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Research article

Effects of layer breeder age and reduced incubator oxygen concentrations on embryo development, hatching events, chick quality, embryonic mortality and hatchability of fertile eggs

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ABSTRACT

The combined effects of breeder age and oxygen (O_2) concentrations on embryo development, hatching events, chick quality, embryonic mortality and hatchability were investigated. A total of 900 hatching eggs of average weight of 53.85 ± 2.40 g and 60.42 ± 2.02 g from 33 to 50 wks ISA layer breeders were incubated for six days at 37.7 °C temperature and 56 % relative humidity **(RH)** before exposure to hypoxic stimulation of 15 % and 17 % O_2 (experimental groups) and 21 % O_2 (control group). In a 2 x 3 factorial experiment, air-N_2 flushing to reduce O_2 was 1 h daily from embryonic day (ED)7–9. The study investigated fresh egg weight before setting, egg weight loss and embryonic parameters at ED11, hatching events, chick quality, embryonic mortality and hatchability. Results showed that regardless of breeder age, early mild hypoxia reduces ($P < 0.001$) embryonic growth rate immediately after exposure and 50 wks breeders were more susceptible because of higher ($P < 0.001$) egg weight loss. There was an interaction effect ($P < 0.05$) on hatching durations, hatch time, hatch window, chick weight, yolk-free chick weight and pipping muscle. Results also showed a combined effect ($P < 0.05$) on high early and pipping embryonic mortality while causing a decrease in the hatch of fertile eggs. Early hypoxic stimulation decreased ($P = 0.05$) yolk sac weight at hatch due to the catch-up growth mechanism during embryogenesis. Conclusively, an early mild hypoxic stimulation can potentially improve chick quality in both young and old layer breeders if an optimal condition can be achieved.

1. Introduction

Gas exchanges during incubation are critical for embryonic development, hatching performance and chick quality. Adequate oxygen levels and the removal of sufficient carbon dioxide are essential to achieve normal embryonic development [1]. This complex process is influenced by various factors such as egg structure, environmental contaminants, and energy sources. The spherical shape of avian eggs enhances gas transfer between the embryo and the external environment [2], primarily facilitated by the chorioallantoic

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membrane (**CAM**) and the eggshell's porosity [3] Oxygen concentration levels are vital for maintaining metabolic processes during development [4]. Increased oxygen **(O_2)** consumption and carbon dioxide (**CO_2**) production as the embryo grows make these gas levels crucial for development and performance post-hatching [5,6]. Hypoxia, or reduced oxygen levels, can disrupt gas exchange, leading to insufficient oxygen supply, metabolic imbalances and potential developmental abnormalities. However, controlled hypoxic conditions may enhance cardiovascular and respiratory system development, improving chick quality and post-hatch performance [7–9].

Pre-incubation and incubation factors influence oxygen consumption and embryo development. Factors like parental flock age, egg weight, storage, eggshell conductance, temperature, humidity, O_2 and CO_2 concentrations, and altitude play significant roles [10,11, 12–14]. At high altitudes, which have reduced O_2 levels [15], observed that high-altitude incubator chicks hatched earlier and had higher weights than low-altitude incubator chicks. High-altitude environments offer Tibetan chickens the ability to produce better-quality chicks due to their genetic adaptations, physiological characteristics, and intestinal microflora [16–19].

The age of breeders may impact hatching performance because according to Ref. [20] the composition of hatching eggs in terms of the ratio of albumen to yolk and eggshell quality changes as the breeder flock ages. Both absolute and relative yolk values are larger for older breeder flocks than younger ones [21]. [22] also reported that the yolk-to-albumen ratio was negatively correlated with egg weight irrespective of the age of the breeder flock. Breeder age-related changes in egg composition can also affect embryonic mortality rates and, consequently, hatch rates [23,24]. Similarly, breeder age influences nutrient availability and subsequent embryonic development [25] through a difference in embryonic metabolism caused by oxygen availability during incubation [26]. The primary energy source during incubation comes from lipids, and factors like hypoxia and hyperoxia can impact egg metabolic rates [27]. Maternal immunoglobulin transfer which can be influenced by age into egg yolks contributes to providing passive immunity to neonates [28].

The developmental environment of avian embryos significantly influences their growth, survival, and overall quality post-hatch. Two critical factors that play a pivotal role in this context are the age of the layer breeders and the oxygen concentration within the incubator. Older breeder hens are known to produce eggs with different characteristics compared to those from younger hens, impacting moisture loss, shell integrity, and ultimately the developing embryo. Concurrently, oxygen concentration within the incubator can dramatically affect the physiological and metabolic processes of the embryo. The combined effect of breeder age and O_2 level in the incubator has been reported to influence yolk-free embryo weight on embryonic day **(ED)** 18 of incubation [29]. The oxygen demand and hypoxic tolerance of the embryo are lowest in the first five days of incubation and increase over time [30,31]. The number of pores and eggshell conductance also affect this situation. As eggshell conductance rises with the increase in egg size, gas

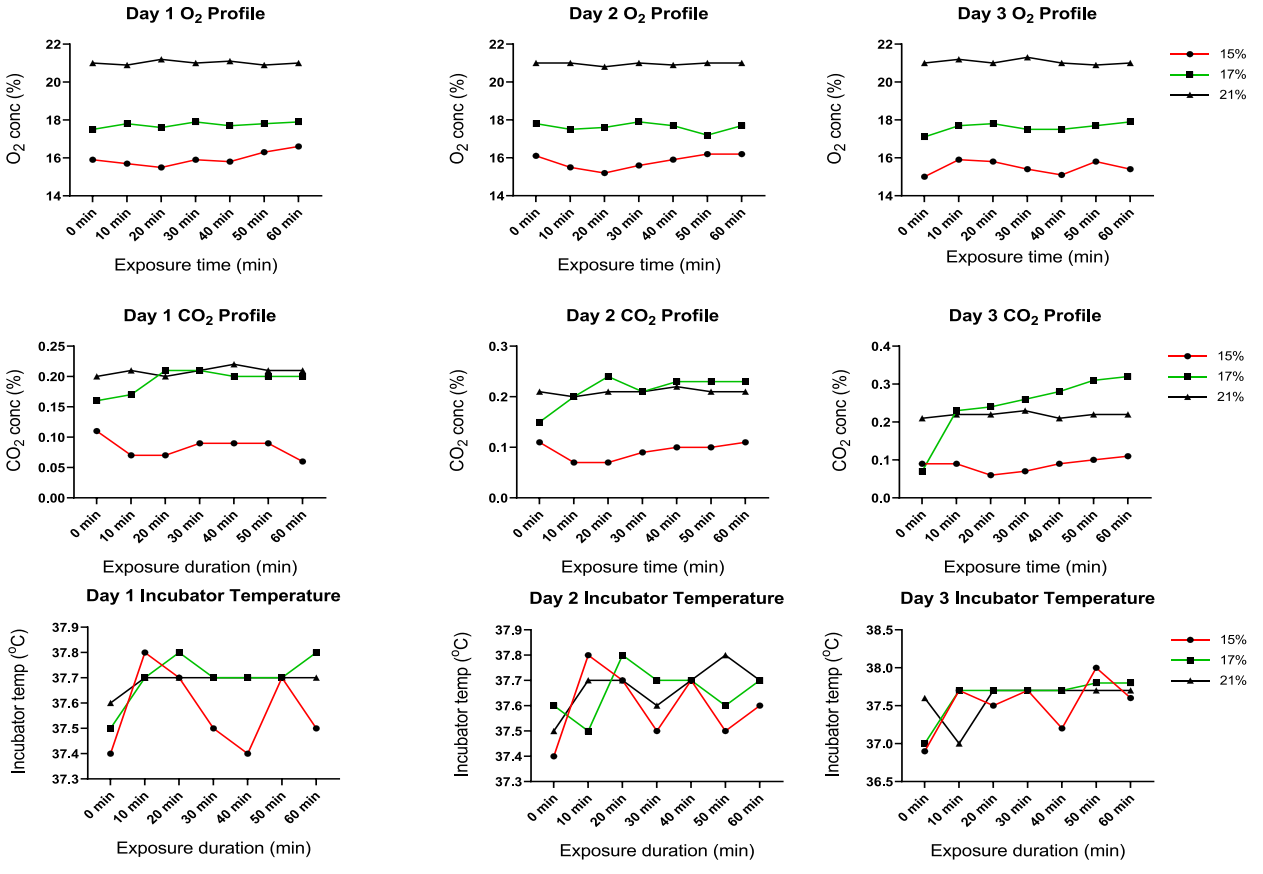

Fig. 1. O_2, CO_2 and incubator temperature (T) profile during the 1-h air-N_2 exposure.

exchange also increases [32,33]. However, the optimal levels and timing of oxygen reduction are not well established, particularly for layers and breeder ages. It is therefore hypothesized that the combined effect of altered oxygen concentration in the incubator and breeder age will show distinct effects on the hatching profile and chick quality of incubated eggs. The purpose of this study was to investigate the combined effects of layer breeder age and reduced incubator oxygen concentrations at an early stage of embryonic development on embryonic characteristics, hatching events, hatchability of fertile eggs, chick quality and embryonic mortality.

2. Material and methods

2.1. Experimental site, ethics and facilities

This research was conducted at the Regional Center of Excellence for Poultry Sciences **(CERSA)** hatchery, research farm and laboratory facilities at the University of Lomé, Togo. All experimental procedures were approved by the Animal Ethics and Scientific Committee following the guidelines of the Regional Center of Excellence for Poultry Sciences, University of Lomé **(CERSA-UL)** (028/2021/BC-BPA/FDS-UL). The experimental site and incubators used were in a geographical location with coordinates as latitude 6°1′95″N, longitude 1°2′53″E and an elevation of 26m above sea level [34].

2.2. Experimental design

A total of 900 hatching eggs were used in a 2 x 3 factorial experimental design, involving two breeder flock ages (33 and 50 wks) and three oxygen concentration **(O_2)** levels. The O_2 levels were set as follows.

1. Experimental groups: 15 % and 17 % O_2
2. Control group: 21 % O_2

For each breeder age group, 450 eggs were allocated, with 150 eggs assigned to each O_2 level. Each O_2 group was divided into three replicates of 50 eggs, placed on separate setter trays in a completely randomized design. From embryonic day **(ED)** 7 to 9, a controlled flow of air-N_2 mixture was used to flush the experimental incubators (PasReform, Zeddam, Netherlands, SmartPro Combi model) for 1 h per day to achieve the target O_2 levels of 15 % and 17 %. An O_2 gas detector (Model: HFP-1201 BX, Xi'an Huafan Technology Co., Ltd., China) was used to continuously monitor and maintain the O_2 levels inside the incubators, following the protocols of [35,36]. Temperature **(T)** and carbon dioxide **(CO_2)** concentrations in the incubators were automatically recorded from the control panel at 10-min intervals during the 1-h air-N_2 flushing period. The profiles of O_2, CO_2, and temperature for the 15 % and 17 % O_2 levels during the flushing period are shown in Fig. 1.

2.3. Hatching eggs, storage and incubation conditions

Hatching eggs of the average weight of 53.85 ± 2.40 g and 60.42 ± 2.02 g were respectively collected from 33 to 50 wks ISA Brown breeder flocks and stored at 18 °C temperature **(T)** and 75 % relative humidity **(RH)** for 4 days. Following storage, the eggs were prewarmed at 24 °C for 6 h, weighed and individually numbered before setting for incubation. During the first 6 days, all groups of hatching eggs were incubated at 37.7 °C T, 56 % RH and automated turning at a 90° angle every hour before being introduced into air-N_2 treatment to reduce O_2 level (ED 7–9). On day 18 of incubation, all the eggs were candled, and those with evidence of living embryos were weighed and transferred from the turning trays to hatching baskets, where they were subjected to normal conditions until hatching on ED 21.

Table 1
Composition of fresh eggs for 33 (younger) and 50wks (older) breeder flock before setting.[c].

Parameters	Egg weight (g)	Eggshell thickness (mm)	Eggshell weight (%)	Albumen weight (%)	Albumen index (%)	Yolk weight (%)	Yolk index (%)	Yolk colour	Yolk: albumen ratio	HU
33wks	53.85[b]	0.37	10.14	56.69	9.51	33.17	35.32	7.67[a]	59.20	84.43
50wks	60.42[a]	0.40	9.82	57.93	9.82	32.25	40.46	5.83[b]	55.69	76.15
SEM[a]	0.905	0.021	0.270	1.000	1.185	0.885	4.000	0.470	2.645	4.550
P-value[b]	0.001	0.317	0.423	0.495	0.865	0.573	0.384	0.014	0.485	0.237

Abbreviations: HU, haugh unit; wks., weeks.
a-b Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.
Results were expressed as a percentage of egg weight.
[a] SEM, pooled standard error of means.
[b] P, probability values.
[c] For all variables measured, n = 25 eggs per breeder flock age group.

4. Results

Effect of breeder age on fresh egg composition before setting: Table 1 illustrates how breeder age impacts the composition of fresh eggs before incubation. Eggs from older breeders (50 wks) had a significantly higher weight compared to those from younger breeders (33 wks) ($P = 0.001$). However, the yolk colour was notably more intense in eggs from younger breeders ($P = 0.014$). Breeder age did not significantly influence other parameters such as albumen weight ($P = 0.479$), yolk weight ($P = 0.561$), eggshell thickness ($P = 0.314$), and eggshell weight ($P = 0.418$). Additionally, no significant effects were observed on the albumen index ($P = 0.865$), yolk index ($P = 0.382$), yolk-to-albumen ratio ($P = 0.485$), or the Haugh unit ($P = 0.237$), which measures egg quality.

Egg weight loss and embryonic characteristics: Table 2 presents the effects of breeder age and oxygen concentration **(O_2)** level on egg weight loss and embryonic characteristics at embryonic day (ED) 11 after the period of air-N_2 exposure. A significant interaction effect was observed on egg weight loss ($P < 0.001$), embryo length ($P = 0.001$), embryo ($P < 0.001$), residual albumen ($P < 0.001$) and residual yolk ($P < 0.001$) weights. Egg weight loss varied significantly with both breeder age and O_2 level, with older breeders showing greater weight loss compared to younger breeders ($P < 0.001$). Specifically, embryos from older breeders exposed to 15 % and 17 % O_2 exhibited higher weight loss ($P < 0.001$), while those exposed to 21 % O_2 experienced less. Embryos from younger breeders also had significantly higher embryo weights and lengths compared to those from older breeders ($P < 0.001$). The interaction between breeder age and O_2 levels revealed that younger breeders, particularly under the 21 % O_2 condition, had embryos with the highest embryo length ($P < 0.001$), whilst the older breeders under hypoxic conditions (15 % and 17 % O_2) had the lowest embryo length ($P < 0.001$). Notably, embryos from the control group (21 % O_2) demonstrated higher embryo weights compared to those in the hypoxic groups (15 % and 17 % O_2) ($P < 0.001$). Residual albumen weight showed a similar trend, with the control group having the highest values ($P < 0.001$), while the residual yolk weight was notably higher in the 15 % and 17 % O_2 groups ($P < 0.001$).

Hatching events: Table 3 displays the effects of breeder age and O_2 levels on pipping time and hatching duration. There was a significant interaction between breeder age and O_2 level on internal pipping **(IP)** and external pipping **(EP)** durations ($P = 0.008$ and $P < 0.001$, respectively), as well as hatch time and hatching window ($P = 0.007$ and $P < 0.001$, respectively). Specifically, the hatching window was narrower, and EP duration was longer in the 50 wks breeders exposed to 17 % O_2 compared to other groups. The hatching time for the 50 wks breeders exposed to 17 % O_2 was significantly longer than that of the 33 wks breeders at 17 % O_2, 33 wks breeders at 21 % O_2, and 50 wks breeders at 15 % O_2 levels. Both chick emergence **(CE)** time and EP duration were higher in the 50 wks breeders compared to the 33 wks breeders ($P = 0.002$). Interestingly, EP time was shorter in eggs exposed to 17 % O_2 compared to 15 % and 21 % O_2. However, the EP time in eggs exposed to 21 % O_2 was comparable to the 15 % and 17 % O_2 groups ($P = 0.048$).

In Fig. 2, the spread of hatch expressed in percentiles of chicks hatched is presented. A significant interaction between breeder age and O_2 level was observed at both the 25th percentile ($P = 0.003$) and the 50th percentile ($P = 0.001$) during hatching. No significant effect of O_2 level ($P > 0.05$) was observed on its own, but the hatching time was earlier ($P < 0.001$) in the 33 wks group compared to the 50 wks group across the 25th, 50th, 75th, and 100th percentiles. No main effect of O_2 level was observed for the other percentiles ($P >$

Table 2
Egg weight loss and embryo characteristics of 33 and 50wks layer breeder eggs on embryonic day (ED) 11 after exposure to reduced oxygen concentration levels.

Parameters	Egg weight loss (%) [c]	Embryo length (cm)	Embryo weight (%)	Residual albumen weight (%)	Residual yolk weight (%)
Age (A)					
33wks	5.93[b]	62.67[a]	8.16[a]	20.21	11.12
50wks	7.42[a]	53.76[b]	6.03[b]	19.71	10.39
SEM[a]	0.142	0.565	0.086	0.466	0.453
Oxygen level (O_2)					
15 %	7.09[a]	50.93[b]	6.73[b]	17.09[c]	14.34[a]
17 %	7.26[a]	61.65[a]	7.08[b]	19.96[b]	11.13[b]
21 %	5.67[b]	62.07[a]	7.47[a]	22.83[a]	6.79[c]
SEM[a]	0.174	0.692	0.106	0.571	0.555
Interaction (A * O_2)					
33wks * 15 %	5.64[b]	57.86[b]	8.02[b]	13.58[c]	11.39[b]
33wks * 17 %	6.38[b]	65.36[a]	7.42[b]	19.04[b]	15.09[c]
33wks * 21 %	5.76[b]	64.80[a]	9.04[a]	28.02[a]	6.89[c]
50wks * 15 %	8.55[a]	44.01[c]	5.44[d]	20.60[b]	17.29[a]
50wks * 17 %	8.13[a]	57.93[b]	6.73[c]	20.89[b]	7.17[c]
50wks * 21 %	5.575[b]	59.33[b]	5.90[d]	17.65[b]	6.70[c]
SEM[a]	0.246	0.979	0.149	0.808	0.785
P-value[b]					
A	<0.001	<0.001	<0.001	0.458	0.263
O_2	<0.001	<0.001	<0.001	<0.001	<0.001
A * O_2	<0.001	0.001	<0.001	<0.001	<0.001

Abbreviations: wks., weeks.
[a-d] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.
[a] SEM, pooled standard error of means.
[b] P, probability value.
[c] Expressed as a percentage of initial egg weight and the data were first transformed to arcsine before analysis.

Table 3
Effect of layer breeder age and early reduced incubator oxygen concentration levels on embryo pipping time and duration.

Parameters	IP Time (hr)	EP Time (hr)	CE Time (hr)	IP Dur (hr)	EP Dur (hr)	Hatch Time (hr)	Hatch Window (hr)
Age (A)							
33wks	448.97	462.93	474.67[b]	13.95[a]	12.86[b]	26.93	21.60[a]
50wks	451.96	463.59	480.39[a]	11.62[b]	16.81[a]	28.43	18.80[b]
SEM[a]	1.030	1.220	1.180	0.561	0.506	0.600	0.600
Oxygen level (O_2)							
15 %	452.54	465.63[a]	478.30	13.09	14.34[b]	27.60	20.10[ab]
17 %	448.43	460.20[b]	477.43	11.77	17.22[a]	28.99	18.60[b]
21 %	450.44	463.94[ab]	476.87	13.50	12.94[b]	26.44	21.90[a]
SEM[a]	1.260	1.490	1.450	0.687	0.620	0.735	0.735
Interaction (A * O_2)							
33wks * 15 %	451.07	466.45	476.20	15.375[a]	13.08[b]	28.80[ab]	19.20[a]
33wks * 17 %	447.62	461.35	474.17	13.73[abc]	12.83[b]	26.55[b]	22.80[a]
33wks * 21 %	448.22	460.98	473.65	12.75[abc]	12.68[b]	25.43[b]	22.80[a]
50wks * 15 %	454.00	464.80	480.40	10.80[bc]	15.60[b]	26.40[b]	21.00[a]
50wks * 17 %	449.24	459.06	480.68	9.82[c]	21.62[a]	31.44[a]	14.40[b]
50wks * 21 %	452.65	466.90	480.10	14.25[ab]	13.20[b]	27.45[ab]	21.00[a]
SEM[a]	1.790	2.110	2.050	0.971	0.877	1.040	1.040
P-value[b]							
A	0.052	0.704	0.002	0.007	<0.001	0.089	0.003
O_2	0.092	0.048	0.784	0.200	<0.001	0.067	0.015
A * O_2	0.737	0.117	0.815	0.008	<0.001	0.007	<0.001

Abbreviation: IP, internal pipping; EP, external pipping; CE, chick emergence; Dur., duration; hr, hour, wks., weeks.
[a-c] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.
[a] SEM, pooled standard error of means.
[b] P, probability value.

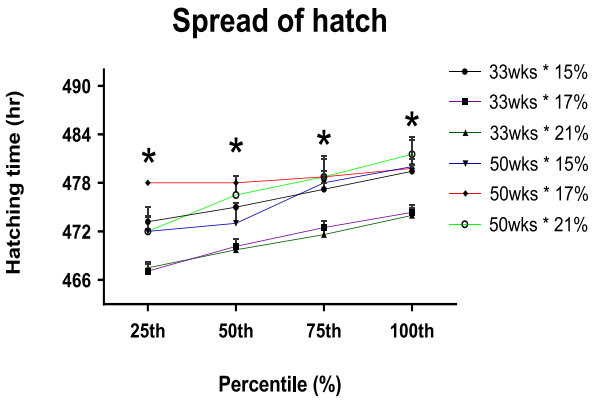

Fig. 2. Spread of hatch.

0.05).

Post-hatch chick quality assessment: The effect of breeder age and O_2 level on chick weight, yolk-free chick weight **(YFCW)** and yolk sac weight at hatch are summarized in Table 4. A significant interaction was observed between breeder age and O_2 level on chick weight ($P = 0.030$), yolk-free chick weight ($P = 0.036$) and yolk sac weight ($P = 0.050$). Chicks from 50 wks breeders exhibited the highest body weight ($P < 0.001$), while those from the 33 wks group had the lowest. The influence of O_2 level was only apparent on yolk sac weight, where a significant effect was noted ($P = 0.046$). However, no significant differences in chick weight or YFCW were observed when expressed as a percentage of initial egg weight at setting.

Additionally, a significant interaction between breeder age and O_2 level was also noted for chick length ($P = 0.001$) and pipping muscle weight ($P = 0.002$), as shown in Table 5. Breeder age significantly affected tibia ($P = 0.017$) and femur ($P = 0.007$) length, with longer lengths observed in the 50 wks breeders compared to the 33 wks group. Oxygen levels only significantly influenced shank length ($P = 0.001$) and toe length was neither affected by breeder age nor O_2 levels ($P = 0.060$).

Hatching performances and embryonic mortality: Table 6 summarizes the effects of breeder age and O_2 level on embryonic mortality, hatchability profiles and Tona chick score. There was a significant interaction between breeder age and O_2 level on the hatch of fertile eggs **(HOF)** ($P = 0.009$), early embryo mortality ($P = 0.029$) and total pipping embryo mortality ($P = 0.05$). Early embryo mortality was lowest in the 33 wks breeders exposed to 21 % O_2 and highest in both the 50 wks breeders exposed to 15 % and 21 % O_2 levels. Hatchability of fertile eggs was lower in the 50 wks breeder groups compared to the 33 wks groups. The 50 wks breeder group

Table 4
Effect of layer breeder age and early reduced oxygen concentration levels on chick quality, yolk-free weight and yolk sac weight.

Parameters	Chick weight (g)	Chick weight[c] (%)	YFCW (g)	YFCW[c] (%)	Yolk sac weight (%)
Age (A)					
33wks	31.72[b]	58.92	28.78[b]	53.45	9.41
50wks	36.42[a]	60.28	32.57[a]	53.91	10.48
SEM[a]	0.381	0.650	0.375	0.656	0.521
Oxygen level (O_2)					
15 %	34.32	60.12	31.40	54.99	8.55[b]
17 %	33.82	59.11	30.20	52.81	10.60[a]
21 %	34.08	59.57	30.43	53.25	10.59[a]
SEM[a]	0.467	0.796	0.459	0.803	0.638
Interaction (A * O_2)					
33wks * 15 %	32.82[b]	60.94	29.94[bc]	55.59	8.84[ab]
33wks * 17 %	31.07[b]	57.69	27.94[c]	51.89	10.11[ab]
33wks * 21 %	31.29[b]	58.11	28.47[c]	52.87	9.02[ab]
50wks * 15 %	35.83[a]	59.31	32.86[a]	54.39	8.26[b]
50wks * 17 %	36.57[a]	60.52	32.46[ab]	53.73	11.08[ab]
50wks * 21 %	36.87[a]	61.02	32.40[ab]	53.62	12.17[a]
SEM[a]	0.660	1.130	0.650	1.140	0.903
P-value[b]					
A	<0.001	0.146	<0.001	0.621	0.119
O_2	0.746	0.668	0.167	0.145	0.046
A * O_2	0.030	0.086	0.036	0.409	0.050

Abbreviations: YFCW., yolk-free chick weight, wks., weeks.
[a-c] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.
[a] SEM, pooled standard error of means.
[b] P, probability value.
[c] Weights are expressed as a percentage of initial egg weight for the various breeder flock ages.

Table 5
Effect of layer breeder age and early reduced oxygen concentration levels on external chick quality measurements and pipping muscle.

Parameter	Chick length (cm)	Shank length (cm)	Toe length (cm)	Tibia length (cm)	Femur length (cm)	Pipping muscle (%)
Age (A)						
33wks	17.33	2.31	2.03	3.11[b]	2.10[b]	1.12
50wks	17.59	2.27	2.07	3.32[a]	2.26[a]	1.10
SEM[a]	0.191	0.021	0.021	0.059	0.038	0.063
Oxygen level (O_2)						
15 %	17.50	2.32[a]	2.07	3.22	2.20	1.20
17 %	17.22	2.20[b]	2.03	3.15	2.13	1.12
21 %	17.67	2.35[a]	2.05	3.28	2.20	0.99
SEM[a]	0.234	0.025	0.025	0.073	0.047	0.077
Interaction (A * O_2)						
33wks * 15 %	18.17[a]	2.30	2.07	3.20	2.07	1.46[a]
33wks * 17 %	16.67[b]	2.27	1.97	2.93	2.07	0.97[b]
33wks * 21 %	17.17[ab]	2.37	2.07	3.20	2.17	0.92[b]
50wks * 15 %	16.83[ab]	2.33	2.07	3.23	2.33	0.94[b]
50wks * 17 %	17.77[ab]	2.13	2.10	3.37	2.20	1.27[ab]
50wks * 21 %	18.17[a]	2.33	2.03	3.37	2.23	1.06[ab]
SEM[a]	0.331	0.036	0.036	0.103	0.066	0.109
P-value[b]						
A	0.351	0.136	0.259	0.017	0.007	0.761
O_2	0.399	0.001	0.647	0.439	0.515	0.161
A * O_2	0.001	0.077	0.060	0.157	0.319	0.002

Abbreviations: wks., weeks.
[a-b] Means within the same column with different superscripts indicate significance at $P < 0.05$ within treatments.
[a] SEM, pooled standard error of means.
[b] P, probability value.

also exhibited the highest levels of late embryonic mortality ($P < 0.001$), internal pipping dead ($P = 0.031$), external pipping dead ($P = 0.017$) and total embryo mortality compared to the 33 wks breeder group. The individual effect of O_2 level was significant, as higher pipping dead rates were recorded in the 15 % and 17 % O_2 groups compared to the control, 21 % O_2 group ($P = 0.031$). In contrast, the HOF was higher in the control group (21 % O_2) compared to the hypoxic groups (15 % and 17 % O_2).

There was no significant interaction between breeder age and O_2 level for the Tona chick score ($P = 0.110$). However, breeder age

Table 6
Effect of layer breeder age and early reduced oxygen concentration levels on embryonic mortality, hatchability profile and chick score.

Parameters	Early dead	Mid dead	Late dead	Internal pipping dead	External pipping dead	Total pipping dead	Hatchability		
							Fertility	Hatch of fertile	Tona chick Score
Age (A)									
33wks	3.41	1.89	2.99[b]	4.68[b]	6.15[b]	10.83[b]	52.67	81.57[a]	96.84[a]
50wks	4.96	3.57	14.80[a]	7.93[a]	13.49[a]	20.08[a]	49.67	56.84[b]	95.28[b]
SEM[a]	0.533	0.596	0.997	0.943	1.850	1.860	2.080	0.785	0.290
Oxygen level (O_2)									
15 %	5.13	2.12	8.60	5.85	9.17	15.02[ab]	49.67	69.51[a]	96.84[a]
17 %	2.91	2.28	8.69	8.48	12.14	20.62[a]	51.33	65.82[b]	95.78[ab]
21 %	4.53	3.79	9.39	4.58	8.16	10.74[b]	52.50	72.28[a]	95.56[b]
SEM[a]	0.651	0.731	1.220	1.160	2.260	2.280	2.550	0.962	0.355
Interaction (A * O_2)									
33wks * 15 %	3.94[ab]	1.99	3.97	4.68	3.40	8.08[bc]	50.67	82.02[a]	97.60
33wks * 17 %	3.75[ab]	1.93	1.88	6.76	10.77	17.53a[bc]	53.33	75.55[b]	96.04
33wks * 21 %	2.55[b]	1.76	3.12	2.59	4.29	6.88[c]	54.00	87.14[a]	96.88
50wks * 15 %	6.31[a]	2.25	13.23	7.02	14.93	21.95[ab]	48.67	57.00[c]	96.08
50wks * 17 %	2.06[ab]	2.64	15.51	10.19	13.51	23.70[a]	49.33	56.09[c]	95.52
50wks * 21 %	6.51[a]	5.83	15.66	6.58	12.02	14.59[abc]	51.00	57.41[c]	94.24
SEM[a]	0.921	1.027	1.730	1.630	3.185	3.230	3.600	1.360	0.502
P-value[b]									
A	0.065	0.078	<0.001	0.031	0.017	0.004	0.328	<0.001	<0.001
O_2	0.074	0.256	0.884	0.090	0.456	0.031	0.737	0.002	0.026
A * O_2	0.029	0.187	0.446	0.878	0.390	0.050	0.962	0.009	0.110

Abbreviations: wks., weeks.
[a-c] Means within the same column with different superscripts indicate significance at $P \leq 0.05$ within treatments.
[a] SEM, pooled standard error of means.
[b] P, probability value.

alone significantly influenced Tona chick score, with the 33 wks breeder group showing higher scores than the 50 wks group ($P < 0.05$). A lower O_2 level of 15 % resulted in a higher Tona chick score compared to the control, 21 % O_2 group, although this was not significantly different from the 17 % O_2 group.

5. Discussion

This study aimed to evaluate the effect of layer breeder age and early reduced incubator oxygen concentration **(O_2)** level on embryo development, hatching performances and chick quality. Results from the fresh egg composition analysis before incubation showed that while older (50 wks) breeders lay heavier eggs, the proportions of yolk and albumen remain relatively unchanged across age groups. The increased egg weight observed in the 50 wks breeders compared to the 33 wks flock is attributed to enhanced reproductive system development and efficiency. It is well-documented that egg size increases with breeder age [37–40], due to increased nutrient deposition [41]. As breeders age, their ability to synthesize and deposit lipids and proteins into eggs improves, resulting in larger eggs with more yolk and albumen [42–45]. However, in this study, yolk and albumen weights were not significantly affected, contrasting with findings by Ref. [46] who reported a 40 % increase in yolk weight and a 13.3 % increase in albumen weight in eggs from 50 wks breeders compared to those from 30 wks breeders. The more vibrant yolk color observed in eggs from younger breeders could be attributed to differences in dietary absorption, metabolic efficiency, or carotenoid allocation. Younger breeders may process dietary pigments more efficiently, leading to deeper yolk pigmentation. This variation in yolk color with breeder age is likely due to physiological changes that occur over time.

Egg composition parameters such as albumen weight, yolk weight, eggshell thickness, yolk-to-albumen ratio, and Haugh Unit **(HU)** showed no significant variation across age groups. This stability could be due to the physiological maturation or nutritional status of the hens [47]. also reported no change in yolk or albumen weights despite increased egg size in older breeders. The findings of this study also contrast with [48] who observed higher yolk-to-albumen ratios in older breeders (73 wks) compared to younger ones (32 wks), likely reflecting differences in growth due to the physiological developmental stages.

Following the exposure to air-N_2 to reduce O_2 level, embryo quality at embryonic day **(ED)** 11 showed an interaction between breeder age and O_2 levels. Embryo weights from 50 wks breeders were significantly affected by hypoxic conditions compared to those from 33 wks breeders and the control groups. Specifically, lower relative embryo weights and lengths were observed in older breeders, with a more pronounced effect in eggs from 50 wks breeders incubated under 15 % and 21 % O_2 levels. This restriction in the growth of

older flocks is likely due to insufficient oxygen for cellular metabolism, which is essential for cell division and organ development. The more substantial impact in the 50 wks breeder eggs under both 15 % and 21 % O_2 may also be due to increased egg weight loss or variability in the eggs used for analysis, warranting further investigation.

Older breeders often exhibit thinner eggshells, which increases water loss during incubation, as supported by findings from Refs. [49,50]. In contrast, eggs from younger flocks have thicker eggshells which reduces moisture loss [44,51]. Hypoxic conditions during the time of exposure, particularly at 15 % O_2 level, exacerbated these effects by altering metabolic processes and increasing the demand for water and gas exchange. The higher weight loss observed in 50 wks breeder eggs under these oxygen conditions suggests that the embryos are compensating for reduced oxygen availability, leading to higher respiratory water loss.

In the current study, the reduced embryo weight coupled with higher residual yolk and lower residual albumen weights a day after exposure to 15 % O_2 compared to those exposed to 17 % and 21 % O_2 levels indicates that severe hypoxic conditions delay yolk utilization, likely due to reduced energy production during the period of exposure. This metabolic conservation of yolk nutrients under hypoxia helps ensure embryo survival by slowing the growth rate, a phenomenon corroborated by Ref. [52] who found that exposure to 15 % O_2 for six days reduced embryo growth. The increase in yolk sac weight in embryos exposed to hypoxia was attributed to lower metabolic heat production which eventually slowed nutrient utilization [52]. [29] indicated that these hypoxic effects limit nutrient metabolism for growth in both young and older broiler breeder flocks. However, the current study reports a more severe impact on older layer flocks compared to the younger ones, due to high susceptibility to weight loss and the increased metabolic demand, a physiology that requires more oxygen to support development [53]. suggested that reduced oxygen consumption during hypoxia is an adaptive mechanism which only suppresses non-essential functions like thermogenesis and tissue growth, rather than a direct consequence of oxygen deficiency.

Contrary to the findings of immediately reduced embryo weight [35], reported that daily exposure to 17 % O_2 level during CAM development resulted in similar embryo weights as control embryos. This suggests a catch-growth mechanism is possible when embryos return early to normoxic incubation conditions [54]. Evidence is seen in the embryo and chick organ development as [55] observed an increase in CAM vascularization and vessel dilation of layer chick organs at hatch as an adaptive response to hypoxia. In broilers, while [29] found no interaction between breeder age and O_2 level on yolk-free body weight at ED 14, an interaction was noted at ED 18, emphasizing developmental stage response to hypoxia. Different stages of development respond variably to hypoxic conditions in terms of metabolic and morphological adaptations [56,57] However, the intensity and duration of hypoxia exposure are crucial in determining its impact on embryonic growth [36,52].

At hatch, chick weight, yolk-free chick weight, and relative yolk-sac weight were found to interactively depend on breeder flock age and the O_2 level during incubation. Chick weights from 50 wks breeders were higher than those from 33 wks breeders. This finding aligns with research indicating increased hatchling weights in older flocks [58,59,60] suggesting a positive correlation between breeder age and chick weight. Early-stage reduced O_2 levels alone did not affect chick or yolk-free chick weights **(YFCW)** in the current study, but breeder age interacted with O_2 level to increase chick weight and YFCW. This confirms the findings of [15] who reported that eggs incubated at higher altitudes—mimicking a hypoxic environment—displayed significantly higher body weights compared to those incubated at lower altitudes. The aftermath adaptability of embryos led to enhanced chick weight through alterations in cardiac output and redistribution of oxygenated blood to vital organs [61]. However, chronic hypoxia on the other side has been reported to decrease chick weight [5,57]. The fact that chicks from young breeders had a higher quality compared to chicks from older breeders in the current study could be attributed to the deterioration rate of albumen quality [58] and the increase of yolk cholesterol content which both could increase the percentage of chicks of low quality obtained from older breeders [62,63]. In early chicken development, breed-specific genotypes and yolk environments interact synergistically [64] The current result agrees with [48]'s finding which showed an increased percentage of chicks of low quality in eggs from older breeders.

Reduced oxygen (O_2) levels during incubation have been shown to shorten the femur, tibia, and shank lengths in chickens [65]. However, only the shank length was significantly affected by the level of oxygen reduction in the current study. In the current study, embryos exposed to 15 % and 17 % hypoxia had higher Tona chick scores likely due to the amount of remaining yolk and the well-closed navel score.

The level of O_2 and CO_2 in the air space of the egg determines the time of external pipping of an embryo [66]. The slow physiological and morphological trajectories of developing embryos in the current study explain why EP time was higher in the 15 % O_2 level group. Mild hypoxia has been reported to cause a decline in O_2 consumption at internal pipping [67] but the responses to different levels of hypoxia increased during the external pipping phase [68]. The severity of hypoxia in the 17 % O_2 group and time of exposure may not be sufficient to increase EP time in the ISA Brown strain as observed in the present study. Concerning the breeder age effect, the current results showed an increase in EP time and duration in young breeders. The fact that embryos from young breeder eggs grew much faster than embryos from old breeder eggs in the current study explains why chicks from younger breeder eggs hatched earlier than chicks from older breeder eggs. The hatching window in the old breeders group compared to young breeders in this study disagrees with [25] which showed a longer hatching window in eggs from 51 wks breeders compared to 38 wks breeders' eggs. The fertility and hatch of fertile eggs are also possible factors which could be considered to have influenced the occurrence of longer hatch windows for younger breeders than the older ones especially because there was a weak interaction effect for breeder age and oxygen levels. Besides, the earlier hatching times observed in the 33 wks group, across all percentiles (25th, 50th, 75th, 100th), suggest that the superior physiological condition of younger breeders facilitated more robust hypoxic resistance during embryonic development under varying oxygen conditions. While oxygen levels influenced early hatching events at 25th and 50th percentiles, breeder age was the dominant factor influencing overall hatching performance. As embryos grow, oxygen demand rises, and possibly the thinner eggshells of older breeder eggs could not resist hypoxic environments compared to the younger breeders. Additionally, higher pipping mortality in the 50 wks group was due to delayed or prolonged pipping time which was caused by oxygen insufficiency (15 % and 17 %

O_2), which impaired essential processes like pipping muscle activity required for hatching. This evident as 50 wks breeders interactively had lower pipping muscle compared to the 33 wks breeders.

The reduced hatchability of fertile eggs **(HOF)** in the 50 wks group can be attributed to increased dehydration because of high weight loss as a result of impaired gas exchange in older breeder eggs. This eventually leads to higher embryo mortality and lower hatch of fertile. The lower fertility and HOF observed for 50 wks breeders in the present study align with the results of [48,69] which demonstrated reduced fertility, lower total hatchability and decreased hatchability of fertile eggs in older breeder ducks (73 wks) compared to younger breeders (32 wks) [70]. also attributed the low fertility and high embryonic mortality in ISA breeders to low roosters among breeder flocks but in the current finding, the decline in HOF in older breeders was primarily attributed to increased embryonic mortality. It is well known that embryos are at increased risk of early embryo death when exposed to low O_2, high CO_2, and elevated temperatures [71] Changes in O_2 levels can significantly impact embryonic development and survival. Low oxygen conditions may alter the cardiovascular and metabolic functions of embryos, affecting their developmental trajectories [72,73] and with increased embryonic mortality, emphasizing the vulnerability of embryos to oxygen fluctuations [74], particularly at the early embryonic stage of development. According to Ref. [75] early embryos adopt a simple strategy of survival or death, and individuals' survival capacities are determined by interindividual differences rather than compensatory mechanisms.

5.1. Implications and limitations of the study

The study suggests that early mild hypoxia may stimulate growth afterwards and eventually improve chick quality at hatch. However, this is more evident in younger than older breeders as they showed less susceptibility to the impact of hypoxia compared to the older flock. Hypoxia, particularly in older breeders extends the hatching window and increase embryonic mortality. The findings also emphasized the importance of customizing hypoxic exposure in incubation protocols. Oxygen levels during critical stages of incubation, particularly between ED 7–9, should be fine-tuned based on breeder age to maximize outcomes because controlled hypoxia has the potential to improve chick quality. It is worth noting that irrespective of the outcome, embryos are at risk of mortality and that necessitates caution. Overall, the study underscores the potential for age-specific incubation practices that optimize hatchery efficiency and productivity by accounting for the varying needs of breeder flocks at different ages.

The limitations of this study are; that it focused on only two breeder age groups (33 and 50 wks) which are not wide apart, therefore, the sample size of eggs used for fresh egg composition determination was the reason for the non-significant influences of the breeder age on most egg components. A larger sample size would have been preferable and would have given a clear understanding of which composition parameter of the fresh egg was influenced by reduced oxygen level, leading to the effects observed in the present study. Also, chick dimensions as always used were not expressed as egg weight. Expressing the dimensions relative to egg weight will give a concise impact on these chick quality parameters. In addition, the study only employed a fixed hypoxic exposure period (ED 7–9) and duration (1 h/day), though hypoxia's effects may vary with changes in timing, intensity and exposure length. Future studies should investigate different hypoxic windows and durations to better understand their impact on embryonic development, chick quality and post-hatch outcomes particularly in layer breeders. Finally, during the period of flushing with air-N_2, there was no control over the level of RH, T and CO_2 level in the incubator. This indicates that controlled experimental conditions of the study may not fully reflect environmental variability in commercial hatcheries. Factors such as incubation conditions, ventilation systems and altitude could modify hypoxia's effects on embryo development and chick quality. Therefore, further research is needed to confirm the applicability of these findings in commercial settings and different geographical regions.

6. Conclusion

Mild hypoxic exposure at an early stage of embryonic development reduces embryo growth rate irrespective of breeder age. Older breeders are more susceptible due to higher egg weight loss. However, the embryo undergoes a catch-up growth mechanism to restore growth when it returns to normoxic conditions. An interaction effect between layer breeder age and reduced oxygen levels was observed in extending hatching durations, hatch time and windows, increased chick weight and yolk-free chick weight in the current study. However, there is no clear and strong evidence of the main effect of oxygen level except for the decreased yolk sac weight at hatch which indicates a better yolk absorption rate during embryogenesis after the exposure period. This has the potential to improve chick quality if an optimum hypoxic duration and concentration for layer breeders can be achieved. Negatively, the present results also showed a combined and main effect of breeder age and oxygen levels on high early and pipping embryonic mortality while causing a decrease in the hatchability of fertile eggs.

CRediT authorship contribution statement

Richard Koblah Agbehadzi: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hezouwe Tchilabalo Meteyake:** Writing – review & editing. **Benjamin Adjei-Mensah:** Writing – review & editing. **Prince Sasu:** Writing – review & editing. **Achiamaa Asafu-adjaye Koranteng:** Writing – review & editing. **Nideou Dassidi:** Writing – review & editing. **Jacob Alhassan Hamidu:** Writing – review & editing, Supervision, Conceptualization. **Kokou Tona:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization.

3. Data collection

3.1. Fresh egg compositions, egg weight loss, embryo and embryonic characteristic measurements

At the onset of incubation, twenty-five (25) hatching eggs of each breeder age (33 and 50 wks) were randomly selected for the determination of the egg characteristics that include: egg weight, eggshell thickness, eggshell weight, albumen weight, yolk weight, albumen index, yolk index, yolk colour, yolk:albumen and Haugh unit **(HU)**. Eggshell thickness was measured using a digital electronic Vernier caliper (YIERYI, Model No: RTO05102, Guangdong, China). Following exposure on ED 11, a total of nine (9) live embryos per treatment were used for embryo and embryonic characteristics measurements which included; egg weight loss, embryo weight, embryo length, residual albumen weight and residual yolk weight.

All absolute weight(s) including fresh egg compositions, embryo and embryonic characteristics were measured using a sensitive weighing scale (Ohaus STX8200 Scout) and expressed as percentages of egg weight using the formulas below [47].

- Eggshell weight (%) = [(eggshell weight (g))/(egg weight (g))] × 100;
- Albumen weight (%) = [(albumen weight (g))/(egg weight (g))] × 100;
- Yolk weight (%) = [(yolk weight (g))/(egg weight (g))] × 100;
- Albumen index (%) = [(albumen height (g))/(albumen width (g))] × 100;
- Yolk index (%) = [(yolk height (g))/(yolk width (g))] × 100;
- HU = 100 × log(H-1.7W0.37 + 7.6); H - albumen height, W – egg weight
- Egg weight loss (%) = [(final egg weight, ED 11 (g))/(initial egg weight, ED 0 (g))] × 100;
- Embryo weight (%) = [(embryo weight (g))/(egg weight (g))] × 100;
- Residual albumen weight (%) = [(residual albumen weight (g))/(egg weight (g))] × 100;
- Residual yolk weight (%) = [(residual yolk weight (g))/(egg weight (g))] × 100;

The 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 [76–78].

3.2. Hatching events

Between 445 and 490 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 [79]. 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 [80].

3.3. 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, pipping muscle weight, external quality measurements and Tona chick score [81]. The yolk sac weight was expressed as a percentage of chick weight while the chick weight and YFCW were relative to the initial average egg weight at setting. The formulas used are below.

- Chick weight (%) = [(chick weight (g))/(initial egg weight (g))] × 100;
- YFCW (%) = [(YFCW (g))/(initial egg weight (g))] × 100;
- Yolk sac weight (%) = [(yolk sac weight (g))/(chick weight (g))] × 100.
- Pipping muscle (%) = [(pipping muscle weight (g))/(chick weight (g))] × 100.

Nine (9) chicks per treatment were randomly selected for measurements of external qualities, including chick length, shank length, toe length, and tibia and femur lengths. 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 [82,77,78]. Chick quality was scored using the Tona scoring method, 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 summing all the scores observed from each parameter.

3.4. Hatching performances and embryo mortality

Chicks hatched at the end of incubation were recorded according to treatments to determine the hatch of fertile **(HOF)** based on the number of fertile eggs. The estimation formula is as follows.

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

Non-hatched eggs were counted and categorized based on embryonic mortality stages. Following a modified classification from Ref. [83] embryos were classified into early (0−10 ED), middle (11−14 ED), and late (15−19 ED) stages of death, as well as internally pipped and externally pipped dead. Total pipping mortality was calculated by summing all internal and external pipped deaths. The stages were distinguished using the development of the chorioallantoic membrane **(CAM)** observed during the study. Each stage of embryonic mortality was expressed as a percentage of fertile eggs using the following formula.

- **Embryonic dead (stage)** = [(number of deads counted/fertile eggs number) x 100]

3.5. Statistical analysis

The experimental samples in the current study were eggs, embryos and chicks. The data collected on egg composition before incubation was subjected to an independent *t*-test model in Minitab Statistical Software, version 21.2 (Minitab, LLC, NY, US, 2021) using the general linear model (GLM) procedure: $\mathbf{Yijk = \mu + Ai + eijk;}$ where **Yijk** is the variable measured, **μ** is the general mean, **Ai** is the main effect of breeder age (i = 33 or 50 wks) and **eijk** is the random residual error term. All other data were arranged as completely randomized in a 2 x 3 factorial design and subjected to a two-way ANOVA using the model: $\mathbf{Yijk = \mu + Ai + Oj + AO_2ij + eijk}$; where **Yijk** is the variable measured, **μ** is the general mean, **Ai** is the main effect of breeder age (i = 33 or 50 wks), $\mathbf{O_2j}$ is the effect of oxygen concentration $\mathbf{(O_2)}$ levels (j = 15 %, 17 % or 21 %), $\mathbf{AO_2ij}$ is the interaction term between breeder age and O_2 in the incubator and **eijk** is the random residual error term. Embryo and embryonic weight(s), chick and yolk-free chick weight(s) and yolk sac weight (s) were adjusted using analysis of covariance with initial egg weights. Data expressed as percentages was first subjected to square root arc sine transformation before analysis. Mean comparisons were performed using the Tukey Test, with significance determined at $P < 0.05$.

Ethics statement

The entire experimental procedures were conducted following the guidelines of the Animal Ethics and Scientific Committee and were approved by the Regional Center of Excellence for Poultry Sciences at the University of Lomé (CERSA-UL), under approval number 028/2021/BC-BPA/FDS-UL.

Data availability statement

All analysed data supporting the findings of this study are provided within the main text and the accompanying supplementary information. Original data from the field are available with the corresponding author and can be made available upon request.

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Declaration of competing interest

The authors 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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