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Movement of chick embryo (*Gallus domesticus* L.) towards the blunt end of the egg in relation to shell pore density and air cell size

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Abstract: The chicken (*Gallus domesticus* L.) eggshells not only protect the contents of the egg from the microbes and physical stress but also control the exchange of water and gases through pores during the extra-uterine growth and development of embryo. The number of pores in blunt end statistically differed ($p<0.01$) from that in the equatorial region and sharp end of the egg. Increase in the air cell circumference had significant association with the pore density ($r=0.39$; $p<0.01$) of the eggshell. The pores and the underlying shell membranes form conductance which is gradually increased for the developing embryo in accordance with onward incubation day resulting on the gradual increase of air cell membrane circumference and thus the chorioallantoic membrane (CAM) fused with air cell membrane. There were traces of some holes at the 10th day of incubation on the inner shell membrane which might be a sign of the initiation of internal pipping. The developing embryo gradually changed its position towards the air cell at the blunt end side of the egg for the optimum exchange of respiratory gases facilitated by the highest number of pores. Thus, it could be noted that the embryo movement towards the blunt end is related to eggshell pore density and air cell size.

Key Words: *Gallus domesticus*, Eggshell pore, Air cell, Development, Pipping, Embryonic movement

Introduction

The avian eggshell protects the developing embryo (Ar *et al.,* 1979) through exchange of gas and water (Burley & Vadehra, 1989) and its functional and structural properties ensure the success of embryonic development in birds (Wagner-Amos & Seymour, 2002, 2003; Nys *et al.*, 2004; Massaro & Davis, 2005). The porosity of the eggshell is very important feature for the development of embryo. During development, the embryo respires by oxygen and carbon dioxide through diffusion across the pores in the eggshell and thus the necessary communication between the developing embryo and the external environment become established (Ar & Rahn, 1985) and their density is equal over the entire eggshell surface (Visschedijk & Rahn, 1983). But the other studies provide clear evidence that there are regional changes in pore number in the egg (Booth, 1989; Soliman *et al.,* 1994) *i.e.* the basal region of egg contains increased number of pores compared to that of equator and apex (Rokitka & Rahn, 1987). This structure of eggshell helps to provide available oxygen which limits the growth of birds' embryo (Zimmerman & Hipfner, 2007) and thus the porosity is important for the hatchability of eggs (Mao *et al.,* 2007).

There are two fibrous eggshell membranes attached to the inner side of the eggshell, which separate the shell proper from the egg contents (Romanoff & Romanoff, 1949). After egg laying, cooling enables the air cell, an air space between the inner and outer eggshell membranes to form by their separation at the blunt end of the shell (Brand *et al.,* 2013). The air cell then expands as water is lost from the egg and serves as the site for embryonic gas exchange during incubation (Rokitka & Rahn, 1987; Rahn & Paganelli, 1990; Mao *et al.,* 2007).

The chorioallantoic membrane (CAM), a specialized vascular membrane, is a fusion of the chorion and the allantois that lies attached to most of the inner eggshell membrane (Romanoff, 1960; Rol'nik, 1970). During avian embryonic development, gas exchange essential to growth and survival of the embryo occurs *via* the CAM (Rokitka & Rahn, 1987). It grows primarily in the equatorial and apical regions of the egg, eventually expands to cover nearly the entire inner surface of the eggshell (Paganelli, 1980; Tazawa, 1980).

The air cell volume increases in size during incubation which can potentially be influenced by any deviation from normal embryonic development (Brand *et al.,* 2013). It is thus hypothesized that measurement of the air cell circumference on specific days of the artificial

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incubation process may indicate whether the embryonic development is proceeding normally or not. The presence of maximum number of pores in the eggshell on the air cell enhances the volume of air cell. Thus, the aim of the study was to compare the numerical density of pores in the sharp, equatorial and blunt ends and to determine the size of air cell membrane during onward incubation days which might have relationship with the embryo movement towards the blunt end of chick egg.

Materials and Methods

Study specimens

Forty six fertile eggs (unhatched and hatched) of layer chicken (RIR, Rhode Island Red) were used for this study. Only one pair was hatched eggs (21st incubation day) and two pairs were unincubated eggs for blastoderm observation. The incubated eggs were purchased daily for 21 incubation days from a local poultry farm under the Rajshahi City Corporation in Bangladesh. Twenty one eggs were used for pore counts and 19 (the egg at the 1st day of incubation for indistinct size and another one at the 21st day for rupturing of the air cell membrane during hatch were excluded) for air cell circumference and embryo movement study. After embryo removal the shells were washed with distilled water and dried at room temperature (28 ± 2^0C) for further uses.

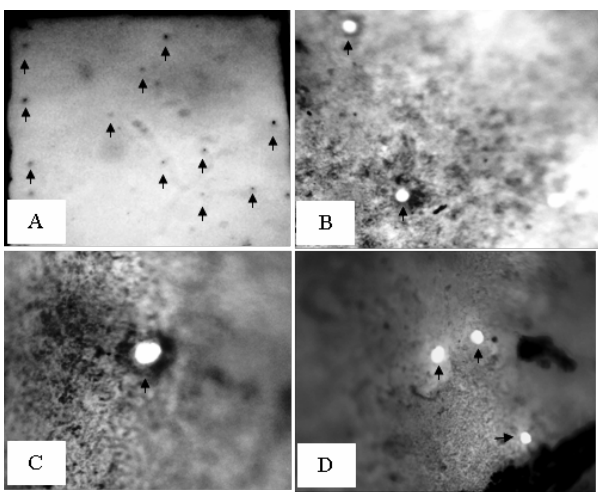


Fig. 1. Eggshell pores (arrows) in the representative eggshell portion (A) after removal of cuticle. B for equatorial region, C for sharp end and D for blunt end of the eggs. A= 0.5 cm^2; B, C and D at 10x magnification each.

Collection of eggshell

The incubated but unhatched eggs were decanted carefully and eggshells were collected from them. The residual albumin was removed from the eggshell with the flow of running water and the eggshells were kept open on the table at room temperature mentioned earlier for drying.

Removal of cuticle

The dried eggshells of the blunt end, equatorial region and sharp end were cut into several pieces (0.5 cm^2) and kept in 200 mM EDTA (ethylene diamine tetraacetic acid) solution for cuticle removal. The cuticle was removed for the clearance of pores in the representative eggshells.

Counting of pores and measurement of air cell circumference

The counting of pores in each piece of the eggshell (tip of both ends and any unit randomly selected from the equatorial region) was done through light microscopic observations (Fig. 1). The air cell circumference was measured by a piece of yarn (Fig. 2 F) with the help of a measuring scale (Fig. 3 G).

Fig. 2. Air cell in the blunt end of egg (E) and a piece of yarn around the air cell to measure its circumference (F).

Pipping of embryo

On the 10th day the incubated eggs were broken carefully and the possible trace of internal pipping (by egg tooth) on the air cell membrane of the embryo side was observed by small holes. It continued and followed by scratching in the inner shell membrane (*i.e.,* air cell membrane) on the 19th day until external pipping which started on the 20th incubation day. The first appearance was just holes like structure on air cell membrane (Fig. 3 H) and later as crack in the shell (Fig. 7 h). After the 20th day (21st day), the chick hatched through breaking of eggshell lining of the equatorial region.

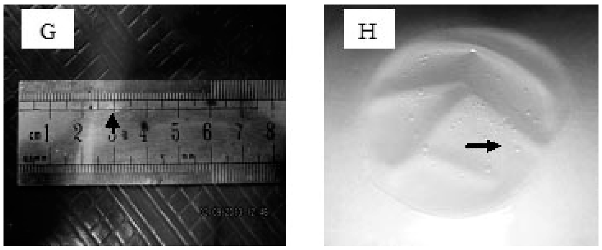


Fig. 3. A measuring scale to record the circumference of air cell with the help of a piece of yarn (small arrow) (G) and the holes indicated by large arrow (H) are the trace of probable internal pipping by egg tooth during the 10th day of incubation.

Developmental changes and movement of embryo towards the blunt end

The embryo development occurred day by day with the onward incubation day (Rahman *et al.,* 2014) and the position of the developing embryo was monitored. The embryo occupied the space inside the egg and showed gradual position change towards the air cell membrane at the blunt end of egg (Fig. 7 a-h).

Photography

A Cannon IXY camera (10 mega pixels; made in Japan) was used to take photographs of the experimental events.

Statistical analysis

Descriptive statistics, one way ANOVA and coefficient of correlation were used to analyze the data of interest.

Results and Discussion

Number of eggshell pores and air cell circumference

The mean ± SD values of pores in the blunt end, equatorial region and sharp end were 32.52±5.87, 24.09±5.12, 18.00±3.04 respectively per 0.5cm^2 of eggshell (Table 1). The number of the pores varied significantly ($p<0.01$; $F_{2,60}=47.83$). The pores from the sharp end and equator were prominent and light intensive compared to those of the blunt end (Fig. 1, B - D). The air cell circumference was 8.18±1.76 cm (Table 1). The pores from the blunt end of eggs showed higher in number than that of the equatorial region and the sharp end (Fig. 5). The air cell circumference showed gradual increase with the onward incubation days (Fig. 6).

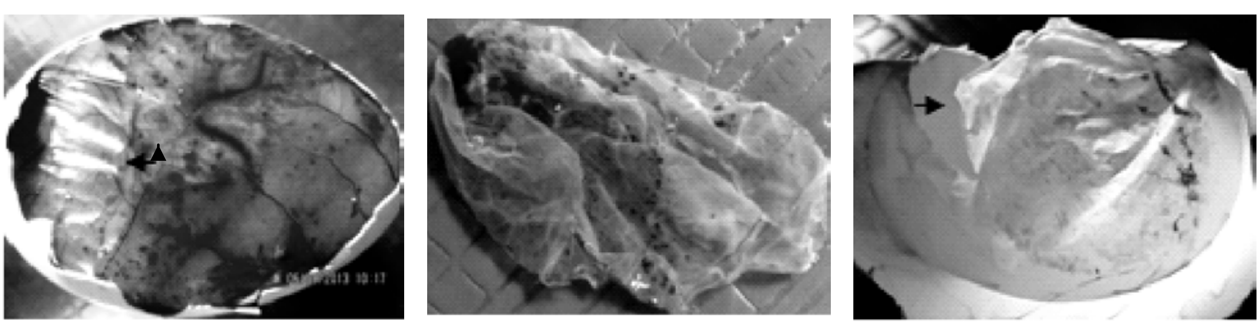
Increase in the air cell circumference

The incubation day-wise air cell circumference and its estimated gradual increase are shown in Fig. 6. The air cell circumference was increased with the gradual expansion of air cell volume. This is happened for the optimum respiratory gases exchanged by embryo during development.

Table 1. Descriptive statistics for traits recorded from eggs of hen.

Part of egg	Traits	Mean±SD	Range		Count (N)
			Min	Max	
Blunt end	Pore number	32.52±5.87	21	42	21
Equatorial region	Pore number	24.09±5.12	15	34	21
Sharp end	Pore number	18.00±3.04	13	25	21
Air cell	Circumference	8.18±1.76	5.5	11	19

SD: standard deviation; Min: minimum; Max: maximum.



(a) CAM adhered to the air cell membrane (b) CAM (c) Air cell membrane

Fig. 4. Adherence of air cell membrane to chorioallantoic membrane (CAM) (arrow) in a; b and c (arrow) show CAM and air cell membrane, respectively.

Fig. 5. Changes in the number of eggshell pores in different parts of the egg at different incubation days.

Adherence of CAM to the inner shell membrane (air cell membrane)

Increase in the air cell circumference had significant association with developmental periods and pore density of eggshell. The coefficient of correlation (r) between the air cell circumference and pore density at the blunt end was 0.39 ($p<0.01$). The pore density was the highest at the blunt end of egg (Fig. 5) and where CAM (Fig. 4b) adhered to the inner face of air cell membrane (Fig. 4a).

The movement of embryo towards the blunt end

On the 10th day of incubation, the embryo possibly made several hole like structures in the air cell by egg tooth (Fig. 3H). The embryonic position at 14 days of incubation seemed to be attached with interior portion of the air cell membrane (Fig. 4a). As the incubation days proceeded, the distance between the CAM and air cell membrane gradually decreased (Fig. 7 a-h).

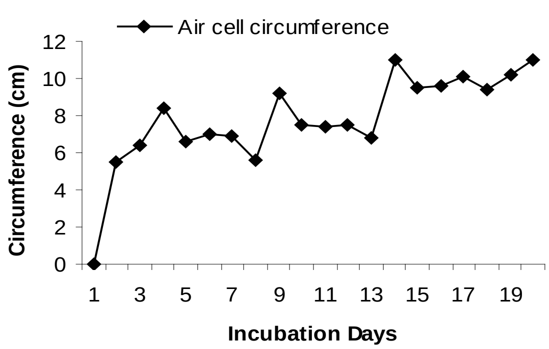


Fig. 6. Changes in the air cell circumference at different incubation days.

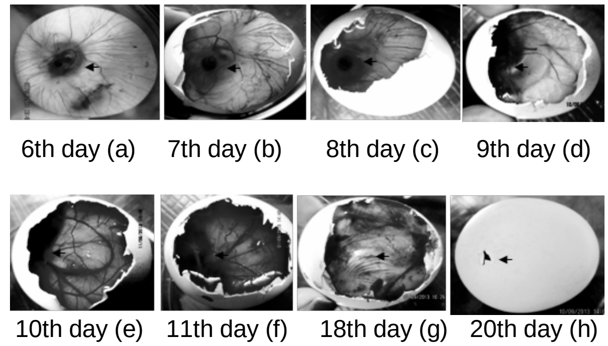


Fig. 7. Gradual movement of embryo towards the blunt end and air cell. Arrows (a-g) indicate the gradual changes of the position of embryos during the incubation period. The arrow of 'h' indicates the cracking of eggshell started before hatch.

The pore density increased with the increase of incubation day in the peking duck eggs *Anas platyrhynchos* f. *dom.* (Balkan *et al.,* 2006) resulting in increased egg permeability (El-Hanoun & Mossad, 2008), especially at the blunt end in comparison to that of the sharp end or the equatorial region (Zhang & Wang, 2012) which is also recently reported in chicken eggshell (Rodríguez-Navarro *et al.,* 2013). Rahman *et al.* (2009) reported that the pore formation in eggshell occurred in the uterus of Japanese quail (*Coturnix japonica*) during laying cycle. This is why the density would be the same in different incubation period of the same egg in the same portion of egg from the same hen *Gallus domesticus* (Simkiss, 1961). As the blunt end of egg contains the highest pore density, it facilitates much air into and out of the eggshell. The findings of this study explored that there is a quantitative difference in pore density in different portions (*i.e.* blunt end>equatorial region>sharp end) in the artificially incubated chick eggs which is in agreement with a number of previous reports (Christensen, 1983; Booth & Seymour, 1987; Rokitka & Rahn, 1987; Booth, 1989; Soliman *et al.,* 1994; Balkan *et al.,* 2006; Iwasawa *et al.,* 2010).

The air cell is most important during the later stages of development just before hatching and during internal pipping, the process in which the chick punctures the air-cell and pulmonary respiration begins (Mao *et al.,* 2007). Later it has been reported that the embryo remains closer to the air cell by which it can facilitate easy transport of respiratory gases and waste products in the blunt end of eggs (Kjelland *et al.,* 2012). The air cell enlarges consequently during incubation, due to evaporation from the egg content (Brand *et al.,* 2013). This is consistent with the present study where the gradual increase of air cell circumference occurred concomitantly with the onward incubation days. It occurred due to the optimum ventilation of respiratory air through the highest pore numbers at the blunt end of egg which is similar to the reports of Balkan *et al.* (2006).

The gas exchange between the embryo and the air diffused into the egg takes place through specialized and highly vascularized CAM in avian eggs (Ackerman & Rahn, 1981; Rokitka & Rahn, 1987; Onagbesan *et al.,* 2007). The oxygen for respiration diffuses in from the environment *via* pores in the eggshell to the CAM and its thickness under the air cell is always lower than the rest of the CAM implies that respiratory gas exchange is higher in this portion (Reizis *et al.,* 2005). Carbon dioxide waste from the embryo accumulated by diffusion in the air cell through the capillaries of chorioallantois (Tazawa, 1980). The blood vessel numerical density under the air cell on different incubation day was higher than the rest of the CAM and this is very important for the developing embryo because the living embryo adapts by varying angiogenesis in relation to the shell permeability to gases (Reizis *et al.,* 2005). The present study revealed that the inner surface of air cell membrane fused together with the CAM (Fig. 4a) and it might be resulted from the enlargement of that membrane during incubation in a day-dependent manner (Fig. 6). Thus it is assumed that this adherence may be for the position change of embryo resulted from the continuous increasing uptake of respiratory gases.

The internal and external pipping started at the 19th day and the 20th day of incubation, respectively (Rahman *et al.,* 2014) where there was a scratch opened externally through eggshell in this species. In contrast, Meir & Ar (1996) reported that drilling holes into the air cells on days 15 to 22 of incubation increased hatchability of goose eggs. At the 10th day of incubation, traces of several holes (Fig. 3H) were found to be present on the membrane (inner side) of air cell in this study. A temporary 'egg tooth' on the dorsal tip of the upper beak (Rahman *et al.,* 2014) developed at this incubation day that might enable the chick to 'pip', which might be an initiation of 'internal pipping'.

The pores of the egg are plugged inside the shell surface when the egg is laid and during incubation, embryonic ossification leads to the unplugging of these pores making them functional. It indicates that the inside mouth of the plugged pores contain loosely packet crystals of calcium carbonate and during incubation these crystals are dissolved away (Booth, 1989). The present study explored that the light pass images through the eggshell pore were less prominent in the blunt end in comparison with that of equatorial region or the sharp end. It might be for the uptake of calcium carbonate by the embryo residing vicinity of the sharp or equator region of the egg during incubation.

The gas diffusion under the air cell increased and there is a increased blood vessel numerical density in chick embryo during incubation (Reizis *et al.,* 2005) whereas Dunker (1978) previously reported that the movement of the blood capillaries occurred towards the inner shell membrane and lay close to the outer surface of it from day 14 onwards. The movement of the chick occurred throughout the internal pipping until external pipping in ostrich egg (Brand *et al.,* 2013). Embryonic mortality resulted from insufficient pore number of the eggshell (Peebles & Brake, 1985) and embryo development occurred next to the air chamber (Kjelland *et al.,* 2012). Thus, this embryo positioning was related with the availability of optimum respiratory gaseous exchange performed by the highly permeable eggshell of the blunt end of egg. After incubation the embryo oriented in quail egg and the anterior posterior axis of embryo appeared in day three of incubation (Rahman & Yoshizaki, 2012). Then the embryo might start to change its position gradually from the site of orientation towards the blunt end of egg. It is because of gradual increase of demand of respiratory gases by developing embryo in the incubated eggs. This is accompanied by the gradual expansion of air cell circumference resulted from the gradual increase of air cell volume by high density of eggshell pores at the blunt end of egg. A slow journey through this position change towards the blunt end might be the first time reporting of embryo movement from its origin to blunt end of the hen's egg.

Hatchability gets it optimum success by smooth embryo development and researchers have investigated many parameters for the evaluation of hatchability in chicken or poultry species. These parameters have been incorporated into commercial selection programs aimed at improving the success and reduction of hatching. Many scientific studies during the past century have shown a strong relationship between eggshell pores and gaseous exchange and also gas conductance through air cells. The gradual increase of air cell circumference provides continuous expansion of air spaces with respiratory gases can be used to predict embryo development throughout the hatching period in ostrich egg (Brand *et al.,* 2013) and this is accomplished by highest number of pores at the blunt end of egg. To meet up the gradual demand of respiratory gases, the embryo moves gradually towards the air cell at the blunt end as incubation day proceeds. This is why the developing embryo movement has relation to the increase of air cell membrane and pore number in eggshell during incubation of chick egg.

Conclusion

A significant relationship between air cell circumference and pore density indicated an optimized embryonic gas exchange. The results of the present study seem to explain that the living embryo adapts by varying air cell circumference in relation to the pore density at the blunt end of chicken egg. As the embryo grows with the onward incubation day, its gradual demand of respiratory gases induces the gradual increase of air cell circumference. The embryo moves towards the blunt end of egg and takes gradual position next to air cell on the basis of incubation day. Thus, results of this study could be helpful to understand the smooth development of chick embryo for the determination of optimum hatchability. Whether this is applicable to other bird species, however, remains to be discovered.

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