sciendo

DOI: 10.2478/fv-2019-0004

FOLIA VETERINARIA, 63, 1: 24—31, 2019

PRE-INCUBATION AND ITS EFFECT ON THE DEVELOPMENT AND MALFORMATIONS OF THE CHICK EMBRYO

Janikovičová, L.[1], Demčišáková, Z.[2], Luptáková, L.[3], Petrovová, E.[1]

[1]Institute of Anatomy
[3]Institute of Biology, Zoology and Radiobiology
University of Veterinary Medicine and Pharmacy in Kosice, Komenskeho 73, 04181 Kosice
[2]Department of Biology, Faculty of Humanities and Natural Sciences
University of Presov, 17. November 6600/1, 080 01 Presov
Slovakia

eva.petrovova@uvlf.sk

ABSTRACT

This study was conducted to evaluate the effect of eggs stored with and without pre-incubation on chick embryos with emphasis on: embryo body, heart weight, malformations, and mortality. For this study, a total of 120 chick embryos were divided into three groups, based on the length of storage before hatching (3, 7 and 10 days). Observations of the weight of chick embryo bodies, chick embryo hearts, and the level of mortality and appearance of malformations were noted. With an increase in days stored, the chick embryo's weight decreased. The pre-incubation period had a positive effect on the weight of chick embryo, and chick hearts. Malformations, including: hydrocephalus, open body cavity and underdeveloped wings, were observed in all three groups, with the highest proportion seen in the pre-incubated hatching eggs stored for 10 days; this group also displayed the highest level of mortality. Non-pre-incubated eggs showed the most promise with better results in all experimental groups. In conclusion, the research suggests the optimal storage for chick embryos to be 3 days, with lowest levels of mortality, malformations and limited effects on the body and heart weight.

Key words: chick embryo; malformation; pre-incubation; storage

INTRODUCTION

Recent preventative medicine research has focused attention on studying the impact of external environmental factors on the growth of the individual, with potential teratogens and their effects on organism growth being largely unstudied.

Research into understanding their influence on physiological function and abnormal growth of an organism can lead to the identification and clarification of certain pathological conditions.

The animal model plays an important role in basic medical research with animal testing done in combination with *in vitro* methods in accordance with ethical, economic and scientific parameters. Alternative methods in animal

testing include: mathematical models, lower organism use and mammals in early stages of development; these reduce unnecessary stress and suffering to the model [13]. Chick embryos have been used as an alternative animal model for many types of experiments. It is also thanks to the fact that with chicken embryos the maternal metabolism does not have any impact. Therefore, we can better understand some of the processes underlying the chick embryo [12]. Chick embryos have also been used for the interpretation of morphogenesis in higher vertebrates as they require less space and storage requirements [4]. Hatching is a biological process of a fertilised egg, where the embryo develops into an individual. To ensure this process, the correct climate conditions including temperature, humidity and egg rotation are paramount. The success of this process is also impacted by: the biological value of the egg (correlated to storage time), the hatching technique used along with age of hen, egg weight and environmental conditions during the incubation. The time of the storage is mainly important for the biological value of the eggs. The highest hatching rate is observed in eggs stored within a 7 day period; as storage periods longer than 14 days there is a significant decrease in hatchability. Pre-incubating (preheating) the eggs can minimalize the negative effect of long storage, by providing uniform development for the eggs in storage. Preheating is helpful for a better uniformity of the development of the chicken embryo in the storage eggs, which will be incubated later together [9, 18]. Other factors that affect increased hatchability and chick quality are: genetic, and the environment through the storage and incubation (volume and gas exchange) [11]. Post-hatching, chick quality can be influenced by transportation induced stress [1]. With correct storage and pre-incubation, the impact of external factors on chick embryo development can be decreased. Therefore, we decided to evaluate the effect of the eggs storage with and without pre-incubation on chick embryos with emphasise on the embryo body, heart weight, malformations, and mortality.

MATERIALS AND METHODS

For this study, 120 eggs of Lohmann Brown chicken breed were obtained from the farm Parovske Haje (Nitra, Slovakia). These eggs were divided into groups according to their storage duration (3, 7 and 10 days at 15 °C and 75 % relative humidity). Approval was not needed to use 9-day-old chick embryos, as they are not included in the legislation on the protection of animals used for scientific purposes (2010/63/EU).

Each group contained 20 hatching eggs; half pre-incubated for 12 hours at room temperature (22—23 °C). All eggs were stored in the incubator under standard conditions for 9 days (37.5 ± 0.5 °C, 60 % humidity). On the 9th day, a hole was cut from the broad end of the egg and the embryo removed using a retractor. Foetal membranes were removed from the chick embryo using tweezers. The treatment was repeated twice.

Macroscopic changes were focused upon: malformations (open body cavity, beak deformations, upstretched wing/limb, two-headed embryos etc.) and haemorrhages. Subsequently, the bodies of embryos were weighted as well as the hearts of embryos were removed and weighted. Dead embryos were discarded without their use.

Statistical analysis of the data was performed using the programme GraphPad Prism 6.0. A two-pass ANOVA test and multiple comparison tests (Sidak test) were used for analysis. Values of $P < 0.05$ were deemed statistically significant.

RESULTS

This study concentrated on the effects of pre-incubation in relation to the incidence of embryonic mortality and malformations as well as its effect on embryonic weight. The results showed that storing hatching eggs although beneficial, repeatedly resulted in problems associated with premature embryonic mortality and variations in morphology and weight.

Embryonic mortality

Embryonic mortality was observed in all storage groups on days 3, 7 and 10. In the group stored for 3 days without pre-incubation, 10 % embryonic mortality was observed. However, in the case of pre-incubation of hatching eggs, mortality was not observed. In the group stored for 7 days without pre-incubation, 0 % embryonic mortality was observed; with pre-incubation, 10 % mortality was seen. The highest mortality occurred in eggs with 10 days of storage. In the group stored for 10 days without pre-incubation almost 32 % mortality was seen; with pre-incubation, 35 %

Table 1. Storage of fertilized eggs and effect of pre-incubation

Storage	No[2]	Live	Dead	Mortality [%]	Malformations	Weight [g]	SD[3]
3-day eggs	20	18	2	10.00	0	1.710	0.259
3-day eggs P[1]	20	20	0	0.00	1	1.835	0.298
7-day eggs	20	20	0	0.00	2	1.620	0.227
7-day egg P	20	18	2	10.00	1	1.731	0.235
10- day egg	19	13	6	31.00	3	1.534	0.226
10-day egg P	20	13	7	35.00	4	1.539	0.195
Total	**119**	**102**	**17**		**11**		

[1] — pre-incubation; [2] — number of chicken embryos; [3] — standard deviation

Table 2. Effect of pre-incubation on the development of the chick embryo

	Mortality [%]	Number of malformations	Body Weight [g]	Heart weight [g]
Eggs	13.00	3	1.632	18.770
Eggs with pre-incubation	14.00	2	1.744	18.890

Table 3. Effect of pre-incubation on heart weight of the chick embryo

Storage	Weight of heart [mg]	SD[2] [mg]
3-day eggs	18.0	3.9
3-day eggs pre-incubation	21.0	6.4
7- day eggs	18.6	5.2
7-day eggs pre-incubation	18.9	4.9
10-day eggs	18.1	3.9
10-day eggs pre-incubation	15.0[1]	3.5[1]

[1] — significant difference *P ≤ 0.05; [2] — standard deviation

mortality was recorded. Total embryonic mortality after 3 and 7 days of storage was 5 % and after 10 days of storage, the embryonic mortality was 33 %. For pre-incubation, total embryonic mortality in eggs without pre-incubation was 14 % and 15 % with pre-incubation. These results show embryonic mortality was not significantly influenced by pre-incubation. Embryonic mortality on the total number of hatching eggs represented about 14 % on average (Fig. 1, Tables 1 and 2).

Spectrum of malformations

Malformations were observed in all groups. Three-day storage without pre-incubation had no malformations present; however, with pre-incubation one case of single-

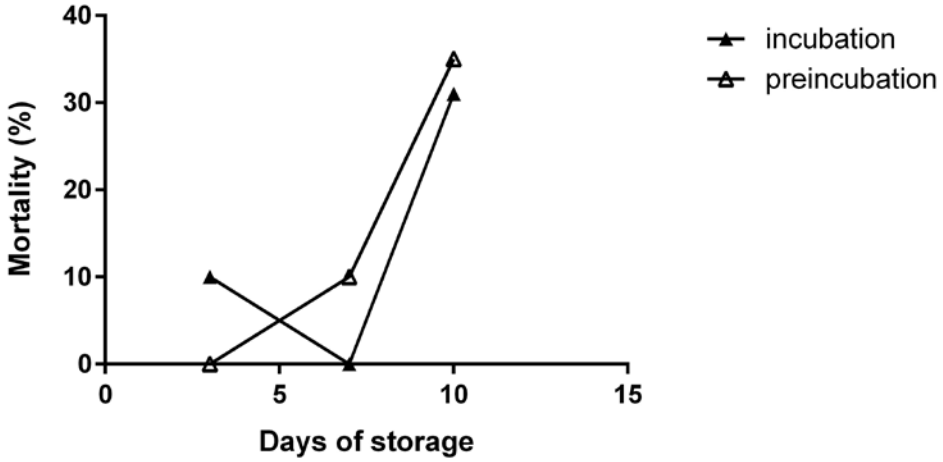

Fig. 1. Storage of fertilized eggs and its effect on the mortality

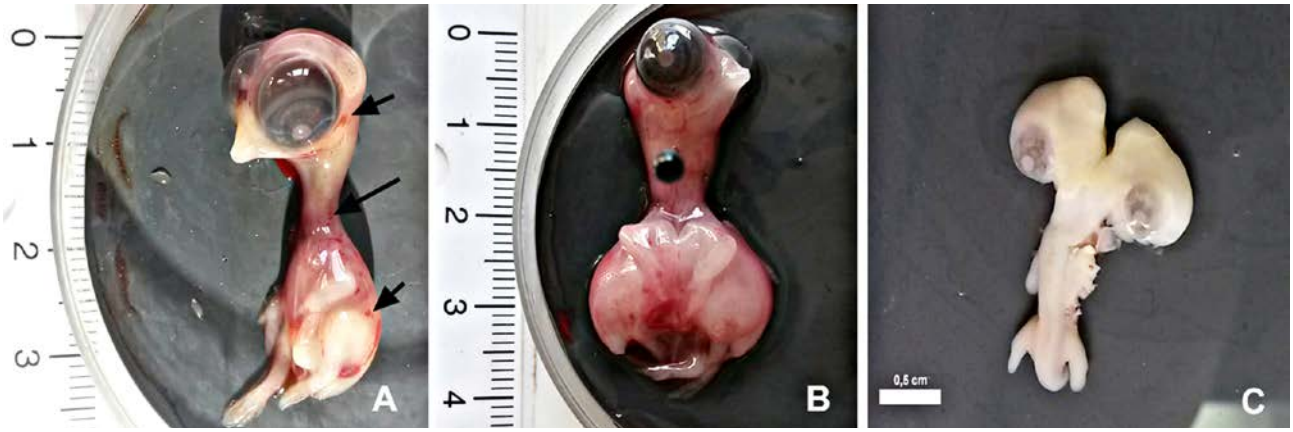

Fig. 2. Malformations observed on the embryonic day 9 in relation to the storage day of fertilized eggs
A) *Haemorrhage* (arrows) are present on the body after 3 days of storage; B) Double body of embryo after 3 days of storage and using pre-incubation; C) Double-headed embryo after 7 days of storage and using pre-incubation

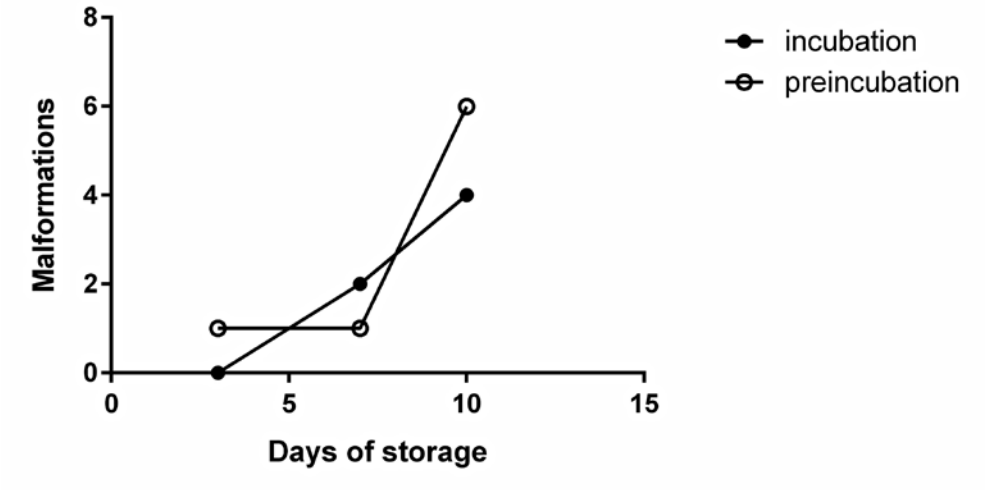

Fig. 3. Storage of fertilized eggs and its effect on the occurrence of malformations

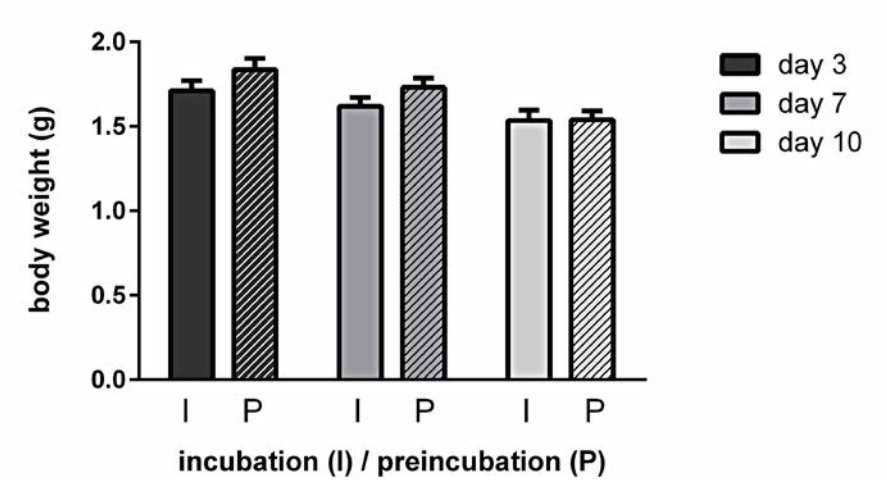

Fig. 4. Storage and pre-incubation of fertilized eggs and the body weight of the chick embryo

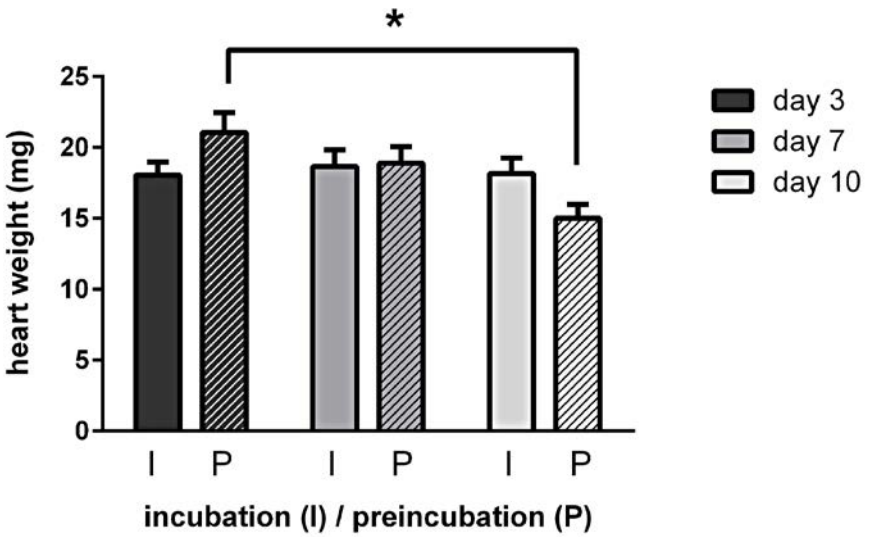

Fig. 5. Storage and pre-incubation of fertilized eggs affect the heart weight of the chick embryo

headed twins sharing organs and cardiomegaly was discovered. Twins weight reached up to 2.58 g and the heart weighed 0.037 g (Table 1).

Seven-day storage without pre-incubation had 2 malformed embryos, one with an open body cavity the other with an undeveloped wing; with pre-incubation one underdeveloped malformed embryo with two heads was found (Table 2).

The highest numbers of malformations were observed in the group of hatching eggs with a 10-day storage. Without pre-incubation, three embryos with malformations including cyanosis, open body cavity, deviations of limb and head were revealed. Hatching eggs with pre-incubation before the incubation contain 4 malformed embryos with malformations (lacked the wings and tail, open body cavity, double-headed embryo, haemorrhagically coloured body, undeveloped beak, and curled limbs (Figs. 2 and 3).

In all experimental groups of hatching eggs, various amounts of haemorrhaging was observed—mainly in the head and pelvic region. It cannot be ruled out that these haemorrhages were a result of removing the embryo from the egg.

Monitoring the difference in body weight of chick embryos

The average weight of all embryos without pre-incubation reached 1.62 g; with pre-incubation it was 1.70 g. Within the groups divided by days of storage, the weight average decreased with an increase in storage time. The greatest weight was reached by the eggs stored for 3 days, 1.835 ± 0.298 g with pre-incubation; 1.710 ± 0.259 g without. The mean average weight of hatching eggs was achieved by embryos with 7 days of storage (1.620 ± 0.227 g, with pre-incubation 1.731 ± 0.235 g). The lowest average weight was seen by the embryos of 10 days of storage, weighing only 1.534 ± 0.226 g without pre-incubation and 1.539 ± 0.195 g with pre-incubation (Table 1, Fig. 4).

Monitoring the difference in heart weight of chick embryos

Similarly, to the body weight correlation, the average weight of the chick hearts decreased in direct correlation with the increase in storage time. The lowest weights were observed in the chick embryos stored for 10 days. The average heart weight from the 3 day stored eggs were 18 ± 3.9 mg; with pre-incubation 21 ± 6.4 mg. The average weight from the 7 day stored eggs were 18.6 ± 5.2 mg; with pre-incubation 18.9 ± 4.9 mg. The 10 day stored eggs had the lowest average weight of 18.1 ± 3.9 mg, with pre-incubation of only 15 ± 3.5 mg (Table 3, Fig. 5).

In all examined hearts, those pre-incubated reached a higher weight than those that were not pre-incubated. The exceptions to this were those embryos retrieved from eggs stored for 10 days. Overall, we achieved the higher weight of all pre-incubated embryos in comparison to the weight of hatching eggs without pre-incubation.

DISCUSSION

Our results showed that eggs stored 7 days and longer had a decrease in hatching quality; the reason for this negative effect has not been resolved. Suggestion for this reduced hatchability could be due to the decreased viability of the embryo caused by the higher grade of cell death; another could be optimal time of pre-incubation could differ according to a length of storage (as the storage length correlates to embryo viability) [14]. The reduced heart and embryo weights could be the result of delayed re-initiation of development following longer storage. The storage of hatching eggs seven days or longer often induced cell death, which in turn led to: increased embryo mortality, decreased hatchability and decreased the development of the embryo metabolism. Embryo mortality of eggs stored at 4 days averaged reached 10.7 % increasing to 27.7 % for eggs stored at 14 days [6].

Chicken embryos from the broilers were examined to prove whether storage had any impact on the development of the chick embryo before incubation; results showed that the longer the egg was stored, the lower the embryo weight, which in turn lead to an increased embryonic mortality [5].

Hatching eggs from the broiler Ross 308 were divided into two groups and stored for 4 and 14 days at the same temperature and the humidity. The conclusion of this testing was that with the increased time of the storage the mechanisms of the apoptosis in the level of the blastoderm were activated. This may be an attributing factor to the reduced weight gain observed during incubation [3, 8].

Eggs stored for 14 days saw an increase in apoptosis, resulting in a growing number of abnormal embryos or dead embryos. These results clarified the importance of optimal conditions for normal growth and development [2].

Embryos retrieved from eggs stored for 7 and 14 days saw retarded growth after 42 hours, compared to the eggs that were not stored. It is likely not caused by late embryonic development but the reduced rate of embryonic growth, which was significantly lower in the first two days of incubation of the eggs stored for 14 days (compared to those not stored). No evidence of a relationship between the developmental stages of the embryo and time of incubation was apparent. A positive correlation was seen between the number of dead and malformed embryos and the storage length [10].

This study suggests eggs (from Cobb 500 broilers) pre-incubated 6 hours before storage provides better quality as it reduces the incubation period and level of embryonic mortality. The worst results were seen in eggs preheated for 12 hours then stored for 14 days; these results recommend that this method not to be used in commercial management [17].

The influence of pre-incubation, broiler breeder age and the impact of storage period on hatching efficiency were all evaluated. Eggs from the broilers Ross 344 x broiler Ross 308 (age of 28, 38 and 53 weeks) were stored follow-

ing 10 days in the storage room, others were removed on the fifth day of storage. They were pre-incubated firstly at 26 °C for 2 hours, 37.8 °C for 3 hours and finally at 26 °C for 2 hours. Eggs that were pre-incubated on the 5th day after 11 day storage had increased hatchability; research suggests this was due to the eggs coming from young broilers and being in the hypoblast stage at the start of the study [7].

Eggs stored longer than 7 days saw changes to: albumen characteristics, decreased the quality of incubation, increased the duration of the incubation period and halted embryo development. This study suggests longer storage times, conditions should be modified with the eggs thin pole ventral and the eggs rotated. In addition to these requirements, pre-incubation can be used which together should reduce the negative impacts seen in long storage [16].

When the hypoblast is completely formed (during the silent developmental state of an embryo), the embryo seems to be more resistant to the larger storage periods. Within the storage the differences in the albumen viscosity, pH of the yolk and albumen are visible. Optimal pH of albumen for the right development is 8.2. It is assumed that the embryo with completely developed hypoblast is able to make a stronger barrier between the embryo and its external environment, and/or it is more capable of producing enough amounts of CO_2, which reduce the pH in the microenvironment of the embryo for the optimal 8.2 [15].

Based on our results it can be concluded that the increased mortality is linked to increased storage periods of hatching eggs. Each studied group displayed malformations regardless of pre-incubation. Results also showed that pre-incubation increased overall bodyweight along with heart weight. The exceptions to this were the eggs stored for 10 days; to which pre-incubation did not play a significant role in the embryonic development. This research suggests ensuring good hatchability and chick quality after long periods of storage; the embryo should be in the stage of a completely formed hypoblast. This material may provide insight into enhancing the storage of experimental hatching eggs. It will allow avoiding false-induced malformations or mortality resulting from incorrect storage or pre-incubation of hatching eggs.

ACKNOWLEDGEMENT

This study was carried out within the framework of the VEGA project No. 1/0050/19.

REFERENCES

1. **Bergoug, H., Burel, C., Guineberetire, M., Tong, Q., Roulston, N., Romanini, C. E. B., et al., 2013:** Effect of pre-incubation and incubation conditions on hatchability, hatch time and hatch window, and effect of post-hatch handling on chick quality at placement. *World's Poultry Science Journal*, 69, 313—334. DOI: 10.1017/S0043933913000329.
2. **Bloom, S. E., Muscarella, D. E., Lee, M. Y., Rachlinski, M., 1998:** Cell death in the avian blastoderm: Resistance to stress-induced apoptosis and expression of anti-apoptotic genes. *Cell Death Differ.*, 5, 529—538. DOI: 10.1104/pp.120.2.529.
3. **Branum, S. R., Tazawa, H., Burggren, W. W., 2016:** Phenotypic developmental plasticity induced by preincubation egg storage in chicken embryos (*Gallus gallus domesticus*). *Physiol. Rep*., 4 (4): pii: e12712. DOI: 10.14814/phy2.12712.
4. **Darnell, D. K., Schoenwolf, G. C., 2000:** The chick embryo as a model system for analysing mechanisms of development. *Methods Mol. Biol.*, 135, 25—29.
5. **Fasenko, G. M., 1992:** Variability in preincubation, embryonic development in domestic fowl, effects of duration of egg storage period. *Poult. Sci.*, 71 (12), 2129—2132.
6. **Fasenko, G. M., 2007:** Egg storage and the embryo. *Poult. Sci.*, 86 (5), 1020—1024.
7. **Gucbilmez, M., Özlü, S., Shiranjang, R., Elibol, O., Brake, J., 2013:** Effects of preincubation heating of broiler hatching eggs during storage, flock age, and length of storage period on hatchability. *Poult. Sci.*, 92 (12), 3310—3313. DOI: 10.3382/ps.2013-03133.
8. **Hamidu, J. A., Uddin, Z., Li, M., Fasenko, G. M., Guan, L. L., Barreda, D. R., 2011:** Broiler egg storage induces cell death and influence embryo quality. *Poult. Sci.*, 90 (8), 1749—1757. DOI: 10.3382/ps.2011-01361.
9. **Hrnčár, C., Bujko, J., 2012:** Effect of pre-storage incubation of hatching eggs on hatchability of poultry (In Slovak, English abstract). *Acta fytotechnica et zootechnica*, 2, 34—37.
10. **Mather, C. M., Laughlin, K. F., 1977:** Storage of hatching eggs: The effect on early embryonic development. B*r. Poult. Sci.*, 18 (5), 597—603. DOI: 10.1080/00071667708416408.

11. **Onagbesan, O., Bruggeman, V., De Smit, L., Debonne, M., Witters, A., Tona, K., et al., 2007:** Gas exchange during storage and incubation of avian eggs: effects on embryogenesis, hatchability, chick quality and post-hatch growth. *World's Poultry Science Journal*, 63 (4), 557—573. DOI: 10.1017/S00 43933907001614.
12. **Petrovová, E., Sedmera, D., Mísek, I., Lesník, F., Luptáková, L., 2009:** Bendiocarbamate toxicity in the chick embryo. *Folia Biol.* (Praha), 55, 61—65.
13. **Rashidi, H., Sottile, V., 2009:** The chick embryo: hatching a model for contemporary biomedical research. *Bioessays*, 31 (4), 459—465. DOI: 10.1002/bies.200800168.
14. **Reijrink, I. A. M., Berghmans, D., Meijerhof, R., Kemp, B., Van den Brand, H., 2010:** Influence of egg storage time and preincubation warming profile on embryonic development, hatchability, and chick quality. *Poult. Sci.*, 89 (6), 1225—1238. DOI: 10.3382/ps.2009-00182.
15. **Reijrink, I. A. M., Meijerhof, R., Kemp, B., Van Den Brand, H., 2008:** The chicken embryo and its micro environment during egg storage and early incubation. *World's Poultry Science Journal*, 64 (4), 581—598. DOI: 10.1017/S0043933908000214.
16. **Rocha, J. S. R., Baiáo, N. C., Barbosa, V. M., Pompeu, M. A., 2013:** Negative effect of fertile egg storage on the egg and the embryo and suggested hatchery management to minimise such problems. *World's Poultry Science Journal*, 69 (1), 35—44. DOI: 10.1017/S0043933913000044.
17. **Silva, F. H. A., Faria, D. E., Torres, K. A. A., Faria Filho, D. E., Coelho, A. A. D., Savino, V. J. M., 2008:** Influence of egg pre-storage heating period and storage length on incubation results. *Rev. Bras. Cienc. Avic.* (Brasilian Journal of Poultry Science), 10 (1), 17—22. DOI: 10.1590/S1516-635X 2008000100003.
18. **Wineland, M. J., 2013:** Almacenamiento de huevos: Manteniendo la viabilidad embrionaria de los huevos almacenados. *Revista Plumazos*, 43, 16—18.

Received January 25, 2019
Accepted February 28, 2019