

Avian embryos in hypoxic environments

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Abstract

Avian embryos at high altitude do not benefit of the maternal protection against hypoxia as in mammals. Nevertheless, avian embryos are known to hatch successfully at altitudes between 4000 and 6500 m. This review considers some of the processes that bring about the outstanding modifications in the pressure differences between the environment and mitochondria of avian embryos in hypoxic environments. Among species, some maintain normal levels of oxygen consumption (\dot{V}_{O_2}) have a high oxygen carrying capacity, lower the air cell-arterial pressure difference ($P_{A_{O_2}} - P_{a_{O_2}}$) with a constant pH. Other species decrease \dot{V}_{O_2} , increase only slightly the oxygen carrying capacity, have a higher $P_{A_{O_2}} - P_{a_{O_2}}$ difference than sea-level embryos and lower the P_{CO_2} and pH. High altitude embryos, and those exposed to hypoxia have an accelerated decline of erythrocyte ATP levels during development and an earlier stimulation of 2,3-BPG synthesis. A higher Bohr effect may ensure high tissue P_{O_2} in the presence of the high-affinity hemoglobin. Independently of the strategy used, they serve together to promote suitable rates of development and successful hatching of high altitude birds in hypoxic environments.

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1. Introduction

Respiration involves multiple and well defined steps of oxygen (O_2) transport from the environment to cells. In adult vertebrates, these steps are represented by *ventilation*, in which air flows by convection from the environment to the alveolar spaces of the lung; *pulmonary diffusion*, by which O_2 in the alveoli comes into contact with the alveolar–capillary walls and passes through them to reach the blood; *blood trans-*

port by which gas is carried from the lung capillaries to those of tissues; finally, in the *tissue diffusion* stage, O_2 passes from systemic capillaries to the cells. In the avian embryo, respiration also requires similar steps of O_2 transport from the environment to the tissues; however, some differences exist. The first stage reflex the most important difference from that of other vertebrates, because the lungs of the avian embryo are not functional until the end of incubation; hence, this step must rely on diffusion from the environment to the air cell of the egg. In the second step, O_2 diffuses across the shell and the attached membranes into the capillary bed of the chorioallantois, passes through them and reaches the systemic blood. As the embryo grows,

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the fixed conductance of the shell and the limited diffusion capacity of the chorioallantoic membrane (CAM) imposed restrictions, which compared with the lungs, are expressed in a lower surface area for diffusion capacity. In the third step, *blood transport* carries the gas from the chorioallantoic-capillaries to those of the tissues. Finally, at the fourth step, O_2 passes from the systemic capillaries to the respiratory enzymes of the mitochondria. Despite these differences, that occur mainly in the first two steps, the process of transporting oxygen to the mitochondria functions efficiently in both systems because the difference between the O_2 partial pressure (P_{O_2}) of the atmosphere and that required in the mitochondria in the avian embryo is as large as in other vertebrates.

At each step of respiration there is a fall in O_2 tension and an increase in CO_2 tension. The various steps of O_2 transport represent the “oxygen cascade”, which sees a progressive drop in O_2 partial pressure (P_{O_2}) (Fig. 1). At high altitude, barometric pressure (PB) is diminished and with it the pressure difference of O_2 available for gas transport to the mitochondria. Thus, this shortfall in air P_{O_2} needs to be compensated, either by the modification of tissue metabolism or by the adjustment of the O_2 cascade (making it less steep), so that the loss of O_2 pressure in the environment has less impact on cellular function. In this review, we shall consider some of the processes of adaptation that bring about these remarkable modifications in the steepness of the O_2 cascade in high altitude avian embryos. In fact, birds are known to breed, and embryos to hatch successfully, even at altitudes between 4000 and 6500 m (Carey et al., 1982). An effort is made to integrate existing data on avian embryos in hypoxic conditions, although very little information on the O_2 transport characteristics of avian embryos from high altitude species in natural conditions has been obtained up to date.

At altitude, the O_2 pressure gradient between the environment and the cells decreases. The decreased gradient should lower the rate of diffusion from ambient air through the shell into the air cell, then through the inner shell membrane and chorioallantoic membrane into the blood, and from the blood into the cell. The gas flux of oxygen (\dot{V}_{O_2}) is proportional to the P_{O_2} difference across the different barriers and to the conductance (permeability) of the different bar-

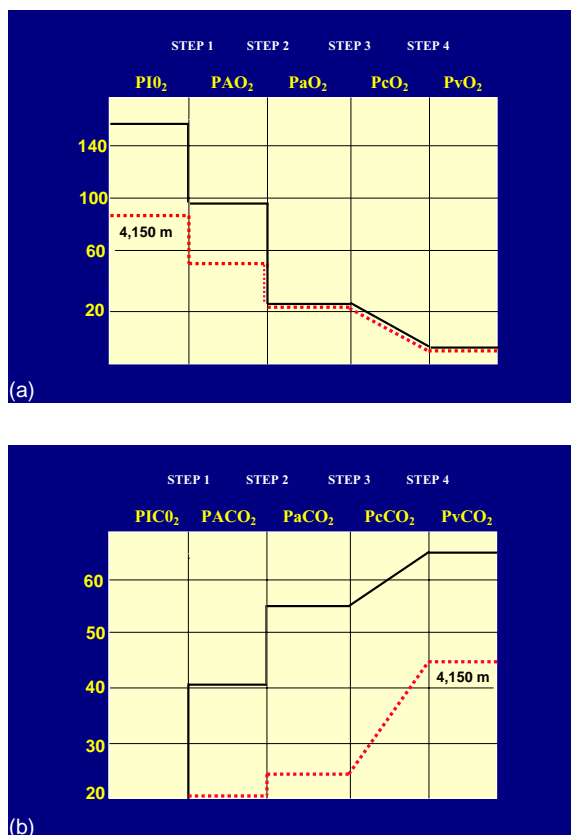


Fig. 1. Mean O_2 and CO_2 pressure gradients (Torr) from ambient air (P_{iO_2} , P_{iCO_2}) to mixed venous blood (P_{vO_2} , P_{vCO_2}) in lowland and Andean coot embryos collected at 4150 m. Air cell (P_{AO_2} , P_{ACO_2}), arterial (P_{aO_2} , P_{aCO_2}) and hypothetical capillary (P_{cO_2} , P_{cCO_2}) O_2 and CO_2 values are also shown.

riers between the ambient environment and the cells ($\dot{V}_{O_2} = G_{O_2} \times \Delta P_{O_2}$) (Table 1). From these equations it is apparent that, for a constant O_2 conductance, a decrease in O_2 consumption (\dot{V}_{O_2}) or metabolic rate would be an indication of the reduction of the pressure difference between the ambient air and the air cell ($P_{iO_2} - P_{AO_2}$), and thus in the conservation of the gradient between air cell and tissues. The combination of a \dot{V}_{O_2} maintained roughly at the sea level value with a large G_{O_2} , in any step of the O_2 cascade, would suggest a ΔP_{O_2} unchanged or even reduced. The components of the oxygen cascade are numerous and adaptations to enhance O_2 transport under hypoxic conditions may occur at any level of the oxygen cascade.

Table 1

Diffusion and convection equations which govern the oxygen flux (\dot{V}_{O_2}) from the environment to the mitochondria in an avian egg

Stages of the oxygen cascade	Oxygen transport equations	Oxygen transport is affected by
Step 1: from the atmosphere (P_{O_2}) to the air cell of the egg	$\dot{V}_{O_2} = G_{O_2}(P_{O_2} - P_{ACO_2})^a$, $G_{O_2} = (Dg/RT)(Ap/Lp)$	Dg, diffusion coefficient of gas; Ap, functional pore area of the shell; Lp, shell thickness; ΔP_{O_2} .
Step 2: from the air cell (P_{ACO_2}) to the chorioalantoic-capillary walls	$\dot{V}_{O_2} = G_{O_2}(P_{ACO_2} - P_{aO_2})^b$	G_{O_2} , the area surface of the CAM, thickness of the CAM, capillary volume and density, hemoglobin concentration and affinity for O_2 ; ΔP_{O_2} .
Step 3: from the chorioalantoic membrane (P_{aO_2}) to tissues	$\dot{V}_{O_2} = G_{O_2}(C_{aO_2} - C_{vO_2})$ $G_{O_2} = V_b\beta(P_{aO_2} - P_{vO_2})$	G_{O_2} , the cardiac output, affinity of hemoglobin for O_2 , allosteric effectors, blood flow distribution, hemoglobin concentration; ΔP_{O_2} .
Step 4: from systemic capillaries (P_{cO_2}) to the mitochondria (P_{tO_2})	$\dot{V}_{O_2} = G_{O_2}(P_{cO_2} - P_{tO_2})$	G_{O_2} , cell area surface, capillary volume and density; mitochondrial density; respiratory enzymes concentration and activity; ΔP_{O_2} .

\dot{V}_{O_2} , oxygen transfer or oxygen consumption; ΔP_{O_2} , oxygen pressure difference in each step of the cascade; R , universal constant of gases; T , temperature; $(C_{aO_2} - C_{vO_2})$, arterial–venous content difference; V_b , blood volume; β , coefficient of capacitance ($\Delta C/\Delta P$); $(P_{aO_2} - P_{vO_2})$, arterial–venous pressure difference.

^a G_{O_2} , conductance of the eggshell plus the outer shell membrane.

^b G_{O_2} , conductance of the CAM plus the inner shell membrane.

Bird embryos exchange respiratory gases with the environment through a barrier that has several layers. These are commonly grouped into an outer barrier, formed by the shell and the outer shell membrane, and an inner barrier formed by the inner shell membrane, and the chorioalantoic membrane, the major respiratory organ of the avian embryo (Piiper et al., 1980; Seymour and Visschedijk, 1988; Wangenstein, 1972; Wangenstein and Rahn, 1970). The major contributor to the diffusive resistance of the outer barrier is the eggshell. The outer shell membrane contributes only little resistance to gas flow (Piiper et al., 1980; Wangenstein and Weibel, 1982; Wangenstein and Rahn, 1970). During incubation, the continual loss of water through the membranes and shell favors the formation of an air pocket ('air cell') at the blunted end of the egg. Toward the end of incubation, the embryo pierces the air cell membrane with its beak ('internal pipping') and after a period of time, which in the chicken embryo lasts approximately 24 h (Burton and Tullett, 1985), it begins to rupture the eggshell ('external pipping').

2. Step 1: from the environment to the air cell

Before the external pipping, oxygen movement through the eggshell is exclusively driven by diffusion and is best described by a modification of the

Fick equation (Ar et al., 1974; Metcalfe et al., 1981; Paganelli, 1980; Rahn et al., 1987; Wangenstein and Rahn, 1970). The O_2 flux at this point is proportional to the difference in P_{O_2} between the environment and the air cell ($P_{I_{O_2}} - P_{A_{O_2}}$) and to the conductance of the diffusion barrier, $\dot{V}_{O_2} = G_{O_2}(P_{I_{O_2}} - P_{A_{O_2}})$.

2.1. Conductance of the eggshell and the outer shell membrane

As the calcareous material of the shell is impermeable to gas, shell conductance depends on the number and size of its pores and on its thickness (Ar et al., 1974; Ar and Rahn, 1985; Rahn et al., 1987). While the average G_{O_2} per pore is quite constant (Rahn et al., 1987), pore density differs between different regions of the chicken egg (Rokitka and Rahn, 1987; Seymour and Visschedijk, 1988).

The conductance of a barrier for a gas also depends on the diffusion coefficient (D , cm^2/s) and on the capacitance coefficient of the gas (Δ concentration/ Δ pressure or $1/RT$, where R is the gas universal constant and T is the temperature). D is inversely proportional to PB, therefore, gases exchanged between an embryo and the environment will diffuse more rapidly as PB decreases with altitude. This effect would partially compensate for the decrease in $P_{I_{O_2}}$ (Visschedijk et al., 1980), but would also increase the rate of loss of water vapor and carbon dioxide (CO_2) from the egg

(Ar and Rahn, 1980; Paganelli et al., 1975; Rahn and Ar, 1974).

The gas conductance of the eggshell differs between lowland and highland eggs (Carey et al., 1983, 1987, 1989a,b; Packard et al., 1977; Rahn et al., 1977; Sotherland et al., 1980; Wangensteen et al., 1974). The degree and direction of the variation depends on the altitude at which the eggs were laid. The conductance (standardized to 760 Torr) of eggs laid up to 3000–3500 m decreases in approximate proportion to the reduction in PB at the breeding site (Carey et al., 1983; Packard et al., 1977; Rahn et al., 1977; Sotherland et al., 1980). Therefore, the increased diffusion of water vapor and CO₂ is offset in some species breeding up to 3500 m. Nevertheless, when eggs laid above 3500 m in the Peruvian Andes were studied, the conductance was found to change not in proportion with PB; actually, the egg conductance exceeded the sea level value (Carey et al., 1987, 1989a,b; León-Velarde et al., 1984a; Rahn et al., 1977; Wangensteen et al., 1974) (Table 2). As a result, in these eggs water loss is higher than at sea level, but air cell oxygen tension is higher as well. This phenomenon could reflect a change in priority for shell design at altitudes above 3000–3500 m towards improvement of O₂ availability at the expense of greater rates of water vapor and CO₂ losses (Carey et al., 1987, 1989a,b; León-Velarde et al., 1984a,b; Monge-C et al., 1988). Since eggshell thickness of most species does not vary with altitude, most of the variation in conductance in eggs collected in hypoxic environments results from changes in the total pore area, which in turn is attributable to changes in the number of pores rather than in pore dimensions (Carey et al., 1987, 1989a,b).

These changes may reflect processes of physiological acclimatization or evolutionary adaptation. On one hand, the changes on shell conductances have been found to reflect long-term selection for females which lay genetically fixed shell structures appropriate for the high altitude habitat (Carey et al., 1984; León-Velarde et al., 1984b). On the other hand, in chickens exposed to elevations of 3800 m ($P_{\text{I}_{\text{O}_2}}$ 90 Torr), 3800 m with supplementary O₂ ($P_{\text{I}_{\text{O}_2}}$ 140 Torr), and 1200 m ($P_{\text{I}_{\text{O}_2}}$ 125 Torr), it has been shown that physiological acclimatization of eggshell conductance can occur and is probably stimulated by hypoxia. The time lag in the response to changes in altitude or $P_{\text{I}_{\text{O}_2}}$ is approximately 2.5 weeks (Hempleman et al., 1993).

2.2. Oxygen consumption, oxygen pressure in the air cell and the $P_{\text{I}_{\text{O}_2}} - P_{\text{A}_{\text{O}_2}}$ difference

Oxygen consumption of precocial sea level embryos increases throughout the incubation until just before pipping of the internal membrane (Vleck et al., 1980). Since the G_{O_2} of the eggshell to O₂ diffusion remains fairly constant during incubation in most species (Carey et al., 1983), the increase in \dot{V}_{O_2} with embryonic mass results in a progressive decrease in O₂ within the air cell ($P_{\text{A}_{\text{O}_2}}$) (Rahn et al., 1974; Wangensteen and Rahn, 1970). $P_{\text{A}_{\text{O}_2}}$ of avian eggs just before pipping has an average of 101 Torr, and $P_{\text{A}_{\text{CO}_2}}$ of 40 Torr (Hoyt and Rahn, 1980), similar to the alveolar O₂ and CO₂ pressures of many vertebrates, including man. The difference between the $P_{\text{I}_{\text{O}_2}}$ and the $P_{\text{A}_{\text{O}_2}}$ ($P_{\text{I}_{\text{O}_2}} - P_{\text{A}_{\text{O}_2}}$) across the shell approximates 42 Torr. Despite the increase in D , at high altitude, the low $P_{\text{I}_{\text{O}_2}}$ reduces the $P_{\text{A}_{\text{O}_2}}$ below the corresponding value of sea level eggs at all embryonic masses (Wangensteen et al., 1974).

Oxygen consumption in high altitude coot embryos (*Fulica americana peruviana*) at 4150 m (PB = 459 Torr), initially increases, then reaches a plateau, and later decreases slightly. Absolute levels of \dot{V}_{O_2} of high altitude embryos are lower than those of lowland coots at any stage of incubation. The $P_{\text{I}_{\text{O}_2}} - P_{\text{A}_{\text{O}_2}}$ difference across the shell remains relatively constant at about 30 Torr. Thus, the depression in \dot{V}_{O_2} seems to be an adaptation that results in minimization of the $P_{\text{I}_{\text{O}_2}} - P_{\text{A}_{\text{O}_2}}$ difference and maintenance of $P_{\text{A}_{\text{O}_2}}$ at the highest possible level. This also seems to be the case in the Andean gull embryo (*Larus serranus*), breeding at 4650 m (PB = 439 Torr), which maintains a $P_{\text{A}_{\text{O}_2}}$ analogous to the Andean coot at the end of incubation, and a $P_{\text{I}_{\text{O}_2}} - P_{\text{A}_{\text{O}_2}}$ difference also similar to that of the coot embryo (Table 3). As at sea level, also at high altitude is remarkable the similarity between the $P_{\text{A}_{\text{O}_2}}$ values of the air cells and the alveolar P_{O_2} values of Andean human natives. In this regard, Monge-C and León-Velarde (1994) have pointed out that some avian embryos, despite the absence of air convection, are able to generate $P_{\text{A}_{\text{O}_2}}$ values in the air cell remarkably similar to the alveolar values of resting birds and mammals, whether the comparison is made at sea level or at high altitude. Because the $P_{\text{A}_{\text{O}_2}}$ is the starting value of the O₂ cascade, like the increase of ventilation in man, the maintenance of a

Table 2
Characteristics of shells of high altitude and sea level eggs

	\dot{G}_{H_2O} (mg per day per Torr)	Pore area (mm ²)	Permeability ($\times 10^{-6}$ cm ³ (STP)/(s cm ² Torr))	Shell thickness (mm)	Surface area (cm ²)
Sea level eggs					
Coot ($n = 4$)	6.04 \pm 1.15	0.88 \pm 0.18	1.55 \pm 0.28	0.33 \pm 0.006	55.85 \pm 2.81
Willow ptarmigan ($n = 4$)	2.92 \pm 0.17	–	1.02 \pm 0.017	0.19 \pm 0.004	37.94 \pm 0.47
High altitude eggs					
Andean coot ($n = 6$)	6.44 \pm 0.80	0.95 \pm 0.1	1.47 \pm 0.18	0.33 \pm 0.004	63.07 \pm 1.04
White-tailed ptarmigan ^a ($n = 7$)	2.50 \pm 0.16	–	1.11 \pm 0.014	0.18 \pm 0.004	35.30 \pm 0.50
Andean goose ^a ($n = 14$)	11.89 \pm 0.23	2.31 \pm 0.05	1.66 \pm 0.04	0.44 \pm 0.008	103.31 \pm 1.2
Speckled Teal ($n = 2$)	6.35 \pm 0.15	0.69 \pm 0.02	1.55 \pm 0.17	0.24 \pm 0.001	58.71 \pm 0.95
White-tufted grebe ($n = 3$)	14.37 \pm 0.17	1.58 \pm 0.01	5.65 \pm 0.10	0.25 \pm 0.006	36.70 \pm 0.31

Values are mean (\pm S.E.M.). Values for \dot{G}_{H_2O} are standardized for the correspondent PB. All eggs were collected at 4150 m, with the exception of the White-tailed ptarmigan eggs, which were collected between 3600 and 4100 m (from Carey et al., 1989a,b, 1991, 1997).

^a Samples sizes are averages.

high $P_{A_{O_2}}$ is beneficial for O₂ transport. It can be hypothesized that the diminished metabolic rate could be due to several reasons: a reduced capillary blood flow, as a consequence of a reduced cardiac output, a diminished tissue vascularization; and/or a reduced aerobic metabolic rate. Modest levels of anaerobiosis could also accompany the plateau in \dot{V}_{O_2} showed by montane coot embryos in the latter stages of incubation. In fact, high altitude coot embryos are hypocapnic and acidotic when compared to lowland coots, and the acidity may be contributed by lactic acid production (Carey et al., 1993). However, no conclusive data exists for high altitude species at present. Hatchling

masses of high altitude coots are similar or slightly larger than sea level coots (Carey et al., 1989a). In contrast, it has been shown that embryonic growth, organ growth, assessed by tissue protein/DNA, and development are strongly influenced by O₂ availability (Asson-Batres et al., 1989). In general, exposure to hypoxia causes retarded development, increased mortality, and a lower embryonic metabolism (Baumann et al., 1983a; Beattie and Smith, 1975). The depressed \dot{V}_{O_2} in absolute values of chicken embryos (*Gallus domesticus*) at 3800 m has been associated with prolonged incubation periods and smaller hatchling masses (Wangensteen et al., 1974).

On the contrary, Puna teal (*Anas versicolor puna*) breeding at 4150 m (PB = 459 Torr) has a \dot{V}_{O_2} which is approximately twice the \dot{V}_{O_2} of the Andean coot and of the Andean gull (Carey et al., 1989a,b, 1994) (Table 3). When compared with chicken embryos at sea level, which have a \dot{V}_{O_2} of approximately 21 ml/h before prior initiation of pulmonary ventilation (Hoyt and Rahn, 1980), it can be seen that Puna Teal embryos \dot{V}_{O_2} is very near this value, because their \dot{V}_{O_2} is almost 18 ml/h, well before prior the beginning of ventilation. \dot{V}_{O_2} of White-tailed ptarmigan (*Lagopus leucurus*) at 3600 (PB = 487 Torr) and 4100 m (PB = 461 Torr) (Carey and Martin, 1997); and Red-winged blackbirds (*Agelaius phoeniceus*) at 2900 m (PB = 531 Torr) (Carey et al., 1982) is independent of altitude, despite their different hatchling masses and incubation periods. Embryos of the bar-headed goose (*Anser indicus*), a species native from the Himalayas, are able to maintain normal rates of \dot{V}_{O_2} when transiently exposed to

Table 3
Gas exchange in three high altitude native species, calculated for 22-g embryos

	Andean coot	Puna Teal	Andean gull
PB (Torr)	459	459	439
\dot{V}_{O_2} (ml/h)	9.46	17.9	7.58
$P_{I_{O_2}}$ (Torr)	86.5	86.5	82.3
$P_{A_{O_2}}$ (Torr)	53.2	25.5	48.3
$P_{I_{O_2}} - P_{A_{O_2}}$ (Torr)	33.3	61.0	34.0
$P_{A_{CO_2}}$ (Torr)	19.6	38.8	20.9
Hematocrit (%)	40.8	48.4	–

PB, barometric pressure; \dot{V}_{O_2} , oxygen consumption; $P_{I_{O_2}}$, air O₂ pressure; $P_{A_{O_2}}$, air cell O₂ pressure; $P_{A_{CO_2}}$, air cell CO₂ pressure. The values for the Andean coot and the puna teal were calculated from the regression equations describing the relation between the different variables as a function of embryo weight from Carey et al. (1989a,b, 1993, 1994). The values for the Andean gull are from Monge-C and León-Velarde (1994).

ambient P_{O_2} as low as 85 Torr (Snyder et al., 1982a). In the high altitude puna teal, the $P_{I_{O_2}} - P_{A_{O_2}}$ difference across the shell averaged 40 Torr, similar to the average value of lowland species. The continuous increase in \dot{V}_{O_2} during development causes a progressive decrease in $P_{A_{O_2}}$ and an increase in the $P_{I_{O_2}} - P_{A_{O_2}}$ difference. Additionally, $P_{A_{CO_2}}$ is low in the coot and gull embryos and high in the puna teal embryo (Table 3). The larger the $P_{I_{O_2}} - P_{A_{O_2}}$ difference across the eggshell, the faster the rate of diffusion. Thus, the high altitude avian embryos which do not drop \dot{V}_{O_2} nor the $P_{A_{O_2}}$ do not “protect” the O_2 cascade or the rate of O_2 diffusion. Therefore, in these cases, a modification of tissue metabolism or other changes further down in the O_2 cascade must occur as a protection against tissue hypoxia.

3. Step 2: from the air cell to the chorioallantoic-capillary walls

The normal development of the avian embryo inside the egg is ensured by the gas exchange provided by the CAM (Wangensteen and Rahn, 1970). This highly vascular structure, in conjunction with the porosity of the eggshell, permits diffusion of O_2 and CO_2 between the environment and the blood (Tullett and Deeming, 1982), a function which could be paralleled to that of the placenta for the mammalian fetus.

Lateral diffusion in the gas-filled shell membranes is insufficient to homogenize gas tensions under the shell (Visschedijk et al., 1988). Thus, to achieve energy-efficient gas exchange between the sub-shell gas and the blood in the CAM, perfusion should match local shell conductance. However, Wagner-Amos and Seymour (2003) have shown that an even eggshell pore distribution and uniformity of chorioallantoic perfusion are not required for successful avian development. In an experimental model where eggs were incubated in hyperoxia with half of the egg shell waxed to stop diffusion, the embryo was able to utilize the increase of environmental O_2 to compensate for the loss of respiratory surface area. These critical experiments show that uniformity of chorioallantoic perfusion seems not to be crucial for avian development; thus, even if hypobaric hypoxia modifies perfusion, this effect should not alter the O_2 transport from the air cell to the chorioallantoic-capillary walls.

However, experimental models do not necessarily simulate the situation posed by the increased air cell–arterial diffusion gradient found in high altitude embryos. O_2 diffuses across the shell and the attached membranes into the capillary bed of the chorioallantois, which serves as the “lung” of the chicken embryo and which replaces the yolk sac as the gas exchange organ at the end of the first week of development. As the embryo grows, the restrictions imposed by the fixed conductance of the shell and the limited diffusion capacity of the CAM result in increasing the air cell–arterial diffusion gradient ($P_{A_{O_2}} - P_{a_{O_2}}$). As a result, progressive hypoxia and hypercapnia are normal features of later stages of embryonic development. It would be of interest to know the developmental evolution of the gradient during embryonic growth at high altitude, where, in addition to the ever-changing conditions related to growth itself, the embryo has to adapt to the lower $P_{I_{O_2}}$. In adult vertebrates in hypoxic environments, as the blood leaves the alveolar–capillary system there is a larger transit time along the alveolar capillaries characteristic of oxygen diffusion limitations. This could be hazardous in certain conditions of decreased oxygen delivery due to the increase of the $P_{A_{O_2}} - P_{a_{O_2}}$ difference. Unfortunately, no data exists in regard to some of the critical components of the CAM capillary system at this stage of the oxygen cascade for avian embryos in hypoxic environments.

4. Step 3: from the chorioallantoic membrane to the tissues

The capacity for O_2 transport from the CAM to the tissue depends on the ΔP_{O_2} at the different levels of the O_2 cascade, but it also depends on the O_2 delivery by the blood, which is a function of the number of red blood cells (RBC), the saturation of the hemoglobin (Hb) with O_2 (which depends on the Hb affinity for O_2), and the blood flow.

4.1. Arterial pressure of oxygen and carbon dioxide

Arterialized oxygen pressure ($P_{a_{O_2}}$) refers to the partial pressure of the oxygenated blood leaving the CAM in chorioallantoic veins (Piiper et al., 1980). Over the embryos’ body mass of approximately

6–21 g, the P_{aO_2} decreased from 60 to 17 Torr, and similarly in high altitude and sea level coot embryos. Arterial CO_2 pressure (P_{aCO_2}) of lowland coots increased significantly with growth from 14 to 60 Torr, within that range of body mass. P_{aCO_2} of mountain coot embryos also increased significantly from 20 to 27 Torr (Carey et al., 1993). Puna teal, which only nests at high altitude, have slightly low values of P_{aO_2} when compared with similarly-sized chicken embryos incubated at sea level (Carey et al., 1994; Tazawa et al., 1971). These lower values are the consequence of the lower P_{AO_2} of the eggs. P_{aCO_2} rose significantly from 8 to 21 Torr over the 3–13 g range in embryo's body mass (Carey et al., 1994). Because measurements in the coot and puna teal were not obtained for embryos of similar body masses, it is difficult to compare the P_{aO_2} values of these two species directly. However, neither the intercepts nor the slopes of the regression lines calculated to describe the relation between P_{aO_2} and body mass differ significantly between the two species (León-Velarde et al., 1997).

4.2. The $P_{AO_2} - P_{aO_2}$ difference

In general, a large $P_{AO_2} - P_{aO_2}$ difference exists in avian eggs in hypoxic circumstances. This has been attributed to diffusion limitation, arterial–venous shunt, varying conductance/perfusion ratios in different parts of the egg, rate of O_2 binding by Hb and O_2 consumption of the CAM (Piiper et al., 1980; Seymour and Visschedijk, 1988; Wakayama and Tazawa, 1988; Wangenstein, 1972).

The $P_{AO_2} - P_{aO_2}$ difference of lowland coots does not vary significantly with body mass, averaging 65 ± 5 Torr. In contrast, the $P_{AO_2} - P_{aO_2}$ difference of mountain coot embryos increased significantly from 16 to 24 Torr with the increase in embryonic body mass. The different $P_{AO_2} - P_{aO_2}$ gradient between species results in part from the influence of reduced P_{IO_2} on P_{AO_2} , and in part from the decreased \dot{V}_{O_2} observed in some avian embryos like coots and chickens (Carey et al., 1993). The $P_{AO_2} - P_{aO_2}$ difference of puna teal is the lowest recorded for any avian embryo. Embryos exposed to hypoxia tend to have lower $P_{AO_2} - P_{aO_2}$ gradients than normoxic embryos (Carey et al., 1989a; Wakayama and Tazawa, 1988). Therefore, the strategy used by the puna teal embryo to maintain a high \dot{V}_{O_2} is that of increasing the CAM-inner shell membrane conductance

by increasing the blood oxygen carrying capacity, the Hb affinity for oxygen, blood flow, increased vascularization, or other components of the CAM-inner shell membrane conductance, thus avoiding a marked drop in P_{aO_2} (León-Velarde et al., 1997).

4.3. Oxygen carrying capacity

The increase in \dot{V}_{O_2} of avian embryos during incubation at sea level correlates with an increase in the O_2 carrying capacity of blood (Tazawa et al., 1971; Temple and Metcalfe, 1970). At high altitude, hematocrits of white-tailed ptarmigan embryos (4200 m) are significantly higher than sea level ptarmigans at all developmental stages (Carey and Martin, 1997). The Hb concentration and hematocrit of lowland and mountain coot embryos increased significantly with the developmental increase in body mass, and high altitude coot embryos measured at 4200 m have significantly higher values of Hb and hematocrit than sea level coot embryos (Carey et al., 1993). The hematocrit values of puna teal embryos determined at 4150 m are significantly higher than mountain or hypoxic embryos of similar size, and higher than coot embryos; these differences could help support to the high \dot{V}_{O_2} of this species (Burton and Smith, 1969; Carey et al., 1993). In contrast to adult high altitude vertebrates, which have no, or a very modest, erythremia, the altitude avian embryos have been found consistently to have a larger number of red blood cells. It can be considered that at the embryonic stage, it is important not only to raise the number RBC in the blood in order to increase the capacity of the blood for carrying O_2 , but also, increasing the Hb concentration, to enhance the buffering capacity of the blood (Tazawa, 1986).

4.4. Oxygen binding properties of embryonic blood

It is well established that embryonic RBCs of the chick and other avian embryos show rapid and extensive changes of the gas transport properties during the later stages of development. This is interpreted as a response to the hypoxia and hypercapnia which normally occur with the developmental growth of the embryo. The Hb affinity for oxygen increases substantially (Lapennas and Reeves, 1983), allowing continuous adjustment to the arterial hypoxia. In addition, the

induction of erythroid carbonic anhydrase II (CAII) improves O₂ and CO₂ transport properties (Baumann et al., 1986), since its change is coordinated with the modification in RBC phosphate pattern and because it opposes the negative effects of hypercapnia on Hb oxygen binding (Birchard and Black, 1986). As in other vertebrates, the oxygen affinity of avian embryonic Hb is predominantly regulated by organic phosphates composition (Baumann and Meuer, 1992).

The mechanisms that control the change from embryonic to adult Hb production in the embryo have not been identified at the molecular level, but there is conclusive demonstration that ambient P_{O₂} can modulate the timing of the switch. When embryos are grown under hypoxic conditions definitive RBC and adult Hb appear in the circulation ~24 h earlier than they do in normoxic controls (Baumann et al., 1983b).

In several mountain species, specific amino acid substitutions cause a small increase in oxygen affinity of adult Hb (barheaded geese, *A. indicus* and Andean geese, *Chloephaga melanoptera*). Inositol pentaphosphate (IPP) and other allosteric effectors (ATP; 2,3-biphosphoglycerate (BDG)) amplify this effect substantially (Hiebl et al., 1987; Jessen et al., 1991; Petschow et al., 1977). The normal developmental changes of ATP and 2,3-BPG levels in RBC of chick and other avian embryos cause an increase in the oxygen affinity of the hemoglobins. During the chick embryonic development adult Hb first appears in the definitive red cell line by day 6. These cells contain adult Hb and subsequently replace the primitive red cells containing the specific embryonic Hb (Bruno and Ingram, 1973). Initially, definitive red cells contain predominantly high-affinity component HbD in excess of HbA, which has a lower intrinsic oxygen affinity.

Dragon et al. (1999) analyzed the developmental changes of the red cell phosphate pattern of mountain white-tailed ptarmigan embryos. Ptarmigans breed in arctic and alpine tundra habitats that range in elevation from 750 to 4250 m (Martin et al., 1993). The eggs were collected from nests located between 3600 and 4100 m altitude in the Rocky Mountains (Colorado). The eggs were incubated at two different altitudes to see if the organic phosphate pattern was affected by different ambient oxygen pressures. The Hb pattern of white-tailed ptarmigan embryos was compared to the pattern of embryos of their lowland counterpart,

the willow ptarmigan (*Lagopus lagopus*). The results showed that the embryos incubated at 3600–4100 m had an accelerated decline of the erythrocyte ATP levels during development compared to embryos incubated at 1600 m. The difference in red cell ATP between the two groups is substantial in the mass range 3–6 g (Dragon et al., 1999). These authors also observed an earlier stimulation of 2,3-BPG synthesis in the high altitude group. However, electrophoretic mobility of Hb of white-tailed ptarmigan embryos measured at 4200 m was similar to sea level ptarmigans of comparable body masses. The results are analogous to the findings obtained in chick embryos exposed to chronic hypoxia, where differences in ATP and 2,3-BPG concentration of normoxic and hypoxic embryos became apparent after day 7 of incubation (Baumann et al., 1983a, 1986). The mechanism seems to consist of a P_{O₂}-dependent control system that, in normoxia, can suppress the action of a “factor” presumably involving the phospholipase C and the protein kinase C (Million et al., 1991). In conclusion, these results suggest that the same oxygen-tension-dependent mechanism controls the red cell organic phosphate pattern of mountain and lowland embryos.

Regarding the respiratory properties of the whole blood during development, Snyder et al. (1982b) studied the affinity of Hb for oxygen in embryos of the bar-headed and Canada geese (*Branta canadensis*). In both species, the affinity of blood for O₂ (expressed as O₂ half-saturation pressure, or P₅₀) increased with development until a steady value was reached. The P₅₀, measured at pH 7.4, of the bar-headed goose, 20.1 ± 0.3 Torr, was significantly lower than that for the Canada goose, 26.9 ± 0.8 Torr. Hill's coefficients, buffering capacity, red cell 2,3-BPG, and blood Hb concentrations were similar between species. The authors also suggested that these embryos have a higher Bohr effect, which should raise the slope of the Hb dissociation curve and increase the P_{O₂} in the tissue capillaries. In general, the available data suggest that red cell ATP level is the principal modulator of Hb–O₂ affinity in the avian embryo (Baumann and Baumann, 1977). Thus, as previously described for adults of these two species of geese (Petschow et al., 1977), the differences in the whole blood oxygen affinity must be due to the intrinsic properties of the Hb itself or in its interaction with the organic phosphates that modulate the Hb–O₂ affinity.

In regard to other possible allosteric effects of the Hb dissociation curve, as mentioned above, Carey et al. (1993) have found that the blood of coot embryo is acidic. This phenomenon parallels the plateau in \dot{V}_{O_2} exhibited by mountain coot embryos during the later stages of incubation (Carey et al., 1989a). The light acidosis may cause a rightward shift in the blood O_2 dissociation curve and facilitate the unloading of O_2 to tissues. Unfortunately, there is no information about the Hb affinity of the coot embryo. Nevertheless, the adult coot, either native to sea level or at high altitude, have indeed a Hb with high affinity for oxygen (Monge-C and León-Velarde, 1991). The affinity of the whole blood for O_2 is an important, genetically based adaptation to ensure a high O_2 content in the blood in the face of the reduction in ambient P_{O_2} when nesting at high altitudes. The higher Bohr effect may ensure high tissue P_{O_2} in the presence of Hb with high O_2 affinity.

In order to determine the importance of β -adrenergic and adenosine receptor activation in the control of red cell ATP and 2,3-BPG concentration, Dragon et al. (1999) investigated the effect of β -adrenergic and adenosine A2 receptor blockade in red cell 2,3-BPG and ATP levels of chick embryos. The combined administration of β -adrenergic and A2 receptor blockers induced considerable mortality of the chick embryos, which indicates the importance of this hormonal system during normal embryonic development. These results suggested to the authors that catecholamines (and adenosine) not only are involved in the control of cardiovascular development but are also critical for the adaptive regulation of embryonic blood gas transport properties. The hormonal regulation of RBC function enables a flexible adjustment of the RBC gas transport properties to the actual P_{O_2} . This fact may represent a prerequisite for the ability of birds to breed in regions above 4000 m. In fact, in embryos of birds exposed to chronic hypoxia and adapted to high altitude, the RBCs are able to switch their organic phosphate pattern depending on the altitude of incubation (Baumann et al., 1983a, 1986; Dragon and Baumann, 2003). If we consider that the rapid decay of red cell ATP is indicative of a Hb with high affinity for oxygen, this characteristic may represent one of the most important modifications to successful breeding at high altitude. In this sense, it is important to mention that a very selected cluster of chickens in

the Andes (Puno, Perú) with a Hb having an exceptionally high affinity for oxygen is the only group of chickens described to date which successfully breed above 3000 m (León-Velarde et al., 1991; Mejia et al., 1994).

4.5. Vascularization

In experimental conditions, Wagner-Amos and Seymour (2003) have proved that angiogenesis reacts negatively to a strong hypoxic environment at any given time of incubation. This contrasts the studies by Dusseau and Hutchins (1988) and Strick et al. (1991) showing that hypoxia caused an overall increase in vascular density and a 'corkscrew' growth of the vessels, and that by Burton and Palmer (1992) where the capillary surface area has not been modified by hypoxia or hyperoxia. However, it is difficult to extrapolate these experimental findings to the natural settings. In fact, Snyder et al. (1984) have found that bird embryos respond to hypoxia by increasing capillarity. The increased capillarity was found in species native to high altitude even when incubated under conditions of normoxia and can be induced in species native to sea level by exposure to hypoxia during development. These authors determined tissue capillarity in the gastrocnemius and myoglobin concentrations in the gastrocnemius and heart for hatchling Canada geese, following incubation of the embryos under either normoxic ($P_{O_2} = 120$ Torr) or hypoxic ($P_{O_2} = 94$ Torr) conditions. Similar observations were made on a limited number of hatchling bar-headed geese. This study showed that capillary densities were higher and diffusion distances shorter in the hypoxic Canada geese and the bar-headed geese than in the normoxic Canada geese. The concentrations of myoglobin in the heart and gastrocnemius increased with mass, but not as a function of hypoxia.

5. Step 4: from the systemic capillaries to the mitochondria

Venous oxygen partial pressure ($P_{\dot{V}O_2}$) refers to the oxygen pressure of the blood flowing from the embryonic tissues to the CAM in the chorioallantoic arteries (Piiper et al., 1980). Within the body mass range of approximately 10–21 g, $P_{\dot{V}O_2}$ of both sea level and

high altitude (4150 m) coot embryos is below 12 Torr, without significant variation during the incubation period. Also the venous CO₂ pressure ($P_{\bar{V}CO_2}$) of lowland coots does not vary significantly with body mass, averaging 45.3 ± 4.6 Torr. In contrast, $P_{\bar{V}CO_2}$ of mountain coot embryos increased significantly from 19 to 42 Torr over the same range of body mass (Carey et al., 1993). Since tissue P_{O_2} cannot exceed $P_{\bar{V}O_2}$, the tissue P_{O_2} of the coot embryo might be below 10 Torr at least during the latter third of incubation. Despite this, it is important to mention the remarkable ability of these birds to grow and develop normally. In fact, at hatching, the body mass of coot embryos in the Peruvian Andes do not differ from those of the sea level coot. The $P_{\bar{V}O_2}$ of puna teal embryos averaged 8–10 Torr below those of similar-sized chicken embryos incubated at sea level. The $P_{\bar{V}CO_2}$ averaged slightly lower (4–9 Torr) than corresponding values of chicken embryos at sea level (Carey et al., 1994; Tazawa et al., 1971) (Table 4). The abilities of these birds to maintain normal functions at such low tissue P_{O_2} , certainly must involve cellular and biochemical characteristics that promote O₂ utilization.

Histochemical and biochemical studies have been performed on the muscles of animals exposed to short or long-term hypoxia. However, few studies of this type have been devoted to animals living at high altitude and even less to avian embryos. The oxidative capacity as reflected by citrate synthase activities in heart and muscle of white-tailed ptarmigan was studied by Carey and Martin (1997). Embryos measured

at 4200 m had significantly higher citrate synthase activity values than sea level ptarmigans at comparable body masses. This would mean that high altitude embryos make more efficient use of substrate by complete oxidation via the citric acid cycle. Indeed, it is common to find higher oxidative capacities in more active muscles and for long periods of time (Barrie et al., 1975; León-Velarde et al., 1993). However, extrapolations from adult muscles in different species to avian embryos must be interpreted with caution. More studies are needed to understand the real participation of cellular adaptation in the presence of other important extra-cellular adaptations.

6. Conclusions

The integrated mechanism that contributes to the oxygen transfer of embryos of mountain species is not completely understood. Much more remains to be learned about how the physiological and cellular design of the avian respiratory system have evolved under the selective pressure of multiple, and in some cases conflicting, requirements for gas exchange. In addition, it should be kept in mind the effect of gene flow between sea level and high altitude species, and the evolutionary time of high altitude exposure. For example, in humans and domestic mammals, whether newcomers or native to high altitude, the degrees of erythrocytosis at high altitude are quite variable. In addition, the higher resting ventilation (higher $P_{A_{O_2}}$) and hypoxic ventilatory responsiveness and the lower Hb values in Himalayans compared to Andeans living at similar altitudes have been interpreted as better adaptive characteristics to hypoxia in the former group (Beall et al., 1990; Monge-C and León-Velarde, 1991; Moore, 2001). Hence, it seems that natural selection has not produced the same results on Andean humans and domestic animals as in high altitude species living in other regions. Presumably, the differences are attributable to migratory habits, greater admixture with lowland groups and less evolutionary time of high altitude exposure (Moore, 2001).

Similarly, the type of specializations adopted by avian embryos of species breeding over 4000 m can be quite different. In fact, although all the species studied so far seem to have a Hb with high affinity for O₂ at the end of incubation, some species maintain

Table 4
Gas exchange and blood gases in two high altitude native species, calculated for the last half of incubation

	Andean coot	Puna Teal
$P_{a_{O_2}} - P_{A_{O_2}}$ (Torr)	33.3	61.0
$P_{a_{CO_2}} - P_{A_{CO_2}}$ (Torr)	35.4	0
$P_{a_{O_2}}$ (Torr)	21.6	26.3
$P_{\bar{V}O_2}$ (Torr)	Below 10	Below 10
$P_{a_{CO_2}}$ (Torr)	24.2	29.9
$P_{\bar{V}CO_2}$ (Torr)	44.3	42.8

$P_{I_{O_2}}$, air O₂ pressure; $P_{A_{O_2}}$, air cell O₂ pressure; $P_{a_{O_2}}$, arterial O₂ pressure; $P_{a_{CO_2}}$, arterial CO₂ pressure; $P_{\bar{V}O_2}$, venous O₂ pressure; $P_{\bar{V}CO_2}$, venous CO₂ pressure. The values were calculated from the regression equations describing the relation between the different variables as a function of embryo weight from Carey et al. (1993, 1994).

high levels of \dot{V}_{O_2} , increase the hematocrit, lower the $P_{A_{O_2}} - P_{a_{O_2}}$ difference and maintain a normal pH value. Other species lower \dot{V}_{O_2} , moderately increase the hematocrit, increase the $P_{A_{O_2}} - P_{a_{O_2}}$ difference and lower both the P_{CO_2} and pH values. Hence, to contrast the problems raised by hypoxia, different adaptive strategies have evolved all leading to successful embryonic development and hatching even at altitudes well above 4000 m, indicating that similar problems can be solved in several manners.

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