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Interactive Effects of Incubation Temperature and In Ovo Feeding on Hatchability, Organ Development, Bone Minerals and Blood Metabolites in Broiler Chicks

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ABSTRACT

Background: Optimal incubation temperature and in ovo feeding strategies during incubation are crucial for ideal performance and welfare of chicks.

Objective: This study investigated the effects of incubation temperature and early in ovo feeding on hatchability, internal organ development, bone mineralisation and blood metabolites in broiler chicks.

Methods: A total of 1200 eggs from 58-week-old Arbor Acre breeders, averaging 62 ± 1 g, were incubated in separate dual-system automatic incubators (Senjie series DZ 47-53) at specified temperatures and 60% humidity. A total of 400 eggs were randomly assigned to three incubation temperature treatments, with four replicates per treatment, based on predetermined air temperatures of 36.5°C, 37.0°C and 37.5°C, maintained from embryonic day (ED) 1 until ED 18. On ED 10, eggs were candled, and each temperature group was further divided into three subgroups: the first serving as a control, where eggs were perforated but not injected; the second receiving an in ovo injection of glucose solution (5 mg/mL); and the third receiving an in ovo injection of vitamin-D3 solution (25 mg/mL). The supplements were administered 0.2 mL at egg level in the air sac.

Results: Hatchability showed a clear temperature-dependent response, reaching a maximum of 89.86% in glucose-injected eggs at 37.5°C and decreasing to 48.33% in vitamin-D3 injected eggs at 36.5°C ($p < 0.05$). Bone mineral content was influenced by interactions between incubation temperature and feeding, with calcium highest in glucose-fed chicks incubated at 37.0°C, while phosphorus was highest in control eggs incubated at 36.5°C ($p < 0.05$). By Day 21, chicks fed vitamin-D3 consistently demonstrated lower cholesterol levels, whereas those fed glucose and controls showed higher alkaline phosphatase activity under increased incubation temperatures ($p < 0.05$).

Conclusion: Optimal results were achieved with glucose injection at 37.0°C and 37.5°C, improving hatchability, intestinal development and bone mineralisation, whereas vitamin-D3 supplementation at lower temperatures negatively impacted hatchability and metabolic balance.

1 | Introduction

Efforts to combat the rising hunger crisis have led to increased demand for broiler and layer products worldwide. As a result, the poultry industry has prioritised optimising chicken production and efficiency. Producing and distributing high-quality chicks is the first step towards successful poultry ventures, and this heavily depends on management decisions and practices at the hatchery. While fertile eggs are designed to protect and nourish developing embryos until hatching, factors such as genotype and nutrient deficiencies in eggs can influence these functions (Kuka et al. 2023, 2024; Okai et al. 2025). In such cases, exogenous nutrients can be injected through the eggshell into the egg to provide supplementary nourishment. This process, known as in ovo feeding (Chen et al.), involves introducing nutrients into breeder eggs via the eggshell. The technology has demonstrated varying but significant effects on the development and physiological functions of broiler chickens' organs (Naeem Asa et al. 2022). In ovo supplementation has also enhanced hatchability, post-hatch growth rates and feed conversion efficiency (Das et al. 2021; Arian et al.; Nabi et al. 2023, 2024). As noted by Li et al. (2024), providing essential amino acids in ovo has resulted in marked improvements in bone mineral density and overall growth performance. In ovo feeding may also increase organ weights and mineral content in post-hatch chicks by improving nutrient absorption and metabolism during embryonic development (Yair et al. 2015).

On the other hand, the effectiveness of in ovo injection varies and seems to be impacted by a number of interrelated variables, including the nutrition, incubation temperature and duration of thermal adjustment. For instance, the development of the skeletal system in broilers can be strongly influenced by the timing and composition of the in ovo nutrients supplied (Zhou et al. 2023). Additionally, organ development, bone mineralisation and blood biochemical properties may suffer from deviations in optimal incubation temperatures (Wijnen et al. 2020; Das et al. 2021). According to Khalil et al. (2024), temperatures that are too low or too high can negatively affect the growth and development of various organs. The interaction between incubation temperature and in ovo feeding impacts the performance and welfare of broiler chickens (Das et al. 2021; Zhao et al. 2023), along with blood biochemical parameters such as lipid, protein and glucose profiles, which influence metabolic activities (Yehia et al. 2024). Therefore, maintaining an appropriate incubation temperature in conjunction with suitable in ovo feeding strategies is vital for enhancing embryogenesis, hatchability and overall post-hatch quality.

Despite these insights, the specific interactive effects of in ovo nutrient supplementation and incubation temperature during the mid-phase of embryonic development remain poorly understood. Most previous studies have examined either the effects of in ovo feeding alone or temperature variations in isolation (Fatemi et al. 2022; Okai et al. 2025), leaving a gap in understanding how these two critical factors jointly influence organ development, bone mineralisation and blood biochemical parameters in broiler chicks. Moreover, the optimal combination of incubation temperature and nutrient supplementation that maximises embryogenesis, hatchability and post-hatch performance is yet to be established, particularly under tropical hatchery conditions. Therefore, this study aims to explore how specific incubation temperatures interact with early (mid-phase) in ovo feeding of selected supplements, affecting internal organ development, bone mineralisation and blood biochemical properties in broiler chicks.

2 | Materials and Methods

2.1 | Sterilisation, Preparation and Application of In Ovo Feed Supplements

The equipment used was sterilised in a Panasonic MLS-3781 autoclave, loaded into a sterilisation frame and dried in an Electric Blast Drying Oven (Shanghai-Heng Scientific Instrument Co. Ltd.). A glucose solution was obtained by dissolving glucose in phosphate buffer solution (PBS) to a concentration of 5 mg/mL. The vitamin-D3 solution was also obtained by mixing vitamin-D3 and anhydrous ethanol (aided solubilisation), and then PBS was added to the mixture to form a concentration of 25 mg/mL. The concentrations of glucose and vitamin-D3 solutions employed in this study were based on pre-trial results, which did not adversely affect hatching rate and chick quality. On incubation Day 10, each egg was injected with 0.2 mL of the supplements at egg level through the air sac using an automatic syringe (Kuka et al. 2023). The injection was performed on a UV-sterilised table. The injection sites were sealed with wax, and the eggs were returned to their respective incubators.

2.2 | Experimental Design

The experimental chicks were obtained from a batch of 1200 eggs laid by a 58-week-old Arbor Acres broiler breeder flock. Breeder eggs that weighed an average of 62 ± 1 g were sampled into the various treatment groups and incubated in separate Dual-system Automatic Incubators (Senjie series incubator DZ47-53) of 60% humidity. The eggs were randomly assigned to three incubation temperature treatments (400 eggs per treatment with 4 replicates) based on machine-set air temperatures of 36.5°C, 37.0°C and 37.5°C, maintained from ED 1 of incubation until ED 18. On the 10th day of incubation (mid-incubation phase) after candling, each temperature group was further divided into three subgroups: the first subgroup in each of the three groups served as the control, with which eggs were perforated but received no injection; the second subgroup received an in ovo injection of glucose solution; and the third subgroup received an in ovo injection of vitamin-D3 solution. On the 444th hour of incubation, hatching events (external pipping and chick emergence) were monitored at every 3-h period and recorded. From each of the 9 groups, 10 chicks (90 chicks in total) were randomly selected at hatch for internal organs harvesting and blood sampling. Additionally, at 21 days of age, 10 chicks from each treatment were selected for right tibia bone harvest and blood sampling.

2.3 | Post-Hatch Husbandry

During post-hatch, the chicks were kept in a 38 × 47 × 35 cm battery cages at a temperature of 32°C and gradually reduced to 23 ± 2°C with a humidity of 65 ± 3%. The chicks in each group were randomly allotted to 5 replicates with 10 chicks per cage, which

were enclosed in a poultry house. The chicks were fed a starter diet that was formulated using the National Research Council's recommendations (National Research Council 1994) with crude protein of 23% and metabolisable energy of 3000 kcal/kg. Feed and water were provided ad libitum. Gumboro and 1st Newcastle vaccines were administered to the birds during the first and second weeks, respectively, but no prophylaxes were provided.

2.4 | Tissue Sampling and Measurement of the Internal Organs

At hatch and on Day 2, 10 chicks were selected and humanely sacrificed and eviscerated. At hatch, the internal organs, including liver, kidney, thigh muscles, duodenum, ileum and jejunum at hatch were removed; however, at Day 21, the right tibia bones were removed. The weight and length of each organ were immediately weighed and measured, respectively, using an Ohaus external chemical balance (model SP602AM; 165 mm × 142 mm; 2000 g; resolution 0.1 g), a ruler and a pair of dividers (Agyekum et al. 2022). The weight of the internal organs was expressed as a percentage of the body weight at hatch.

2.5 | Testing for Blood Metabolites

At 8:00 a.m. on both experimental days (at hatch and Day 21), five selected chicks from each treatment group were euthanised by exsanguination following cervical dislocation (Ripplinger et al. 2024), after which blood was drawn from each bird through the wing vein. Serum was immediately separated from the blood samples by centrifugation in a euthanising tube at 4°C, 3000r/rpm for 10 min and then kept at −20°C in a deep freezer for 7 days. Using commercial kits, serum concentrations of calcium, phosphorus, albumin, glucose, cholesterol, triglycerides and alkaline phosphatase were determined at the Sichuan Michael Biotechnology Co., Ltd. using an automated 7160A Hitachi Biochemical Analyser (Hitachi Tokyo, Japan) as directed by the manufacturer.

2.6 | Testing for Calcium and Phosphorus in the Tibia Bone

The right tibia bone of the 10 chicks per treatment group was degreased with alcohol and benzene for 72 h and dried at 105°C in a Qixin Oven (DHG-9070A, China). The bones were then burned to ash at 550°C for 6 h in a Muffle Furnace (Xianke, KSJ and China) to prevent flames and smoke. The calcium and phosphorus levels were determined using the potassium permanganate and spectrophotometric methods.

2.7 | Calcium Level Determination

Two grams of the bone ash was mixed with 10 mL of hydrochloric acid and 30 μL of concentrated nitric acid. The mixture was heated on an electric hot plate until the boiling point. The solution was then pipetted into separate 200 mL and 100 mL beakers, each and then diluted with deionised water (ddH_2O) to about 100 mL. Two drops of methyl red indicator were then added to each of the diluted solutions. Ammonia was added to each of the solutions until it turned orange, and then hydrochloric acid was added until it turned pink. The solutions were heated, and 10 mL of 4.2% ammonium oxalate solution was dropped into each of them to lower the pH for the calcium to precipitate. The solutions were heated for an additional 2 min to ensure complete precipitation of the calcium oxalate. The solutions were cooled overnight to maximise the precipitation and then filtered with a quantitative filter paper. The filtrates (precipitates) were rinsed with ammonia solution (1 part of water to 50 parts of ammonia oxalate) and transferred into their original 250 mL beaker, and dissolved with 10 mL of sulphuric acid solution (1 part of water to 3 parts of sulphuric acid) and 100 mL of ddH_2O. Each solution was heated up to 75°C–80°C on an electric heater. The dissolved solution from each treatment group was then titrated with standardised potassium permanganate (KMnO4) solution using methyl red as the indicator. The titration of each sample was ended when a light pink colouration persisted for more than 1 min. The calcium content of each sample was calculated based on the volume and known concentration of the $KMnO_4$ solution used (Song et al. 2022). The formula used was:

$$\%x = \frac{(V - V_0) \times c \times 0.02}{m \times \left(\frac{V'}{100}\right)} \times 100 = \frac{(V - V_0) \times c \times 200}{m \times V'}$$

where x (%) is the calcium concentration given as a mass percentage (%); V is the volume of the samples' consumption of the potassium permanganate standard solution (mL); V_0 is the amount of standard potassium permanganate solution (mL) that the blank solution consumed; c is the standard potassium permanganate solution concentration (mol/L); V' is the volume of the sample decomposition solution pipetted during the titration (mL); m is the mass of the sample (g); and 0.02 is the calcium expressed in grams equivalent to 1.00 mL of standard potassium permanganate solution.

$$c \times \left(\frac{1}{5}\mathrm{KMnO_4}\right) = 1.000\,\mathrm{mol/L}$$

2.8 | Phosphorus Level Determination

Different volumes (0, 1, 2, 4, 8 and 10 mL) of phosphorus standard solution were correctly pipetted into 50 mL volumetric flasks, then 10 mL of ammonium vanadyl aluminate colour reagent was added to the solution in each flask. The solutions were diluted with water to the 50 mL mark on the flask, shaken well and left to settle at room temperature for 10 min. In a 1 cm cuvette, the absorbance of each solution was determined with a spectrophotometer (BioTek, Winooski, VT, USA) at 400 nm. The concentration of phosphorus was used as the abscissa and absorbance as the ordinate to draw the working curve below, according to Song et al. (2022).

$$\%X = \frac{m_1 \times V}{m \times v_1 \times 10^6} \times 100 = \frac{m_1 \times V}{m \times v_1 \times 10^4}$$

where X is the mass proportion (%) representing the phosphorus content; m_1 is the phosphorus content of the sample breakdown solution based on the working curve (g) calculated; V is the sample breakdown solution's total volume (mL); m is the sample's

mass (μg); and V_1 is the sample decomposing solution's measured volume (mL).

Ammonium vanadate molybdate colour reagent was prepared by adding 200 mL of water to 1.25 g of ammonium metavanadate. The samples were heated to dissolve the ammonium metavanadate and cooled, after which 250 mL of nitric acid was added. Under cold conditions, 25 g of ammonium molybdate was dissolved in 400 mL of water and the two solutions were combined, diluted to 1000 mL with water and stored in the dark. Where precipitation occurred, the sample was not used. Potassium dihydrogen phosphate was dried at 105°C for an hour and cooled in a desiccator, and then 0.2195 g of it was dissolved in water and the solutions were carefully transferred into a 1000 mL volumetric flask and topped up with 3 mL of nitric acid. The solutions were diluted to the 1000 mL mark each with water and shaken to obtain 50 μg/mL standard phosphorus solutions. Two parallel samples were obtained from each sample for measurement, and the arithmetic mean was used as the result.

2.9 | Statistical Analysis

Data collected were subjected to the two-way analysis of variance in a completely randomised design that was arranged in a 3 × 3 factorial design with the incubation temperatures and in ovo feeding supplements as the fixed factors. The Generalised Linear Model Procedure in Minitab (version 19; LLC, NY, US, 2019) was used. The post hoc Tukey Test was used to separate and compare the means at 5% level of significance. Data were presented as means and standard error of the mean of a repeated experiment. The model used was:

$$Y_{ijk} = \mu + \mathrm{IOF}_i + \mathrm{TEMP}_j + \mathrm{IOFTEMP}_{ij} + e_{\mathrm{ijk}}$$

where Y_{ijk} is the variable measured, μ is the general mean, IOF_i is the main effect of in ovo feeding, TEMP_j is the effect of incubation temperatures, $\mathrm{IOFTEMP}_{ij}$ is the interaction between in ovo feeding and incubation temperature and e_{ijk} is the random residual error term.

3 | Results

3.1 | Effect of Incubation Temperature and Early In Ovo Feeding on Internal Organs at Hatch and Hatching Rate

The influence of varying machine temperature and in ovo feeding on body weight, internal organs at hatch and hatching rate is presented in Table 1. There was no interactive effect of machine temperature and in ovo feeding on body weight at hatch ($p > 0.05$). However, machine temperature significantly affected body weight at hatch such that eggs incubated at 37.5°C were heavier than those incubated at 36.5°C ($p = 0.047$). Among the relative internal organs measured, the liver, kidney, thigh muscle and ileum weights were not significantly affected by the thermal treatment and in ovo feeding ($p > 0.05$). There was a significant interaction between injection treatment and incubation temperature on duodenum weights. Eggs injected with glucose and incubated at 36.5°C, and those injected with Vitamin D3 and incubated at 37.5°C, had the highest duodenum weights, whereas control groups at the lower temperatures had the lowest weights. Concerning relative jejunum weight, no significant interactive effect was recorded ($p > 0.05$), but the main effect of in ovo vitamin-D3 injection had superior jejunum weight compared to the control group ($p = 0.042$). Likewise, machine temperatures at 37.0°C and 37.5°C significantly increased jejunum weight compared to 36.5°C ($p = 0.031$). Furthermore, duodenum and jejunum lengths were not affected by thermal treatment and in ovo feeding ($p > 0.05$). However, there was a significant interactive effect on the ileum length such that glucose injection at 36.5°C had the longest ileum compared to in ovo injection of vitamin-D3 groups and the control group at 36.5°C ($p = 0.047$). There was a significant ($p < 0.0001$) interactive effect of incubation temperature and in ovo feeding on hatchability. Across the feeding groups, hatchability increased with increasing incubation temperature, with the highest (89.86%) for the glucose-injected eggs incubated at 37.5°C and the lowest (48.33%) for the vitamin-D3-fed eggs incubated at 36.5°C.

3.2 | Effect of Incubation Temperature and Early In Ovo Feeding on Bone Mineral Concentration in the Right Tibia

In Table 2, the interaction of incubation temperature and early in ovo feeding had a significant effect on the amount of calcium and phosphorus in the tibia bone of the 21-day-old chicks ($p = 0.028$; $p = 0.035$). Calcium was mostly concentrated in chicks hatched from the glucose-fed eggs that were incubated at 37.0°C (13.72%), but lowest in chicks produced from the control eggs incubated at 37.0°C. The concentration of phosphorus was significantly highest in chicks hatched from the control eggs incubated at 36.5°C (22.81%) but lowest in chicks hatched from the control and glucose-fed eggs incubated at 37.0°C (9.44%).

3.3 | Effect of Incubation Temperature and Early In Ovo Feeding on Blood Metabolites of Chicks at Hatch

Results presented in Table 3 show no significant variation in the serum biochemical parameters of the day-old chicks in terms of alkaline phosphatase, total protein, globulin and phosphorus ($p > 0.05$). Triglycerides had no significant interactive effect; however, significantly higher concentration was found in the in ovo glucose-fed chicks compared to the control and vitamin-D3 groups ($p = 0.012$). There was a significant interactive effect of thermal treatment and in ovo feeding on albumin, total cholesterol and calcium concentrations ($p = 0.005$; $p < 0.0001$; $p = 0.003$). At incubation temperature 36.5°C, the day-old chicks from the control eggs had the highest concentration of serum albumin, followed by those from the vitamin-D3 and then the glucose-fed eggs. Total cholesterol was statistically lowest in chicks obtained from the control group eggs incubated at 36.5°C and 37.0°C compared to those incubated at 37.5°C and in ovo glucose at 37.0°C. Calcium level was highest for chicks hatched from the control group eggs at 37.5°C compared to the control and in ovo vitamin-D3 groups at 36.5°C and 37.0°C.

TABLE 1 | Effect of incubation temperature and early in ovo feeding of glucose at 5 g/mL and vitamin-D3 at 25 g/mL injected at 0.2 mL at egg level on internal organs of chicks at hatch.[a]

		Internal organ parameters										
Factors	**Feeding group**	**Initial body weight (g)**	**Liver (%)**	**Kidney (%)**	**Thigh muscle (%)**	**Duodenum (cm)**	**Jejunum (cm)**	**Ileum (cm)**	**Duodenum (%)**	**Jejunum (%)**	**Ileum (%)**	**Hatchability (%)**
In ovo feeding	CON	48.6	2.42	0.32	2.61	7.91	13.3	12.6	0.31	0.43^{b}	0.30	63.4^{a}
	GLU	46.9	2.45	0.34	2.63	7.55	16.0	12.4	0.58	0.84^{ab}	0.34	68.6^{b}
	VIT	48.9	2.41	0.35	2.49	7.60	16.4	11.0	0.57	0.95^{a}	0.30	61.5^{c}
	SEM	0.99	0.11	0.051	0.19	0.39	1.24	0.86	0.086	0.15	0.11	0.057
Temperature (°C)	36.5	46.3^{b}	2.37	0.26	2.85	7.57	13.6	12.3	0.44	0.40^{b}	0.26	48.0^{c}
	37.0	48.7^{ab}	2.48	0.41	2.45	7.60	17.0	11.4	0.41	0.91^{a}	0.46	63.6^{b}
	37.5	49.9^{a}	2.43	0.34	2.43	7.89	15.0	12.2	0.61	0.90^{a}	0.53	81.9^{a}
	SEM	0.99	0.11	0.051	0.19	0.39	1.2	0.86	0.086	0.15	0.11	0.057
In ovo feeding * temperature	CON*36.5	47.3	2.24	0.26	3.0	7.32	11.0	10.9^{bc}	0.21^{d}	0.21	0.21	44.1^{i}
	CON*37.0	48.5	2.57	0.41	2.90	7.60	13.4	13.7^{ab}	0.21^{d}	0.21	0.21	71.2^{d}
	CON*37.5	50.1	2.44	0.30	1.93	8.80	15.5	13.1^{ab}	0.51^{b}	0.86	0.47	75.0^{c}
	GLU*36.5	45.4	2.69	0.22	2.74	7.62	14.9	15.5^{a}	0.89^{a}	0.57	0.35	51.6^{g}
	GLU*37.0	47.3	2.27	0.42	2.32	7.94	20.1	9.12^{c}	0.43^{c}	1.37	0.21	64.4^{e}
	GLU*37.5	47.9	2.38	0.38	2.84	7.08	12.8	12.4^{b}	0.43^{c}	0.57	0.45	89.9^{a}
	VIT*36.5	46.26	2.16	0.30	2.80	7.76	14.8	10.4^{bc}	0.22^{d}	0.43	0.22	48.3^{h}
	VIT*37.0	48.7	2.59	0.41	2.14	7.26	17.6	11.3^{b}	0.60^{b}	1.14	0.97	55.2^{f}
	VIT*37.5	51.7	2.48	0.34	2.52	7.78	16.7	11.1^{b}	0.89^{a}	1.27	0.65	80.9^{b}
	SEM	1.7	0.19	0.089	0.33	0.69	2.2	1.5	0.15	0.26	0.18	0.099
p values	IOF	0.307	0.969	0.923	0.844	0.787	0.177	0.365	0.051	0.042	0.087	< 0.0001
	Temp	0.047	0.783	0.126	0.238	0.822	0.157	0.710	0.226	0.031	0.193	< 0.0001
	IOF*Temp	0.910	0.206	0.923	0.177	0.502	0.266	0.047	0.008	0.071	0.149	< 0.0001

Note: Means with different superscripted letters in a column are significantly different ($p < 0.05$).

Abbreviations: CON, control; GLU, glucose; IOF, in ovo feeding; SEM, standard error of means; Temp, temperature; VIT, vitamin.

[a]Relative values are expressed as a percentage of the live weight of chicks at hatch.

TABLE 2 | Effect of incubation temperature and early in ovo feeding of glucose at 5 g/mL and vitamin-D3 at 25 g/mL injected at 0.2 mL on bone mineral concentration of the chicks at Day 21.

Factors	Feeding group	Mineral level[a]	
		Calcium (%)	Phosphorus (%)
In ovo feeding	CON	9.4[a]	16.4[a]
	GLU	11.8[a]	9.9[b]
	VIT	11.3[ab]	15.3[a]
	SEM	0.67	1.3
Incubation temperature (°C)	36.5	10.5	16.1[a]
	37.0	10.6	10.8[b]
	37.5	11.4	14.6[ab]
	SEM	0.67	1.3
In ovo feeding* incubation temperature (°C)	CON*36.5	11.0[ab]	22.8[a]
	CON*37.0	6.66[b]	9.44[b]
	CON*37.5	10.6[ab]	16.85[ab]
	GLU*36.5	9.53[ab]	10.2[b]
	GLU*37.0	13.7[a]	9.44[b]
	GLU*37.5	12.4[ab]	10.0[b]
	VIT*36.5	11.0[ab]	15.3[ab]
	VIT*37.0	11.4[b]	13.4[ab]
	VIT*37.5	11.4[ab]	17.1[ab]
	SEM	1.1	2.2
p values	IOF	0.047	0.005
	Temp	0.574	0.026
	IOF*Temp	0.028	0.035

Note: Means with different superscripted letters in a column are significantly different ($p < 0.05$)

Abbreviations: CON, control; GLU, glucose; IOF, in ovo feeding; SEM, standard error of means; Temp, temperature; VIT, vitamin.

[a]Values are percentage of bone-dry matter.

3.4 | Effect of Incubation Temperature and Early In Ovo Feeding on Blood Metabolites of Chicks at Day 21

The data presented in Table 4 show the influence of thermal treatment and in ovo feeding on blood metabolites of 21-day-old chicks. No significant changes were observed in the serum concentrations of total protein, globulin, triglycerides and phosphorus ($p > 0.05$). At incubation temperatures of 37.0°C and 37.5°C, respectively, the concentrations of alkaline phosphatase were significantly higher in the control and glucose-fed eggs than in the vitamin-D3-fed eggs at 37.0°C and 37.5°C, glucose-fed eggs at 37.0°C and control eggs at 36.5°C ($p < 0.0001$). A significantly higher interactive effect of concentrations of albumin was observed in the control and vitamin-D3 fed eggs at 36.5°C than those in the remaining vitamin-D3 groups ($p = 0.022$). Total cholesterol concentrations were significantly lower in the vitamin-D3-fed eggs across all incubation treatments and glucose-fed eggs at 36.5°C and 37.0°C than those in the control group at 37.5°C ($p = 0.004$). Furthermore, there was no significant interactive effect on calcium concentration, but the main effect of the control group of eggs had a higher concentration than that of the vitamin-D3-fed group ($p = 0.012$).

4 | Discussion

The post-hatch performance of birds mostly depends on the external and internal quality of day-old chicks (Hamidu et al. 2018; Tona et al. 2022). Whereas chick quality is dependent on many factors, measures such as in ovo feeding have been shown to improve the quality of various portions of their small intestine (Bakyaraj et al. 2012; Araújo et al. 2019). However, results of this work indicate that whether or not broiler breeder eggs are supplemented with exogenous nutrients during incubation, the weight and length of the internal organs of chicks that hatch from them will not vary. Nonetheless, Al-Shammari (2025) has indicated that in ovo feeding of vitamin-D3 and sugars can significantly improve embryo growth by ensuring fast tissue and organ development. Chicks with the heaviest jejunum were produced from the vitamin-D3-fed eggs over those produced from the control and glucose-injected eggs, in opposite to the findings of Cöner and Saçaklı (2023), which indicated improved small intestinal development in chicks hatched from eggs that were in ovo injected with glucose. Similar variability in intestinal responses to different in ovo nutrients has been reported: in ovo vitamin-D3 and 25(OH)D3 often improve intestinal histomorphology and nutrient absorption, whereas other nutrients such as amino acids, sugars or vitamins C/E may have more pronounced or different region-specific effects (Gonzales et al. 2013; Sun et al. 2025; Ghane et al. 2021). The present data also show that the breeder eggs that were incubated at 37.5°C produced day-old chicks that generally had better-developed internal organs than the ones produced from eggs incubated at the lower temperatures of 36.5°C and 37.0°C. The current findings support the observations made when some hatching eggs were exposed to a higher incubation temperature (39.0°C) for increased embryonic growth, yolk absorption and enhanced metabolic rate, which caused the internal organs to develop faster than when the incubation was done at a lower temperature of 37.5°C (Kuzmina 2023). Meanwhile, when some breeder eggs were also incubated at a suboptimal temperature of 37.0°C (Xie et al. 2018), growth and tissue (organ) development were retarded (Vaishnav et al. 2024) as was generally found for the chicks that had their eggs incubated at 36.5°C in the present work. Even though the current findings peg 37.5°C as the best incubation temperature for better organ development, hatchery operators must be cautious when incubating beyond this temperature because Sgavioli et al. (2016) have reported lower absolute and relative heart weights in eggs incubated at 39.0°C than those incubated at 37.5°C. Also, the proposed humidity of 50%–60% needed to ensure proper gas exchange and moisture retention in incubators (Noiva et al. 2014) can be compromised at very high temperatures. Additionally, numerous studies indicate that moderately elevated temperatures during the late stages of incubation can positively affect chick body weight and the development of certain organs (Leksrisompong et al. 2007; Wijnen et al. 2020; Kaneko et al. 2021). However, the pattern and timing of temperature fluctuations, whether continuous or intermittent, are crucial factors. Short-term thermal stimulation or slight

TABLE 3 | Effect of incubation temperature and early in ovo feeding of glucose at 5 g/mL and vitamin-D3 at 25 g/mL injected at 0.2 mL on serum metabolites of chicks at hatch.

Factors	Feeding group	Blood parameters							
		ALP (u/L)	TP (g/L)	ALB (g/L)	GLUB (mol/L)	TG (mol/L)	TCHO (mol/L)	Ca (mol/L)	P (mol/L)
In ovo feeding	CON	1573	28.9	7.02^{a}	10.4	0.92^{b}	9.93	4.84	2.33
	GLU	1778	31.0	5.37^{b}	11.6	1.35^{a}	10.3	4.92	2.19
	VIT	1708	26.9	5.94^{ab}	10.5	0.89^{b}	9.04	4.75	2.29
	SEM	88.1	2.58	0.422	0.517	1.33	0.649	0.056	0.198
Incubation temperature (°C)	36.5	1666	26.0	5.99	11.0	1.03	8.71	4.74^{b}	2.18
	37.0	1769	27.7	5.71	10.4	1.08	9.84	4.79^{ab}	2.35
	37.5	1625	33.2	6.61	11.0	1.06	10.7	4.98^{a}	2.28
	SEM	88.1	2.58	0.422	0.517	1.33	0.649	0.0555	0.198
In ovo feeding * incubation temperature (°C)	CON*36.5	1433	26.6	8.18^{a}	11.2	1.03	7.95^{b}	4.56^{b}	2.49
	CON*37.0	1686	24.6	4.60^{b}	8.65	0.84	7.78^{b}	4.73^{b}	2.00
	CON*37.5	1603	35.5	8.30^{a}	11.3	0.88	14.1^{a}	5.23^{a}	2.50
	GLU*36.5	1908	27.0	4.70^{b}	12.1	1.03	8.92^{ab}	4.91^{ab}	1.81
	GLU*37.0	1974	29.6	6.04^{ab}	11.7	1.58	13.55^{a}	4.97^{ab}	2.40
	GLU*37.5	1452	36.6	5.40^{b}	11.0	1.44	8.28^{ab}	4.87^{ab}	2.40
	VIT*36.5	1658	24.4	5.14^{b}	9.74	1.02	9.25^{ab}	4.77^{b}	2.24
	VIT*37.0	1648	28.8	6.50^{ab}	10.9	0.82	8.22^{ab}	4.67^{b}	2.66
	VIT*37.5	1821	27.5	6.18^{ab}	10.8	0.86	9.70^{ab}	4.82^{ab}	1.98
	SEM	153	4.47	0.731	0.896	1.78	1.12	0.0962	0.343
p values	IOF	0.262	0.532	0.028	0.187	0.012	0.407	0.119	0.874
	Temp	0.499	0.133	0.318	0.646	0.945	0.114	0.014	0.829
	IOF*Temp	0.125	0.715	0.005	0.187	0.298	<0.0001	0.003	0.322

Note: Means with different superscripted letters in a column are significantly different ($p < 0.05$).

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; Ca, calcium; CON, control; GLU, glucose; GLUB, globulin; IOF, in ovo feeding; P, phosphorous; SEM, standard error of means; TCHO, total cholesterol; Temp, temperature; TG, triglycerides; TP, total protein; VIT, vitamin.

temperature increases during specific developmental windows can enhance post-hatch performance, helping to mitigate the adverse effects associated with prolonged exposure to elevated temperatures (Leksrisompong et al. 2007; Wijnen et al. 2020; Kaneko et al. 2021). The present results show that the interaction of incubation temperature and in ovo feeding did not affect the development of the organs studied, apart from the length of the ileum and the weight of the duodenum. Nevertheless, the structure of the internal body organs was generally top-notch for the day-old chicks that were hatched from eggs supplemented with vitamin-D3 and incubated at the highest temperature of 37.5°C in the present work. It must, however, be known that individual internal organs would relate differently to different in ovo feed nutrients and incubation temperatures. Therefore, the choice of incubation temperature and feed supplements during the application of the technology must be guided by production objectives and research findings, if improving internal body organs is the purpose. However, for faster embryonic organogenesis, feeding hatching eggs with vitamin-D3 and incubating them at 37.5°C is suggested. This assertion aligns with reports that in ovo administration of vitamin-D3 or its metabolites can enhance bone and intestinal development and elevate serum vitamin D status at hatch, effects that may be potentiated when eggs are incubated at optimal temperatures (Fatemi et al. 2021, 2022; Badri et al. 2023). The interactive effect of incubation temperature and in ovo feeding on hatchability of the broiler eggs was significant. Nevertheless, whether or not in ovo feeding is applied, hatchability increased as the incubation temperature was increased. However, when broiler eggs are injected with glucose and incubated at 37.5°C, a hatchability of 89.86% should be expected, while at the same temperature, 80.90% of vitamin-D3-fed eggs may hatch. Incubating vitamin-D3-fed eggs at 36.5°C is not recommended, but generally, incubating at 37.5°C would optimise eggshell temperature for improved hatchability. The positive effect of in ovo carbohydrate supplementation on hatchability and hatch weight has been documented in multiple studies (Salmanzadeh 2012; Tufarelli et al. 2021), whereas some vitamin injections can have neutral or dose- and timing-dependent effects on hatchability (Fatemi et al. 2021, 2022). Serum calcium was observed by Akter et al. (2020) to be significantly and positively associated with glucose markers, which was inversely associated with serum. On the other hand, vitamin D is known to regulate

TABLE 4 | Effect of incubation temperature and early in ovo feeding of glucose at 5 g/mL and vitamin-D3 at 25 g/mL injected at 0.2 mL on serum metabolites of chicks at Day 21.

Factors	Feeding group	Blood parameters							
		ALP (u/L)	TP (g/L)	ALB (g/L)	GLUB (mol/L)	TG (mol/L)	TCHO (mol/L)	Ca (mol/L)	P (mol/L)
In ovo feeding	CON	2309	32.2	8.01	12.4	0.46	3.78^{a}	5.26^{a}	2.28
	GLU	2223	33.8	8.10	13.3	0.50	3.45^{ab}	5.26^{a}	2.53
	VIT	2186	31.7	7.41	12.6	0.43	3.35^{b}	5.10^{b}	2.39
	SEM	129	0.685	0.273	0.343	0.0202	0.109	0.0399	0.0935
Incubation temperature (°C)	36.5	2142	32.0	8.17^{a}	13.2	0.48	3.44	5.24	2.40
	37.0	2167	33.5	8.21^{a}	12.7	0.44	3.48	5.15	2.51
	37.5	2409	32.1	7.13^{b}	12.4	0.46	3.66	5.22	2.30
	SEM	129	0.685	0.273	0.343	0.0202	0.109	0.0399	0.0935
In ovo feeding * incubation temperature (°C)	CON*36.5	1575^{bc}	32.8	9.38^{a}	12.5	0.44	3.60^{ab}	5.30	2.31
	CON*37.0	3180^{a}	32.5	7.78^{ab}	12.6	0.44	3.66^{ab}	5.20	2.25
	CON*37.5	2173^{abc}	31.2	6.86^{b}	12.1	0.48	4.10^{a}	5.28	2.30
	GLU*36.5	2261^{abc}	33.2	8.20^{ab}	14.4	0.57	3.10^{bc}	5.36	2.60
	GLU*37.0	1222^{c}	33.5	8.38^{ab}	12.7	0.47	3.35^{bc}	5.15	2.81
	GLU*37.5	3186^{a}	34.7	7.70^{ab}	12.9	0.44	3.94^{ab}	5.27	2.20
	VIT*36.5	2590^{ab}	30.1	6.94^{a}	12.7	0.43	3.65^{bc}	5.10	2.31
	VIT*37.0	2100^{bc}	34.5	8.46^{b}	12.9	0.41	3.42^{bc}	5.22	2.46
	VIT*37.5	1867^{bc}	30.4	6.84^{b}	12.3	0.45	2.98^{c}	5.12	2.40
	SEM	224	1.19	0.472	0.593	0.0351	0.190	0.0692	0.162
p values	IOF	0.787	0.085	0.173	0.145	0.118	0.024	0.012	0.178
	Temp	0.286	0.250	0.012	0.259	0.371	0.307	0.245	0.297
	IOF*Temp	< 0.0001	0.131	0.022	0.506	0.147	0.004	0.486	0.284

Note: Means with different superscripted letters in a column are significantly different ($p < 0.05$).

Abbreviations: ALB: albumin; ALP: alkaline phosphatase; Ca: calcium; CON: control; GLU: glucose; GLUB: globulin; IOF: in ovo feeding; P: phosphorous; SEM: standard error of means; TCHO: total cholesterol; Temp: temperature; TG: triglycerides; TP: total protein; VIT: vitamin.

calcium and phosphorus homeostasis and bone mineralisation (Durá-Travé and Gallinas-Victoriano et al. 2024) and so might be responsible for the low levels of phosphorus in the bone of the respective chicks. The amount of minerals in the bone of the broiler chickens at week 3 was significantly affected by the interaction of the incubation temperature and in ovo feeding. At this age, the concentration of calcium was highest in birds from eggs injected with glucose and incubated at 37.0°C. However, at the same incubation temperature, the calcium level was lowest in chicks from the control eggs that had no in ovo feed supplementation. On the other hand, while the amount of phosphorus was significantly highest in the 3-week-old chickens hatched from the non-supplemented eggs incubated at 36.5°C, it was lowest in the birds that were produced from the non-supplemented and glucose-fed eggs and incubated at 37.0°C at the same age. The increased calcium concentration in chicks from glucose-injected eggs supports the greater skeletal development observed when glucose was administered in ovo (Winkens et al. 2025). In the past, minerals have been made available through in ovo feeding to improve the mechanical characteristics of the tibia bone (Laboissiere et al. 2025), while their availability has affected bone development in broilers at the embryonic and post-hatch stages (Tianyang et al. 2025). The present results indicate different effects of the two in ovo feed supplements and varying incubation temperatures on the mineral contents of the tibia bone in broiler chickens. Therefore, the choice of feed supplements and the temperature at which breeder eggs should be incubated must be guided by production objectives and research findings to ensure strong bone and shell formation. However, for better bone mineralisation of chicks at 3 weeks of age, broiler eggs can be incubated without in ovo feeding; otherwise, they should be injected with glucose, but can be incubated at 36.5°C in both cases. These findings echo previous work showing that in ovo minerals and vitamin-D3 can improve hatchling bone parameters and later bone strength (Sun et al. 2025; Laboissiere et al. 2025), while the source, dose and timing of vitamin-D3 determine whether benefits are realised under different post-hatch dietary calcium/phosphorus regimens (Fatemi et al. 2021). Findings of the current investigation suggest that the concentration of most of the blood metabolites and compounds would not change significantly between day-old chicks that are hatched from in ovo-treated and untreated eggs, apart from albumin and triglyceride levels. However, chicks produced from in ovo fed eggs could have reduced levels of blood albumin when compared

to those from the non-supplemented eggs. Excess glucose is converted into fatty acids in the liver and adipose tissue and subsequently esterified to triglycerides (Alves-Bezerra and Cohen 2018). This could have led to the increased level of triglyceride in the day-old chicks hatched from the glucose-injected eggs. This may enhance the physiological adaptation of the birds to store energy for subsequent growth (Vaishnav et al. 2024), but the high levels of triglyceride can increase the levels of low-density lipoprotein cholesterol (Kosmas et al. 2023). Meanwhile, the lower concentration of triglyceride detected in the day-old chicks produced from the vitamin D3-fed eggs than the control eggs means that the levels of total and low-density lipoprotein cholesterol and their possible effects can be minimised in day-old chicks through vitamin-D3 injection. This will help to produce low-fat broilers that can live well and survive heat stress (Almeida et al. 2022). On the other hand, high triglyceride levels have been linked to high levels of total and LDL cholesterol (Kosmas et al. 2023; Balling et al. 2023). The current results, however, show that while the application of glucose in ovo could increase the concentration of most of the blood biochemical components, the application of vitamin-D3 could generally cause them to decrease. These observations indicate that, application of in ovo feeding technology to improve the quantity of specific blood biochemical elements or compounds in broiler day-old chicks would depend on the type of feed supplement used. Support for nutrient-specific effects on blood metabolites comes from multiple in ovo studies: in ovo glucose or carbohydrate injections often raise energy-related metabolites and lipids at hatch (Salmanzadeh; Abdel-Halim et al. 2020), whereas in ovo vitamin-D3 or its hydroxylated forms can alter calcium/phosphorus balance and influence lipid metabolism indirectly via altered mineral status or gene expression (Laboissiere et al. 2025; Fatemi et al. 2021). The interaction of incubation temperature and in-ovo feeding had a significant impact on the amount of blood albumin, total cholesterol and calcium of the broiler day-old chicks, but not the concentration of alkaline phosphatase, globulin, triglycerides and phosphorus. Nonetheless, for increased calcium concentration for stronger bone development, in ovo feeding, particularly with glucose and incubating eggs at 36.5°C, is recommended per the findings of the current investigation. Eggs that were in ovo supplemented particularly with vitamin-D3 had the blood albumin content of their day-old chicks increased. This could improve or otherwise reduce the functionality of blood albumin because high albumin levels have been linked to severe dehydration and diarrhoea, while low albumin levels have been linked to malnutrition and liver or kidney diseases (MedlinePlus 2024). Though the current data portrays that the concentration of most of the blood components increased in the day-old chicks from the glucose-fed eggs that were incubated at 37.5°C, the conditions may not be suitable to improve every biochemical element or compound because the results of the present work show different relations between the various blood constituents and the factors. Meanwhile, when incubating eggs beyond 37.5°C, measures must be put in place to control eggshell temperature, which increases with increasing incubation temperature, to maintain bone calcification in a previous trial (Okai et al. 2025). It is important to emphasise that in ovo effects on blood biochemistry can be transient; some changes evident at hatch may normalise or evolve by later ages, depending on post-hatch diet and environment (Panda et al. 2015; Niloofar et al. 2024). Moreover, in ovo application of other vitamins (C, E) or bioactive compounds has been shown to modulate oxidative status, immunity and some blood parameters, underscoring the breadth of possible in ovo interventions (Ghane et al. 2021; Ebrahimi et al. 2024; El-Kholy et al. 2019). The interactive effect of incubation temperature and in ovo feeding on the levels of albumin, total cholesterol and alkaline phosphatase of the 3-week-old broiler chickens was significant. The results reveal that the concentration of albumin can be increased for quality composition and proper functionality of total blood protein if breeder eggs are incubated at 36.5°C. However, though in ovo application may not be needed, injecting vitamin-D3 and incubating eggs at 37.0°C would produce the best results and is so recommended when the technology must be applied. To produce broilers that are devoid of high levels of cholesterol and its associated effects, in ovo feeding with glucose and incubating broiler eggs at 36.5°C would be most useful. This must, however, be done with care because excess glucose level in the body has been linked to the generation of triglyceride (Alves-Bezerra and Cohen 2018), which has also been associated with increased low-density lipoprotein cholesterol (Balling et al. 2023). For broiler chickens that require a high amount of alkaline phosphatase for proper physiological functioning, applying glucose in ovo and incubating broiler chicken eggs at 37.5°C may yield the best results. However, when the incubation is done at 37.0°C, supplementing with glucose would significantly reduce the concentration of alkaline phosphatase, because increased levels of alkaline phosphatase may indicate membrane damage in the liver or cancer and heart infections (Balabonova et al.). These age-dependent and parameter-specific outcomes are consistent with the literature showing that early-life manipulations (temperature or nutrient) can have persistent, selective effects on metabolism, bone and blood chemistry, but effects depend on the nutrient form and incubation protocol (Agyekum et al. 2022; Fatemi et al. 2021). The interaction of the two factors did not change the concentration of total protein, globulin, triglycerides, calcium and phosphorus of the chickens at 3 weeks of age. At large, the majority of the blood compounds increased in the 3-week-old birds hatched from the glucose-fed eggs incubated at 36.5°C because genes responsible for the metabolism of the various chemical elements and compounds can be changed if eggs are incubated at different temperatures. This was revealed in an experiment that showed that both high and low incubation temperatures (temperatures that were 1.5°C below or above 37.8°C) caused a reduction in the expression of PEPT1 and ApoA1 genes that are responsible for oligopeptide uptake and lipid metabolism and altered glycogen storage of the yolk sac tissue toward the hatching period (Dayan et al. 2020). Likewise, in this current study, most of the biochemical elements increased when broiler eggs were in ovo injected with glucose and incubated at 37.0°C.

Overall, the study covered a limited range of incubation temperatures and supplement types and post-hatch evaluation focused mainly on early developmental stages. Moreover, the work was conducted under controlled experimental conditions that may not fully represent commercial hatchery settings. Despite these constraints, the findings provide valuable insights into the interactive effects of incubation temperature and in ovo feeding, offering a foundation for future applied research aimed at improving hatchery management and chick performance.

5 | Conclusion

Incubation temperature and in ovo feeding showed significant interactive effects on hatchability, intestinal development, bone mineralisation and blood metabolites of chicks. Glucose injection at higher temperatures (37.5°C) enhanced hatchability, intestinal growth and bone mineralisation, whereas vitamin-D3 supplementation at lower temperatures impaired these traits and disrupted metabolic balance. These findings emphasise that the success of in ovo feeding depends on aligning nutrient type with optimal incubation conditions. Practically, integrating temperature management with targeted nutrient supplementation can improve chick quality, early growth and production efficiency. Hence, the application of in ovo technology should be guided by production goals and expert recommendations to ensure sustainable and cost-effective poultry performance.

Author Contributions

Maxwell Ansong Okai: conceptualisation, writing – manuscript and editing. **Francis Kruenti**: writing – manuscript and editing. **Zheng Liu**: writing – manuscript. **Ma Yingfa**: writing – manuscript. **Nadiedjoa Yendouchamtchié**: data collection. **Mijiyawa Ahmed**: data collection. **Roland Yao Apélété Toglo**: data collection. **Maa Maa Temhoul Clarice**: data collection. **Xin Qian**: supervision. **Kokou Tona**: supervision, writing – manuscript and editing. **Jacob Alhassan Hamidu**: supervision, writing – manuscript and editing. **Benjamin Adjei-Mensah**: editing of manuscript. **Hai Lin**: conceptualisation, supervision, writing – manuscript and editing.

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Ethics Statement

The Animal Care Committee of the Shandong Agricultural University of China approved all the procedures utilised in this study, and they were carried out in compliance with the standards for animal experimentation as outlined by the Ministry of Science and Technology, China.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

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