In the current study, hypoxia-induced reductions in the physiological and morphological growth trajectories of developing embryos resulted in significant increases in internal and external pipping and hatching events. Visschedijk (1968) highlighted that oxygen and carbon dioxide concentrations in the egg's air space play a pivotal role in determining the timing of external pipping. Mild hypoxia has been shown to reduce oxygen consumption at internal pipping (Szdzuy et al., 2008) while responses to varying hypoxic levels are more pronounced during external pipping (Menna and Mortola, 2003). The difference in effects of hypoxia exposure observed in the present study likely explains the variation in pipping and hatching times among the 15 %, 17 %, and 21 % (control) oxygen groups. Furthermore, embryos from older breeders exhibited longer hatching times than those from younger breeders, a finding consistent with previous studies by Ulmer-Franco et al. (2010). However, the interaction effects identified in the present study suggest that oxygen levels in the incubator significantly influence hatch time, particularly through their impact on external pipping duration. Embryos from younger breeders exhibited higher resilience to hypoxia, faster recovery, and accelerated development, contributing to shorter hatching and pipping durations than the older breeder flocks.

Although breeder age did not significantly affect the hatch window in the current study, Machado et al. (2020) reported a wider hatch window in eggs from 51-week-old breeders compared to those from 38-week-old breeders. The irregular hatch window observed at the 15 % oxygen level in the present study can likely be attributed to the increased embryonic mortality associated with severe hypoxic exposure at the mid-stage of embryo development. The delayed hatching events observed under the 15 % O_2 condition in the present study were also reflected in the timing of internal pipping, which was analyzed across different percentiles. A greater proportion of embryos incubated under 15 % O_2 exhibited delayed internal pipping at the 25th, 50th, 75th, and 100th percentiles compared to those in the control group (21 % O_2). Embryos incubated under 17 % O_2 displayed internal pipping patterns that only differed significantly from the 15 % O_2 group at the 25th percentile. At the 100th percentile, a significant interaction between breeder age and O_2 level influenced the total number of embryos that were internally pipped. For external pipping, the impact of reduced O_2 levels (15 % and 17 %) was only evident at the 100th percentile. The consistent delay in hatching time across all percentiles in the 15 % O_2 group suggests that hypoxic conditions predominantly affect embryos during the internal pipping stage rather than during external pipping. The interaction between breeder age and O_2 concentration was primarily observed in overall hatching time.

Chick weight and relative yolk sac weight were significantly affected by the interaction between breeder age and O_2 level. Chicks from 50-week-old breeders exhibited greater body weights and yolk-free chick weights (YFCW) compared to those from 33-week-old breeders. Previous studies by Tona et al. (2004); Ulmer-Franco et al., 2010; Koppenol et al. (2015); Iqbal et al., 2016; Damaziak et al. (2018) have consistently shown that hatchling weight increases with breeder age, largely due to differences in egg weight. In the present study, the higher chick weights observed under 15 % and 17 % O_2 conditions, during ED 13-15, align with the findings of Bahadoran et al. (2010) who reported that chicks incubated at high altitudes (hypoxic environments) exhibited significantly greater body weights than those incubated at lower altitudes (normoxic environment). This weight increase under hypoxia is likely attributable to the greater yolk sac weight, which suggests reduced embryonic metabolism. However, Agbehadzi et al. (2024) reported lower yolk sac weights following early-stage hypoxic stimulation (ED 7-9), potentially due to prolonged yolk metabolism during embryo development. Embryos exposed to mild hypoxia during early stages may exhibit compensatory growth upon return to normoxia, demonstrating adaptability to hypoxic conditions (Zamudio, 2003). This adaptation is evidence by physiological processes adjustment including vascular development, the redistribution of oxygenated blood to vital organs and increased cardiac output which may further contribute to increased chick weight when embryos are re-exposed to normoxia following early hypoxic stimulation (Mulder et al., 1998; Galli et al., 2023). In contrast, chronic hypoxia has been shown to reduce chick weight in some studies (Altimiras and Phu, 2000; Burton and Palmer, 1992; Dzialowski et al., 2002; Sharma et al., 2006; Lock et al., 2024).

During avian embryogenesis, the development and structural integrity of chicken bones relies on the embryo's ability to absorb Ca and P from the eggshell and yolk during embryogenesis. The present study found no significant interaction between breeder age and incubator oxygen levels on chick shank and toe length; however, significant interactions were observed on tibia length, diameter, femur weight, and seedor index. Hypoxic conditions (15 % O_2) significantly reduced tibia weight, length and diameter compared to normoxic conditions (21 % O_2). These findings are consistent with previous research indicating that reduced oxygen levels are associated with shorter femurs, tibias, and shanks in chickens (Oviedo-Rondón et al., 2008). Chicks from embryos incubated under hypoxic conditions (15 % and 17 % O_2) exhibited lower bone Ca and P content than those incubated under normoxic conditions. This suggests that while hypoxic treatment may lead to an increase in chick weight, it compromises bone quality, as evidenced by reduced mineralization and altered bone morphometry. The observed reduction in Ca content aligns with studies by Chen et al. (2022) and Wawrzyniak and Balawender (2022) who suggested hypoxia impaired calcium metabolism and deposition in developing bones, leading to weaker skeletal structures. The mechanism underlying these effects likely involves reduced energy availability due to decreased oxidative phosphorylation during tissue development under hypoxic conditions (Oviedo-Rondón et al., 2008; Solaini et al., 2010). Oxidative stress may disrupt endochondral ossification, thereby impairing the rate and extent of bone mineralization, particularly in long bones (Glimcher, 2006). In chickens, tibial development is a key indicator of overall bone quality, as negative alterations in tibia growth, mineralization, and strength are frequently observed (Aguado et al., 2015). According to Almeida Paz and Bruno (2006), seedor density determines bone density. Tibia and femur weights, lengths and seedor index were more affected in chicks from older breeders compared to younger ones.

Exploring the relationship between O_2 and breeder age holds immense significance for stimulating CAM and influencing mineral absorption during embryonic development. Higher plasma Ca and P levels in chicks from younger breeders have been linked to greater eggshell Ca content (Ahmed, 2016). Some contradictory results exist regarding the influence of eggshell quality on mineral transfer, with some studies suggesting that thinner eggshells in older breeders may facilitate greater Ca and P transfer to the embryo. Further research is needed to confirm whether eggshell quality due to breeder age affects Ca and P transfer to the embryo.

Avian embryos heavily rely on eggshell Ca for bone mineralization, with up to 80 % of their Ca requirements met through this source (Crooks and Simkiss, 1975; Carey, 1983; Uni et al., 2012; Kaweewong et al., 2013). Mobilization of Ca from the eggshell occurs mainly between days 10 and 12 of incubation (Torres and Korver, 2018) and peaks around day 17 (Obara et al., 2022). From ED 0, Halgrain et al. (2022) observed a decrease in P but a significant decrease in eggshells Ca and Mg at ED12 and ED16 of embryo development. In a nutshell, Ca, P and Mg levels in eggshells decrease until day 19 of embryonic development (Halgrain et al., 2021; Varol Avcılar et al., 2024).

In addition to breeder age and oxygen levels, genetic factors also play a crucial role in Ca and P absorption during incubation. Genes involved in the mobilization of these minerals differ between the yolk sac and CAM extraembryonic structures (Halgrain et al., 2021, 2022, 2023). The efficiency of Ca mobilization from the eggshell is positively correlated with the number of mammillary tips on the shell (Karlsson and Lilja, 2008). Ca^{2+} transport via chorionic epithelial cells to the embryonic circulation occurs at a rate of approximately 100 nmol/cm^2/h on the CAM surface (Sys et al., 2013). Hypoxia may alter calcium signaling pathways, especially in chronic conditions, preventing adequate ionization of Ca from the blood into bone tissue before hatching (Pearce, 2006; Quan et al., 2021). This is the case in the present study as less ca and P is observed in the bone but more in the blood (although not significant) under hypoxic condition.

Booth et al. (2020) demonstrated that early-stage exposure to low oxygen, high carbon dioxide, and elevated temperatures increases the risk of early embryonic death. Oxygen levels play a critical role in embryonic development, with hypoxic conditions adversely affecting cardiovascular and metabolic functions, leading to developmental trajectories (Souchet et al., 2020, 2023). Hypoxia has been linked to elevated embryonic mortality, underscoring embryos' susceptibility to oxygen fluctuations (Umaoka et al., 1992). In the present study, 15 % oxygen exposure during the late CAM maturation stage significantly compromised embryo survival and hatch of fertile compared to 17 % and 21 % oxygen. While older breeders showed the lowest hatch of fertile rates and highest mid-stage mortality, the increase in mid-stage embryonic death coincided with hypoxic exposure. At this stage (ED 13-15), embryos possess a fully developed respiratory system with heightened oxygen demands. Sudden hypoxic conditions likely induced physiological shock, contributing to elevated mid-stage mortality, a pattern observed in both 33- and 50-week-old breeder groups.

Conclusion

This study demonstrated a novel interaction between late-stage CAM growth (ED 13-15) hypoxic stimulation and breeder age to influence embryonic development, hatchability, and post-hatch chick quality. Under controlled conditions, hypoxia may slow development rates and conserve yolk nutrients, potentially improving organ maturation, despite its negative perception. It is especially relevant in temperate climates, where high ambient temperatures can accelerate embryonic metabolism. Moreover, the study also showed that eggs from older breeders are more susceptible to hypoxic stress due to their greater oxygen demands and thinner eggshells. In addition, hypoxic conditions have an adverse effect on bone mineralization and morphometry, resulting in compromised bone quality despite increased chick weights. These findings not only contribute to our understanding of avian embryology but also offer practical insights into optimizing hatchery conditions for enhanced chick quality and adaptability.

Disclosures

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Richard Koblah Agbehadzi reports financial support was provided by World Bank Group IDA 5424. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

The data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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