

RESEARCH ARTICLE

Differential effects of maternal dietary and *in ovo* L-arginine supplementation on late embryonic mortality in Alope Unhas 1 chickens



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ABSTRACT

Background and Aim: Improving embryonic survival and hatchability is essential for increasing the productivity of indigenous chicken breeds. L-arginine is a functional amino acid involved in nitric oxide synthesis, angiogenesis, protein metabolism, and immune regulation, and researchers have investigated it as a nutritional intervention to improve embryonic development and post-hatch performance. However, information regarding the combined effects of maternal dietary and *in ovo* L-arginine supplementation in indigenous chickens remains limited. This study evaluated the effects of dietary and *in ovo* L-arginine supplementation on embryonic development, hatchability, embryonic mortality, growth performance, intestinal morphology, and muscle histomorphology in Alope Unhas 1 chickens.

Materials and Methods: A total of 108 Alope Unhas 1 breeder chickens (90 hens and 18 roosters) were allocated to four treatments in a completely randomized design with three replicate pens per treatment. A total of 120 fertile eggs (30 per treatment, with 10 eggs per replicate) were allocated for hatchability and embryonic mortality assessments. The treatments consisted of a basal diet without L-arginine supplementation or *in ovo* injection (T0); a basal diet with *in ovo* injection of 2% L-arginine (T1); a basal diet supplemented with 0.25% L-arginine (T2); and combined dietary supplementation and *in ovo* injection (T3). The *in ovo* injection was administered on day 18 of incubation using 0.5 mL of 2% L-arginine solution delivered into the amniotic cavity through the air cell. Embryonic development, hatchability, embryonic mortality, post-hatch growth performance, intestinal morphology, and muscle histomorphology were evaluated. The replicate pen was considered the experimental unit for statistical analysis. Data were analyzed using one-way analysis of variance followed by Duncan's multiple range test, with statistical significance set at $p < 0.05$.

Results: Maternal dietary and *in ovo* L-arginine supplementation did not significantly affect embryonic development, hatchability, hatch weight, post-hatch growth performance, intestinal morphology, or muscle histomorphology ($p > 0.05$). However, late embryonic mortality differed significantly among treatments ($p = 0.024$). The dietary L-arginine supplementation group (T2) exhibited the lowest late embryonic mortality ($0.00 \pm 0.00\%$), compared with the untreated control group (T0; $7.49 \pm 1.45\%$) and the combined dietary and *in ovo* supplementation group (T3; $15.61 \pm 9.27\%$). Late embryonic mortality was significantly lower in T2 than in T3, whereas the difference between T2 and T0 was not statistically significant. Although hatchability was numerically highest in T2 ($83.89 \pm 21.10\%$), no significant differences were observed among treatments ($p > 0.05$). These findings suggest that maternal dietary L-arginine supplementation may influence late embryonic survival, whereas combining dietary and *in ovo* supplementation did not provide additional benefits under the experimental conditions.

Conclusion: Maternal dietary L-arginine supplementation may improve late embryonic survival in Alope Unhas 1 chickens without significantly affecting post-hatch growth, intestinal morphology, or muscle histomorphology. In contrast, combining dietary and *in ovo* L-arginine supplementation did not confer additional biological advantages and may adversely affect late embryonic survival. These findings provide useful baseline evidence for optimizing L-arginine supplementation strategies in indigenous chicken breeding programs and warrant further dose-response, mechanistic, and long-term studies to establish effective supplementation protocols.

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Keywords: Alope Unhas 1 chickens, embryonic development, embryonic mortality, hatchability, *in ovo* feeding, intestinal morphology, L-arginine, muscle histomorphology.

INTRODUCTION

Poultry production plays a pivotal role in meeting the increasing global demand for animal-derived protein, particularly in developing countries where indigenous chicken breeds contribute substantially to household nutrition, rural livelihoods, and local food security. Alope Unhas 1 is an Indonesian indigenous chicken strain developed to enhance local poultry production in tropical environments. This strain is recognized for its adaptability, suitability for smallholder farming systems, and potential to conserve and sustainably use indigenous poultry genetic resources. Nevertheless, like many native chicken strains, reproductive efficiency, hatchability, and early growth performance remain suboptimal, limiting productivity and broader use of this genetic resource [1].

Embryonic development critically determines hatchability, chick quality, and subsequent growth performance. Throughout incubation, the developing embryo depends primarily on nutrients stored within the egg, particularly amino acids required for protein synthesis, tissue differentiation, and organ development. Among these nutrients, L-arginine is considered a functional amino acid because it participates in several physiological processes, including protein metabolism, immune regulation, polyamine synthesis, and nitric oxide production [2, 3]. Mechanistically, L-arginine is the primary substrate for nitric oxide synthase, which catalyzes its conversion to nitric oxide and L-citrulline. Nitric oxide is an important signaling molecule involved in vascular regulation, angiogenesis, cellular proliferation, and blood flow, thereby facilitating oxygen and nutrient delivery to developing embryonic tissues. These functions become particularly important during late incubation, when embryonic growth, oxygen consumption, and metabolic activity increase markedly.

In recent years, *in ovo* feeding has emerged as a promising approach to enhance embryonic nutrition by directly administering nutrients into the egg during incubation. Depending on the nutrient type, dose, and timing, *in ovo* supplementation has been shown to improve hatchability, intestinal development, immune competence, and post-hatch performance in poultry [4, 5]. Similarly, dietary supplementation with functional amino acids, including L-arginine, has been associated with improvements in growth performance, nutrient utilization, intestinal morphology, muscle development, antioxidant capacity, and immune function [6]. These findings indicate that both maternal nutrition and embryonic nutritional interventions may influence developmental programming and subsequent physiological performance.

Despite these advances, previous studies on L-arginine supplementation in poultry have predominantly investigated dietary supplementation and *in ovo* administration as separate nutritional strategies. Dao *et al.* [7] evaluated dietary L-arginine supplementation in laying hens, whereas Dai *et al.* [8] investigated the effects of *in ovo* L-arginine administration on intestinal development, microbial composition, and metabolic responses in layer chicks. Similarly, Lu *et al.* [9] examined the effects of *in ovo* L-arginine supplementation on hatchability, growth performance, and antioxidant capacity in slow-growing chickens, while Taufik *et al.* [10] investigated embryonic development and hatching performance following *in ovo* L-arginine administration in Indonesian local chickens. Although these studies offer valuable insights into physiological and developmental responses to L-arginine supplementation, evidence directly comparing maternal dietary supplementation, *in ovo* administration, and their combined use within the same production cycle remains limited. Consequently, it remains unclear whether simultaneous supplementation through both routes provides additional developmental benefits or produces unfavorable responses compared with either strategy alone.

This knowledge gap is particularly relevant to indigenous chicken strains, which may differ from commercial poultry in genetic background, growth rate, metabolic requirements, embryonic development, nutrient utilization, and physiological responses to amino acid supplementation. Therefore, findings obtained from intensively selected commercial breeds cannot necessarily be extrapolated to Alope Unhas 1 chickens. Furthermore, evidence on the combined effects of maternal dietary and *in ovo* L-arginine supplementation on multiple developmental and physiological outcomes, including embryonic development, hatchability, embryonic mortality, post-hatch growth performance, intestinal morphology, and muscle histomorphology, remains limited. Although both supplementation routes may improve embryonic nutrient availability, their combined use could elicit different biological responses depending on timing, dosage, and route of administration. Addressing these gaps is essential to determine whether combined supplementation offers measurable advantages over individual administration or potentially compromises embryonic survival. Accordingly, this study comparatively evaluates these supplementation strategies in Alope Unhas 1 chickens, with particular emphasis on hatchability and late

embryonic mortality.

This study aimed to evaluate the individual and combined effects of maternal dietary and *in ovo* L-arginine supplementation on embryonic development, hatchability, embryonic mortality, post-hatch growth performance, intestinal morphology, and muscle histomorphology in Alope Unhas 1 chickens. The primary objectives were to determine the effects of these supplementation strategies on hatchability and embryonic mortality, with particular emphasis on late embryonic mortality as a key indicator of embryonic survival. The secondary objectives were to assess their effects on embryonic growth, hatch weight, early post-hatch performance, intestinal development, and muscle structural characteristics. We hypothesized that maternal dietary and *in ovo* L-arginine supplementation would improve embryonic survival and physiological development when administered individually, whereas their combined application was expected to provide greater developmental benefits than either strategy alone, consistent with a potential additive effect rather than a presupposed synergistic interaction. This comparative approach was intended to determine whether combined supplementation offers additional biological advantages or produces less favorable developmental responses in Alope Unhas 1 chickens.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving animals were conducted in accordance with internationally accepted principles for the ethical care and use of animals in research and complied with the institutional guidelines of Hasanuddin University. The experimental protocol, animal handling procedures, housing conditions, and sampling methods were reviewed and approved by the Animal Ethics Committee of Hasanuddin University, Makassar, Indonesia, under approval number 016/UN4.12/EC/VI/2025, before the commencement of the study. Throughout the experiment, all efforts were made to minimize animal stress, discomfort, and unnecessary suffering during handling, husbandry, sampling, and tissue collection. Animals were monitored regularly by trained personnel to ensure their health and welfare were maintained throughout the study.

Study period and location

The study was conducted from June to November 2025 at the Poultry Production Laboratory, Faculty of Animal Science, Hasanuddin University, Makassar, South Sulawesi, Indonesia. The experiment was conducted using Alope Unhas 1 breeder chickens maintained under intensive management conditions at the experimental poultry facilities of Hasanuddin University, Makassar, South Sulawesi, Indonesia. Egg incubation, *in ovo* injection, hatchability evaluation, and post-hatch observations were performed under controlled experimental conditions at the same facility.

Study design

This study employed a completely randomized design (CRD) comprising four treatment groups with three replicates per treatment. The experimental design was developed to evaluate the individual and combined effects of maternal dietary and *in ovo* L-arginine supplementation on embryonic development, hatchability, embryonic mortality, and selected physiological characteristics of Alope Unhas 1 chickens.

Experimental design

A total of 108 Alope Unhas 1 breeder chickens, comprising 90 females and 18 males at 36 weeks of age, were randomly allocated to four experimental treatments. Each treatment consisted of 27 birds, which were further divided into three replicates of nine birds each. Two replicate-pen configurations were used: eight hens with one rooster or seven hens with two roosters. Across the 12 replicate pens, six pens contained eight hens and one rooster, whereas the remaining six pens contained seven hens and two roosters, maintaining an overall female-to-male ratio of 5:1 across the breeder population.

A total of 120 fertile eggs (30 eggs per treatment, with 10 eggs per replicate) were collected for hatchability and embryonic mortality assessments. An additional 36 fertile eggs, comprising nine eggs per treatment and three eggs per replicate, were independently selected for destructive embryonic development assessment on day 18 of incubation. These eggs were excluded from the hatchability and embryonic mortality analyses. Thus, a total of 156 fertile eggs were allocated across the two assessment procedures, ensuring that destructive embryo sampling did not affect the denominator used to calculate hatchability. The sample size was determined based on the available Alope Unhas 1 breeder population and the replicate-pen experimental unit used in this study. Three replicate pens per treatment were included to permit estimation of experimental error within the CRD, with each

replicate pen considered the experimental unit. Similar replicate-based experimental designs have been widely adopted in poultry nutrition studies [11, 12]. Nevertheless, the relatively small number of replicates may have reduced the statistical power to detect subtle treatment effects, particularly for variables exhibiting high biological variability. Therefore, non-significant results were interpreted cautiously, considering the potential occurrence of Type II error.

The experimental treatments were as follows:

- T0: Basal diet without L-arginine supplementation and no *in ovo* injection.
- T1: Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine.
- T2: Basal diet supplemented with 0.25% L-arginine without *in ovo* injection.
- T3: Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection of 2% L-arginine.

The methodological novelty of this study lies in the simultaneous evaluation of maternal dietary supplementation and embryonic-stage *in ovo* administration of L-arginine within the same production cycle, thereby enabling assessment of both their individual and combined effects in an indigenous chicken breed.

Animals and housing

A total of 108 Alope Unhas 1 breeder chickens (90 females and 18 males) at 36 weeks of age, were used as breeder stock. Birds were reared under an intensive management system in floor 12 floor pens measuring 3.0 × 2.4 m with litter flooring, following standard husbandry practices for indigenous chickens [13]. The breeder flock was maintained under a natural tropical photoperiod of approximately 12 h light and 12 h dark, with ambient temperatures ranging from 26°C to 30°C and relative humidity maintained between 70% and 80% throughout the study.

Each replicate pen housed nine birds, comprising either eight hens and one rooster or seven hens and two roosters, according to the treatment allocation described in the Experimental design subsection. The overall female-to-male ratio was 5:1 across the entire breeder population. All replicate pens were maintained under comparable environmental and management conditions throughout the experiment to minimize variation in reproductive performance attributable to housing and husbandry practices. Each pen was equipped with feeders, drinkers, and nesting boxes to facilitate egg production. Birds were maintained under uniform environmental and management conditions throughout the experimental period. Feed and drinking water were provided *ad libitum*, and routine husbandry practices, including health monitoring, sanitation, and general flock management, were implemented consistently to maintain optimal bird health and performance.

Diet preparation and L-arginine supplementation

The experimental basal diet consisted of a commercial breeder feed formulated for indigenous breeder chickens (Product Code 534 RJ, PT Charoen Pokphand Indonesia Tbk, Jakarta, Indonesia). According to the manufacturer's nutrient specifications, the diet contained 16% crude protein, 2,700-2,900 kcal/kg metabolizable energy, 3% crude fat, 6% crude fiber, 0.9% calcium, 0.6% phosphorus, 0.8% lysine, 0.4% methionine, and 0.95% total arginine. The calcium concentration reflected the manufacturer's formulation for indigenous breeder chickens and aligned with standard poultry nutrition recommendations [14]. The reported nutrient composition was based on the documented feed specifications rather than independent laboratory analysis.

Analytical-grade L-arginine (Sigma-Aldrich, St. Louis, MO, USA) was added to the basal diet at 0.25% according to the treatment allocation. The L-arginine was thoroughly mixed into the feed to ensure uniform distribution before offering it to the birds [7]. Maternal dietary supplementation was initiated 28 days (4 weeks) before egg collection and continued throughout the egg-collection period. This feeding period was intended to provide sufficient maternal exposure to the experimental diets before egg formation and collection. However, the deposition of supplemental L-arginine into egg components was not directly quantified.

The basal diet contained 0.95% total arginine based on the manufacturer's nutrient specifications. Consequently, the calculated total arginine concentration remained 0.95% in T0 and T1, whereas supplementation increased it to 1.20% in T2 and T3 after adding 0.25% analytical-grade L-arginine. These values represented formulated dietary arginine concentrations rather than analytically verified concentrations.

The reported nutrient composition refers to the manufacturer's specifications for the basal diet. No laboratory analyses were performed to verify the complete nutrient composition following L-arginine supplementation. Therefore, the final arginine concentrations of the experimental diets were calculated from the basal dietary arginine content and the amount of supplemental analytical-grade L-arginine added. Individual feed

intake by breeder hens was not measured, and actual daily arginine consumption per hen was therefore not calculated. Accordingly, the reported arginine concentrations represent estimated dietary inclusion levels rather than quantified individual arginine intake.

To preserve L-arginine stability, supplemented diets were prepared periodically in small batches, stored in sealed containers under cool, dry, and shaded conditions, and used within the designated feeding period. No heat treatment or additional feed processing was applied after L-arginine supplementation.

Egg incubation and *in ovo* injection procedure

Eggs were collected daily from each replicate pen, and only clean, intact, normally shaped eggs of uniform size were selected for incubation. Before incubation, each egg was labeled according to its treatment and replicate. Eggs were subsequently randomized within each treatment replicate to ensure equal representation among treatment groups.

Fertility was confirmed by candling on day 7 of incubation, and only fertile eggs were included in the hatchability and embryonic mortality analyses. Fertile eggs were incubated in a commercial incubator (OvaEasy 190 Advance Series II, Brinsea Products Ltd., Weston-super-Mare, UK) under standard incubation conditions at 37.5–37.8°C with a relative humidity of 55%–60%. Eggs were automatically turned at regular intervals throughout incubation.

The *in ovo* injection procedure was performed on day 18 of incubation. Eggs from all treatment groups were removed simultaneously from the incubator and handled for an equivalent duration to minimize handling bias. Non-injected eggs were neither punctured nor injected but were exposed to identical handling conditions. A sham-injected or vehicle-injected control was not included because the experimental design focused on comparing untreated, dietary, *in ovo*, and combined supplementation strategies. Consequently, the potential effects of eggshell puncture and injection handling could not be distinguished from those of L-arginine administration. The L-arginine injection solution was prepared by dissolving analytical-grade L-arginine in sterile distilled water to obtain a final concentration of 2% (w/v; 20 mg/mL), corresponding to 10 mg of L-arginine per egg at an injection volume of 0.5 mL. In this reference protocol, the solution pH was adjusted to 7.0 and the solution was sterilized through a 0.22- μ m membrane filter under aseptic conditions. The injection volume was selected based on the *in ovo* feeding procedure described by Lu et al. [9], with modifications to the L-arginine concentration.

Before injection, the eggshell surface was disinfected with 70% ethanol. The injection site was located at the broad end of the egg, corresponding to the air cell. A sterile 1-mL disposable syringe fitted with a 21-gauge needle was used, with a reference insertion depth of approximately 20 mm. The needle was carefully advanced through the air cell toward the amniotic cavity, using the anatomical position of the developing embryo to guide placement. Needle positioning was anatomically guided rather than independently verified. Following injection, the puncture site was immediately sealed with sterile paraffin wax to minimize contamination and moisture loss. Eggs were then returned promptly to the incubator and maintained under standard hatching conditions until hatch. Post-injection candling was subsequently performed to assess embryo viability and to remove eggs showing evidence of embryonic mortality or contamination.

Embryonic development

Embryonic development was evaluated on day 18 of incubation before the *in ovo* injection procedure between 08:00 and 10:00 h. Therefore, these measurements were intended to assess embryonic development in relation to maternal dietary supplementation rather than the effects of subsequent *in ovo* L-arginine administration. A total of nine fertile eggs per treatment, comprising three eggs from each replicate, were randomly selected for embryonic development assessment. Each egg was weighed before opening, after which the eggshell was carefully broken at the broad end. The embryo was gently separated from the yolk sac, amniotic fluid, and allantoic fluid using sterile forceps. The yolk, amniotic and allantoic fluids, and embryo were weighed separately using a digital analytical balance (Pioneer PX224/E, OHAUS Corporation, Parsippany, NJ, USA). Embryo weight was recorded after complete removal of the yolk sac to avoid overestimation of embryonic mass. The embryo-to-egg weight ratio was calculated as the percentage of embryo weight relative to the initial egg weight [15, 16].

Hatchability and embryonic mortality

Hatchability and embryonic mortality were evaluated to determine the effects of the experimental treatments on hatching success. Fertility was initially assessed by candling on day 7 of incubation, based on visible

evidence of embryonic development, including embryonic vascularization and identifiable embryonic structures. Hatchability was calculated as the percentage of chicks hatched relative to the total number of fertile eggs. At the end of the incubation period, all unhatched fertile eggs were opened to determine the stage of embryonic mortality. Early embryonic mortality was assessed retrospectively based on evidence of previous embryonic development, including blood rings, vascular remnants, or recognizable embryonic tissues. Eggs without identifiable developmental structures could not be reliably distinguished from infertile eggs by candling alone, and this diagnostic limitation was considered when interpreting early embryonic mortality. Embryonic mortality was classified as early (days 1–7 of incubation), middle (days 8–14), or late (days 15–21) according to the estimated stage of embryonic death. Classification was based on embryo size, degree of morphological development, feather development, yolk absorption, and embryonic position within the egg. For each replicate, the numbers of fertile eggs, hatched chicks, and embryos dying at each developmental stage were used to calculate hatchability and stage-specific embryonic mortality. Embryonic mortality for each developmental stage was expressed as a percentage of the total number of fertile eggs within each replicate [17].

Post-hatch performance

Post-hatch performance was evaluated to determine the effects of the experimental treatments on early chick growth. Immediately after hatching, chicks were individually weighed to determine hatch weight and subsequently maintained according to their original treatment and replicate groups. A total of 15 chicks per treatment, comprising five chicks from each replicate, were used for post-hatch performance evaluation.

Performance measurements were conducted from day 1 to day 7 post-hatch. The evaluated variables included hatch weight, egg-to-hatch weight ratio, feed intake, body weight gain, and feed conversion ratio (FCR). The egg-to-hatch weight ratio was calculated as hatch weight divided by the initial egg weight and multiplied by 100. Feed intake was determined at the replicate level by subtracting the amount of feed remaining from the total amount of feed offered during the experimental period. Feed intake was expressed as cumulative consumption per chick (g/chick) over the six-day measurement interval rather than daily consumption. Body weight gain was calculated as the difference between final and initial body weights, and average daily weight gain (g/chick/day) was calculated by dividing total weight gain by six days. FCR was determined by dividing cumulative feed intake by cumulative body weight gain over the corresponding interval. The replicate pen served as the experimental unit for the analyses of feed intake, body weight gain, and FCR [18].

Intestinal morphology and histology

Intestinal morphology and histological characteristics were evaluated on day 7 post-hatch. Three birds per treatment, comprising one bird randomly selected from each replicate, were humanely euthanized for intestinal sampling. The small intestine was excised and divided into the duodenum, jejunum, and ileum. Each intestinal segment was weighed and measured to determine relative intestinal weight and length. Relative intestinal length (%) was calculated by dividing the length of each intestinal segment by the combined length of the duodenum, jejunum, and ileum and multiplying by 100. Similarly, relative intestinal weight (%) was calculated by dividing the weight of each segment by the combined weight of these three intestinal segments and multiplying by 100. Thus, the reported percentages represent the proportional contributions of individual segments to the total small intestinal length and weight, respectively.

For histological evaluation, an approximately 2-cm section was collected from the midpoint of the jejunum, rinsed with physiological saline, and fixed in 10% neutral-buffered formalin. Tissue samples were dehydrated through a graded ethanol series, cleared, embedded in paraffin, sectioned at approximately 5 µm thickness, and stained with hematoxylin and eosin using standard histological procedures.

Representative jejunal sections with intact mucosal architecture were examined under a light microscope (Model CX23, Olympus Corporation, Tokyo, Japan). Villus height was measured from the villus tip to the villus-crypt junction, whereas crypt depth was measured from the villus-crypt junction to the base of the crypt. For each sample, at least 10 well-oriented villi and 10 corresponding crypts were measured. The villus height-to-crypt depth ratio was calculated by dividing villus height by crypt depth. Both measurements were expressed in micrometers (µm), and the resulting ratio was dimensionless. The corrected ratio was determined using measurements expressed in consistent units, with replicate-level values serving as the basis for statistical analysis. Morphometric measurements were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by an observer blinded to the treatment allocation [9, 19].

Muscle histomorphology

Muscle histomorphology was evaluated to determine the effects of the experimental treatments on skeletal muscle development. Sampling was performed on day 7 post-hatch using the same birds selected for intestinal histological evaluation. Three birds per treatment, comprising one bird from each replicate, were included in the muscle histomorphological analysis.

Samples of the *pectoralis major* muscle were collected immediately after euthanasia and fixed in 10% neutral-buffered formalin for at least 24 h. Subsequently, tissue samples were processed using standard histological procedures, including dehydration, paraffin embedding, sectioning at approximately 5 µm thickness, and hematoxylin and eosin staining.

Histological examination was performed at 400× magnification using a light microscope (Model CX23, Olympus Corporation, Tokyo, Japan). Representative transverse sections were selected for quantitative analysis. The evaluated parameters included myofiber number, myofiber cross-sectional area, and myofiber diameter. Myofiber number was expressed as the number of identifiable muscle fibers per microscopic field at 400× magnification rather than as fiber density per unit tissue area. Only intact, well-defined, transversely sectioned muscle fibers were included in the analysis. Myofiber cross-sectional area was defined as the cross-sectional area of individual muscle fibers, whereas myofiber diameter was measured as the average width of each fiber. For each sample, 10-20 randomly selected muscle fibers were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by an observer blinded to the treatment allocation [9, 20].

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) based on a completely randomized design (CRD) to determine the effects of the experimental treatments. Although the experimental treatments followed a 2 × 2 arrangement comprising two levels of maternal dietary L-arginine supplementation (0% and 0.25%) and two levels of *in ovo* administration (without and with 2% L-arginine injection), the four treatment combinations (T0–T3) were analyzed as a single categorical factor. This approach was selected to compare the overall responses among the four predefined supplementation strategies. Therefore, the analysis evaluated differences among treatment combinations rather than separately testing the main effects and interaction between the two supplementation routes. The replicate pen was considered the experimental unit for all statistical analyses. Individual measurements obtained from eggs, chicks, histological sections, and myofibers were treated as subsamples within their respective experimental replicates rather than as independent experimental units. Replicate-level means were used as independent observations for statistical comparisons.

Proportional outcomes, including hatchability and early, middle, and late embryonic mortality, were calculated separately for each replicate as percentages of the corresponding number of fertile eggs. These replicate-level percentages were subsequently used for statistical analysis. Before ANOVA, data were assessed for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Given the limited number of replicates per treatment ($n = 3$) and the occurrence of zero mortality in some treatment groups, the statistical assumptions for proportional outcomes require particular consideration. Consequently, findings from these analyses should be interpreted cautiously, particularly where the assumptions of conventional ANOVA may not be adequately satisfied. When ANOVA indicated a significant treatment effect ($p < 0.05$), differences among treatment means were compared using Duncan's multiple range test as the post hoc multiple comparison procedure. All statistical analyses were performed using IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA; <https://www.ibm.com/support/pages/ibm-spss-statistics270x>). Data are presented as mean ± standard deviation, and statistical significance was declared at $p < 0.05$.

RESULTS

The embryonic development characteristics of Alopecurus Unhas 1 chicken embryos are presented in Table 1. No significant differences were observed among treatment groups in egg weight, yolk weight, amniotic and allantoic fluid weight, embryo weight, or the embryo-to-egg weight ratio on day 18 of incubation (all $p > 0.05$). As these measurements preceded *in ovo* injection, the findings primarily reflect embryonic development under the respective maternal dietary conditions rather than responses to *in ovo* supplementation.

Hatchability and embryonic mortality

The effects of dietary and *in ovo* L-arginine supplementation on hatchability and embryonic mortality are presented in Table 2. A total of 30 fertile eggs per treatment (120 fertile eggs overall) were included in the

evaluation. After hatching, all unhatched fertile eggs were opened to determine the stage of embryonic mortality. Hatchability, early embryonic mortality, and middle embryonic mortality were not significantly affected by the treatments (all $p > 0.05$). However, late embryonic mortality differed significantly among treatment groups ($p = 0.024$). The combined dietary and *in ovo* supplementation group (T3) exhibited the highest late embryonic mortality, whereas the dietary supplementation group (T2) showed no late embryonic mortality and differed significantly from T3. Treatment T1 showed intermediate values and did not differ significantly from either T0 or T3. The significant overall treatment effect was distinguished from individual pairwise comparisons, which were interpreted according to Duncan's multiple range test.

Table 1: Embryonic development characteristics of Alope Unhas 1 chicken embryos under different treatments.

Treatment	Egg weight (g)	Yolk weight (g)	Amniotic and allantoic fluid weight (g)	Embryo weight (g)	Embryo-to-egg weight ratio (%)
T0	45.17 ± 4.16	7.87 ± 2.70	2.43 ± 0.35	24.97 ± 2.76	55.30 ± 3.67
T1	44.37 ± 0.85	5.97 ± 0.96	2.63 ± 0.65	25.27 ± 0.64	56.98 ± 2.52
T2	46.13 ± 1.44	8.03 ± 0.40	3.00 ± 0.52	24.23 ± 0.78	52.53 ± 0.44
T3	45.00 ± 3.00	7.43 ± 1.00	2.80 ± 0.44	24.47 ± 2.24	54.32 ± 1.91
p-value	0.880	0.394	0.582	0.898	0.233

T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection.

Hatchability and embryonic mortality percentages were calculated using the total number of fertile eggs within each replicate as the denominator. These outcomes represented mutually exclusive categories, and their combined percentages were expected to equal 100% for each replicate. The treatment-level mean percentages totaled approximately 100%, with minor deviations attributable to numerical rounding.

Table 2: Hatchability and embryonic mortality of Alope Unhas 1 chicken embryos subjected to different treatments.

Treatment	Hatchability (%)	Early embryonic mortality (%)	Middle embryonic mortality (%)	Late embryonic mortality (%)
T0	63.18 ± 5.82	20.40 ± 7.78	8.93 ± 7.78	7.49 ± 1.45 ^b
T1	69.68 ± 16.08	0.00 ± 0.00	20.63 ± 14.64	9.68 ± 1.46 ^{ab}
T2	83.89 ± 21.10	16.11 ± 21.10	0.00 ± 0.00	0.00 ± 0.00 ^c
T3	70.97 ± 14.79	0.00 ± 0.00	13.42 ± 18.87	15.61 ± 9.27 ^a
p-value	0.464	0.113	0.307	0.024

Different superscripts within the same column indicate significant differences ($p < 0.05$). T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection.

Post-hatch performance

Post-hatch performance data are presented in Figure 1 and Table 3. Dietary and *in ovo* L-arginine supplementation did not significantly affect hatch weight, egg-to-hatch weight ratio, feed intake, body weight gain, or FCR (all $p > 0.05$). Overall, post-hatch growth performance remained comparable among all treatment groups during the first week after hatching.

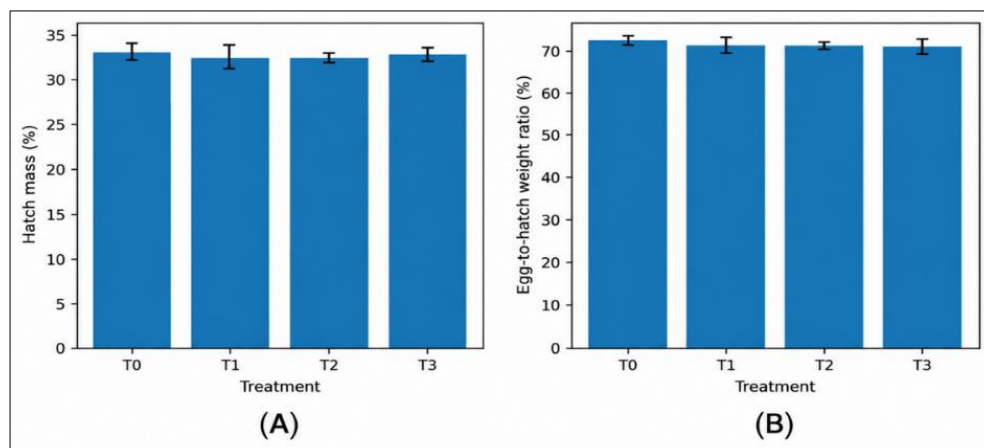


Figure 1: Hatch weight (A) and egg-to-hatch weight ratio (B) of Alope Unhas 1 chickens under different treatments.

Table 3: Post-hatch performance of Alope Unhas 1 chickens subjected to different treatments.

Treatment	Cumulative Feed intake (g/chick)	Body weight gain (g/chick/day)	Feed conversion ratio
T0	119.33 ± 20.49	9.70 ± 1.06	2.04 ± 0.13
T1	113.54 ± 4.49	9.80 ± 0.60	1.94 ± 0.19
T2	110.92 ± 3.17	9.79 ± 0.66	1.90 ± 0.14
T3	111.46 ± 8.76	9.46 ± 1.03	1.97 ± 0.08
p-value	0.728	0.912	0.623

T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection.

Intestinal morphology and histology

The effects of dietary and *in ovo* L-arginine supplementation on intestinal morphology and histology are presented in Table 4 and Table 5, respectively. No significant differences were observed in the relative length or weight of the duodenum, jejunum, or ileum among treatment groups (all $p > 0.05$). Likewise, villus height, crypt depth, and the villus height-to-crypt depth ratio were not significantly influenced by the treatments (all $p > 0.05$), indicating that intestinal development remained comparable among the experimental groups.

Table 4: Intestinal morphology of Alope Unhas 1 chickens subjected to different treatments.

Treatment	Duodenum length (%)	Jejunum length (%)	Ileum length (%)	Duodenum weight (%)	Jejunum weight (%)	Ileum weight (%)
T0	20.85 ± 2.10	40.98 ± 1.40	38.16 ± 1.00	29.40 ± 3.90	40.52 ± 8.90	30.08 ± 8.90
T1	18.35 ± 1.23	43.89 ± 0.46	37.76 ± 1.67	24.78 ± 3.07	45.00 ± 1.13	30.23 ± 2.04
T2	17.76 ± 1.35	40.80 ± 2.42	41.44 ± 3.24	25.38 ± 2.61	39.73 ± 4.61	34.89 ± 3.83
T3	19.69 ± 2.27	40.53 ± 2.97	39.78 ± 1.38	25.65 ± 3.88	42.05 ± 0.98	32.30 ± 3.23
p-value	0.317	0.201	0.145	0.421	0.298	0.267

T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection.

Table 5: Intestinal histological characteristics of Alope Unhas 1 chickens subjected to different treatments.

Treatment	Villus height (μm)	Crypt depth (μm)	Villus height-to-crypt depth ratio
T0	906.51 ± 137.92	249.06 ± 55.10	3.64
T1	924.10 ± 123.60	252.44 ± 52.00	3.66
T2	804.18 ± 272.34	229.90 ± 43.64	3.50
T3	894.55 ± 149.52	306.19 ± 69.37	2.92
p-value	0.533	0.412	0.365

T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection. Data are presented as mean ± standard deviation (n = 3 replicates per treatment).

Muscle histomorphology

The muscle histomorphological characteristics of Alope Unhas 1 chickens are presented in Table 6. Dietary and *in ovo* L-arginine supplementation did not significantly affect myofiber number, myofiber cross-sectional area, or myofiber diameter (all $p > 0.05$). These findings indicate that the supplementation strategies evaluated did not influence early skeletal muscle development in Alope Unhas 1 chickens.

Table 6: Muscle histomorphology of Alope Unhas 1 chickens subjected to different treatments.

Treatment	Number of myofibers (fibers/field)	Myofiber cross-sectional area (μm ²)	Myofiber diameter (μm)
T0	113.67 ± 12.75	2356.03 ± 1091.41	39.83 ± 5.20
T1	161.11 ± 41.66	2934.81 ± 2180.61	46.16 ± 14.98
T2	124.67 ± 46.03	2577.61 ± 1684.91	49.61 ± 15.90
T3	172.33 ± 51.11	2460.28 ± 869.57	45.07 ± 3.90
p-value	0.215	0.873	0.612

T0 = Basal diet without L-arginine supplementation and no *in ovo* injection; T1 = Basal diet without L-arginine supplementation with *in ovo* injection of 2% L-arginine; T2 = Basal diet supplemented with 0.25% L-arginine without *in ovo* injection; T3 = Basal diet supplemented with 0.25% L-arginine combined with *in ovo* injection.

DISCUSSION

Overall effects of dietary and *in ovo* L-arginine supplementation

To our knowledge, this study is the first to comprehensively evaluate the combined effects of maternal

dietary and *in ovo* L-arginine supplementation in Alopecurus Unhas 1 chickens, an indigenous Indonesian chicken strain adapted to tropical production systems. Overall, L-arginine supplementation had limited effects on most evaluated parameters, except late embryonic mortality, suggesting its biological effects were stage-specific rather than uniformly expressed throughout embryonic and early post-hatch development [10, 21].

Compared with previous studies in commercial broilers and slow-growing chickens, the responses observed in the present study appear to be more closely associated with embryonic survival than with growth or tissue development. Previous investigations showed that *in ovo* L-arginine supplementation could improve hatchability, antioxidant status, intestinal development, and muscle-related characteristics in broiler and slow-growing chickens, although these responses varied by supplementation dose, timing, and genetic background [8, 10, 19]. In contrast, the present study showed no significant improvements in post-hatch performance, intestinal morphology, or muscle histomorphology in Alopecurus Unhas 1 chickens. These findings suggest that indigenous chickens may respond differently from commercial strains because of differences in growth rate, metabolic demand, genetic background, and adaptation to tropical production environments.

Effects on embryonic development

The absence of significant differences in embryonic development parameters suggests that maternal dietary L-arginine supplementation was not associated with detectable changes in embryonic growth under the experimental conditions. Importantly, these measurements were obtained on day 18 of incubation before *in ovo* L-arginine administration. Therefore, the findings cannot establish whether subsequent *in ovo* supplementation influenced embryonic development. Avian embryogenesis depends primarily on endogenous nutrient reserves stored in the egg, particularly yolk-derived proteins, lipids, and amino acids, which provide substrates for tissue formation and energy metabolism [22]. Under conditions in which nutrient availability is not limiting, supplementation with exogenous amino acids may not further enhance embryonic growth [2].

Furthermore, embryonic metabolism is tightly regulated throughout incubation, with organogenesis, cellular differentiation, and tissue maturation occurring in a coordinated manner at specific developmental stages. Consequently, the responsiveness of the embryo to nutritional interventions may depend on the timing of nutrient administration relative to these developmental events [8]. Therefore, the lack of measurable changes in embryonic weight and related parameters may reflect the embryo's ability to meet its nutritional needs from endogenous reserves under the experimental conditions used in this study. However, this interpretation applies only to embryonic development before injection, and the potential effects of *in ovo* L-arginine administration on subsequent developmental processes could not be evaluated using these measurements.

Effects on embryonic mortality

Unlike embryonic development, treatments significantly affected late embryonic mortality, indicating differences in embryonic survival among supplementation strategies rather than establishing a specific underlying physiological mechanism. Late embryogenesis is characterized by rapid fetal growth, increased metabolic activity, elevated oxygen consumption, and the transition from chorioallantoic to pulmonary respiration, rendering embryos more susceptible to hypoxia and metabolic disturbances [23].

L-arginine is the principal substrate for nitric oxide synthesis through nitric oxide synthase, and nitric oxide regulates vasodilation, angiogenesis, and embryonic blood flow [24, 25]. These physiological pathways may contribute to the regulation of oxygen and nutrient delivery during late embryogenesis and provide a plausible biological context for the observed differences in embryonic mortality. However, nitric oxide production, vascular function, and related metabolic responses were not directly measured in the present study. Therefore, their involvement remains hypothetical and cannot be established from the current findings. Moreover, the absence of significant treatment effects on embryonic growth, hatchability, intestinal morphology, muscle histomorphology, and post-hatch performance indicates that the observed response was largely confined to late embryonic mortality under the experimental conditions [26].

The highest late embryonic mortality was observed in T3, suggesting that the combined supplementation strategy did not provide additional benefits for embryonic survival. However, this finding cannot be attributed specifically to increased L-arginine exposure because egg arginine concentrations were not quantified and a sham-injected or vehicle-injected control was not included. Consequently, potential contributions from eggshell puncture, injection handling, and solution administration cannot be excluded. Although excessive L-arginine exposure could theoretically influence amino acid homeostasis, osmotic balance, nitric oxide production, or nitrosative stress, these mechanisms were not directly investigated and should be considered possible

explanations rather than demonstrated causes of embryonic mortality. Accordingly, the findings emphasize the need for further studies incorporating appropriate injection controls, direct measurements of arginine exposure, and physiological assessments to determine the mechanisms underlying embryonic survival and optimize supplementation dose, timing, and delivery route.

Effects on post-hatch performance

Although dietary L-arginine supplementation reduced late embryonic mortality, these improvements did not translate into enhanced post-hatch performance. Treatment did not affect hatch weight, feed intake, body weight gain, or feed conversion ratio. These findings suggest that any beneficial effects occurring during embryonic development were either transient or insufficient to influence subsequent growth performance [27].

Post-hatch growth is a multifactorial process influenced by nutritional composition, nutrient digestibility, environmental conditions, management practices, and genetic background [28]. Moreover, the rapid physiological transition from endogenous yolk utilization to exogenous feeding immediately after hatch may diminish the long-term effects of embryonic nutritional interventions [29]. Consequently, improved embryo survival does not necessarily translate into enhanced post-hatch growth.

Effects on intestinal morphology and muscle histomorphology

The absence of significant differences in intestinal morphology and histological characteristics further indicates that L-arginine supplementation exerted only limited systemic effects under the conditions of the present study. Intestinal morphology, particularly villus height and crypt depth, is widely recognized as an indicator of digestive capacity, absorptive efficiency, and epithelial renewal [30]. The lack of structural alterations suggests that L-arginine supplementation did not significantly affect enterocyte proliferation, differentiation, or intestinal functional development [31]. These observations align with unchanged growth performance and indicate that nutrient utilization efficiency remained comparable across treatments.

Similarly, L-arginine supplementation did not significantly affect muscle histomorphology, including myofiber number, myofiber cross-sectional area, or myofiber diameter. Skeletal muscle development in poultry is regulated through prenatal hyperplasia followed by postnatal hypertrophy, both of which depend on nutrient availability and multiple metabolic signaling pathways [32]. The absence of changes in muscle histomorphological characteristics suggests that the supplementation protocol used in the present study did not substantially influence myogenic regulatory pathways or protein accretion [33]. Furthermore, because intestinal development and muscle growth are physiologically interconnected, the lack of intestinal morphological responses may partly explain the absence of measurable effects on muscle development [34].

Biological implications

Collectively, the findings show that the biological response to L-arginine supplementation varies across developmental stages and physiological systems. Although most measured variables remained unchanged, the reduction in late embryonic mortality indicates that L-arginine may selectively support embryo viability during periods of increased metabolic demand [21]. This stage-specific response highlights the importance of optimizing supplementation dosage, timing, and delivery method to maximize biological efficacy while minimizing potential adverse effects [35].

Strengths and limitations

A major strength of the present study is that it simultaneously evaluated maternal diet and *in ovo* L-arginine supplementation within a single experimental model using an indigenous chicken breed. This design enabled assessment of both the individual and combined effects of these nutritional strategies on embryonic development and early physiological responses.

Nevertheless, several limitations should be acknowledged. First, the limited number of replicate pens may have reduced the statistical power to detect subtle biological responses, particularly for variables exhibiting considerable biological variation. Consequently, non-significant findings should not be interpreted as definitive evidence of the absence of biological effects. Second, the study evaluated only a single dietary inclusion level and one *in ovo* concentration of L-arginine, precluding assessment of dose-response relationships. Third, the study did not investigate physiological and molecular biomarkers, including nitric oxide concentration, oxidative stress indicators, amino acid deposition within the egg, and molecular regulators of angiogenesis, intestinal development, and muscle growth. Consequently, the mechanistic basis underlying the observed reduction in late embryonic mortality remains unclear [36]. Future studies should incorporate larger sample sizes, multiple supplementation levels, and integrated physiological and molecular analyses to establish optimal supplemen-

tation strategies for indigenous chicken production systems.

Practical implications

From a practical perspective, maternal dietary L-arginine supplementation showed a potentially favorable response in late embryonic survival, as T2 exhibited no late embryonic mortality. However, this finding should be considered preliminary rather than sufficient evidence to establish dietary supplementation as a recommended nutritional strategy for Alopecurus Unhas 1 breeder chickens. The limited number of experimental replicates, absence of significant improvements in hatchability and post-hatch performance, and lack of dose-response evaluation restrict the practical interpretation of these findings. Furthermore, the higher late embryonic mortality observed with combined supplementation cannot be attributed exclusively to L-arginine exposure because injection-related effects were not independently controlled. Therefore, neither dietary supplementation alone nor combined maternal and *in ovo* supplementation can currently be recommended for routine application without further experimental validation.

This study provides valuable preliminary baseline information for developing nutritional strategies to improve embryonic survival in indigenous chicken breeds. However, further investigations involving larger sample sizes, multiple supplementation doses, appropriate injection controls, and evaluation under practical production conditions are required to optimize supplementation protocols and elucidate the physiological mechanisms responsible for the observed responses.

CONCLUSION

This study demonstrated that maternal dietary and *in ovo* L-arginine supplementation had limited effects on embryonic development, hatchability, post-hatch growth performance, intestinal morphology, and muscle histomorphology in Alopecurus Unhas 1 chickens. However, late embryonic mortality differed significantly among treatment groups ($p = 0.024$). Maternal dietary L-arginine supplementation alone (T2) resulted in the lowest late embryonic mortality ($0.00 \pm 0.00\%$), compared with the untreated control group (T0; $7.49 \pm 1.45\%$) and the combined supplementation group (T3; $15.61 \pm 9.27\%$). These findings indicate treatment-dependent differences in late embryonic mortality, although the overall statistical significance should be distinguished from the significance of individual pairwise comparisons.

Future studies should test different dietary inclusion levels, *in ovo* doses, and injection timings, using larger experimental populations and mechanistic evaluations, including nitric oxide metabolism, oxidative stress biomarkers, angiogenic responses, amino acid deposition, and molecular pathways regulating embryonic development and muscle growth. Further investigations should also incorporate appropriate injection controls and factorial statistical analyses to distinguish the independent and interactive effects of maternal dietary and *in ovo* supplementation. These investigations will help develop optimized supplementation protocols for Alopecurus Unhas 1 chickens before their potential application to broader indigenous poultry production systems.

In conclusion, maternal dietary L-arginine supplementation at 0.25% was associated with the lowest observed late embryonic mortality, whereas its combination with a 0.5 mL *in ovo* injection of 2% L-arginine on day 18 of incubation did not demonstrate additional benefits. These findings should be considered preliminary and specific to the supplementation levels, injection timing, experimental population, and management conditions evaluated in this study. Further validation using larger experimental populations and different supplementation protocols is necessary before these results can be generalized to other indigenous chicken strains or commercial poultry production systems.

DATA AVAILABILITY

The supplementary data are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

No generative AI tools were employed for data analysis, data interpretation, or the generation of original scientific content. During manuscript preparation, AI assistance was limited to English grammar refinement and language clarity. All scientific content, interpretations, and conclusions were reviewed and approved by the authors.

AUTHORS' CONTRIBUTIONS

WP, MIAD, and SP: Conceptualization, methodology, and study design. ASI and IM: Investigation and data

curation. AMD and MIAD: Software development, data visualization, and validation. WP, ASI, IM, and AMD: Formal analysis and writing of the original draft. All authors reviewed, revised, read, and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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