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Improving hatching performance and chick quality in egg-type layers by modulating the incubation temperature

Izboljšanje valilnih rezultatov in kakovosti piščancev pri nesnicah lahkega tipa z uravnavanjem temperature med valjenjem

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ABSTRACT

This study investigated the effects of variations in incubation temperature on hatchability and early post-hatch performance in Prelux-G laying hens. During the first 18 days of incubation, eggs were exposed to one of five temperature regimes: Group A (37.5 °C for six days, then 37.0 °C), Group B (37.5 °C followed by 38.0 °C), Group C (37.0 °C followed by 37.5 °C), Group D (38.0 °C followed by 37.5 °C) and Control Group K (constant 37.5 °C). In the last three days of incubation, all groups were kept at a uniform temperature of 37.2 °C. A total of 3040 eggs were used, and incubation parameters were evaluated with both individually marked and group-monitored eggs. Lower incubation temperatures (groups A and C) resulted in significantly higher egg weight loss ($P<0.001$), with group A experiencing the greatest loss. Group A also had the longest incubation period, while group D had the shortest ($P<0.001$). The chicks in groups B and D had a significantly higher body weight at hatching ($P<0.001$). Longer egg storage (eight to nine days) had a negative effect on hatchability ($P<0.05$), with the lowest hatch rates observed after eight days. Notably, initial exposure to 38.0 °C (Group D) partially counteracted this decline, improving hatchability to 79.2%. Embryonic mortality and sex ratio were not significantly affected ($P>0.05$). Although differences in body temperature and chick length were already observed on the first day ($P<0.05$), these did not persist after days 12 and 27. Early thermal manipulation, especially with initially higher temperatures, can improve hatchability and chick quality in commercial hatcheries.

Keywords: incubation temperature, hatchability, chick development, egg storage, embryonic metabolism, poultry production

IZVLEČEK

V tej študiji je bil preučen vpliv različnih temperatur med valjenjem na valilnost in rast piščancev po izvalitvi pri komercialnih nesnicah Prelux-G. V prvih 18. dneh valjenja so bila jajca izpostavljena enemu izmed petih temperaturnih režimov: v skupini A 37,5 °C prvih šest dni, nato 37,0 °C; v skupini B 37,5 °C, nato 38,0 °C; v skupini C 37,0 °C, nato 37,5 °C; v skupini D 38,0 °C, nato 37,5 °C; v kontrolni skupini K pa stalno 37,5 °C. V zadnjih treh dneh valjenja so bila jajca v vseh skupinah izpostavljena enotni temperaturi 37,2 °C. Skupno je bilo uporabljenih 3040 jajc, parametri valjenja pa so bili spremljani pri individualno in skupinsko označenih jajcih. Nižje temperature valjenja (skupini A in C) so povzročile večjo izgubo mase jajc ($P<0,001$), največjo v skupini A. Najdaljše obdobje valjenja je bilo ugotovljeno v skupini A, najkrajše pa v skupini D ($P<0,001$). Višja telesna masa ob izvalitvi je bila izmerjena pri piščancih iz skupin B in D ($P<0,001$). Daljše skladiščenje jajc (osem do devet dni) je negativno vplivalo na valilnost ($P<0,05$), ki je bila najnižja po osmih dneh. Začetna izpostavljenost temperaturi 38,0 °C (skupina D) je ta upad deloma omilila in izboljšala valilnost na 79,2%. Temperatura med valjenjem ni statistično značilno vplivala na embrionalni pogin in razmerje med spoloma

(*P*>0,05). Čeprav so bile razlike v telesni temperaturi in dolžini piščancev prisotne prvi dan (*P*<0,05), teh po 12. in 27. dnevu ni bilo več. Rezultati kažejo, da lahko zgodnja izpostavljenost višji temperaturi izboljša valilnost in kakovost piščancev, kar je pomembno za komercialne valilnice.

Ključne besede: temperatura valjenja, valilnost, razvoj piščancev, skladiščenje jajc, embrionalna presnova, perutninska prireja

INTRODUCTION

Incubation temperature plays a crucial role in the embryonic development of chickens and has a direct influence on hatchability, chick quality and post-hatch performance. Although the optimal incubation temperature for chicken eggs is around 37.5 °C, even small deviations within the generally accepted range of 37.0 °C to 38.0 °C can have a significant impact on metabolic activity and developmental outcomes (Yalcin et al., 2022). Suboptimal thermal conditions during incubation have been associated with altered hatchability, impaired thermoregulation and reduced chick viability (Joseph et al., 2006). New evidence suggests that even minor thermal manipulations during critical windows of embryogenesis may have long-lasting effects on metabolism, growth trajectory and thermoregulatory function (Al Amaz and Mishra, 2024). Temperature-induced shifts in embryonic metabolic rate have been associated with changes in hatching time, chick weight and post-hatch performance (Noiva et al., 2014). However, the literature on this topic is contradictory. Some studies report positive effects of increased incubation temperatures on early development and growth (Tzschentke and Halle, 2009), while others point to potential drawbacks, including increased embryonic mortality and developmental abnormalities (Yalcin et al., 2022), highlighting the need for further systematic investigations. Importantly, most studies on thermal manipulation during incubation have been conducted on broilers (meat type chickens), while relatively few studies have focused on layer breeds (egg-type chickens). Given the potential for breed-specific physiological responses, it is critical to evaluate the effects of variations in incubation temperature in layer lines to ensure the applicability of incubation protocols in all poultry production systems.

In addition to the influence of incubation temperature on embryonic development, the culling of male day-old chicks remains a major challenge in modern laying hen husbandry. In recent years, considerable progress has been made in sexing technologies. Optical and spectral techniques such as near-infrared spectroscopy (Schreuder et al., 2024) and hyperspectral imaging combined with machine learning (Ji et al., 2024; Ahmed et al., 2025) have achieved high accuracy even before incubation or in the earliest stages of embryogenesis. Promising results have also been reported for the imaging assessment of embryonic morphology using computer vision techniques (Zhang and Jacobs, 2025). In addition to optical methods, molecular diagnostic methods such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) (Van Der Hofstadt et al., 2025) enable rapid and highly specific sex identification. Importantly, several of these methods are already in industrial use (Associated Press, 2024). Although the present study did not directly address sex ratio manipulation, it provides valuable insights into how incubation conditions affect embryonic development and chick quality, both of which are critical for the successful implementation and continuous improvement of in-ovo sexing technologies.

The present study, therefore, aims to investigate how incubation temperatures at the lower (37.0 °C) and upper (38.0 °C) limits of the optimal range, applied during different periods of the setter phase, affect hatchability, chick weight, body temperature at hatch, incubation duration, sex ratio and post-hatch growth in a layer breed. A notable feature of this study is the use of a dual tracking method that includes both individually and group-marked eggs to improve the accuracy and reliability of the data. In addition, this study investigates whether early

exposure of embryos to elevated temperature (38.0 °C) can counteract the detrimental effects of prolonged egg storage - an increasingly relevant problem in commercial hatcheries. By integrating these components, the study provides valuable insights for refining incubation strategies to support chick viability and optimize production efficiency.

MATERIALS AND METHODS

Ethics statement

This study was reviewed by the Institutional Animal Ethics Committee of the Department of Animal Sciences, Biotechnical Faculty, University of Ljubljana. The committee confirmed that the experimental design complies with Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament and Council of the European Union, 2010) and found that the procedures performed within a standard production cycle are categorised as non-experimental agricultural practises (Chapter I, Article 1, point 5). Therefore, no formal ethics approval was required for the present research.

Study design and experimental procedure

The experiment was conducted with hatching eggs from a Slovenian commercial egg-type hybrid, Prelux-G, in which the sex can be determined at hatching based on feathering speed. The Prelux-G hybrid is a two-way cross developed at the Poultry Educational and Research Centre of the Department of Animal Sciences of the Biotechnical Faculty of the University of Ljubljana, by mating roosters of the Slovenian Barred hen with hens of the Slovenian Brown hen. Both parent breeds were systematically selected for more than 50 years, with the main focus on egg production, egg weight, and shell colour. The use of this hybrid was appropriate as it represents a locally important laying hen genotype with well-documented performance characteristics and is routinely used in regional poultry production. The parent flock from which the hatching eggs for the Prelux-G layers came was 28 weeks old at the start of egg collection. Fresh, clean eggs were collected from the breeding flock, maintained under a lighting programme (14L:10D), with the light switched on early in the morning, to stimulate egg laying predominantly in the morning hours. Eggs were collected three times a day for eight consecutive days at approximately 08:00, 10:30, and 13:30. The breeder farms supplying the hatching eggs were located approximately 7 km from the hatchery, and the eggs were transported daily in a combined vehicle equipped with a refrigerated chamber. Upon arrival at the hatchery, the eggs were taken to an egg grading facility, where they were weighed, and only medium-sized eggs (size M, 53 – 63 g) were selected for the study. The eggs were then stored under controlled conditions at 15.0 °C and 75% RH (relative humidity) until incubation. During the storage period, the eggs were turned once a day. After nine days of storage, a total of 3040 eggs were randomly assigned to five treatment groups. To control for the effects of pre-incubation egg age, each group contained 76 eggs from each of the eight collection days (608 eggs per group). Of these, 264 eggs per group were individually labelled, weighed and placed in the incubators, while the remaining 344 eggs per group were weighed in groups and tracked together.

Incubation conditions and temperature regime

The incubation process was carried out using three S168 setters and one H168 hatcher (Petersime, Zulte, Belgium) over a total period of 21 days, consisting of an 18-day setter phase followed by a 3-day hatcher phase. During the setter phase, the RH was kept at 60%, and the eggs were automatically turned at an angle of 45° every hour. Three hours after the start of incubation, once the target temperatures had stabilized, all eggs were fumigated with formaldehyde (21 g potassium permanganate and 43 ml formalin per m^3) according to a standardized protocol. Five experimental groups were subjected to different temperature regimes during the setter phase. For the hatcher phase (days 18–21), all groups were transferred to a common H168 hatcher set at 37.2 °C and 70% RH. Table 1 gives an overview of the temperature profiles to which the individual groups were exposed during the incubation period.

Table 1. Temperature conditions during the setter (days 1–18) and hatcher (days 18–21) phases

Group	Days 1–6 (setter phase)	Days 7–18 (setter phase)	Days 18–21 (hatcher phase)	Notes
K (Control)	37.5 °C	37.5 °C	37.2 °C	Constant temperature throughout the setter phase
A (Lower final T)	37.5 °C	37.0 °C	37.2 °C	The temperature lowered after day 6
B (Higher final T)	37.5 °C	38.0 °C	37.2 °C	The temperature increased after day 6
C (Lower starting T)	37.0 °C	37.5 °C	37.2 °C	Low starting temperature, then increased
D (Higher starting T)	38.0 °C	37.5 °C	37.2 °C	High starting temperature, then lowered

Observations on the transfer of the eggs and the hatching of the chicks

On the 18th day of incubation, the 264 individually marked eggs per group were weighed again and placed in individual wire trays for hatching. The group-marked eggs (344 per group) were weighed together by treatment and age of the eggs before incubation and placed in separate hatching trays. Hatching of chicks was first monitored 469.5 hours after the incubator reached the set temperature, and then hatching was recorded at 12-hour intervals. Hatching times were recorded at 481.5, 493.5, 505.5, 517.5, 529.5 and 541.5 hours. All individually hatched chicks were weighed, sexed according to the degree of feathering (early or late feathering) and their hatching times recorded. The chicks hatched in groups were sexed and counted according to the treatment and the age of the eggs before incubation, and the average weight was calculated separately for males and females. At the final hatching time (541.5 hours), body temperature and body length were measured in 20 randomly selected male and 20 female chicks from each treatment. The unhatched eggs were opened to determine fertility and embryonic mortality stage. Clear eggs are classified as infertile or contain embryos that have not started to develop. Confirmation was made by analyzing the breakout of the eggs, where only the yolk and albumen were present, with no visible signs of embryonic structures. Early embryonic death is defined as mortality occurring within the first 0–7 days of incubation. On examination, a small or degenerated embryo is usually observed, often accompanied by an intact yolk and partially developed extraembryonic membranes. Late embryonic death is thought to occur in the second half of the incubation period, usually shortly before the expected hatching date. The diagnosis was made by opening the egg and revealing a fully developed embryo or chick, often with an unabsorbed yolk sac or other developmental anomalies.

Monitoring growth after hatching

One day after hatching, 240 female chicks from each treatment group (1200 in total) were transferred to a floor rearing facility. Each group was divided into two replicates of 120 chicks housed in separate compartments (10 in total). Only female chicks were reared, as is common in commercial egg production. All compartments were equipped with heat sources, automatic feeders, and hand drinkers. The chicks were fed a complete commercial starter feed developed for laying-type chickens (Jata Emona, Ljubljana, Slovenia). According to the manufacturer, the feed contained 20.5% crude protein, 4.5% crude fat, 4.0% crude fibre, and 6.0% crude ash, with a metabolisable energy content of 11.3 MJ/kg. The premix contained calcium (1.0%), phosphorus (0.7%), sodium (0.2%), lysine (1.2%), methionine + cysteine (0.9%), threonine (0.7%), vitamin A (10.000 IU/kg), vitamin D_3 (2,000 IU/kg), vitamin E (20 mg/kg), and trace elements (Mn, Zn, Fe, Cu, Se, I), in accordance with the EU feeding standards for layer-type chickens. The temperature in the rearing facility was set at 33 °C in the first week and then gradually lowered by around 2–3 °C per week until it reached 22 °C at the end of the experiment. The RH was kept between 60–70%. A lighting programme of

23L:1D was used for the first week, after which the photoperiod was gradually reduced to 16L:8D by day 21 and maintained until day 27. On days 12 and 27 post-hatching, 10 chicks per compartment (20 per treatment) were randomly selected, and body weight, body length and body temperature were measured to assess early growth performance.

Statistical analyzes

All data analyses were performed using SAS/STAT software (SAS Institute, 2016). The effects of treatment (K, A, B, C, D) and age of eggs before incubation (two to nine days) within treatment on egg weight loss, hatching time and chick weight at hatching were assessed using ANOVA based on the following model:

$$y_{ijk} = \mu + T_i + S_{ij} + e_{ijk}$$

where y_{ijk} is the trait of interest; μ is the overall mean; T_i is the fixed effect of temperature treatment; S_{ij} is the effect of the age of the eggs before incubation within treatment; and e_{ijk} is the residual error. The distribution of sexes across hatching intervals was analyzed using the chi-square test via the SAS procedure FREQ. Chi-square analysis was also applied to assess differences in fertility and early/late embryonic mortality between treatments. To evaluate the effects of treatment on chick sex ratio, multivariate logistic regression was performed using the LOGISTIC procedure. The GLM procedure was used to analyze the effects of treatment and sex on chick body weight, temperature and length at hatching with the model:

$$y_{ijk} = \mu + T_i + S_j + e_{ijk}$$

where y_{ijk} is the trait of interest, T_i is the effect of temperature treatment, S_j is the effect of sex, and e_{ijk} is the residual error. Growth traits at days 12 and 27 post-hatching were also analyzed using GLM, with the model:

$$y_{ij} = \mu + T_i + e_{ij}$$

where y_{ij} is body weight, body temperature or body length; μ is the overall mean; T_i is the treatment effect; and e_{ij} is the residual error.

RESULTS

Effects of the treatment on the incubation parameters

The application of different thermal incubation treatments significantly affected four incubation parameters evaluated: egg weight at transfer (P<0.001), relative egg weight loss during the setter phase (P<0.001 for group-marked eggs), incubation duration (P<0.001) and relative chick weight at hatching (P=0.027; P<0.001) (Table 2). For individually marked eggs, treatment D yielded the highest egg weight at transfer (51.20 g), which was significantly higher than that of treatments A (50.63 g) and C (50.76 g), while treatments B (51.09 g) and K (51.02 g) yielded intermediate values. A similar pattern was observed in group-marked eggs, with treatment D (51.25 g) yielding significantly heavier eggs at transfer than treatment A (50.37 g), while treatments B (51.19 g) and K (51.06 g) were not significantly different (P>0.05) from treatment D (Table 2). The relative weight loss of eggs up to day 18 of incubation did not differ significantly between treatments for individually marked eggs (P=0.112). The mean values ranged from 9.87% (treatment D) to 10.87% (treatment A), but the differences were not statistically significant. However, in the group-labelled eggs, the effect of treatment was significant (P<0.001): The greatest relative weight loss was observed in treatment A (10.83%), intermediate values in treatments C (10.23%) and K (9.61%), and the least in treatments B (9.38%) and D (9.29%) (Table 2). The incubation period of the individually marked eggs was longest in treatment A (512.99 h) and was significantly (P<0.01) longer than that of all other treatments. The shortest incubation times were found for treatments B (499.11 h) and D (497.62 h), while treatments C (507.95 h) and K (506.08 h) had intermediate values. For group-marked eggs, treatment A (509.41 h) also resulted in a significantly (P<0.001) longer incubation period than treatments B (496.72 h) and D (496.62 h). Treatments K (504.57 h) and C (506.53 h) had intermediate durations (Table 2).

Table 2. Effect of treatment on five incubation parameters

Incubation parameters	Treatment	Individually marked eggs (LSM ± SE)	Group - marked eggs (LSM ± SE)
Weight of eggs at transfer (g) (P<0.001)	A	50.63 ± 0.072^{a}	50.37 ± 0.11^{a}
	B	51.09 ± 0.071^{c}	51.19 ± 0.11^{bc}
	C	50.76 ± 0.073^{ab}	50.72 ± 0.11^{ab}
	D	51.20 ± 0.086^{c}	51.25 ± 0.11^{c}
	K	51.02 ± 0.072^{bc}	51.06 ± 0.11^{bc}
Egg weight loss during setter phase (%) Individually marked: (P=0,112) Group marked: (P<0.001)	A	10.87 ± 0.294^{a}	10.83 ± 0.036^{a}
	B	10.06 ± 0.288^{a}	9.38 ± 0.035^{b}
	C	10.64 ± 0,296^{a}	10.23 ± 0.036^{c}
	D	9,87 ± 0.290^{a}	9.29 ± 0.037^{b}
	K	10.19 ± 0.296^{a}	9.61 ± 0.036^{d}
Incubation duration (hours) (P<0.001)	A	512.99 ± 0.517^{c}	509.41 ± 1.02^{a}
	B	499.11 ± 0.507^{a}	496.72 ± 1.00^{bc}
	C	507.95 ± 0.522^{b}	506.53 ± 1.01^{ad}
	D	497.62 ± 0.618^{a}	496.62 ± 1.01^{b}
	K	506.08 ± 0.519^{b}	504.57 ± 1.01^{cd}
Weight of the hatched chicks (%)[1] Individually marked: (P=0,027) Group marked: (P<0.001)	A	80.19 ± 0.150^{a}	80.31 ± 0.118^{abc}
	B	80.72 ± 0.148^{b}	80.13 ± 0.116^{a}
	C	80.02 ± 0.152^{a}	80.15 ± 0.117^{ab}
	D	80.31 ± 0.149^{ab}	80.70 ± 0.119^{c}
	K	80.32 ± 0.151^{ab}	80.59 ± 0.117^{bc}
Weight of the hatched chicks (%)[2] (P<0.001)	A	71.47 ± 0.160^{a}	71.61 ± 0.109^{a}
	B	72.59 ± 0.157^{b}	72.61 ± 0.107^{b}
	C	71.50 ± 0.162^{ac}	71.96 ± 0.108^{a}
	D	72.38 ± 0.158^{bd}	73.22 ± 0.111^{c}
	K	72.14 ± 0.161^{bc}	72.84 ± 0.108^{b}

[1] Chick weight expressed as % of the egg weight at transfer – day 18

[2] Chick weight expressed as % of the egg weight at setting – day 0

LSM = Least Squares Mean; SE = Standard Error

[a, b, c] Values within the same column for each incubation parameter with different superscripts indicate significant statistical differences at P≤0.05

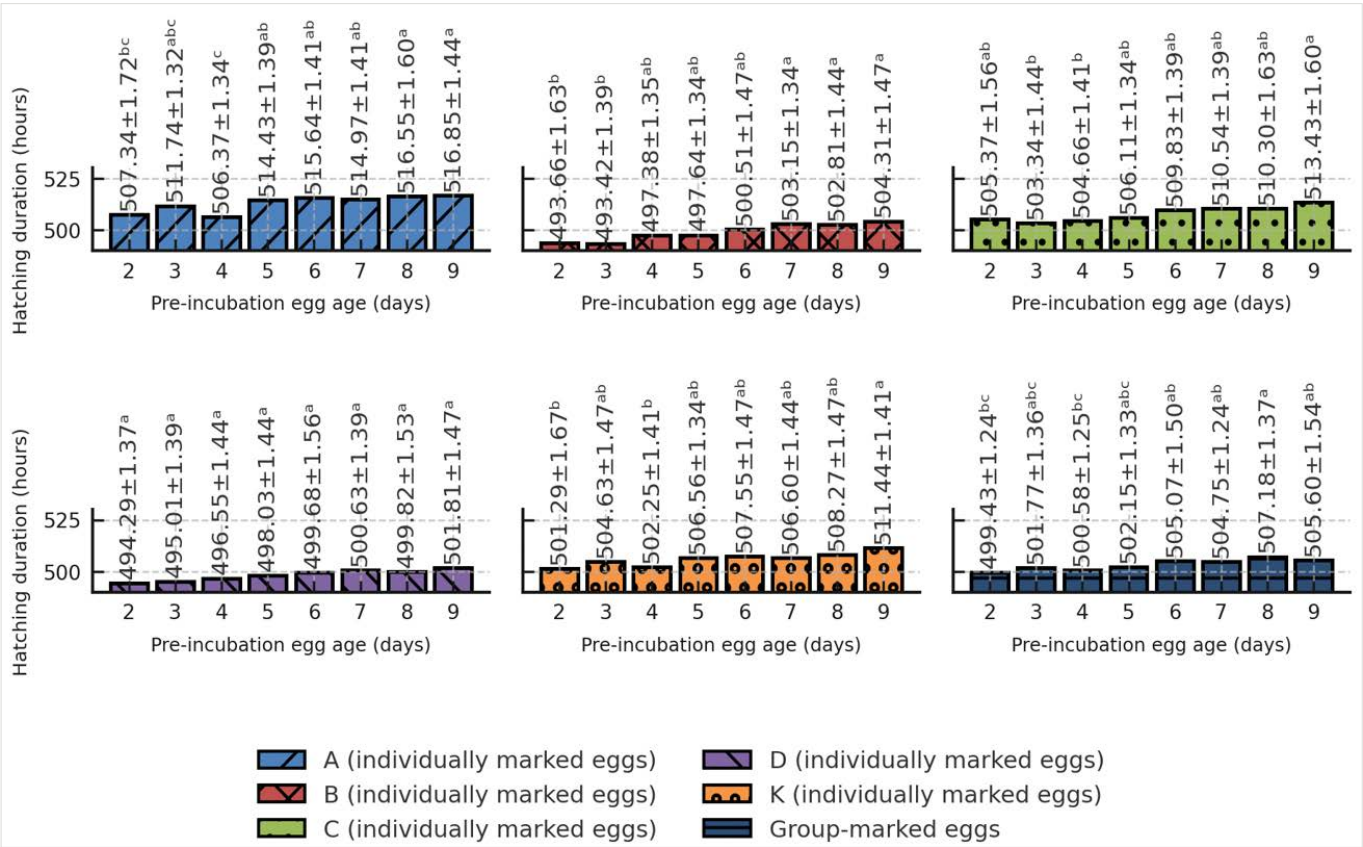
The relative weight of chicks at hatching (expressed as % of egg weight at transfer, day 18) was significantly higher in the individually marked eggs of treatments B (80.72%) and D (80.31%) than in treatments A (80.19%) and C (80.02%), with treatment K (80.32%) having an intermediate value. For the group-marked eggs, the highest relative chick weight was obtained in treatment D (80.70%), which was significantly higher than treatment

B (80.13%) and C (80.15%) (Table 2). When chick weight was expressed in relation to egg weight at setting (day 0), a similar pattern emerged, with the highest values being achieved in treatments B (72.59%) and D (72.38%) for individually marked eggs, and in treatment D (73.22%) ($P<0.001$) for group-marked eggs.

Effect of the age of the eggs before incubation on the incubation duration and hatchability

The effect of the age of the eggs before incubation on the incubation duration was very different in the different treatments (Figure 1). In treatment A, individually marked eggs aged four days had a significantly shorter incubation time (506.37 h) than nine-day-old eggs (516.85 h). Similarly, in group-marked eggs, four-day-old eggs (500.58 h) hatched significantly earlier than eight-day-old eggs (507.18 h) (Figure 1). A similar trend was observed for two-day-old individually marked eggs (507.34 h) compared to nine-day-old eggs. For treatment B, the shortest incubation period was observed for eggs aged three (493.42 h) and two days (493.66 h), significantly shorter than for eggs aged nine (504.31 h), eight (502.81 h) and seven days (503.15 h). Treatment C showed the longest incubation period in nine-day-old eggs (513.43 h), which differed significantly from eggs aged four (504.66 h) and three days (503.34 h).

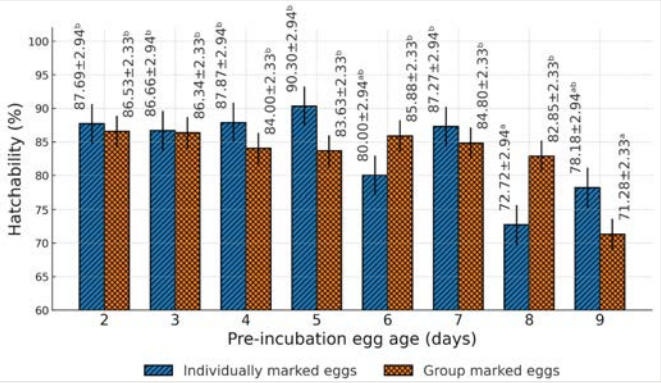
Within treatment D, no statistically significant differences ($P>0.05$) were found in the incubation duration between the different pre-incubation egg ages. In contrast, treatment K showed shorter incubation times for four-day-old (502.25 h) and two-day-old (501.29 h) eggs compared to the nine-day-old eggs (511.44 h) (Figure 1). These results consistently show a decrease in incubation duration with decreasing age of eggs prior to incubation, which is particularly evident in treatments A, B, C and K.



([a, b, c] Least-squares means within the same treatment, marked with different superscript letters, are statistically different at $P<0.05$)

Figure 1. Effect of the age of eggs before incubation within the treatment on the hatching time of individually and group-marked eggs

Although there was no significant overall effect of treatment on hatchability ($P>0.05$), the age of the eggs prior to incubation significantly ($P<0.01$) affected hatchability in all treatments (Figure 2). For individually marked eggs, the hatchability rate was significantly lower for eight-day-old eggs (72.72%) than for eggs aged two to seven days (between 86.66% and 90.30%). A similar pattern was seen in the group-marked eggs, where the hatchability was significantly lower in nine-day-old eggs (71.28%) compared to two- to eight-day-old eggs (between 82.85% and 86.53%). In the individually marked eggs, hatchability was significantly lower in eight-day-old eggs (Figure 2).



([a, b] values within individually marked eggs and within group-marked eggs marked with different superscript letters are statistically different at $P<0.05$)

Figure 2. Effect of the age of the eggs before incubation on hatchability

Effect of treatment on clear eggs and embryo mortality

Treatment had no statistically significant effect on the number of clear eggs or the incidence of embryonic mortality, either in individually marked ($\chi^2=7.1280$, $P=0.8490$) or group-marked eggs ($\chi^2=8.6091$, $P=0.7359$) (Figure 3). Among the individually marked eggs, the highest percentage of clear eggs was found in treatment D (68.00%) and the lowest in treatment A (51.35%). Early embryonic mortality ranged from 4.55% (treatment C) to 10.81% (treatment A), and late embryonic mortality ranged from 22.00% (treatment D) to 40.91% (treatment C). For group-marked eggs, the percentage of clear eggs was highest in treatment B (59.32%) and lowest in treatment C (41.27%). Early embryonic mortality ranged from 6.35% (treatment K) to 17.14% (treatment A), while late embryonic mortality ranged from 27.12% (treatment B) to 42.86% (treatment C) (Figure 3).

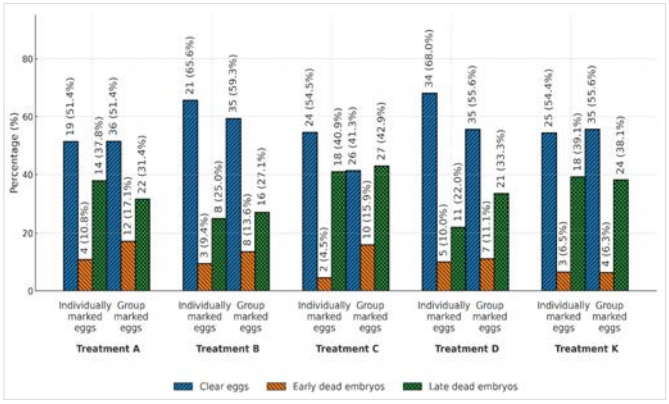


Figure 3. Effect of treatment on the number and percentage of clear eggs and embryonic mortality

Relationship between treatment and sex ratio of chicks

No statistically significant effects of treatment, age of eggs before incubation or their interaction on chick sex ratio were found in either individually marked ($\chi^2=6.405$, $P=0.1708$) or group-marked eggs ($\chi^2=1.551$, $P=0.8176$) (Figure 4). For individually marked eggs, the sex ratio ranged from 45.05% males (treatment B) to 56.62% males (treatment D). For group-marked eggs, the proportion of males ranged from 48.91% (treatment D) to 52.66% (treatment C) (Figure 4).

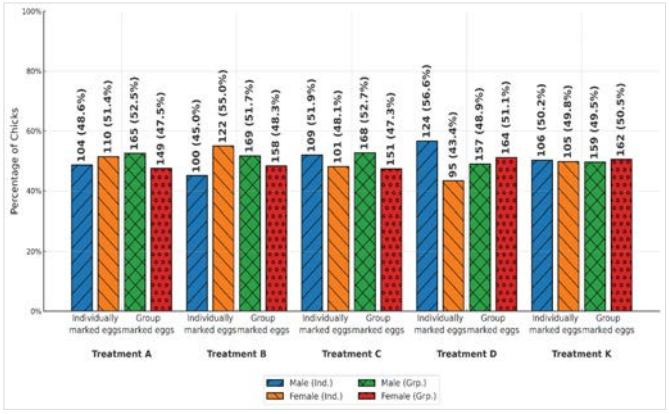


Figure 4. Number and proportion of chicks by sex in all treatments and differently marked eggs

Multivariate logistic regression confirmed the absence of significant effects: Treatment (Wald $\chi^2=5.6201$, $P=0.2294$), age of eggs before incubation (Wald $\chi^2=5.6061$, $P=0.6913$) and their interaction (Wald

χ^2=25.5455, *P*=0.5980) were not significant for individually marked eggs. The same pattern was true for group-marked eggs (treatment: Wald χ^2=3.0319, *P*=0.5525; egg age: Wald χ^2=6.0173, *P*=0.6453; interaction: Wald χ^2=25.4748, *P*=0.7864).

Physical characteristics of chicks and pullets

The rearing period was limited to 27 days in order to capture the early growth phase after hatching, when the influence of incubation temperature is still recognisable before environmental and nutritional factors begin to dominate the growth processes. Several studies have shown that the long-term effects of thermal manipulation of the embryo diminish after the first four weeks post-hatching, when chicks develop their full thermoregulatory capacity (Nord and Giroud, 2020; DuRant et al., 2012). Therefore, the assessment up to day 27 represented a relevant time frame for the evaluation of the biological transfer effects of the incubation treatments. The treatment had a significant effect (*P*<0.05) on the body temperature and body length of the day-old chicks, while no differences were observed at 12 and 27 days of age (Table 3). Day-old chicks in treatment A had the highest body temperature (40.28 °C), which was significantly higher than that of treatment B (39.90 °C), D (40.06 °C) and the control group K (40.08 °C). The initial body weight of the female chicks ranged from 40.47 g (treatment A) to 41.34 g (treatment B), but no significant differences (*P*>0.05) were observed between treatments (Table 3). Total body weight gain from day 1 to day 27 also did not differ significantly (*P*>0.05) and ranged from 263.41 g (treatment B) to 292.96 g (treatment D).

Treatment C (40.21 °C) was not significantly different from A, but was significantly higher than B. Body length was highest in treatment B (18.29 cm), significantly longer than in treatment A (17.82 cm) and C (17.94 cm). Treatment D (18.20 cm) also resulted in significantly longer chicks compared to A and C, while the control group K (18.11 cm) had intermediate values. At 12 days of age, no significant differences (*P*>0.05) were found between treatments in terms of relative body weight (283.37-288.48%), body length (26.97–27.20 cm) or body temperature (40.83–41.10 °C). Relative body weight (638.82–716.61%), body length (36.97–37.76 cm), and body temperature (41.59–41.65 °C) also did not differ significantly (*P*>0.05) between treatments after 27 days. However, a significant increase in mean body temperature was observed between 12 and 27 days (41.00 °C vs. 41.63 °C; *P*<0.001). Mortality was recorded throughout the rearing period. Overall mortality was low up to day 27 and was within the expected range for layer-type chicks (approximately 2–3%). Cumulative mortality rates after treatment were as follows: Group K, 2.1%; Group A, 2.1%; Group B, 2.5%; Group C, 1.8%; and Group D, 3.0%.Fisher's exact test revealed no statistically significant differences between the groups (*P*>0.05).

Table 3. Effect of the different treatments on the physical properties of the Prelux-G pullets at different ages

Treatment	Day 1 (LSM)			Day 12 (LSM)			Day 27 (LSM)			Gain (1-27) (g)
	BW (g)	BL (cm)	BT (°C)	RBW (%)	BL (cm)	BT (°C)	RBW (%)	BL (cm)	BT (°C)	
A	40.47^{a}	17.82^{a}	40.28^{a}	288.48^{a}	26.97^{a}	40.83^{a}	666.35^{a}	37.31^{a}	41.65^{a}	269.28^{a}
B	41.34^{a}	18.29^{c}	39.90^{c}	284.04^{a}	27.20^{a}	41.10^{a}	638.82^{a}	36.97^{a}	41.65^{a}	263.41^{a}
C	40.73^{a}	17.94^{ab}	40.21^{ab}	285.67^{a}	27.20^{a}	41.10^{a}	704.40^{a}	37.76^{a}	41.65^{a}	285.77^{a}
D	41.04^{a}	18.20^{c}	40.06^{b}	283.37^{a}	27.07^{a}	40.97^{a}	716.61^{a}	37.68^{a}	41.60^{a}	292.96^{a}
K	41.06^{a}	18.11^{bc}	40.08^{b}	284.47^{a}	27.00^{a}	41.03^{a}	714.53^{a}	37.48^{a}	41.59^{a}	292.69^{a}

BT = body temperature; BL = body length; BW = body weight; RBW = body weight at a given age expressed as a percentage of body weight on day 1 (relative body weight).

LSM = Least Squares Mean.

^{a,b,c} Values within a column for each parameter marked with different superscript letters differ statistically at *P*≤0.05

DISCUSSION

Influence of the incubation temperature on the incubation parameters

The results of this study confirm that incubation temperature has a significant influence on the most important incubation parameters, including egg weight at transfer (day 18), egg weight loss, incubation duration and chick weight at hatching. Egg weight loss is a key indicator of proper embryonic development as it allows for adequate formation of the air cells required for lung respiration (Boerjan, 2012). The presentation of the weight loss of the eggs as relative values provides additional information on the effects of the incubation methods. For individually marked eggs, relative weight loss did not differ significantly between treatments up to day 18, suggesting that individual egg variability may mask treatment effects. In contrast, significant differences were observed in group-marked eggs, with lower relative losses in treatments B and D suggesting more favourable incubation conditions for water balance and gas exchange. The higher water loss observed in groups A and C can primarily be attributed to the longer incubation period at lower temperatures. It is known that lower incubation temperatures slow down embryonic development (Noiva et al., 2014), prolonging incubation time and increasing cumulative water loss. In addition, lower metabolic water production under cooler conditions may have contributed to a net increase in water loss. Slower embryonic metabolism produces less metabolic water, which normally helps to compensate for evaporative loss (Noiva et al., 2014). Another possible, although less well-documented, factor could be temperature-induced changes in eggshell permeability. Prolonged exposure to lower temperatures could alter the microstructure of the shell pores and affect vapor diffusion rates; however, this hypothesis requires further investigation.

The incubation period was strongly influenced by the temperature regimes. Group B and D eggs, which were exposed to elevated temperatures during either late or early embryonic development, hatched significantly earlier than group A and C eggs. This is consistent with the findings that higher incubation temperatures accelerate cell proliferation and metabolic activity, thereby shortening the incubation period, while lower temperatures slow down these processes and prolong development (Noiva et al., 2014; Nideou et al., 2019). Of all treatments, group A had the longest incubation period, while group D had the shortest.

A strong correlation was found between egg weight at transfer and chick weight at hatching, which is consistent with previous research showing that heavier eggs tend to produce heavier chicks due to greater nutrient and energy reserves (Williams, 1994; Ramaphala and Mbajiorgu, 2013). Interestingly, chicks from groups B and D, both of which were exposed to elevated temperatures during certain developmental windows, were significantly heavier in terms of relative weights than those from groups A and C. This is likely due to optimized metabolic activity during critical developmental periods rather than differences in water loss. Slightly elevated temperatures may have improved energy utilization and nutrient uptake, thereby increasing growth efficiency (Yalcin et al., 2022). Although higher incubation temperatures are often associated with lower hatch weight, these results suggest that a controlled and well-timed increase in temperature can actually promote embryo growth. Lipid metabolism also plays a crucial role in energy supply during embryogenesis. It has been shown that embryos exposed to higher temperatures make greater use of lipid reserves, especially triacylglycerols, to generate energy (Li et al., 2024). This may lead to more efficient energy utilization, allowing for higher chick weights without metabolic exhaustion. In addition, accelerated embryonic development often leads to more complete yolk uptake prior to hatching, which may increase hatch weight, although this is not necessarily accompanied by increased tissue mass (Leksrisompong et al., 2007). Conversely, chicks in groups A and C were significantly lighter, suggesting that suboptimal thermal conditions may affect development by slowing metabolic rate and delaying tissue growth. Colder incubation conditions have also been associated with suppressed thyroid hormone activity, which may further reduce metabolic efficiency

and growth potential (Yalcin et al., 2022). Importantly, no significant differences in hatch weight were observed between the control group and the group exposed to slightly elevated temperatures, suggesting that a moderate increase in temperature can optimize development without negative effects, provided that water loss remains within acceptable limits. In addition, moderate heat exposure has been shown to stimulate muscle fiber formation and promote cardiovascular development, likely due to increased heart rate and oxygen consumption during incubation (Mortola and Labbe, 2005). Nevertheless, temperatures above 39 °C are consistently associated with increased embryonic mortality and developmental abnormalities, highlighting the need for precise thermoregulation throughout incubation (Masia et al., 2024). Overall, these results highlight the complex interplay between incubation temperature, metabolic regulation and embryonic growth and emphasize the importance of maintaining optimal thermal conditions to promote viability and development without causing physiological stress.

Effect of egg storage duration on hatching results

This study confirms that prolonged storage of eggs prior to incubation negatively affects both incubation duration and hatchability, which is consistent with previous findings showing a decrease in hatchability and an increase in incubation time with increasing storage time (González-Redondo et al., 2023; Tona et al., 2003). Eggs stored for nine days showed a significantly longer incubation time than those stored for only two days, especially in groups A, B and K. This delay is probably due to increased water and CO_2 loss during prolonged storage, which disrupts the biochemical environment required for optimal embryonic development. Interestingly, the incubation period in treatment D did not differ significantly between storage durations, suggesting that early thermal stimulation may mitigate the detrimental effects of prolonged storage. Significant differences were observed in incubation duration between treatments, with treatment B showing the greatest variability (10.89 hours), suggesting greater heterogeneity in embryonic development — possibly as a result of metabolic fluctuations or thermal inconsistencies. Comparable variability was observed in treatments A (10.48 hours), C (10.09 hours) and K (10.15 hours), while treatment D showed the least variation (7.52 hours), suggesting a more synchronized hatching process. The increased incubation temperature (38.0 °C) during the first six days in treatment D may have accelerated embryonic metabolism, increased CO_2 production and stabilized the internal biochemical environment, thereby reducing developmental differences (Decuypere and Michels, 1992). Early thermal stimulation is known to modulate the expression of heat shock proteins and metabolic enzymes, which increases embryo resilience and aids recovery from the stress associated with prolonged storage (Ncho et al., 2022).

Hatchability decreased significantly in eggs stored for eight or nine days compared to eggs stored for shorter periods, which is consistent with previous research showing that storage beyond seven days increases water and CO_2 loss, resulting in increased egg yolk pH and a suboptimal embryonic environment (Reijrink, 2010). This alkaline shift promotes oxidative stress, cellular damage and apoptosis, which ultimately impairs embryo viability. In addition, prolonged storage accelerates lipid oxidation in both the yolk and egg white, which has been linked to reduced hatchability due to the accumulation of cytotoxic peroxidation by-products (Romero et al., 2022). Recent evidence suggests that controlled humidity and CO_2 supply during egg storage may help to stabilize the pH of the albumen and preserve embryo integrity, potentially mitigating the adverse effects of prolonged storage (Reijrink, 2010). Overall, these results highlight the importance of minimizing storage time and exploring pre-incubation measures, such as early thermal stimulation or environmental control strategies, to maintain hatchability and uniformity of development.

Effect of incubation temperature on clear eggs and embryo mortality

The results of this study show that variations in incubation temperature during the setter phase had no significant effect on the number of clear eggs or embryonic

mortality. The lack of statistically significant differences between treatments is likely due to the relatively narrow range of temperature variation, which was within the optimal thermal window for embryonic development of the chickens. These results are consistent with previous research suggesting that moderate temperature fluctuations do not seriously affect embryogenesis or reduce hatchability, provided that conditions remain within physiologically acceptable limits (Al Almaz and Mishra, 2024). In addition, the standardized incubation conditions during the last three days of incubation may have played a crucial role in stabilizing embryo survival in all treatment groups. The last phase of incubation is a sensitive developmental period in which uniform heat and humidity conditions are crucial for successful lung transition and hatching. It is plausible that consistent conditions during this period mitigated any transient effects of earlier temperature differences. Previous studies have shown that short-term or mild temperature fluctuations can induce physiological adaptations in the embryo, but significant impairments in hatchability and viability generally only occur when incubation temperatures exceed critical thresholds, e.g. prolonged exposure above 40.0 °C or below 34.0 °C (Almaz and Mishra, 2024). Such extreme deviations are known to disrupt metabolic processes, impede cardiovascular development and impair oxygen exchange, leading to increased early and late embryonic mortality, developmental abnormalities and reduced post-hatching performance (Yalcin et al., 2022). In addition, embryo viability is affected by a variety of factors beyond incubation temperature, such as breeding flock nutrition, male to female ratio, environmental conditions, egg storage duration and handling method (King'ori, 2011). In this study, all eggs were from a single breeding flock kept under standardized housing conditions, minimizing potentially confounding effects associated with maternal or environmental variation. This supports the conclusion that the incubation temperatures tested in this experiment did not significantly affect embryonic survival or fertility.

Influence of incubation temperature on the sex ratio of chicks

Incubation conditions — particularly temperature — can influence the sex-specific survival of embryos, potentially shifting the sex ratio at hatching under extreme thermal conditions (Eiby et al., 2008). The results of this study show that incubation temperature in the range of 37.0 °C to 38.0 °C had no significant effect on the secondary sex ratio of chicks at hatching. Although slight differences in sex ratio were observed among the different temperature treatments, these differences were not statistically significant. The lack of significant sex ratio deviations may be attributed to the moderate temperature fluctuations that remained within the optimal physiological limits for chicken embryonic development. In addition, the application of uniform incubation conditions during the last three days (37.2 °C, 70% RH) may have alleviated earlier thermal stress and promoted synchronous development and balanced survival of the sexes. These results are consistent with research suggesting that moderate temperature fluctuations within standard incubation protocols do not significantly affect secondary sex ratios (Collins et al., 2013). In contrast, more extreme incubation temperatures — particularly those exceeding 39 °C or falling below 36 °C — have been associated with sex-specific embryonic mortality, likely due to differential thermosensitivity between male and female embryos and disruption of metabolic or developmental processes (Al Amaz and Mishra, 2024). Overall, the current results reinforce the conclusion that while incubation temperature under extreme conditions may affect embryo viability in a sex-dependent manner, moderate thermal variation within standard incubation parameters does not significantly affect the sex ratio.

Effects of incubation temperature on chick physiology and growth

This study has shown that variations in incubation temperature significantly affect certain physiological characteristics of day-old chicks, particularly body

temperature and body length. The chicks in treatment A, in which the temperature was lowered in the middle of the embryonic period (from 37.5 °C to 37.0 °C), had the highest body temperature after hatching. This may be attributed to metabolic compensatory mechanisms, including increased thyroid hormone activity, which may increase thermogenic capacity. In contrast, chicks in treatment B, incubated at a moderately elevated temperature (38.0 °C) from day 7 to 18, had the lowest post-hatching body temperatures. This supports previous findings that prolonged exposure to elevated temperatures during incubation can trigger metabolic programming leading to reduced endogenous heat production after hatching (Piestun et al., 2011).

Incubation temperature also influenced skeletal growth. Chicks from treatment B had the greatest body length, followed by those from treatment D, suggesting that elevated temperatures during certain developmental windows may increase metabolic activity and nutrient utilization, thereby promoting skeletal development and bone mineralization. Conversely, chicks in treatments A and C, where they were exposed to 37.0 °C during early or mid-embryonic development, had the shortest body lengths, likely due to delayed chondrogenesis and ossification associated with lower metabolic activity (Shim and Pesti, 2011; Sözcü et al., 2022).

A significant sex-specific difference was also observed in body temperature, with pullets showing higher temperatures than cockerels. This is consistent with previous findings suggesting that thyroid hormone activity may contribute to sex-specific differences in thermoregulation (Fernie and Marteinson, 2016). However, incubation treatments had no significant effect on relative body weight, temperature or length of pullets at 12 and 27 days of age, suggesting that the early thermal effects were transient and likely overshadowed by post-hatching environmental factors such as diet and environmental conditions. These observations are consistent with previous research showing that the physiological effects of incubation temperature diminish over time as chicks develop independent thermoregulatory capacity (Nord and Giroud, 2020; DuRant et al., 2012). A significant increase in body temperature was observed between day 12 and 27, which can be explained by age-related metabolic changes, improved thermoregulatory abilities and increased thyroid activity. In addition, the shortening of the photoperiod during this period may have contributed to metabolic adaptations, as shorter light exposure has been shown to affect circadian temperature rhythms and energy expenditure (Yu and Li, 2023; Wu et al., 2022). Overall, these results emphasize the complex interplay between incubation conditions and post-hatching environmental factors and highlight the remarkable physiological adaptability of chicks to moderate temperature fluctuations. The results of the present study showed that the treatments had only a limited effect on the growth performance of the Prelux-G chickens. Initial body weights were comparable between groups (40.47–41.34 g), indicating that randomisation was successful and that possible treatment effects could not be attributed to differences in initial conditions. In addition, body weight gains from day 1 to day 27 did not differ significantly (263.41–292.96 g), indicating that the treatments neither affected nor improved the growth potential of the chicks during the early rearing phase. This conclusion is also supported by the relative body weight data, which also showed no significant differences between treatments, with chicks in all groups reaching approximately 640–720% of their day 1 body weight by day 27. These results are consistent with previous studies reporting that short-term manipulations of incubation or early rearing conditions can affect certain physiological traits, such as body temperature or body length, but do not necessarily result in measurable differences in growth performance (Wijnen et al., 2020).

CONCLUSIONS

This study provides new insights into the effects of precise thermal manipulation during the setter phase of incubation on embryonic development, hatchability and early growth in a Prelux-G chicken hybrid. In contrast to most of the existing literature, which focuses mainly on broiler lines and individual temperature interventions, this study systematically investigated dynamic temperature shifts — both upward and downward — during defined developmental windows in a layer genotype. The application of a higher initial incubation temperature (38.0 °C for six days, followed by 37.5 °C) was found to be particularly effective, as it significantly improved hatchability, chick weight and post-hatching growth, while mitigating the negative effects of prolonged egg storage. These results are particularly important for commercial hatcheries that require longer egg storage due to logistical constraints. In addition, the dual methodology using both individually and group-marked eggs provides a robust level of data validation rarely used in incubation studies, increasing the reliability of the results. The lack of negative effects on embryonic mortality or sex ratio despite moderate thermal interventions highlights the potential for safe and targeted optimization of incubation protocols. This work is a contribution to the limited body of knowledge on thermal manipulations in layer breeds and demonstrates that a well-timed, moderate increase in incubation temperature can result in positive metabolic programming without compromising viability. By investigating both physiological and production-oriented parameters under controlled, yet industry-relevant conditions, this study provides a valuable framework for the development of more precise, breed-specific incubation strategies aimed at improving chick quality and performance in egg-type layers.

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