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Interactions between broiler parent stock age and egg pre-incubation duration: effects on embryo development, hatchability, day-old chick weight, and yolk sac weight

M. Gregrova [a], M. Lichovnikova [a,*], M. Foltyn [b], Z. Tvrdon [b], D. Hampel [c]

[a] Department of Animal Breeding, Mendel University in Brno, Zemedelska 1, 61300 Brno, Czech Republic
[b] Vykrm Trebic, s.r.o., Karlov 196, 28401 Kutna Hora, Czech Republic
[c] Department of Statistics and Operation Analysis, Mendel University in Brno, Zemedelska 1, 61300 Brno, Czech Republic

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ABSTRACT

Egg storage is a common practice in commercial hatcheries, but prolonged storage can negatively impact hatchability, causing a significant problem for the poultry industry. Repeated pre-incubation could mitigate the decline depending on the age of the parent stock. The study investigated the interactions between parent stock age (30, 45, and 58 weeks) and repeated pre-incubation on the hatchability and embryo development of 15-day stored eggs from Ross 308 parent stock. Three different pre-incubation durations were employed during storage: no pre-incubation and twice at the 5th and 10th days of egg storage for either 4 or 8 h. For each parent stock age, 3 600 eggs collected on the same day from the same parent stock were used for hatchability assessment. The duration of pre-incubation was determined as the eggshell temperature increased from 28 °C to a maximum of 35 °C and then cooled to 28 °C; the persistency at 35 °C was either 1 or 4 h for the total pre-incubation period. The hatchability of both set and fertile eggs and early, middle, and late embryonic mortality depended on the parent stock's age ($P < 0.001$). Pre-incubation alone did not have any impact on hatchability. Conversely, a significant interaction was observed between parent stock age and pre-incubation duration on the hatchability of fertile eggs ($P = 0.001$). At 30 weeks of parent stock age, both durations of pre-incubation positively influenced the hatchability. At 45 weeks of parent stock age, pre-incubation length had no effect on hatchability. At 58 weeks of parent stock age, a longer pre-incubation period, 2 × 8 h, was associated with decreased hatchability. Early embryonic mortality was not influenced by pre-incubation. However, a significant interaction was observed ($P = 0.003$). At 30 weeks of parent stock age, both short and long lengths of pre-incubation were associated with a decrease in early embryonic mortality. However, at 45 and 58 weeks, pre-incubation did not significantly affect early embryonic mortality. Additionally, longer pre-incubation periods significantly increased middle mortality compared to untreated eggs ($P = 0.035$). The median of embryo development for untreated eggs was the same across all ages (stage 10). In the older parent stock (45 and 58 weeks), repeated pre-incubation increased variability in embryo development, while in the younger parent stock, repeated pre-incubation decreased variability in embryo development. In conclusion, the duration and the frequency of pre-incubation should be specified based on the age of the parent stock.

Implications

Egg storage is a prevalent practice in hatcheries. A significant limitation of egg storage is reduced hatchability. Repeated pre-incubation, depending on the age of the parent stock, could mitigate the decline. A significant interaction between parent stock age and pre-incubation duration on the hatchability of 15 days of stored eggs we observed. Consequently, this method should be specifically designed for the specific age of the parent stock. Accurate implementation of pre-incubation duration can enhance the hatchability of chicks from stored eggs, contributing to improved economic efficiency of day-old chick production.

Introduction

Every fertile egg is expected to yield a healthy hatchling; however, achieving this optimal situation is challenging for stored eggs.

* Corresponding author.
E-mail address: martina.lichovnikova@mendelu.cz (M. Lichovnikova).

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Commercial hatcheries may need to store hatching eggs for various reasons, such as synchronising hatching times, adjusting production to market demand, or accumulating a sufficient quantity of parent stock hatching eggs (Fasenko, 2007; Gonzalez-Redondo, 2010). Nevertheless, storing hatching eggs adversely affects hatchability (Fasenko et al., 2001; Dymond et al., 2013; Okur et al., 2018). In a field study conducted by Yassin et al. (2008), an additional day of storage for broiler eggs up to 7 days reduced hatchability by an average of 0.2%, and from day 7–14, it further decreased by 0.5%. Embryos can only be stored within a specific developmental stage known as the "diapause developmental window" to ensure normal subsequent development (Pokhrel et al., 2021). Reijrink et al. (2009) proposed that there exists an optimal developmental stage or a range of stages for maintaining embryo viability during extended egg storage. According to Fasenko et al. (2001), embryos that have reached developmental stages XII or XIII according to **EG&K** (Eyal-Giladi and Kochav, 1976) exhibit greater resistance to prolonged egg storage than less developed or more advanced embryos.

Embryo development initiates following ovulation and fertilisation, progressing during egg formation within the hen's body for 24–26 h (Christensen et al., 2001). In an analysis of freshly laid blastomeres from Ross 308 eggs, Pokhrel et al. (2017) demonstrated that the total nucleus count increases with developmental stage, ranging from 60 000 at stage X EG&K to 130 000 at stage XIII EG&K. Additionally, staging embryos from young (32 weeks) and old (63 weeks) flocks indicated that blastoderms from older flocks were more advanced, with predominant embryonic stages being XI and XII EG&K in young and old flocks, respectively. In contrast, Özlü et al. (2021) reported embryo development in young (29 weeks) and old (58 weeks) parent stock at stage 10.27 in young and stage 11.1 EG&K in old stock, respectively. At both ages, the stage was lower than suggested for egg storage (Fasenko et al., 2001).

Consequently, two methods, prestorage incubation (warming hatching eggs before storage) and pre-incubation (known also as **SPIDES** - short periods of incubation during egg storage), have been proposed to mitigate the adverse effects of long-term storage on hatchability and enhance embryo development. However, considering differences in embryo development postoviposition (Pokhrel et al., 2017), these methods may have variable impacts on hatchability based on parent stock age (Damaziak et al., 2018) and the duration of heat treatment combined with parent stock age (Reijrink et al., 2009). Results obtained from applying both methods to eggs from different parental stock ages generate contradictory findings. Özlü et al. (2021) found no correlation between parent stock age (29 vs 58 weeks) and SPIDES but confirmed a positive effect of this method on hatchability. Conversely, Damaziak et al. (2018) reported no effect of SPIDES on the hatchability of eggs from parent stock aged 49–52 weeks but observed a positive effect at parent stock aged 70–73 weeks. In addition, Reijrink et al. (2009) observed a negative effect of prestorage heat treatment on hatchability at a parent stock age of 61 weeks but a positive effect at a parent stock age of 28 weeks.

Given these contradictory results, the objective of this study was to evaluate, within the same parent stock flock, the impact of three different parent stock ages and three pre-incubation durations on embryo development, hatchability, and the weights of day-old chicks and yolk sacs after a 15-day egg storage period at 14 °C. The goal was to cover the entire parent stock production period, utilising equipment designed for pre-incubation with an appropriate cooling system for pre-incubated eggs and maintaining a lower recommended temperature for egg storage. The tested hypothesis was that repeated pre-incubation significantly affects hatchability, day-old chick and yolk sac weights, and embryo development before egg setting to the setter in 15-day stored eggs regardless of the age of the parent stock flock.

Material and methods

Broiler parent stock management and performance

The research utilised hatching eggs sourced from Ross 308 parent stock. Birds were housed in commercial conditions within enclosed buildings, with litter covering the floor and approximately 1/3 of the area equipped with grates near the nests. Automatic nests, providing about 1 m^2 per 100 hens, were available. The stocking density in the hen house ranged from 6.4 to 6.8 birds per square meter. The lighting schedule consisted of 16 h of light and 8 h of darkness. Watering and feeding lines were automated. Birds were fed according to the recommendations of genetic material producers (Aviagen). At 30, 45, and 58 weeks of age, when eggs were collected for the experiment, the production parameters were as follows: laying intensity of 84.9, 69.9, and 55.0%, egg weight of 56.4, 65.0, and 69.0 g, daily female feed intake of 173.4, 154.4, and 167.2 g, female live BW of 3.68, 3.98, and 4.13 kg, daily male feed intake of 100.2, 125.8, and 142.9 g, male live BW of 4.12, 4.42, and 4.91 kg, and male-to-female ratio of 1:9, 1:10.1, and 1:10.6, respectively.

Study design

The study was conducted at the hatchery of Vykrm Trebic s.r.o. A total of 11 610 hatching eggs were utilised, originating from the same parent stocks of ages 30, 45, and 58 weeks. A total of 10 800 eggs were utilised for the hatchability test, and 810 were used for the analysis of embryo development.

At each parent stock age, 3 870 eggs were collected on the same day. Thirty eggs were used for embryo development analysis, and the remaining eggs were randomised among the treatments. Hatching eggs were divided into three experimental groups: a control group without pre-incubation comprising 1 200 eggs and two pre-incubated groups comprising 1 320 eggs, with 120 eggs from each pre-incubation used for embryo development stage analysis. The eggs were randomly arranged in 20 or 22 trays (control or pre-incubated eggs), with 60 eggs per tray, where one tray represented one replication. Each treatment had 20 replications for the hatchability test. Each treatment had its own trolley during storage, where all trays were placed together. Three different lengths of pre-incubation were applied during storage: none, twice on the 5th and 10th day of egg storage for 4 h, and twice on the 5th and 10th day of storage for 8 h. The effect of factors such as parent stock age, duration of pre-incubation, and interactions were evaluated.

No pre-incubation for stored eggs was chosen because many hatcheries store eggs without using pre-incubation. The study aimed to determine whether pre-incubation could improve the hatchability of stored eggs, and for this assessment, no pre-incubation was crucial. Repeated pre-incubation was chosen based on the recommendations of the producer of the Re-Store machine (Petersime, Belgium), who advises repeating pre-incubation at intervals of 5–7 days for longer-stored eggs. Pre-incubation durations of 4 and 8 h were selected because the experiment aimed to observe the effect of repeated pre-incubation on flocks of different ages, and according to Damaziak et al. (2018), age influences embryo development. Thus, it was important to ensure that the embryos in younger flocks were pre-incubated for a sufficiently long period.

Egg storage and pre-incubation

All eggs were stored for 15 days at a room temperature of 14 °C and 80% RH.

Pre-incubation was conducted using special Re-Store machines (Petersime, Belgium). The length of pre-incubation (4 or 8 h) was measured when the eggshell temperature reached 28 °C, then at the maximum of 35 °C, and after cooling to 28 °C; persistency at 35 °C was 1 or 4 h for the total length of pre-incubation of 4 or 8 h, respectively. Time measurement from a temperature of 28 °C was established based on Petersime's recommendations for Re-Store. The temperature was monitored by OvoScan (Petersime, Belgium) at three positions on the trolley: upper, middle, and lower trays. The total eggshell temperature of twelve eggs was monitored each time. Fig. 1 shows the temperature profile of treated eggs, including both heating and cooling. Re-Store machines are developed to heat and actively cool hatching eggs. They are specifically designed for pre-incubation. Active cooling is conducted directly in the machines and monitored using OvoScans. Cooling is performed using water in radiators and active air exchange. The negative effect of storing hatching eggs on hatchability is well-documented (Fasenko, 2007; Yassin et al., 2008; Okur et al., 2018). Therefore, this experiment did not include fresh eggs (4 days of storage).

Egg incubation condition

Stored eggs were placed in a Biostreamer 24S incubator (Petersime, Belgium) after 15 days. Heating of hatching eggs took place in the setter 24 h before incubation according to the following scheme: the initial temperature was 23.9 °C; after 12 h, it was increased to 25°C; after 21 h to 32.2 °C, and after 24 h, the temperature reached 38.3 °C. All eggs were then heated to the incubation temperature of 38.3 °C within 6 h and then incubated for the first 3 days at 38.3 °C, followed by a temperature of 37.8 °C. OvoScan controlled the temperature in the setters until day 18 of incubation. On day 18, the eggs were moved to the Biostreamer 8H hatcher and incubated until handling, which occurred 518 h after setting, following the standard programme specified by the technology manufacturer. The incubation was conducted at total capacity, with the setter holding 115 200 eggs and the hatcher 38 400 eggs. The remaining space in the incubator and hatcher was filled with hatching eggs not part of the experiment, ensuring consistent airflow across all eggs. The incubation system remained consistent across all ages of the parent stock.

Hatchability and 1-day-old chick and yolk sac weight

On day 7 of incubation, all hatching eggs underwent candling, and clear eggs were opened to determine embryonic mortality and fertility macroscopically. Differentiation between blastoderms and blastodiscs was carried out according to Bakst (2014). Upon pulling, live hatched chicks were counted per basket. Unhatched eggs were opened to determine the stage of embryo mortality, with early-stage embryonic mortality assessed up to 9 days of incubation (black eye visible, embryo without feathers), middle-stage embryonic mortality from 10 to 17 days of incubation (tiny embryo with feathers), and late-stage embryonic mortality from 18 to 21 days (full-grown embryo with yolk out or full-grown embryo dead or alive with yolk subtracted, Reijrink et al., 2009). Hatchability was calculated as percentages of set and fertile eggs. Embryonic mortality was calculated as percentages of fertile eggs. Twenty randomly selected chicks per treatment were weighed, and after euthanasia by decapitation, the residual yolk was weighed, too.

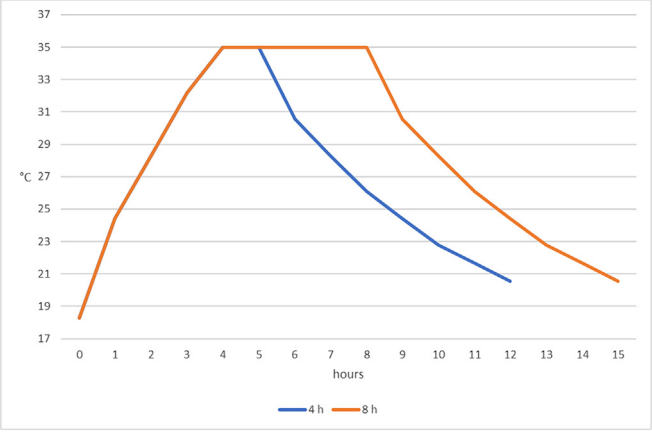

Fig. 1. Temperature profile during pre-incubation for both 4 and 8 h treatments in broiler hatching eggs during storage.

Embryo development staging

Thirty fresh eggs (after transportation to the hatchery) and sixty treated eggs after each pre-incubation at each age were staged to determine embryo development, totalling 810 eggs. Embryos were isolated from the yolk using the filter ring technique, as described by Gupta and Bakst (1993). The staging of embryo development followed the criteria of Eyal-Giladi and Kochav (1976) or Hamburger and Hamilton (1951). These methods are based on categorising embryonic development stages by specific morphological characteristics. The Eyal-Giladi and Kochav (1976) criteria are used to assess early embryonic stages before the egg is laid, focusing on the cellular organisation in the blastoderm. In contrast, the Hamburger and Hamilton (1951) method is applied to later stages of development postoviposition, evaluating the progressive anatomical and structural changes as the embryo advances towards hatching.

Statistical analysis of data

The effects of parent stock age (30, 45, or 58 weeks) and the duration of pre-incubation (0, 2 × 4 h, or 2 × 8 h) on fertility, hatchability, and embryo mortality were evaluated using a generalised linear model for binomial proportions, considering 20 replications of batches of 60 hatching eggs for each factor combination. The significance of individual factors and their interactions was assessed through the analysis of deviance. Homogeneous groups were determined using the LSD *posthoc* comparison. The Genstat 22 software was employed for these analyses. The effects of these factors on day-old chicks and yolk sac weights, yolk proportion, and yolk free body mass were analysed by two-way ANOVA using the Unistat 10 software. Using the Kruskal-Wallis rank test, the embryological development levels for different combinations of parent stock age and pre-incubation duration were compared, considering the ordinal nature of embryo development levels. Homogeneous groups were established by the Nemenyi *posthoc* test. Calculations were performed in the Matlab R2023a computational system. The significance level for all analyses was set at a *P*-value of 0.05.

Results

Hatchability and embryonic mortality

The effects of parent stock age, pre-incubation, and their interactions on apparent fertility, hatchability, and embryonic mortality are shown in Table 1. The apparent fertility exhibited a significant decrease as the age of the parent stock increased ($P < 0.001$). At a parent stock age of 58 weeks, the apparent fertility was 16.8% lower than the fertility observed at 30 weeks. No significant

Table 1
Effects of broiler parent stock age and duration of pre-incubation on the hatchability and embryonic mortality (%).

Item	Age (weeks)			Pre-incubation (h)			Age × Pre-incubation (weeks × h)								*P*-value		
	30	45	58	0	2x4	2x8	30x0	30x2x4	30x2x8	45x0	45x2x4	45x2x8	58x0	58x2x4	58x2x8	A	P	A × P
n	60	60	60	60	60	60	20	20	20	20	20	20	20	20	20			
Apparent fertility[1] (%)																< 0.001	–	–
	98.3[a] (2.26)	94.9[b] (2.68)	81.5[c] (5.39)	–	–	–	–	–	–	–	–	–	–	–	–			
Hatchability set eggs (%)																		
	85.1[a] (5.11)	80.0[b] (5.07)	61.7[c] (6.21)	75.7 (9.97)	75.9 (11.7)	76.9 (12.5)	82.8[b] (5.40)	86.8[a] (3.37)	86.0[a] (5.44)	79.6[c] (5.07)	78.8[c] (5.19)	81.5[bc] (4.80)	64.3[d] (6.82)	61.6[de] (5.65)	59.6[e] (5.29)	< 0.001	0.96	0.003
Hatchability fertile eggs (%)																		
	86.6[a] (4.80)	84.3[b] (4.69)	75.9[c] (7.21)	82.7 (6.41)	82.7 (7.05)	82.7 (8.27)	84.4[b] (5.42)	87.7[a] (3.74)	87.7[a] (4.42)	84.1[b] (5.30)	83.1[b] (4.49)	85.6[ab] (4.08)	78.7[c] (6.98)	76.0[cd] (6.55)	73.0[d] (7.28)	< 0.001	0.99	0.001
Embryonic mortality (%)																		
Early 1–9 d																		
	8.8[b] (4.55)	8.5[b] (3.85)	14.6[a] (5.22)	11.2 (4.91)	10.5 (5.18)	9.4 (5.67)	11.6[b] (4.67)	7.6[c] (3.40)	7.2[c] (4.22)	8.9[c] (3.94)	9.4[bc] (4.19)	7.2[c] (3.18)	13.6[ab] (5.06)	15.3[a] (4.75)	14.9[a] (6.04)	< 0.001	0.053	0.003
Middle 10–17 d																		
	0.1[c] (0.57)	0.6[b] (1.27)	3.2[a] (2.20)	0.9[b] (1.71)	1.1[ab] (1.89)	1.6[a] (2.29)	0.1 (0.38)	0.2 (0.55)	0.2 (0.79)	0.4 (0.97)	0.6 (1.61)	0.9 (1.18)	2.5 (2.10)	2.8 (1.97)	4.3 (2.23)	< 0.001	0.035	0.98
Late 18–21 d																		
	4.0[b] (2.39)	6.5[a] (2.85)	6.2[a] (3.89)	5.0 (3.04)	5.5 (3.31)	6.1 (3.44)	3.5 (2.32)	3.9 (2.42)	4.6 (2.44)	6.5 (2.67)	6.9 (3.41)	6.3 (2.49)	4.9 (4.89)	6.0 (3.37)	7.7 (4.58)	< 0.001	0.14	0.32

Abbreviations: A = age, P = pre-incubation, A × P = interaction age × pre-incubation
Predictions and SD in parentheses are presented.
Values within a column with different superscripts differ significantly at $P < 0.05$.
[1] For the characteristic "apparent fertility," only the age factor was statistically evaluated.

interaction was found between pre-incubation and parent stock age, and pre-incubation did not affect apparent fertility evaluation. The hatchability of both set and fertile eggs was found to be dependent on the age of the parent stock ($P < 0.001$). However, pre-incubation alone did not have any impact on hatchability. Conversely, a significant interaction was observed between parent stock age and pre-incubation duration regarding both set eggs' hatchability ($P = 0.003$) and fertile eggs' hatchability ($P = 0.001$). At 30 weeks of parent stock age, both lengths of pre-incubation positively influenced the hatchability of both set and fertile eggs. At 45 weeks of parent stock age, pre-incubation length had no significant effect on either set or fertile eggs' hatchability. At 58 weeks of parent stock age, a more extended pre-incubation period of 2 × 8 h was associated with decreased hatchability of both set and fertile eggs.

Early embryonic mortality was significantly influenced by the parent stock age factor ($P < 0.001$). The factor of pre-incubation had a *P*-value of 0.053, indicating a potential effect, although it was not statistically significant. However, a significant interaction was observed between these two factors ($P = 0.003$). At 30 weeks of parent stock age, both short and long lengths of pre-incubation were associated with a decrease in early embryonic mortality. However, pre-incubation did not significantly affect early embryonic mortality at 45 and 58 weeks of parent stock age. Middle embryonic mortality demonstrated a significant increase with an increase in parent stock age ($P < 0.001$). Additionally, more extended pre-incubation periods (2 × 8 h) significantly increased mortality compared to untreated eggs ($P = 0.035$). However, no significant interaction was observed in this regard. Parent stock age significantly influenced late embryonic mortality ($P < 0.001$). The mortality rate was higher at 45 and 58 weeks compared to the mortality rate at 30 weeks. Pre-incubation did not affect late embryonic mortality, nor was any observed interaction between parent stock age and pre-incubation about late embryonic mortality.

1-day-old chicks and yolk sac weight

The impact of parent stock age and pre-incubation on 1-day-old chicks and yolk sac weight is presented in Table 2. The live weight of 1-day-old chicks increased with the advancing age of the parent stock ($P < 0.001$). No significant effect of pre-incubation or interaction between age and pre-incubation was observed. Yolk sac weight was influenced by both parent stock age ($P < 0.001$) and the duration of pre-incubation ($P = 0.003$). Compared to chicks from parent stock age 30 weeks, yolk sac weights increased by 1.3 or 1.2 g in chicks from parent stock age 45 or 58 weeks, respectively. Pre-incubation for 2 × 4 h or 2 × 8 h led to a decrease in yolk sac weights by 0.6 and 0.9 g, respectively, compared to the yolk sac weight of chicks hatched from untreated eggs. Yolk sac proportion followed the same pattern as yolk sac weight in response to age ($P = 0.003$). The length of pre-incubation decreased yolk sac proportion ($P < 0.001$). Yolk-free body mass increased with the age of the parent stock ($P < 0.001$).

Embryonic development

The effects of parent stock age and pre-incubation on embryo development are depicted in Fig. 2. The independent variable on the x-axis represents the length of the pre-incubation treatment. The y-axis illustrates the embryo development stage according to Eyal-Giladi and Kochav (1976) or Hamburger and Hamilton (1951). Embryo development was assessed after each pre-incubation, so the figure also indicates the embryonic stage after the first pre-incubation (1 × 4 h or 1 × 8 h).

Table 2
Effects of broiler parent stock age and duration of pre-incubation on the 1-day old chicks and yolk sac weights (g).

Item	Age (weeks)			Pre-incubation (h)			Age × Pre-incubation (weeks × h)								*P*-value		
	30	45	58	0	2x4	2x8	30x0	30x2x4	30x2x8	45x0	45x2x4	45x2x8	58x0	58x2x4	58x2x8	A	P	A × P
n	60	60	60	60	60	60	20	20	20	20	20	20	20	20	20			
Live weight (g)																		
	39.7^{c} (2.51)	45.9^{b} (3.48)	47.4^{a} (4.08)	43.7 (4.78)	43.4 (4.44)	43.4 (4.89)	39.9 (2.42)	39.8 (3.03)	39.4 (2.06)	45.4 (2.73)	45.8 (3.36)	46.5 (4.28)	48.8 (4.92)	46.9 (2.97)	46.6 (4.09)	<0.001	0.82	0.37
Yolk sac weight (g)																		
	3.6^{b} (1.10)	4.9^{a} (1.51)	4.8^{a} (2.10)	4.8^{a} (1.62)	4.2^{b} (1.38)	3.9^{b} (1.75)	4.2 (1.24)	3.7 (0.98)	2.9 (0.66)	5.0 (1.32)	4.9 (1.41)	4.9 (1.82)	5.8 (2.19)	4.3 (1.59)	4.3 (2.22)	<0.001	0.003	0.17
Yolk sac proportion (%)																		
	9.0^{b} (2.48)	10.7^{a} (2.72)	9.9^{a} (3.83)	10.9^{a} (2.83)	9.6^{b} (2.61)	8.8^{c} (3.09)	10.4 (2.72)	9.1 (2.15)	9.5 (1.57)	11.0 (2.42)	10.6 (2.62)	10.3 (3.14)	11.7 (3.64)	9.0 (3.15)	8.9 (4.25)	0.003	<0.001	0.22
Yolk–free body mass (g)																		
	36.1^{c} (2.15)	40.9^{b} (2.60)	42.6^{a} (2.87)	38.9 (3.76)	39.2 (3.68)	39.5 (3.74)	35.7 (1.91)	36.2 (2.60)	36.5 (1.90)	40.4 (2.02)	40.8 (2.55)	41.6 (3.11)	43.0 (3.48)	42.6 (2.17)	42.3 (3.02)	<0.001	0.43	0.70

Abbreviations: A = age, P = pre-incubation, A × P = interaction age × pre-incubation
Means and SD in parentheses are presented.
Values within a column with different superscripts differ significantly at $P < 0.05$.

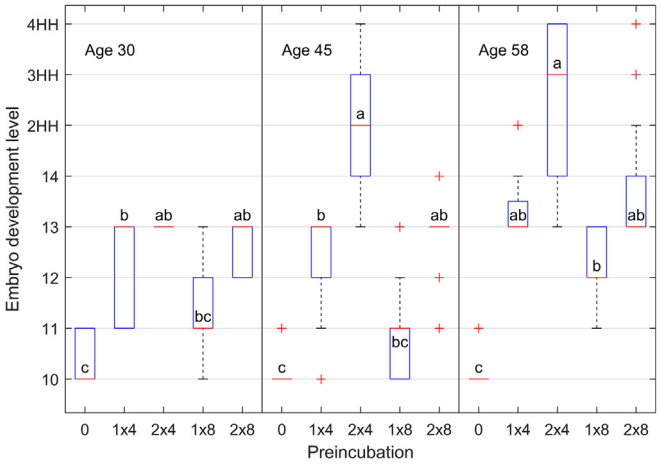

Fig. 2. Effect of pre-incubation duration (h) on embryo development depending on broiler parent stock age (Eyal-Giladi and Kochav, 1976 or Hamburger and Hamilton, 1951). Redline segments represent the median; blue lines outline the lower and upper quartiles; dashed sections indicate non-outlier minimum and maximum, and plus symbols denote outliers. Different letters indicate significant differences ($P < 0.05$).

The median of embryo development for untreated eggs was the same across all ages (stage 10). In the older parent stock (45 and 58 weeks), repeated pre-incubation increased variability in embryo development, while in the younger parent stock, repeated pre-incubation decreased variability in embryo development. At this age, after both 2 × 4 h or 2 × 8 h pre-incubations, the embryos reached a significantly higher stage 13 ($P < 0.05$) compared to untreated eggs, corresponding to higher hatchability and lower early embryonic mortality. At the age of 45 weeks, repeated pre-incubation for 4 or 8 h increased embryo development to stage 2HH or 13. After applying 2 × 8 h pre-incubation, the embryos reached stage 13, and hatchability was the highest at this age, although not significantly so. At the parent stock age of 58 weeks, repeated pre-incubation for 4 or 8 h increased embryo development to stage 3HH or 13. After applying 2 × 8 h pre-incubation, some outliers reached even stage 4HH, which likely resulted in significantly lower hatchability from eggs treated in this manner.

Discussion

Eggs from older hens exhibited lower apparent fertility and hatchability, in accordance with the findings of several researchers (Damaziak et al., 2018; Özlü et al., 2018; Özlü et al., 2021). Fasenko et al. (1992) attributed the decreased hatchability in older hens to the increased prevalence of first-of-sequence eggs, which they found to have lower viability (hatch of fertile eggs). Pre-incubation did not enhance hatchability; however, positive interactions between parent stock age and pre-incubation were identified. A positive effect ($P < 0.05$) of pre-incubation length on hatchability was observed in a young flock, consistent with the results of Dymond et al. (2013). In their study, repeated 4 × 4 h pre-incubation increased hatchability in eggs stored for 21 days from parent stock aged 33, 35, and 36 weeks. Gucbilmez et al. (2013) also demonstrated a positive effect of pre-incubation in younger flocks (27 and 29 weeks) with eggs stored for 11 days. However, Özlü et al. (2021) reported a positive effect of pre-incubation on hatchability irrespective of parent stock age (29 and 58 weeks). Conversely, Damaziak et al. (2018) showed increased hatchability after pre-incubation in older parent stocks (2 × 4 h in 12 days stored eggs). However, they compared eggs from substantially older parent stock age groups (49 and 52 weeks

vs 70 and 73 weeks), which is uncommon in broiler hatchery practice.

More extended pre-incubation periods (2 × 8 h) significantly increased middle embryonic mortality compared to untreated eggs. During the 2nd week of incubation, nutrients are actively taken from albumin and progressively from the yolk (Bellairs and Osmond, 2014). The intensive development of the yolk sac bloodstream in close contact with the yolk membrane, which may be fragile due to prolonged pre-incubation and long-term storage, could cause this mortality. A deeper study of the effect of pre-incubation on the quality of the yolk membrane and the viability of embryos could clarify this impact.

The live weight of chicks and yolk-free body mass increased with parent stock age ($P < 0.001$), with chicks from older hens generally having higher BW. This is associated with higher egg weight and a strong correlation between these traits (Pinchasov, 1991; Willemsen et al., 2008). In this study, no effect of pre-incubation or interaction between parent stock age and pre-incubation was observed on the BW of hatched chickens, consistent with the findings reported by Dymond et al. (2013) using repeated pre-incubation (4 × 4 h) or single heating for 6 or 12 h in eggs stored for 21 days. Pre-incubation decreased yolk sac weights compared to the yolk sac weight of chicks hatched from untreated eggs. Chicks from pre-incubated eggs hatch earlier (Okasha et al., 2023) and thus utilise nutrients from the yolk sac sooner. Yolk contents could pass into the intestines via the lumen of the yolk stalk in at least newly hatched to 3-day-old chickens (Esteban et al., 1991). To account for this, hatching chicks from pre-incubated eggs would be advisable separately.

Fasenko et al. (2001) demonstrated that single prestorage heating allows embryos to complete the formation of hypoblasts, thereby increasing hatching ability, even with 14 days of egg storage. However, repeated heating during egg storage more precisely mimics the natural conditions birds provide to eggs before they begin hatching (Meijer and Siemers, 1993), so we applied repeated heat treatment during hatching egg storage in our study.

Embryos from fresh eggs of all age groups (30, 45, and 58 weeks) were at the same stage of development (X EG&K), in agreement with Fasenko et al. (1992), who showed that embryonic development was not significantly related to hen age from 31 to 54 weeks (10.14 EG&K). Özlü et al. (2018) also observed embryo development in fresh eggs at almost the same stage (10.1 EG&K) with a parent stock age of 50 weeks, but in younger stock (27 weeks), they found an even lower level of embryo development (8.9 EG&K). However, Damaziak et al. (2018) reported higher embryo development in older hens, but they compared embryos of hens at ages 49 and 52 weeks vs 70 and 73 weeks. Fasenko et al. (1992) emphasised that embryonic development was significantly related to the egg sequence position. Embryos of first-of-sequence eggs were more developed than embryos of subsequent eggs, suggesting that Damaziak et al. (2018) might have observed differences in embryo development as a consequence of increased first-of-sequence eggs, depending on the hen ages in broiler parent stock from 49 to 73 weeks. The higher embryo development of first-of-sequence eggs was also confirmed by Reijrink et al. (2009). This phenomenon can clarify the higher variability of pre-incubated embryo development in older parent stock in this study, mainly using repeated pre-incubation. It can explain the incidence of 3HH and 4HH stages, according to Hamburger and Hamilton (1951).

The duration of pre-incubation improved embryo development in this study; however, Damaziak et al. (2018) did not observe any effect of heat treatment (2 × 4 h) on embryo development evaluated after 48 or 72 h of incubation. These researchers used a maximum pre-incubation temperature of 30 °C, while in our study, a maximum temperature of 35 °C was applied. On the other hand, Dymond et al. (2013) utilised a temperature of 37.5 °C and, after just 6 h of heating, observed embryo development at stage 14.0 EG&K. However, Özlü et al. (2021) reported embryo development (10.24–11.13) after the same length of pre-incubation (6 h) depending on parent stock age (29 and 58 weeks), without any interaction between parent stock age and pre-incubation.

There are many controversial results when evaluating the effect of pre-incubation on both hatchability and embryo development. The main issue is the pre-incubation process; researchers used different maximum pre-incubation temperatures from 30 °C (Damaziak et al., 2018), 35 °C (Özlü et al., 2021 and in this study), 37.5 °C (Dymond et al., 2013) to 37.8 °C (Gucbilmez et al., 2013). The length of pre-incubation and its repetition is also crucial. This study demonstrates that pre-incubation of 2 × 4 h is not the same as 1 × 8 h from an embryo developmental point of view. The development of embryos was significantly higher with 2 × 4 h application compared to 1 × 8 h in parent stock aged 45 and 58 weeks. This could be due to the longer cumulative time the embryos were exposed to temperatures higher than physiological zero. Opinions on the temperature at which embryogenesis begins, known as the physiological zero, vary. Proudfoot and Hulan (1990) consider the physiological zero to be 20–21 °C, although Lundy (1969) states that the temperature threshold is between 25 and 27 °C. If the physiological zero is 20–21 °C, the eggs treated 2 × 4 h would be exposed to the temperature above physiological zero for a longer duration than the 1 × 8 h pre-incubation treatment, as shown in Fig. 1.

Conclusion

This study shows that evaluating the effect of pre-incubation on embryo development and hatchability requires precise attention to the whole process, including the duration of pre-incubation, maximum temperature, and cooling system. These factors all influence the duration of temperature exposure above physiological zero. The duration and the frequency of pre-incubation should be specified based on the age of the parent stock.

Ethics approval

Experimental procedures with day-old chicks were in accordance with the Czech national guidelines for animal care for research purposes and were approved by the Institutional Ethics Committee of the Faculty of AgriSciences, Mendel University in Brno, for animal studies (16OZ27083/2014-17214).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Author ORCIDs

Martina Gregrova: https://orcid.org/0000-0002-4930-6276.
Martina Lichovnikova: https://orcid.org/0000-0001-6930-8837.
David Hampel: https://orcid.org/0000-0002-3865-5948.

CRediT authorship contribution statement

M. Gregrova: Writing – original draft, Investigation. **M. Lichovnikova:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **M. Foltyn:** Supervision, Methodology, Investigation, Conceptualization. **Z. Tvrdon:** Supervision, Resources, Methodology, Conceptualization. **D. Hampel:** Writing – original draft, Formal analysis.

Declaration of interest

None.

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