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IMPACT OF *IN OVO* PROPOLIS ADMINISTRATION ON CHICKEN EMBRYO DEVELOPMENT

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Abstract

Contemporary poultry production increasingly prioritizes optimized prenatal nutrition to enhance chick health, immunity, and productivity. This 2024-2025 study conducted at the Institute of Ecology and Technology, Popova Shapka, examined the effects of in ovo-administered aqueous propolis extract (1,5% and 3% concentrations) on chicken embryo development. Fertilized eggs from crossbreed hens (n=30 per experimental group) were disinfected with 70% ethanol and administered precise air cell injections on incubation day 0 using insulin syringes. Incubation was maintained under standard conditions (37.5±0.1°C for days 1-18; 37.2°C for days 19-21) with relative humidity at 65-70% (days 1-18) and 70-75% (days 19-21). Embryonic development was evaluated through serial measurements of weight, length, and morphological characteristics on days 7, 14, and 21 of incubation. The results demonstrate a clear dose-response relationship, with the 1.5% propolis group exhibiting superior developmental outcomes: maximum embryo weight (33.0 g versus 31.0 g in controls at day 21), greatest length (86 mm versus 82 mm in controls), and significantly larger eye diameter (6.0 mm versus 4.0 mm in controls at day 7). These findings indicate that propolis enhances neurogenesis and metabolic efficiency through its bioactive antioxidant and immunomodulatory components, establishing it as an effective natural alternative for improving poultry production outcomes.

Key words: *Propolis, in ovo injection, embryogenesis, natural growth promoters.*

Introduction

Embryonic development in poultry is a complex and highly coordinated process, influenced by genetic factors as well as external conditions such as nutrient availability from the yolk and incubation parameters including temperature and humidity [1, 2]. Adequate prenatal nutrition is essential for proper organ formation,

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maturation of the immune system, and overall post-hatch performance, whereas insufficient nutrient supply can compromise development and reduce survival [3].
Modern poultry production increasingly focuses on strategies that provide embryos with bioactive compounds to enhance early growth, immune competence, and vitality [4]. One of the most efficient approaches is *in ovo* injection, which allows direct administration of nutrients or functional substances into the egg prior to hatching. When applied correctly, this technique can support organ development and facilitate adaptation after hatching [4, 5]. The allantoic cavity, particularly between days 12 and 18 of incubation, is considered an optimal site for injection, as it promotes effective absorption and systemic distribution of the administered compounds [6, 7].
Propolis, a resinous substance collected by honeybees, is rich in flavonoids, phenolic acids, and terpenoids, which are known for their antimicrobial, antioxidant, and immunomodulatory properties. *In ovo* administration of propolis has been shown to enhance embryonic growth, increase hatchability, and improve post-hatch performance [8, 9]. Low concentrations appear to be safe and supportive of development, while excessive doses may compromise embryo viability [10]. Bioactive constituents, such as caffeic acid phenethyl ester and galangin, are thought to reduce oxidative stress during embryogenesis, thereby promoting organ formation and metabolic efficiency [11]. Taken together, these observations highlight the potential of propolis as a natural growth-promoting agent in poultry, offering a promising alternative to conventional antibiotics.

Materials and methods

Egg collection and incubation

For each experimental group, 30 fertilized chicken eggs were carefully selected. Eggs were collected daily from crossbred layer hens and stored at 15 °C for no longer than three days prior to use. The parental stock originated from a private farm in Sinichane village, Polog region. Fertilization was verified on day 3 post-laying using candling with a fluorescent lamp, and any unfertilized eggs were excluded. Selected eggs were individually weighed and labeled according to their assigned treatment groups.
Prior to incubation, the egg surface was disinfected with 70% ethanol. On incubation day 0, 0.1 mL of aqueous propolis extract at concentrations of 1%, 1.5%, and 3% was injected into the air cell using precision insulin syringes. Eggs were incubated in a digital incubator (EVL model) under controlled conditions:

- Temperature: 37.5 ± 0.1 °C (days 1–18), then lowered to 37.2 °C (days 19–21);
- Relative humidity: 65–70% (days 1–18), 70–75% (days 19–21).

Eggs were rotated 3–5 times daily during the first 18 days and kept in a stationary position during the final three days corresponding to the hatching phase.

Embryo assessment and morphological analysis

Eggs were weighed before incubation and on days 7, 14, and 21 using a digital scale with 0.01 g accuracy. Ten eggs per treatment group were sampled at each interval, and embryos were carefully removed, fixed in 70% ethanol, and stored in sterile conical tubes until evaluation.

The following parameters were examined:

a. Degree of vascularization and organ differentiation
b. Embryonic stage, classified according to established morphological criteria
c. Macroscopic anatomical features
d. Morphometric indices, including embryo weight, limb length, and eye diameter

Linear measurements and eye diameter were recorded under a light microscope (Motic), and representative images were captured to illustrate developmental stages. Embryonic stages were assigned using the standardized Hamburger and Hamilton (1951) system [12]. Data were organized into tables and figures for direct comparison between treatment groups.

Results

Effects of propolis on embryonic development

The impact of aqueous propolis extract on the development of chicken embryos (*Gallus gallus domesticus*) was examined using an *in ovo* approach based on the Hamburger and Hamilton (1951) staging system [12]. Embryonic growth and morphology were evaluated for three propolis concentrations (1%, 1.5%, and 3%) at key incubation stages (days 7, 14, and 21) to determine potential effects on developmental parameters.

Egg weight and embryonic growth

Mean egg weights (± SD) prior to incubation and at successive measurement points are summarized in Table 1. No significant differences were observed between the control group and the groups treated with 1% or 1.5% propolis across all time points ($p > 0.05$). Eggs injected with 3% propolis consistently showed higher initial and subsequent weights than controls, with statistically significant differences ($p < 0.001$). These findings suggest a dose-dependent response of embryonic growth to propolis, with the highest concentration producing marked changes in egg and embryo mass.

Table 1: Mean egg weight (± SD) during *In Ovo* Propolis treatment (n = 30) before and throughout incubation (t-test)

Group	Pre-incubation (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)	Loss to Day 21 (g, %)
Control (0%)	56.79 ± 2.58	54.74 ± 2.82	48.65 ± 2.84	43.49 ± 2.13	13.30 (23.4%)
Propolis 1%	56.65 ± 2.65 ns	53.74 ± 2.51 ns	49.06 ± 2.24 ns	43.26 ± 2.17 ns	13.39 (23.6%)
Propolis 1.5%	56.37 ± 2.79 ns	51.67 ± 2.71 ns	49.18 ± 1.88 ns	42.38 ± 2.53 ns	13.99 (24.8%)
Propolis 3%	62.94 ± 2.86 *	63.15 ± 3.27 *	55.42 ± 1.88 *	49.11 ± 2.70 *	13.83 (22.0%)

Notes: ns: not significantly different compared to control ($p > 0.05$)
**: significantly different compared to control ($p < 0.001$)*

Significance testing was conducted separately for each incubation day, with treatment groups compared against the control within the same column.

Egg mass retention

During incubation, eggs naturally lose mass due to water evaporation through the shell. As shown in Table 1, the average initial egg mass in the control group (56.79 g) was comparable to that of the 1% and 1.5% propolis groups (56.4–56.7 g). A significantly higher baseline mass was recorded only in the 3% propolis group (62.94 g; $p < 0.001$), likely reflecting egg selection rather than treatment effects.

Throughout the incubation period, no statistically significant differences were observed between the control and the 1% or 1.5% propolis groups ($p > 0.05$). By day 21, relative mass loss was similar across these groups: 23.4% in the control, 23.6% in the 1% group, and 24.8% in the 1.5% group, indicating normal physiological water loss and no adverse influence of these treatments.

Eggs injected with 3% propolis showed the lowest relative weight loss (22.0%). Although this might initially appear beneficial, it coincided with increased mortality and developmental abnormalities, suggesting impaired shell permeability and disrupted gas exchange rather than a protective effect [13].

These findings align with prior studies. Elevated concentrations of natural extracts have been reported to interfere with shell properties and compromise embryonic viability [8, 12]. Conversely, low doses (around 1%) have been shown to be safe and may even stabilize the shell and reduce water loss [14]. Overall, moderate propolis concentrations (≤1.5%) appear compatible with normal incubation dynamics, whereas higher concentrations (3%) may be detrimental by altering shell physiology and compromising embryo development.

Morphometric parameters

Morphometric traits, including embryonic body mass, length, and eye diameter, are established indicators of developmental progression. In this study, all measured parameters increased steadily throughout incubation, consistent with the standard growth trajectory outlined by Hamburger and Hamilton [12].

According to Table 2, control embryos showed gradual development, reaching 3.0 g and 25.0 mm at day 7, 14.0 g and 75.0 mm at day 14, and 31.0 g and 82.0 mm by day 21. Comparable trends were observed in the propolis-treated groups, although the magnitude of growth differed by concentration.

Embryos exposed to 1% propolis exhibited slightly higher body mass and length than controls at all stages. Statistically significant differences were observed for eye diameter on day 7 ($p < 0.05$) and for length on days 14 and 21 ($p < 0.05$). At 1.5% propolis, more consistent enhancements were evident, with significantly greater body mass, length, and eye diameter across all incubation points ($p < 0.01$).

The most pronounced effects occurred in the 3% group, where all morphometric traits were markedly elevated compared to controls ($p < 0.001$). Eye diameter on day 7 nearly doubled (8.0 vs. 4.0 mm), and by day 21, body mass and length reached 36.0 g and 95.0 mm, respectively, versus 31.0 g and 82.0 mm in the control group.

Despite apparent growth stimulation, these increases must be interpreted cautiously. The sharp elevations at 3% propolis were accompanied by higher mortality and morphological abnormalities, suggesting that excessive doses may trigger pathological overgrowth or developmental dysregulation rather than genuine physiological improvement [16, 17].

Table 2. Morphometric parameters of chicken embryos during incubation (mean ± SD, n = 10 per group)

Propolis conc.	Incubation day	Body mass (g) ± SD	Embryo length (mm) ± SD	Eye size (mm) ± SD
0% (control)	7	3.0 ± 0.20	25.0 ± 1.20	4.0 ± 0.30
	14	14.0 ± 1.00	75.0 ± 2.50	–
	21	31.0 ± 1.50	82.0 ± 3.10	–
1% propolis	7	3.1 ± 0.30	25.5 ± 1.30	5.2 ± 0.40 *
	14	14.8 ± 1.10	78.0 ± 2.90 *	–
	21	31.8 ± 1.80	84.0 ± 2.80 *	–
1.5% propolis	7	3.2 ± 0.30	26.0 ± 1.50 *	6.0 ± 0.50 **
	14	14.5 ± 1.30	83.0 ± 2.80 **	–
	21	33.0 ± 2.00 *	86.0 ± 3.00 **	–
3% propolis	7	4.0 ± 0.40 **	28.0 ± 1.80 **	8.0 ± 0.70 ***
	14	17.0 ± 1.50 **	90.0 ± 3.20 ***	–

	21	36.0 ± 2.30 **	95.0 ± 3.80 ***	–

*Note: ns – not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control.*

Morphometric development of embryos

Embryonic body mass increased steadily across all groups during incubation. By day 21, control embryos reached 31.0 ± 1.50 g. Administration of 1% propolis resulted in a slight, non-significant increase to 31.8 ± 1.80 g. Higher concentrations led to more pronounced effects, with mean weights of 33.0 ± 2.00 g in the 1.5% group and 36.0 ± 2.30 g in the 3% group. This pattern suggests a dose-dependent anabolic response, likely mediated by the bioactive flavonoids and polyphenols in propolis, which can influence cell proliferation and protein metabolism [15].

Embryo length followed a comparable trend. At day 21, the control group measured 82.0 ± 3.10 mm, whereas the 1% propolis group reached 84.0 ± 2.80 mm. The 1.5% and 3% groups attained 86.0 mm and 95.0 mm, respectively. The increased variability at higher concentrations may indicate interference with homeostatic mechanisms that regulate embryonic growth [16].

Eye diameter, assessed on day 7 when optic vesicles were well-formed, also mirrored this trend. Control embryos averaged 4.0 ± 0.30 mm, while the 1% and 1.5% groups reached 5.2 ± 0.40 mm and 6.0 ± 0.50 mm, respectively. The 3% group exhibited an extreme value of 8.0 ± 0.70 mm ($p < 0.001$), suggesting hyperplastic growth exceeding physiological norms. Lower doses likely promoted standard neuroepithelial proliferation, whereas the highest dose appeared to induce disproportionate organogenesis, consistent with previous observations of developmental abnormalities [16, 17].

The influence of propolis on morphometric traits can be attributed to multiple mechanisms. Propolis enhances cell division through modulation of growth factors, stabilizes the embryonic environment by improving nutrient availability, and promotes vascular development, which may contribute to fluid retention under oxidative stress [18,16]. Therefore, low-to-moderate concentrations (≤1.5%) support normal embryonic development, whereas 3% may disrupt developmental equilibrium, resulting in uncoordinated and disproportionate growth.

Visual assessment of morphological development in chicken embryos treated with aqueous propolis solution

Figure 1 illustrates the morphological and physiological characteristics of chicken embryos incubated with aqueous propolis solutions at concentrations of 1%, 1.5%, and 3%, alongside embryos from the untreated control group (0%). Images were obtained on days 7, 14, and 21, enabling a comparative evaluation of developmental progress over time.

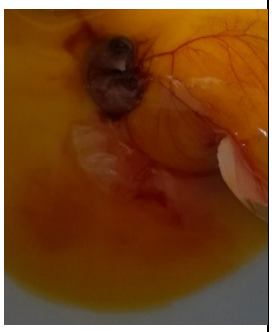
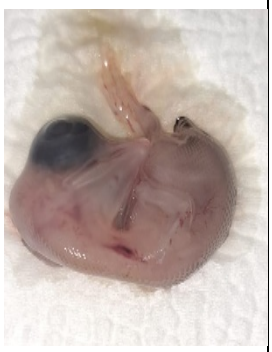
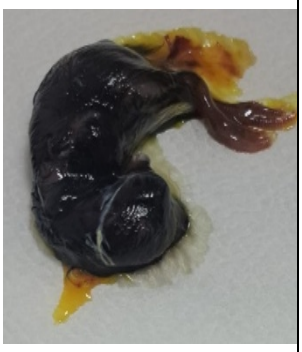
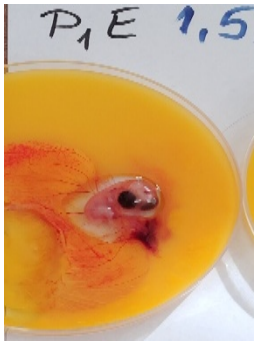
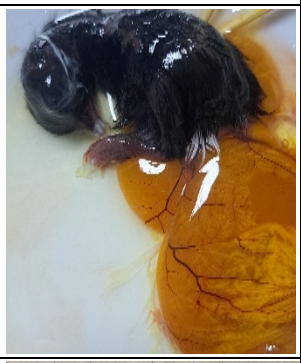
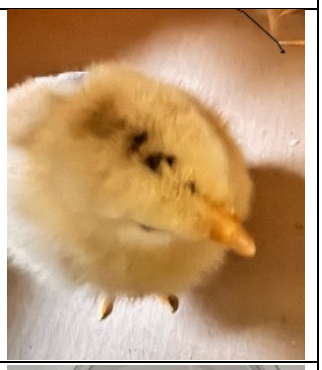
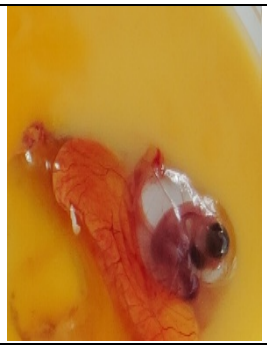
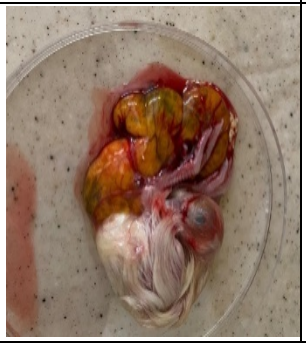
	7th day	14th day	21st day
Control 0%			
Trial 1 Propolis 1%			
Trial 2 Propolis 1.5%			
Trial 3 Propolis 3 %			

Figure 1: Morphological development of chicken embryos treated with aqueous propolis solutions (0%, 1%, 1.5%, 3%)
Original photographs

The photographs provided detailed information on body conformation, overall size, pigmentation, limb formation, and vascularization. Control embryos displayed normal morphology and followed standard growth trajectories.

Embryos treated with propolis exhibited noticeable morphological changes. The 1% concentration resulted in a slight increase in body mass and length. The 1.5% and 3% groups showed more pronounced effects, including enlarged eye diameter and enhanced vascular development.

At the 3% concentration, certain features suggested developmental instability, such as disproportionate organ growth and irregular vascular patterns, indicating that high doses may disrupt normal embryonic development [8, 24].

Table 3: Morphological assessment of chicken embryos based on visual evaluation and Hamburger–Hamilton (HH) stages

Group	Day 7 (HH stage)	Day 14 (HH stage)	Day 21 (HH stage)	Visual assessment
Control (0%)	HH 25–27	HH 34–36	HH 45–46	Well-developed embryos with proper pigmentation and normal hatching. Circulation and vascular patterns were clearly visible, consistent with HH stages [8,9].
Propolis 1%	HH 27–28	HH 35	HH 46	Morphologically healthy embryos with well-defined somites, visible ocular pigmentation, and no pathological signs. Hatched chicks appeared normal [8].
Propolis 1.5%	HH 25–26	HH 36	HH 45–46	Minor abnormalities, including edema, pigmentation disorders, and subcutaneous hemorrhages. Hatched chicks had normal morphology but reduced vitality [9].
Propolis 3%	HH 21–23	HH 30–32	HH 43–44	Clear growth retardation, deformities, poor vascular development, hypoplastic limbs, and delayed yolk sac resorption. Hatched chicks showed weakness and wet plumage, indicative of internal dysfunction [20].

Embryonic morphology offers an objective framework for evaluating somite formation, organogenesis, pigmentation, and vascular development, and it is widely recognized as an international standard for monitoring embryogenesis.

In the control group (0% propolis), embryos progressed according to the expected HH stages (25–46). Morphological development was consistent, with symmetrical limbs, normal pigmentation, and well-organized vascular networks. Yolk resorption was complete, and hatched chicks were viable and free of abnormalities. This group served as a baseline for comparison with treated groups [18].

Embryos exposed to 1% propolis exhibited normal structural features. Head, eyes, limbs, and pigmentation appeared typical, and hatched chicks demonstrated normal vitality and motor activity. These observations indicate that 1% propolis is safe and may exert a bio-stimulatory effect, likely mediated by antioxidant and immunomodulatory actions of flavonoids and phenolic compounds [18–20].

The 1.5% propolis treatment led to mild morphological deviations, such as edema, subcutaneous hemorrhages, and delayed yolk resorption. Minor limb asymmetries were occasionally observed; however, most chicks hatched with adequate vitality. This suggests a subclinical effect at 1.5%, affecting only sensitive embryos [19].

At the highest concentration (3% propolis), pronounced morphological abnormalities were observed. Somite formation was delayed, vascular networks were poorly developed, and pigmentation was weak. Specific anomalies included microcephaly, cranial asymmetry, beak deformities, and shortened or twisted limbs. Many embryos remained at earlier HH stages (≤ HH 30). Hatched chicks showed reduced vitality, diminished responsiveness, and impaired motor function, indicating teratogenic and cytotoxic effects, likely related to oxidative stress and disrupted vascular regulation [16, 21].

Overall, the assessment confirms that 1% propolis supports normal embryonic development, whereas concentrations of 1.5% and 3% provoke morphological deviations and reduce post-hatch vitality. These findings underscore the necessity of precise dosing when applying natural bioactive compounds *in ovo*.

Table 4: Statistical analysis of incubation outcomes and correlation between morphometric and morphological parameters (n = 90 eggs)

Treat-ment	Conc. (%)	Hatched chicks (Mean ± SD)	Mortality (Mean ± SD)	p-value	Correlation (r)	95% CI (Hatchability)	LD_{50} (%)	χ^2 (vs. control)
Aqueous extract of propolis	1.0	89.58 ± 5.1	10.42 ± 5.1	0.112 ns	–0.94	84.5–94.7	2.1	4.2
	1.5	62.96 ± 8.7	37.04 ± 8.7	0.025 *	–	54.3–71.7	–	12.6 **
	3.0	46.15 ± 10.3	53.85 ± 10.3	0.008 **	–	35.9–56.5	–	18.4 ***

Analysis of incubation outcomes (Table 4) revealed that propolis concentration had a significant impact on embryonic viability and mortality. At 1.0% propolis, hatchability remained high (89.58 ± 5.1%) and mortality was low (10.42 ± 5.1%), showing no significant difference compared with the untreated control ($p > 0.05$). A strong negative correlation was observed between propolis concentration and hatchability ($r = –0.94$), indicating that higher doses are associated with reduced hatching success. The calculated LD_{50} of 2.1% defines the approximate threshold for embryotoxicity, with the 95% confidence interval for hatchability ranging from 84.5% to 94.7%.

When the propolis concentration was increased to 1.5%, hatchability declined to 62.96 ± 8.7%, while mortality rose to 37.04 ± 8.7%, showing a statistically significant difference from the control ($p = 0.025$; $\chi^2 = 12.6$, $p < 0.05$). These findings indicate that concentrations above 1.0% may begin to exert detrimental effects on embryonic development.

The highest concentration tested, 3.0%, produced the greatest reduction in embryonic viability (46.15 ± 10.3%) and the highest mortality (53.85 ± 10.3%), with a statistically significant difference relative to controls ($p = 0.008$; $\chi^2 = 18.4$; $p < 0.01$). This

demonstrates a clear dose-dependent toxic effect, where incremental increases in propolis consistently lowered hatchability and increased mortality.

The narrowing of the 95% confidence intervals for hatchability at higher concentrations reflects increased variability and instability in embryonic development. These observations reinforce the relevance of the LD_{50} value as a practical indicator of safety, consistent with earlier studies on propolis effects *in ovo* [22, 23]. Overall, the χ^2 analysis supports the conclusion that propolis exerts a concentration-dependent toxic impact, emphasizing that doses below 1.0% are generally safe, whereas those exceeding approximately 2.1% surpass the margin for normal embryonic growth.

Conclusion

The present study demonstrates that aqueous propolis exerts a clear dose-dependent effect on chicken embryonic development. At low concentrations, up to 1.0%, embryos developed normally, exhibiting standard growth patterns, proper morphological features, and high hatchability. Exposure to 1.5% propolis induced mild alterations, including minor edema, subcutaneous hemorrhages, and slight reductions in vitality, indicating the initial onset of adverse effects.

The highest concentration, 3.0%, caused pronounced developmental disturbances, including delayed embryonic staging, abnormal limb formation, irregular vascularization, and increased mortality, resulting in substantially reduced hatchability. These observations indicate that propolis may act as a natural growth promoter at low doses, but can become detrimental when administered at higher levels.

Overall, the findings highlight the critical importance of precise dose management in *in ovo* applications. Concentrations up to 1.0% are considered safe and supportive of normal embryonic development, while higher doses exceed the safe threshold, leading to compromised viability and morphological instability.

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