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Regional and developmental variations of blood vessel morphometry in the chick embryo chorioallantoic membrane

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Summary

Avian eggs contain all the necessary materials for embryonic development except for oxygen, which diffuses in from the environment *via* pores in the hard, calcified eggshell to the chorioallantoic membrane (CAM), the respiratory organ, which is rich in blood vessels. An air cell is formed at the blunt pole of the egg between the two membranes of the eggshell and enlarges during incubation due to water vapor loss. In this study of the CAM of chicken eggs, we compared blood vessel numerical density [$N_{A(v)}$], area fraction of blood vessels [$A_{A(v)}$], CAM thickness (D_{CAM}), total length of blood vessels (L) and surface area of the CAM attached to the eggshell (CAM_{re}) with those under the air cell (CAM_{ac}) during incubation. We found that $N_{A(v)}$, $A_{A(v)}$, D_{CAM} and L of the CAM increase with embryonic age and development. The $N_{A(v)}$, $A_{A(v)}$ and L under the air cell were higher in relation to the rest of the CAM at all ages tested, while the D_{CAM} under the air cell was always lower than around the rest of the egg. Since the eggshell over the air cell has a relatively greater porosity, and the respiratory gas exchange ratio there is higher than at other areas of the egg, there is a correlation between all the above morphometric data and the eggshell porosity. This suggests optimization of embryonic gas exchange in the chicken egg. We would like to propose that, during natural incubation, an increased gas diffusion under the air cell, together with increased blood vessel numerical density, may compensate for covering of the central part of the eggshell by the incubating parent.

Key words: air cell, blood vessel numerical density, chick embryo, chorioallantoic membrane, morphometry.

Introduction

Avian eggs contain all the necessary materials for embryonic development other than oxygen, which diffuses in from the environment *via* pores in the hard, calcified eggshell. There are two fibrous eggshell membranes attached to the inner side of the eggshell, which separate the shell proper from the egg contents (Romanoff and Romanoff, 1949). During incubation, an air cell is formed at the blunt pole of the egg between the external and the internal eggshell membranes. The air cell enlarges during incubation, due to evaporation from the egg content, and normally occupies ~15% of the egg volume at the end of the incubation (Ar and Rahn, 1980).

There are ~120 pores cm^{-2} of ~17 μm diameter each, which traverse the 300 μm-thick eggshell of the domestic hen (Ar and Rahn, 1985). According to Rol'nik (1970), Rizzo (1899) was the first to study the histology of the eggshell. He found that the pores in the blunt, middle and pointed regions of eggs decline in number from 149 to 131 to 90 pores cm^{-2}, respectively. Romanoff (1943) and Romanoff and Romanoff (1949) found that permeability of the eggshell to an air pressure difference of 200 mmHg (26.7 kPa) is highly variable but averages higher at the blunt pole than at the small pole (14.5 and 10.5 ml cm^{-2} min^{-1} $mmHg^{-1}$, respectively, which are 108.76 and 78.76 ml cm^{-2} min^{-1} kPa^{-1}, respectively).

Rokitka and Rahn (1987) measured water vapor diffusive conductance of eggshells of six avian species and found an average decline of 88 and 63% of blunt end values, for the middle and the pointed end, respectively. These relationships corresponded to the changes in regional eggshell pore density. Romijn (1950) estimated that ~80% of the O_2 consumed towards the end of the incubation is supplied through the shell over the air cell area. However, direct measurements show that ~29% of the total O_2 consumption comes from the area over the air cell and that the respiratory gas exchange ratio (RQ) is higher under the air cell (0.75) compared with the rest of the chorioallantoic membrane (CAM) (0.68) while the total RQ is 0.7 (Visschedijk, 1968a,b). Seymour and Visschedijk (1988) have obtained similar results. Paganelli et al. (1988) analysed these ratios and concluded that they indicate a relative 'hyperventilation' over the air cell area, namely a higher ratio of gas diffusion rate through the shell to the corresponding CAM blood perfusion.

The CAM is a fusion of the chorion and the allantois, which start to develop on the 5th day of the 21 incubation days. On the 11th day, it is completely formed and lies attached under

most of the inner eggshell membrane (Romanoff, 1960; Rol'nik, 1970). In parallel, it begins its role as a respiratory organ. The CAM is rich in blood vessels and its capillaries migrate towards the inner shell membrane and lie close to the outer surface from day 14 (Duncker, 1978).

Dusseau and Hutchins (1988) followed the vascular density of the CAM with time from day 7 and found an increase in CAM vascular density index (VDI = intersections per unit area) of 36% and 68% on days 10 and 14, respectively. They found an additional increase in VDI of ~34% on these days in 15% O_2. Strick et al. (1991), who incubated chicken eggs in 12, 16, 21, 45 or 70% O_2 from day 7 to 14, showed that the graded exposure to O_2 produced a dose-related change in the VDI: hypoxia increased and hyperoxia decreased VDI. Corona and Warburton (2000) covered ~25% of chicken eggshells with beeswax before placing them in an incubator and measured VDI in the formed hypoxic region of the CAM on day 12 of incubation. They did not find VDI differences between the hypoxic and the control regions at this early stage. On the other hand, Wagner-Amos and Seymour (2003) demonstrated that artificially covering half of the shell with wax at the beginning of incubation reduced vessel density of the CAM under the covered area compared with untreated eggs.

As far as we know, there are no quantitative histological studies on the VDI, area fraction and CAM thickness under the air cell in comparison with the rest of the CAM. We hypothesized that local eggshell porosities and conductances, which are known to exist between the two zones, would be correlated with the local CAM blood vessel numerical density, area fraction and thickness. Thus, the aim of this research was to compare the blood vessel numerical density, area fraction of blood vessels and local thickness of the chick embryo CAM under the air cell with those of the rest of the CAM at different embryonic ages.

Materials and methods

Ninety-five fresh fertile chicken eggs (Cobb) were obtained from a commercial source (Y. Brown and Sons Ltd, Hod HaSharon, Israel). Eggs were weighed on a Shimadzu Bx 420H electronic balance (Kyoto, Japan) to the nearest mg and incubated under standard conditions (Victoria model V-34 incubator; Padova, Italy; 37.5°C; 55% RH, turned once every hour).

On days 10, 12, 14, 16, 18 and 20 of the incubation, 9–14 fertile eggs were randomly sampled. The eggshell above the air cell was removed and the CAM, including the shell membrane attached to it, was cut off for further examination. In addition, five pieces of 50–60 mm^2 CAM attached to the shell membranes were taken at random from other locations on the eggshell. All samples were immediately fixed in 10% buffered formaldehyde for at least 24 h. Tissues were embedded in paraffin, thick sectioned (4–6 μm), stained with hematoxylin–eosin and observed using an Olympus BX50 light microscope. Counts and measurements were performed using common morphometric approaches (Williams, 1977; Russ and DeHoff, 2000). Blood vessels from 8 μm (lowest limit of detection) in diameter were counted using the systematic sampling approach (Russ and DeHoff, 2000). In short, the tissue was divided into 5–10 equally distributed fields. Measurements were made in the center of each field.

Blood vessel numerical density

Counts of blood vessel numerical density [number per unit area; $N_{A(v)}$] were done with a 20× objective and using a projected calibrated square grid graticule (5×5) inside the 10× ocular. For each CAM sample (under the air cell and under the rest of the shell), the blood vessel profile was counted at 10 areas and the results were averaged for each sample.

Blood vessel area fraction

Counting of area fraction [$A_{A(v)}$] was done as described above for blood vessel numerical density using a calibrated square grid graticule (10×10). Each CAM sample was point counted at five different locations and the results were averaged. The total number of points hitting blood vessels [$P_{i(v)}$] and the reference tissue [$P_{i(t)}$] were counted. Thus, the area fraction (Williams, 1977) is:

$$A_{A(v)} = [\sum P_{i(v)}] / [\sum P_{i(t)}].$$

Thickness of the CAM

Measurements of the tissue thickness (D_{CAM}) were done with a calibrated objective of 40× and using a 10×10 calibrated grid in the 10× ocular. Each CAM sample was measured by the use of the projected and calibrated grid at five different locations and the results were averaged.

In paraffin-embedded tissue, material shrinkage is estimated to be ~25% relative to the fresh material (Mandarim-de-Lacerda et al., 1985, 1987; Mandarim-de-Lacerda, 2003). We assumed that, since all tissues were prepared similarly, tissue shrinkage is the same in both CAM zones. Thus, shrinkage corrections are unnecessary for tissue comparisons.

Total egg surface area was calculated from initial fresh egg mass (after Paganelli et al., 1974). We assumed that the area of the dome-shaped part of the shell, covering the air cell (blunt end), equals the area of the inner shell membrane to which the CAM under it is attached, since initially the shell membranes of the freshly laid egg adhere to the shell and presumably do not stretch or shrink during incubation. To calculate this area (S_{ac}), we used the following equation: $S_{ac}=2\pi Rh$, where R is the radius of a hemisphere (assuming that the blunt side of the egg is a part of a hemisphere). This R was measured at the widest part of the egg waist. h is the height of the line normal to the plane of an imaginary disc (of a radius r) formed by the line of contact at the edge of the air cell and the CAM and is calculated as: $h=R-\sqrt{R^2-r^2}$. A comparison with measured values of Romijn (1950) demonstrated a high correlation with our calculated values (r^2=0.923).

Since the total length of the blood vessels (L) is calculated as $L=2N_{A(v)}V$ (Williams, 1977; Russ and DeHoff, 2000), and

Table 1. *Chorioallantoic membrane (CAM) thickness (D_{CAM}), surface area (S_{CAM}) (calculated), blood vessel numerical density [$N_{A(v)}$] in the CAM, and area fraction of blood vessels [$A_{A(v)}$] under the air cell and under the rest of the eggshell of the chick embryo*

CAM sample zone	Embryonic age (day)						ANOVA (among days)
	10	12	14	16	18	20	
D_{CAM} (μm)							
Air cell	60.6±22.8	64.9±13.2	35.1±15.7	69.6±28.7	69.6±22.0	89.1±25.3	P<0.001*
Rest	67.4±26.4	79.8±26.1	49.4±20.0	77.0±35.9	85.7±21.0	110±38.3	P<0.001*
t-test	N.S.	P<0.01	P<0.01	N.S.	N.S.	P<0.01	
Air cell/rest ratio	1.0±0.3 (11)	0.8±0.2 (13)	0.7±0.3 (14)	1.0±0.5 (13)	0.9±0.3 (14)	0.8±0.2 (9)	N.S.**
S_{CAM} (cm)							
Air cell S_{ac}	9.6±0.4	10.9±0.4	12.5±0.5	13.9±0.8	15.0±0.5	16.0±0.8	P<0.001*
Rest S_{re}	72.0±3.1	68.4±2.3	67.9±2.7	66.1±3.6	64.0±2.3	57.7±3.0	P<00.01*
t-test	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	
Air cell/rest ratio	0.13±0.06 (10)	0.16±0.05 (13)	0.18±0.06 (13)	0.21±0.08 (12)	0.23±0.06 (13)	0.25±0.07 (6)	
$N_{A(v)}$ (vessels mm^{-2})							
Air cell	102±19	127±25	141±23	164±49	153±24	122±27	P<0.001*
Rest	62±10	84±11	88±11	93±39	83±13	61±17	P<0.001*
t-test	P<0.001	P<0.001	P<0.002	P<0.001	P<0.001	P<0.001	
Air cell/rest ratio	1.7±0.4 (10)	1.5±0.4 (13)	1.6±0.4 (13)	2.0±0.8 (12)	1.9±0.4 (13)	2.3±1.1 (6)	N.S.**
$A_{A(v)}$ (% of total area)							
Air cell	33.5±11.0	32.2±10.5	40.0±10.1	38.1±7.6	36.3±9.8	42.1±14.4	N.S*
Rest	24.0±10.8	18.8±6.1	21.8±4.8	17.6±4.1	18.5±3.9	21.6±7.2	N.S*
t-test	P<0.03	P<<0.01	P<<0.01	P<<0.01	P<<0.01	P<0.01	
Air cell/rest ratio	1.5±0.7 (11)	1.8±0.6 (13)	1.9±0.5 (14)	2.3±0.5 (13)	2.0±0.6 (14)	2.8±1.6 (9)	P<0.05**

Values are means ± S.D. (N). Student's *t*-tests (paired) are between zones for each day. *One-way ANOVA is among days. **Kruskal–Wallis ANOVA, median test is among days for the ratios under the air cell to the rest of the CAM.

the volume (V) of a CAM sample is the product of its surface area (S_{CAM}) multiplied by CAM sample thickness (D_{CAM}), we may calculate the total length of blood vessels within the CAM in different zones as $L = 2\ N_{A(v)}\ S_{CAM}\ D_{CAM}$. Thus, the ratio between the L_{ac} and L_{re} is: $[N_{A(v)ac}\ S_{ac}\ D_{ac}]/[N_{A(v)re}\ S_{re}\ D_{re}]$.

Calculation of total number of pores

Thirteen eggs were used to calculated the total number of pores in the different zones. This was done by calculating for each egg the total eggshell surface area using the initial egg mass according to Paganelli et al. (1974). The area over the air cell of the same eggs was calculated as described above. These values were used to calculate the shell area of the rest of the shell, by subtracting the area of the shell over the air cell from the total surface area for each egg on day 16 of the incubation. These values, multiplied by the pore density taken from Rizzo (1899), gave values for the total number of pores in each area.

Statistical methods

The two different zones were statistically compared for the same eggs using paired Student's *t*-test. The results for different embryonic ages were compared using one-way analysis of variance (ANOVA). All data ratios between the two different zones for every embryonic age were compared using the Kruskal–Wallis ANOVA median test.

Results

The average CAM thickness (D_{CAM}) under the air cell was always less than that of the rest of the CAM, although this difference was significant only on days 12, 14 and 20 of the incubation (Table 1). The CAM thickness was significantly different at different embryonic ages, both under the air cell and for the rest of the CAM (Table 1). On day 14, the CAM thickness was significantly lower than for the other embryonic ages, both under the air cell and for the rest of the CAM.

The calculated surface area of the CAM under the air cell (S_{ac}), increased, and that of the rest of the shell (S_{re}) decreased, from day 10 to 20 (Table 1). The ratio between S_{ac} and S_{re} increased with embryonic age from 0.13 to 0.25.

Blood vessel numerical density [$N_{A(v)}$] varied at different embryonic ages and in the two zones (Table 1). The $N_{A(v)}$ under the air cell on each embryonic age tested was significantly higher (range: 102–164 vessels mm^{-2}) than the $N_{A(v)}$ of the rest of the CAM (range: 61–93 vessels mm^{-2}). $N_{A(v)}$ increased significantly in both zones up to day 16 and then decreased until day 20 (Table 1). The ratio between the $N_{A(v)}$ in the CAM_{ac} and CAM_{re} was not significantly different among the embryonic development days. The overall ratio averaged 1.79±0.59 (N=67).

Table 1 also shows that the mean area density of blood vessels [$A_{A(v)}$], as a percentage of the total CAM area under the air cell, ranged from 32 to 42% with no significant differences among the days. The $A_{A(v)}$ values under the air cell

Table 2. *Various eggshell and chorioallantoic membrane (CAM) parameters in relation to different regions of the chick hen egg*

Parameter	CAM under air cell	Rest of the CAM		References	*N*
	Blunt end	Middle	Pointed end		
Pore density (cm^{-2})	149	131	90	Rizzo (1899)	12
Pore density (%)	100	88	60	Rizzo (1899)	12
Eggshell area specific gas pressure permeability (%)	100	78		Romanoff (1943)	193
Eggshell area specific water vapour diffusive conductance (%)	100	88	63	Rokitka and Rahn (1987)	35
Pore density (%)	100	81	63	Rokitka and Rahn (1987)	35
Respiratory quotient (day 19)	0.82	0.67		Seymour and Visschedijk (1988)	86
Surface area (cm^2), S_{ac}, S_{re} (day 16)	13.2	62.8		Present study	74
Total pore number	1970*	6935*		Present study	74
Total blood vessels length, *L* (cm) (day 16)	2.92×10^3	9.83×10^3		Present study	74
Total blood vessels length/total pores (cm $pore^{-1}$)	1.47	1.42		Present study	74

*For the pore number calculation, the values of the middle and pointed ends from Rizzo (1899) were averaged.

Table 3. *Total length of blood vessels under the air cell (L_{ac}), the rest of the CAM (L_{re}), their ratios (L_{ac}/L_{re}) and the ratio of the corresponding surface areas (S_{ac}/S_{re}) in different days of incubation*

Parameter	Embryonic age (day)						ANOVA (among days)
	10	12	14	16	18	20	
L_{ac} ($\times10^4$ mm)	1.20	1.71	1.18	2.92	3.18	3.67	$P<0.001$*
% of day 10	100	142	98	243	265	306	
L_{re} ($\times10^4$ mm)	5.99	9.56	6.00	9.83	9.11	7.91	$P<0.005$*
% of day 10	100	160	100	164	152	132	
t-test (between zones)	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.005$	$P<0.001$	$P<0.05$	
L_{ac}/L_{re}	0.20	0.19	0.20	0.30	0.35	0.47	$P<0.005$**
S_{ac}/S_{re}	0.13	0.16	0.18	0.21	0.23	0.25	$P<0.001$**
N	10	13	13	12	13	6	

Values are means. Student's *t*-tests (paired) are between zones. *One-way ANOVA among days. **Kruskal–Wallis ANOVA, median test among days for the ratio of air cell to rest.

were almost double and significantly different from those under the rest of the shell (17–24%).

Although our results may be biased due to the fact that they are based on some model assumptions, e.g. calculation of surface area, tissue shrinkage during histological preparation and the kind of morphometric measurements used, the final comparisons would yield bias-free results, since all techniques used would have the same systematic errors. Therefore, for the purpose of comparisons they are still valid.

Discussion

As early as 1930, Riddle (1930; according to Rol'nik, 1970) noted the 'paradox of the shell', where 'the eggshell must be sufficiently permeable to gases in order to secure normal gas exchange; concurrently, it should not be too permeable to another gas, water vapor'. Thus, among other factors, an optimal exchange rate for both O_2 and CO_2 through the shell to and from the blood vessels of the CAM should exist. This suggests that an area of the shell, which potentially evaporates relatively more water, can potentially exchange more respiratory gases (Rokitka and Rahn, 1987). In addition, the CAM blood flow supplying/carrying away the respiratory gases should be regulated, and this requires an appropriate development of blood vessels.

We consider that the potential for CAM gas exchange can be estimated from the ratio between the eggshell pore density (determining eggshell diffusive gas conductance) and CAM blood vessel density (involved in gas transfer by the blood in the CAM circulation), since both structures participate in this gas exchange. Regional differences in shell pore density (Rizzo, 1899; according to Rol'nik, 1970) and shell gas conductance (Rokitka and Rahn, 1987) have been reported, indicating higher area-specific gas conductance over the air cell (Table 2). It can be inferred from Simkiss (1980) that temperature fluctuations in the air cell may enhance gas exchange over it in comparison with the rest of the eggshell. It has been demonstrated that the partial pressure of oxygen in the air cell of goose eggs is higher than in

other areas under the eggshell (Meir et al., 1999). According to Romanoff (1943), the differential-pressure gas permeability of the shell over the air cell is 28% higher than that of the shell over the small end of the egg. These conductance differences manifest themselves in the different RQ over the different regions of the shell (Paganelli et al., 1988).

The CAM reacts differently to *total* or *local* hypoxia (coverage of part of the eggshell) as follows: when the eggshell is entirely exposed to various O_2 atmospheres, there is a dose-dependent change in the vascular density index of the CAM (VDI) on days 7–14; in hyperoxia the VDI decreases, and in hypoxia it increases (Dusseau and Hutchins, 1988; Strick et al., 1990). This corresponds to an increase in embryonic mass and a decrease in CAM mass in hyperoxia, and *vice versa* in hypoxia (Richards et al., 1991–92).

When part of the eggshell of chicken eggs was covered at the beginning of incubation, the CAM VDI under that part showed no changes on day 12, in comparison to non-covered control eggs (Corona and Warburton, 2000). However, the results of Wagner-Amos and Seymour (2003) showed a decrease in CAM VDI under the covered side under similar conditions from day 12 onwards, indicating suppressed angiogenesis of the circulatory system when oxygen supply is limited. This may correspond to the fact that at day 12, although the CAM is complete, the size of the embryo is only 10% of its final size, and thus the rate of oxygen consumption is very low (Romanoff, 1967; Duncker, 1978).

The apparent contradiction in the exposure of the CAM to full hypoxia (VDI *increases*) and partial coverage of the shell (VDI *decreases* under the covered area) may stem from the fact that, in the latter, compensation in the non-affected CAM areas is possible. However, evidence in the literature is not conclusive and it is premature at this stage to speculate further about the reasons.

In view of the natural regional differences in shell conductance and pore density between the eggshell over the air cell and the rest of the eggshell, we discuss the possibility of an appropriate matching of the CAM VDI under them. In contrast to previous work, the present study focuses on and discusses the two different CAM zones, which presumably are exposed to different oxygen levels during normal incubation.

Strick et al. (1990) found that VDI and blood vessel length density increased from day 8 until day 14, after which there were negligible changes until day 18. Similarly, Wagner-Amos and Seymour (2003) found that the density of pre- and post-capillary vessels increased in control eggs, reaching a maximum on day 14. Table 1 shows that blood vessel numerical density in both CAM areas [$N_{A(v)}$] reaches a peak on day 16, which is the time of maximal growth rate of the embryo (Dietz et al., 1998; Romanoff, 1967). It seems that maximal $N_{A(v)}$ (and VDI) is reached on days 14–16 and, from that time on, other mechanisms, such as increased CAM blood flow and blood oxygen affinity (Tazawa, 1980) and the movement of the blood capillaries to a position nearer the inner shell membrane (Duncker, 1978), enhance oxygen delivery rate.

It is interesting to note that, on day 14 of incubation, the D_{CAM} is reduced both under the air cell and the rest of the shell (Table 1). Similar results were found in other studies (Romanoff, 1960; Ar et al., 1987). The reason is not yet known, but day 14 also seems to be a pivotal time in terms of air cell and blood gas pressures (Tazawa et al., 1980; Tazawa, 1980) and marks the beginning of fast growth (Romanoff, 1967).

The values of $N_{A(v)}$ and $A_{A(v)}$ under the air cell are significantly higher than those of the rest of the CAM at all embryonic ages checked (Table 1). From day 16 onwards, $N_{A(v)}$ and $A_{A(v)}$ values under the air cell are almost twice those of the rest of the CAM. Tazawa and Ono (1974) found that the blood vessels show similar area densities for days 12–18 of incubation (33.6–40.8%). We found comparable values (32.2–42.1%) and showed that there is no statistically significant difference in the $A_{A(v)}$ under the air cell on days 10–20 of incubation (Table 1).

Our data show that the total length of blood vessels (L), the ratio of the CAM surface area under the air cell to that of the rest of the CAM (S_{ac}/S_{re}) and the same ratio for total length of blood vessels (L_{ac}/L_{re}) all increase with embryonic age and air cell size (Table 3) and, thus, with the increase of the O_2 consumption rates and embryonic age (Romanoff, 1930, 1967). The relative increase in L_{ac} with age, together with the increase in S_{ac} (Table 1), parallels the increased participation of the eggshell over the air cell in gas exchange during incubation.

As Table 3 shows, the total length of blood vessels under the air cell (L_{ac}) on day 20 is three times that on day 10 (L_{ac}=3.67×10^4 mm and 1.20×10^4 mm, respectively). This increase is more than the increase in S_{ac}. In comparison, the total length of blood vessels under the rest of the CAM (L_{re}) on day 16 is ~1.64 times that on day 10 (L_{re}=9.83×10^4 mm and 5.99×10^4 mm, respectively). This increase is despite a decrease in S_{re} (Table 1). From day 16 onwards, a decrease in L_{re} to 7.91×10^4 mm on day 20 is observed. The final L_{re} value is only 1.32 times that of day 10.

Romijn and Roos (1938) found that the 'allantoic surface, which lines the floor of the air space' comprises 10–20% of the entire respiratory area of the embryo in the last days of the incubation. In our study, the ratio S_{ac}/S_{re} increases from 0.13 (day 10) to 0.25 on day 20 (Table 3). Concurrently, the ratio L_{ac}/L_{re} increases from 0.20 to 0.47 (Table 3). This difference can explain the ratio of embryonic O_2 consumption rate of 0.42 between the air cell and the rest of the eggshell towards the end of the incubation found by Visschedijk (1968a) and the relatively higher CO_2 loss rate through it (Paganelli et al., 1988).

The total number of pores in the different zones was calculated according to the egg surface area (Paganelli et al., 1974) and the relative number of pores in each zone (Rizzo, 1899) (Table 2). The regional length per pore found for the CAM under the air cell and for the rest of the CAM is almost the same (1.47 cm pore^{-1} and 1.42 cm pore^{-1}, respectively; Table 2). This may mean that each pore, whether of the air cell or the rest of the shell, serves a similar length of blood vessels. This corresponds to the almost constant gas exchange rate per pore and the area of service per pore concept advanced by Ar and Rahn (1985) for bird eggs in general.

The relationships between blood vessel density and pore density in the CAM indicate optimization of embryonic gas exchange. We suggest that in natural incubation, the increased gas exchange under the air cell compensates for covering of the central part of the eggshell by the incubating parent (~20% of the eggshell area: Kendeigh, 1973; Yom–Tov et al., 1986; Handrich, 1989).

Our results seem to explain, in part, the apparent 'paradox of the shell' (Riddle, 1930; as cited by Rol'nik, 1970) where the living embryo adapts by varying angiogenesis in relation to the shell permeability to gases. Whether this concept holds for other bird species remains to be seen.

List of symbols/abbreviations

$A_{A(v)}$	area fraction of blood vessels
CAM	chorioallantoic membrane
CAM_{ac}	CAM tissue under the air cell
CAM_{re}	CAM tissue under the rest of the eggshell
D_{CAM}	thickness of the CAM
L	total length of blood vessels
L_{ac}	total length of blood vessels under the air cell
L_{re}	total length of blood vessels under the rest of the eggshell
$N_{A(v)}$	blood vessel numerical density
S_{CAM}	surface area of the CAM
S_{ac}	surface area of the CAM under the air cell
S_{re}	surface area of the CAM under the rest of the eggshell
VDI	vascular density index (intersections per unit area)

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