

The effect of eggshell colour on early embryo development in the ring-necked pheasant (*Phasianus colchicus*)

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Ring-necked pheasant females lay eggs with different shell colours, such as white, blue, olive or brown. Eggshell colour was found to be related to egg quality and hatchability. The experiment was carried out to determine the relationship between pheasant eggshell colour and embryo development during the first 72 hours of incubation. In total, 314 eggs (72 blue, 79 dark-brown, 78 light-brown and 85 olive) were collected and incubated. The Hamburger and Hamilton (HH) staging procedure [1951] was applied in the morphological evaluation of pheasant embryo preparations. After 24, 48 and 72 h of incubation, embryos reached the average stage of 4 HH, 10+ HH and 16 HH, respectively. The highest stage of development after 24 h of incubation was observed in embryos from blue eggs (4.23 HH) and the lowest from light-brown ones (3.96 HH), although the differences were not significant. Values of all the investigated traits of embryos (blastoderm and area vasculosa diameter, embryo length, pairs of somites and stage) from the blue-shelled egg group measured after 48 h were significantly lower in comparison to those of dark-brown eggs with a similar tendency observed in relation to eggs of other colours. These differences were not observed after 72 h of incubation. Egg weight loss after 72 h in the dark- and light-brown groups was significantly lower than in olive-shelled eggs. Moreover, significant correlations were found between some traits of the egg, embryo and extra-embryonic membranes in each time point of observations.

KEYWORDS: eggshell colour / embryo development / incubation / pheasant

Although the ring-necked pheasant is a niche species of poultry, it has been playing a very important role in the hunting economy for many years. However, in order to support the existence of wildlife pheasant populations farm-bred birds need to be introduced continuously.

Cost-effectiveness of breeding in this species mainly depends on the obtained number of healthy chicks. Unfortunately, generally reproduction results of ring-necked pheasants on farms vary and not always satisfactory. It is also well known that good hatchability of these birds and high chick quality depend on genetic and environmental factors. Therefore, there have been many researches aimed at clarifying a number of aspects of pheasant biology to improve ultimately the production results [Richards and Deeming 2001, Kirikçi *et al.* 2005, Krystianiak *et al.* 2005, Nowaczewski and Kontecka 2005, Kożuszek *et al.* 2009a, 2009b, Nowaczewski *et al.* 2013, Kontecka *et al.* 2014].

Pheasant females lay eggs varying in shell colour, from white through blue, olive to dark-brown. It must be emphasised that throughout the reproductive season each female lays eggs of the same colour, but the intensity of eggshell colour decreases as the laying period progresses [Bezděková 2007]. Moreover, a relationship was found between eggshell colour and the quality of eggs, the course of the embryogenesis process and subsequent hatchability [Kirikçi *et al.* 2005, Kożuszek *et al.* 2009a, 2009b]. For example, Kuźniacka *et al.* [2005] reported that pheasant eggs with a lighter shade of shell colour were characterised by lower weight and a thinner eggshell. Higher porosity and water vapour permeability of blue eggshells in pheasant eggs indicate their inferior quality in comparison with those of other colours [Krystianiak *et al.* 2005]. Research showed that blue-shelled eggs were also characterised by lower fertilisation rates and hatchability [Krystianiak *et al.* 2005, Kożuszek *et al.* 2009b]. In order to gain insight into the relationship between the colour of pheasant eggshells and hatchability, an investigation on the early embryonic development has to be carried out. Earlier studies on freshly laid pheasant eggs showed a higher number of blastodermal cells in dark-brown and olive-shelled eggs in comparison with light-brown and blue-shelled ones [Kożuszek *et al.* 2009b]. There are some morphologically distinguishable key traits facilitating the identification of the stage of early embryo development, related directly to the embryo and its extra-embryonic membranes [Hamburger and Hamilton 1961, Grzegorzówka and Michalska 2005]. To date no results of the investigations in this respect have been found in the available literature.

The objective of this study was to determine the relationships between pheasant eggshell colour and embryo development during the first three days of incubation.

Material and methods

Birds and recorded traits

The experimental material comprised eggs obtained from 1-year-old pheasants (*Phasianus colchicus*) kept outdoors in 25 aviaries (702 m² surface area per each) belonging to the Polish Hunting Association – Animal Breeding Center in Moszna, Poland. The reproductive flock consisted of 2600 females and 325 males. The mating sex ratio (male to females) was 1:8 in each aviary. A total of 104 females and 13 males were housed in each aviary (stock density was 6 m²/bird). During the reproductive period of pheasants (April – June) no lighting programme was used. All birds were fed *ad libitum* with a complete diet containing 11.30 MJ/kg metabolisable energy, 17% crude protein, 4% crude fibre, 0.8% lysine, 0.7% methionine, 3.5% calcium and 0.4% total phosphorus.

In total, 314 eggs were collected for the investigation at the beginning of reproduction (11th April) when the laying rate was 64.6%. These eggs were randomly selected from among all eggs laid that morning several hours after laying. Then experimental eggs were weighed (WPS 360C type balance, RADWAG, Poland) and divided into 4 groups according to their colours: blue, dark-brown, light-brown and olive. The eggs were not stored to avoid uncontrolled embryo development during storage as the additional effect. Some earlier papers on poultry suggested such an effect needs to be considered [Stępińska and Olszańska 1983, Grzegorzówka and Michalska 2008]. Prior to incubation all eggs were disinfected (Virkon, Naturan, Poland). The following numbers of eggs from each colour group were set to the incubator: 72 blue, 79 dark-brown, 78 light-brown and 85 olive. It had been planned to use min. 24 eggs per colour group after each incubation period. Incubation was carried out in the setting compartment (La Nationale, France) at a temperature of 37.5°C and 65% relative humidity. Eggs were automatically turned (90° per hour). After 24, 48 and 72 h of incubation, eggs from each group were shortly chilled at 20°C until opening and blastoderm preparations were made using black ink (KOH-I-NOOR HARDTMUTH) for background staining and 1% acetic acid (Sigma) to fix the blastoderm surface [Grzegorzówka 2010]. Using a magnifying binocular (PZO MSt 131, 25×) the morphological evaluation of pheasant embryos was conducted according to the chicken embryo development staging system proposed by Hamburger and Hamilton [1951]. There is no other satisfactory developmental pattern for early pheasant embryos.

The following traits were analysed: initial egg weight, egg weight loss up to 24, 48 and 72 h, blastoderm diameter (average from measurements in the vertical and horizontal embryo axes) after 24 and 48 h, the presence of pairs of somites after 24 h, the length of area pellucida (in the vertical embryo axis) after 24 h, the diameter of area vasculosa (average from measurements in the vertical and horizontal embryo axes) after 48 and 72 h, embryo length (from the highest point of brain to the lowest point on the caudal side) after 48 and 72 h, the number of pairs of somites after 48 and

72 h, the number of somite pairs enveloped by developing the amniotic fold after 72 h and the stage after all the three periods of incubation. Egg weight was measured using an electronic balance (RADWAG, 0.001 g accuracy) and other linear measurements using a digital calliper (PRO L150, 0.01 mm accuracy). Photographic documentation of embryos was prepared using a digital camera (SAMSUNG, PL200).

Statistical analyses

The statistical analysis was carried out using the SAS® package [SAS 2011]. Mean values and the standard error of mean (SEM) on pooled data for each trait of eggs and embryos were estimated within each time point of incubation: 24, 48 and 72 h. The significance of differences between eggs with different shell colours with regard to the analysed traits was individually verified by one-way ANOVA. The significance of differences between the means for each egg group was verified by Duncan's test. Moreover, Spearman's rank correlations were calculated for each pair of embryo and extra-embryonic membrane traits studied after 24, 48 and 72 h of pheasant egg incubation.

Results and discussion

Due to unfertilisation, in some cases of embryo early mortality and developmental anomalies or some problems with embryo preparation, the total number of recorded eggs was finally 222 (Tab. 1).

Table 1. Number of eggs analysed in the experiment

Eggshell colour	Time of incubation		
	24 h	48 h	72 h
Blue	16	17	15
Dark-brown	20	21	16
Light-brown	19	18	19
Olive	20	23	18
Total number	75	79	68

No significant differences in egg weight depending on shell colour were found. The highest fertilisation rate was observed in dark-brown eggs, while in the blue-coloured ones the lowest proportion of fertilised eggs was found. The values of this parameter were as follows: dark-brown (78.5%), olive (77.6%), light-brown (76.9%) and blue eggs (73.6%). Similar results showing the highest fertilisation rate in eggs with dark-brown eggshells were reported by other authors [Kuzniacka *et al.* 2005, Kożuszek *et al.* 2009b]. Incubation time and hatchability may depend on incubation parameters and potentially also other factors [Nowaczewski and Kontecka 2005, Kożuszek *et al.* 2009b, Kontecka *et al.* 2014], which may cause some differences in early embryo development between the pheasant and other poultry species, e.g. chicken, and even within the same species. Egg characteristics and embryo parameters

in each eggshell colour group are summarised in Table 2. Generally the analysed early pheasant embryo development was slightly slower than the chicken [Hamburger and Hamilton 1951, Sellier *et al.* 2006].

Embryos after 24 h of incubation

After 24 hours of incubation, pheasant embryos reached the average stage of 4.14 HH (ranging from 3+ HH to 5+ HH) (Fig. 1). The stage of development in chickens after 24 h of incubation is usually around 6 HH, when the head fold starts to visibly mark the anterior end of the embryo proper, and 7 HH with one pair of somites and neural folds visible in the embryo [Hamburger and Hamilton 1951]. Current studies on pheasant embryos showed an earlier stage of HH in all investigated colour groups. Comparative development described by Sellier *et al.* [2006] showed a later stage in the goose (5 HH) and Japanese quails (6 HH) and lower than in our investigation observed in the turkey (XII EGK), the Muscovy duck (XIV EGK) and the Guinea fowl (3 HH).

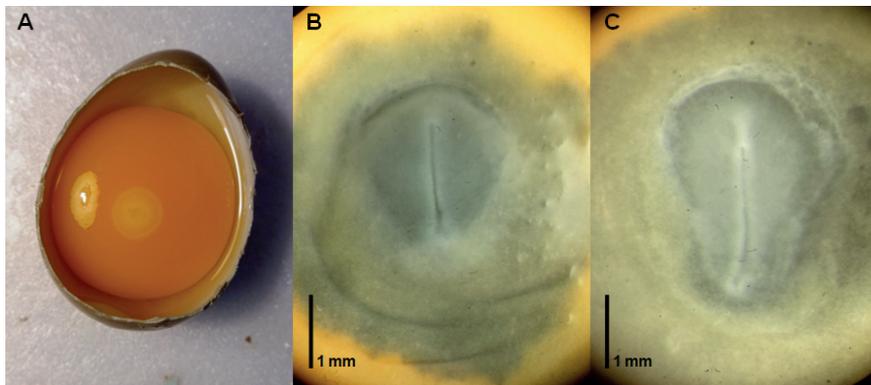


Fig. 1. Pheasant embryos after 24 hrs of incubation, typical stages: A – blastoderm visible in the centre of egg-yolk (\varnothing 8.5 mm), B – embryo in the mean stage 4 HH, C – embryo in the maximum stage 5 HH.

The latest average stage of development was observed in embryos from blue eggs (4.23 HH, with the maximum stage of 5+ HH) and the earliest from light-brown eggs (3.96 HH, max 3+ HH), although the differences were not statistically significant (Tab. 2). The most typical element morphologically distinguishable in embryos after 24 h of incubation was a primitive streak in its maximum length with the present primitive groove in the middle and Hensen's node on the front end (stage 4 HH) (Fig. 1B) or even a head-process visible forward from the caudally regressing node (stage 5 HH) (Fig. 1C). None of the embryos examined at that incubation period had somite-forming structures.

The most advanced extra-embryonic membrane development was observed in olive eggs. Both blastoderm diameter and the length of area pellucida were slightly greater than in differently coloured groups of eggs (Tab. 2). The blastoderm diameter

Table 2. Characteristics of eggs and embryo development traits in the pheasant depending on eggshell colour

Trait	Eggshell colour				
	blue	dark-brown	light-brown	olive	pooled SEM*
Initial egg weight (g)	33.16 ^a	33.04 ^a	32.47 ^a	32.59 ^a	0.49
after 24 h of incubation					
Egg weight loss (%)	0.81 ^a	0.79 ^a	0.81 ^a	0.85 ^a	0.03
Blastoderm diameter (mm)	7.52 ^a	6.96 ^a	6.90 ^a	7.56 ^a	0.36
Area pellucida length (mm)	3.06 ^a	3.11 ^a	3.13 ^a	3.26 ^a	0.11
Stage (HH)	4.23 ^a	4.16 ^a	3.96 ^a	4.21 ^a	0.10
after 48 h of incubation					
Egg weight loss (%)	1.36 ^a	1.37 ^a	1.40 ^a	1.49 ^a	0.04
Blastoderm diameter (mm)	21.13 ^b	24.30 ^a	22.92 ^{ab}	21.99 ^{ab}	0.25
Area vasculosa diameter (mm)	6.60 ^b	7.46 ^a	7.27 ^{ab}	6.89 ^{ab}	0.23
Embryo length (mm)	5.19 ^b	5.94 ^a	5.85 ^a	5.51 ^{ab}	0.17
Pairs of somites (no.)	8.94 ^b	11.86 ^a	11.92 ^a	10.61 ^a	0.57
Stage (HH)	9.65 ^b	10.71 ^a	10.62 ^a	10.33 ^a	0.19
after 72 h of incubation					
Egg weight loss (%)	2.03 ^{ab}	1.87 ^b	1.85 ^b	2.37 ^a	0.11
Area vasculosa diameter (mm)	17.57 ^a	16.68 ^a	16.50 ^a	17.09 ^a	0.43
Embryo length (mm)	8.41 ^a	8.13 ^a	8.12 ^a	8.34 ^a	0.11
Pairs of somites (no.)	26.73 ^a	26.28 ^a	26.18 ^a	26.53 ^a	0.49
Somites under amniotic fold (no.)	14.73 ^a	13.84 ^a	13.82 ^a	14.97 ^a	0.93
Stage (HH)	16.13 ^a	15.95 ^a	15.86 ^a	16.01 ^a	0.17

^{ab}Means bearing different superscripts differ significantly at $p \leq 0.05$.

*Standard error of the mean.

ranged from minimum 4.4 mm (light-brown) to maximum 10.79 mm (blue). The length of area pellucida measured in the longitudinal (anterior-posterior) embryo axis ranged from 2.29 mm (dark-brown) to 4.16 mm (olive).

The differences in all observed traits after 24 hours of incubation were not statistically confirmed. However, there were some significant Spearman's correlations estimated for each pair of traits, indicating the relationship between egg weight loss measured in grams and initial egg weight (0.328, $p=0.0055$), percentage weight loss (0.866, $p<0.0001$), between blastoderm diameter and area pellucida (0.672, $p<0.0001$), as well as stage (0.701, $p<0.0001$).

Embryos after 48 h of incubation

After 48 hours of incubation, new details in developing embryos and extra-embryonic membranes became morphologically distinguishable (Fig. 2). The entire blastoderm expanded to an average diameter of 22.63 mm (min. 13.98 mm and max. 30.88 mm, both in the blue-coloured group) with little difference in each eggshell colour group (Tab. 2). An early stage of area vasculosa formation was observed with extra-embryonic blood islands aggregating to capillaries (prospective blood vessels) close to this area border called sinus terminalis. In more advanced blastoderms, reddish-coloured vessels were visible. However, the heart was not yet present outside

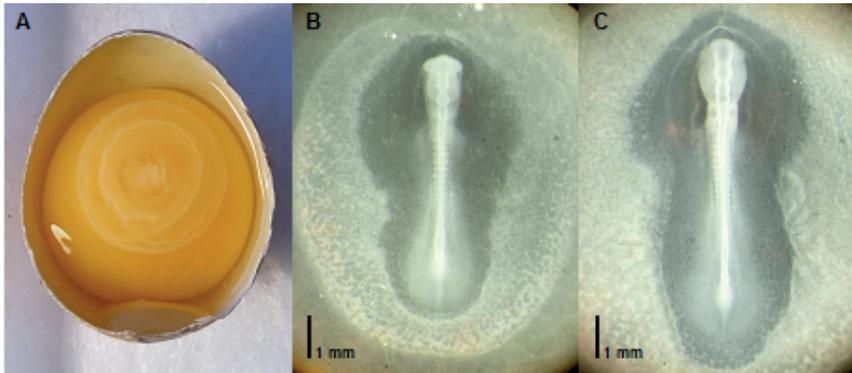


Fig. 2. Pheasant embryos after 48 hrs of incubation, typical stages: A – blastoderm visible in the egg-yolk ($\varnothing 22$ mm), B – embryo in the mean stage 10 HH, C – embryo in the maximum stage 12 HH.

the right side at the level of the head region. The average diameter of area vasculosa was 7.06 mm and varied slightly between groups. The lowest value was observed in blue eggs (with a minimum of 4.57 mm), whereas in the light-brown group sinus terminalis reached the maximum diameter of 9.87 mm.

All investigated traits of the blue-shelled egg group indicating embryo development showed on average significantly lower values in comparison to dark-brown-shelled eggs (Tab. 2). In the case of the other tested pairs of colour groups, the differences were not confirmed statistically.

After 48 h of incubation, the head amniotic fold together with lateral amniotic folds extend caudally and rise, progressively enveloping brain vesicles [Moigne 1999]. The mean stage of development reached by pheasant embryos after 48 h of incubation was 10+ HH, ranging from 8 HH (olive) to 12 HH (found in all groups) (Fig. 2). Chicken and goose embryo stages after this period were more advanced (12 HH each) than in our investigations, whereas those of the turkey, Muscovy duck and Guinea fowl were less advanced (7 HH, 5 HH and 9 HH, respectively) [Sellier *et al.* 2006]. After 48 hours of incubation Japanese quail embryos reached on average the stage of 14 HH [Grzegorzówka and Michalska 2005, Sellier *et al.* 2006].

The embryos were situated in a straight line with length of 5.64 mm on average (ranging from minimum 3.88 mm to maximum 7.41 mm, both in the blue-coloured group). In this period, neurulation and somitogenesis processes are evident. The head region was formed from three main vesicles: the forebrain (prosencephalon) with eye vesicles visible on both sides, the midbrain (mesencephalon) and the hindbrain (rhombencephalon) usually forming a series of smaller neuromeres. In more advanced embryos, the head fold of the amnion started to form as it can be seen in Figure 2C (a light line surrounding the cephalic part). The average number of somites in pheasant embryos after 48 h of incubation was 10.88 pairs with the smallest number of 4 pairs found in blue eggs and the highest in the dark-brown-shelled egg group numbering 16 pairs.

Spearman's correlation estimated for each pair of traits measured after 48 h of incubation indicated highly significant relationships in some cases, such as between the blastoderm diameter and diameter of area vasculosa (0.777, $p < 0.0001$), the number of somite pairs (0.752, $p < 0.0001$), embryo length (0.777, $p < 0.0001$), stage (0.744, $p < 0.0001$); between area vasculosa diameter and somite pair number (0.781, $p < 0.0001$), embryo length (0.884, $p < 0.0001$), stage (0.780, $p < 0.0001$) and between the number of somite pairs and embryo length (0.860, $p < 0.0001$) as well as stage (0.984, $p < 0.0001$). Moreover, significant correlations were found between egg weight loss during 48 h of incubation measured in grams and initial egg weight (0.340, $p = 0.0027$) and percentage egg weight loss (0.813, $p < 0.0001$) at that time point. A greater egg weight loss had no effect on embryo development during the first two days of incubation.

Embryos after 72 h of incubation

The significant differences between eggshell colour in relation to egg weight loss were observed only after 72 hours of incubation. Both dark- and light-brown eggs were characterised by a lower value of the trait (Tab. 2). Earlier investigations on pheasant eggs showed thinner shells and poor hatchability in blue-shelled eggs, which may reflect high rates of weight loss [Richards and Deeming 2001]. For example, Krystianiak et al. [2005] reported that eggs with blue shells were characterised by the highest water vapour conductance. This was probably due to higher porosity of the blue-shelled eggs, because the total number of pores in these shells was significantly higher (by 443) when compared to the other colour groups of eggs.

There was no significant effect of shell colour on the investigated parameters of embryo and extra-embryonic membrane development. The developing blastoderm with the membrane edges covering all the visible sphere of the egg yolk made it impossible to measure the simple diameter as a two-dimensional layer. Area vasculosa was clearly developed with vitelline veins and arteries connecting the embryo with sinus terminalis (Fig. 3). Moreover, the heart beat and blood circulation were visible.

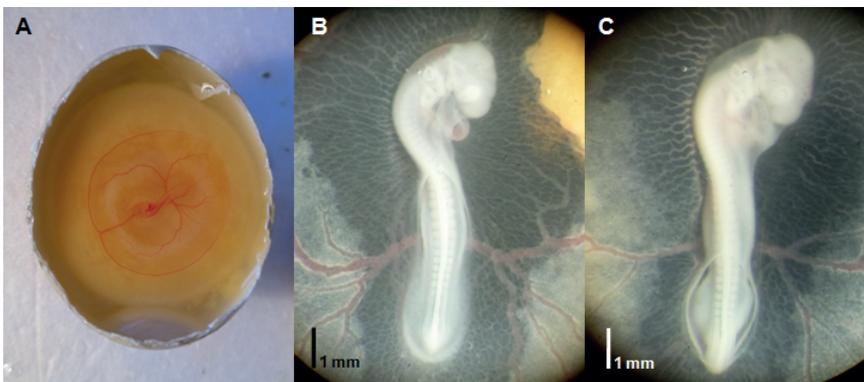


Fig. 3. Pheasant embryos after 72 hrs of incubation, typical stages: A – area vasculosa visible around the embryo (\varnothing 20.6 mm), B – embryo in the mean stage 16 HH, C – embryo in the maximum stage 17 HH.

The average diameter of area vasculosa after 72 h of incubation was 16.92 mm with the range from 11.59 mm (dark-brown eggs) to the maximum value of 22.08 mm (blue eggs). However, the group averages did not statistically differ (Tab. 2).

After 72 hours of incubation, all embryos were seen rotated with their anterior part and lying with their left side of the head turned to the yolk surface. In addition, five brain vesicles were developed and the cranial flexure on the level of mesencephalon vesicle was visible.

Moreover, in the cephalic region the lens vesicle formed from the lens placode with the optic cup (from the optic vesicle) and the auditory pit arising from the auditory placode were commonly distinguishable. In more advanced embryos, fore- and hind-limb buds, an elongating tail bud, and the progressive development of the amniotic fold covering a larger part of embryo were also observed (Fig. 3).

According to these morphological characteristics, the main stage of development was evaluated on 16 HH with the minimal 14 HH stage observed in both brown groups and maximum 17 HH reached in all four investigated groups. For comparison, the average stage of development observed by Sellier et al. [2006] after 72 h of incubation in other species was as follows: 20 HH in the chicken, 19 HH in the Japanese quail, 16 HH in the goose, 15 HH in the Guinea fowl and 12 HH in the Muscovy duck.

An average number of 26.42 somite pairs was observed after 72 hours of incubation, with the minimum number of 21 found in light- and dark-brown eggs and the maximum of 30.5 reached in olive-shelled eggs. The amniotic fold at that time point covered from 3 (light brown) to 22 pairs of somites with an average number of 14.33 pairs. In the chicken at this stage folds reach 17-20 pairs of somites [Hamburger and Hamilton 1951]. After 72 h of incubation, chicken embryos usually reach stage 19 HH, with 37–40 pairs of somites, a curved tail-bud, clearly visible limb buds and a small pocket of the allantois [Hamburger and Hamilton 1951].

There were some slight differences in embryo traits between eggshell colour groups, but they were not confirmed statistically (Tab. 2).

Among the traits studied after three days of incubation, there were significant ($p \leq 0.01$) Spearman's correlations confirmed between all pairs of embryo and extra-embryonic membrane traits (Tab. 3). Most of them exceeded the value of 0.6. Moreover, significant correlations were found between egg weight loss measured

Table 3. Spearman's rank correlations confirmed for embryo and extra-embryonic membrane traits studied after 72 hrs of pheasant egg incubation

Trait	Embryonic length	Pairs of somites	Somites under amniotic fold	Stage
Area vasculosa diameter (mm)	0.645***	0.821***	0.769***	0.769***
Embryonic length (mm)		0.657***	0.543***	0.549***
Pairs of somites (no.)			0.883***	0.943***
Somites under amniotic fold (no.)				0.880***

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

in grams and initial egg weight (0.301, $p=0.0148$) and percentage egg weight loss during incubation (0.886, $p<0.0001$). Other authors [Krzanowska 1959, Coleman *et al.* 1964] found a small or no relationship between egg weight and the rate of early embryonic development in chickens. Researches on the Japanese quail indicated low and in some case negative or non-significant correlations between egg weight and early embryo development traits [Korzyńska-Nowak 1990, Korzyńska-Nowak and Michalska 1992/93, Grzegorzółka and Michalska 2005].

As expected, early embryo development in the pheasant is slower than in the chicken [Hamburger and Hamilton 1951, Sellier *et al.* 2006], especially because of the difference in the incubation period needed for each species. The blue-shelled egg group showed a delayed early embryo development in comparison to the dark-brown group with a similar tendency observed in relation to eggs of other colours. On the contrary, the phenomenon of growth compensation cannot be excluded, so research on later stages of development would complement this issue. Moreover, the diverse embryo growth rate indicates that different shell-coloured eggs might require adjustment of incubation conditions to reach the same level of hatchability as eggs in the other colour groups.

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