Gas exchange during storage and incubation of Avian eggs: effects on embryogenesis, hatchability, chick quality and post-hatch growth

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Embryonic development is a dynamic process that requires a fine balance between several factors in order to achieve an optimum hatchability and chick quality. These factors include the background of the embryo, such as genetic line of the breeders, the age of the breeder, egg weight, and factors related to the environment in which the egg is stored and incubated, such as temperature, humidity, gas levels and altitude. Gas exchanges are of fundamental importance for embryonic development during incubation and may affect the livability of the embryo. This paper reviews the roles of the gaseous environment (*i.e.* O_2 and CO_2) around hatching eggs during storage and during incubation and the effect it might have on the survival of the developing embryos and the chicks that hatch. The state of the art on the different attempts to establish the optimum requirements of different gases that promote the optimal developmental trajectories at different periods during incubation is presented. The roles and consequences of different levels of O_2 and CO_2 during storage and incubation on hatchability, incubation duration, hatching process, embryo growth, embryo mortality, organ development and morphology, metabolism, blood acid-base balance, chick quality and chick post-hatch growth are reviewed.

Introduction

Hatching eggs from chickens require interactions between several factors to promote the development of the embryo and hatch at the appropriate duration for incubation. These factors include the background of the embryo, such as genetic line of the breeders, the age of the breeder, the egg weight, and factors related to the environment in which the egg is

stored and incubated, such as temperature, humidity, gas levels and altitude. Egg handling such as turning also has some effects. Embryonic development is a dynamic process that requires a fine balance between these environmental factors in order to achieve an optimum hatchability and chick quality. Because there are differences in the quality of hatching eggs from different lines of breeders, breeders of different ages, eggs stored under different conditions or even eggs of different weights, it is becoming apparent that different eggs may require different incubation conditions. Recent studies also suggest that gas requirements of developing embryos change during incubation. Thus incubation procedures and incubator designs are now being modified to fulfill these requirements for optimal hatchability.

This paper reviews the roles of the gaseous environment around hatching eggs during storage and during incubation and the effect it might have on the survival of the developing embryos, the hatchability of the eggs and the quality of the chicks hatched. Gas exchanges are of fundamental importance for embryonic development during incubation and may affect the livability of the embryo (Tullett, 1990). The exchange of incubation gases is facilitated by the chorioallantoic membrane (CAM), which is a highly vascular structure in conjunction with the porosity of the egg shell. These two structures however permit the diffusion of oxygen (O_2) and carbon dioxide (CO_2) between the environment and the blood of the embryo (Tullett and Deeming, 1982). During incubation, the egg loses water through the membranes and this culminates in the formation of the air cell between the inner and outer membranes underneath the eggshell at the blunt end of the egg. At internal pipping, the embryo pierces into the air cell through the membrane to gain access to air so that pulmonary ventilation becomes an additional route for gas exchange (Vince and Salter, 1967; El-Ibiary *et al.*, 1966) until the rupture of the eggshell (external pipping) when full pulmonary ventilation is established. Several studies on the chicken have attempted to establish the optimum requirement of different gases that promote the optimal developmental trajectories at different periods during incubation. Using the chicken as a primary example in the following sections (except otherwise stated), the roles of the eggshell and the CAM in gas exchange between the external and internal environment of the egg, the optimal requirements and the effects of O_2 and CO_2 on embryonic development, hatchability and chick quality will also be reviewed.

The role of the eggshell in gas exchange

The avian eggshell forms the barrier between the internal and external environment of the egg. Its structure has been comprehensively described by Tullet (1984). It protects the embryo mechanically against impacts and serves as a barrier against bacterial infection. The inner side of the shell serves as a source of calcium for the development of embryonic bones (Romanoff, 1960). An important feature of the eggshell is its porosity. The avian embryo respires by exchanging oxygen and carbon dioxide across the pores in the eggshell. The pores also provide escape route for water vapour.

Romanoff and Romanoff, (1949), Wangensteen and Rahn, (1970-71), Ar *et al.* (1974) have demonstrated that gases and water vapour are exchanged between the internal and external environments of the egg through the pores in the eggshell according to simple laws of diffusion (Fick's law).

Rahn, (1981) and Rahn *et al.* (1981) have provide evidence that it is the shell conductance that determines the precise amount of O_2, CO_2 and water vapour that is exchanged between the internal and external environment of the egg. Thus diffusive conductance of gases, in and out of the egg, increases if pores are more numerous, wider and shorter. As shell porosity decreases, the amount of CO_2 that escapes across the shell is

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reduced. Ar *et al.* (1974) have reported that egg shell conductance increases with egg weight since larger eggs have more porous shells. Tullet, (1979, 1981) also reported a wide variation in shell porosity between eggs of different hens. Typically the pore numbers in domestic hen range between 7,000 and 17,000 (Solomon, 1991).

Differences in eggshell conductance has been ascribed to differences in age of parent stock, as older parents produce eggs of higher shell conductance (Peebles and Brake, 1987; Kirk *et al.*, 1980; Hamilton, 1978; Roque and Soares, 1994). Other factors are ambient temperature (Bamelis, 2003), and feed composition (Hurwitz, 1987). Thus, in order to create optimum gas exchange in the embryonic environment, Visschedijk (1991) suggested that the functional conductance of the eggshell must be considered along with the gaseous composition and barometric pressure of the ambient fresh air, the incubator ventilation rate and the embryonic O_2 uptake.

Several studies have reported the consequences that eggshell conductance can have on embryonic development during incubation. Tullett and Deeming (1982) reported that low porosity limits embryonic respiration and Burton and Tullett (1983) showed that this results in lower growth rate. The metabolic rate of the embryo increases during the second half of incubation and thus is the oxygen requirement. O_2 supply is limited by the conductance of the shell and other physiological characteristics of the embryo. The result is a plateau phase of O_2 input and CO_2 output. Bamelis *et al.* (2001) reported that this phase is reached earlier in eggs with lower eggshell conductance (especially eggs from young breeders).

Burton *et al.* (1989), reported that air cell pO_2, just before pipping, increases with increasing conductance of the eggshell and the reverse is true for pCO_2. With increasing conductance of the shell, pipping by embryos is delayed. Hatchability has also been reported to be inversely related to eggshell conductance. Eggs with the lowest eggshell conductance achieve the highest hatchability irrespective of the age of the parent stock (McDaniel *et al.*, 1979; Roque and Soares, 1994; Visschedijk, 1968b). The stage of embryonic mortality has been related to eggshell conductance; late embryonic mortality is higher in eggs with high eggshell conductance (Bamelis, 2003).

The role of yolk sac membrane and cam in gas exchange

Gas exchange between the embryo and the air diffused into the egg takes place through specialized and highly vascularized embryonic organs. During early incubation, the yolk sac membrane is the respiratory organ. Later, at d 5 of incubation of chicken eggs, the CAM which is a fusion of the chorion and allantois, starts to develop to assume the respiratory function of the embryo. By d 11 of incubation, the CAM is fully developed lying attached under most of the inner eggshell membrane and fully functional (Romanoff, 1960). The gas fluxes between the embryo and the environment depend on 1) the gas partial pressures in the blood of the extra-embryonic circulation, 2) the effective gas exchange area and 3) the thickness and diffusive properties of the material separating the red blood cells from the environment.

The primary gas transport system of the yolk sac membrane begins to function after the onset of circulation at d 2 of incubation. Meuer and Baumann (1988) showed that the major part of the diffusion resistance between the environment and the gas vessels at this period is provided by the albumen layer that separates the yolk sac from the inner shell membrane. The low pO_2 values in the intraembryonic venous vessels of young embryos at d 4 – d 6 further suggest that the gas transport system is working at the limit of its capacity and could be determinative for early embryonic development.

When the allantoic sac develops and fuses with the chorion (at about d 8), respiratory

function is successively transferred to the blood vessels of the newly formed CAM. The outer surface of the organ forms a very dense capillary bed. Dusseau and Hutchins (1988) studied the vascular density of the CAM in chicken eggs with time from d 7 and found an increase in CAM vascular density index (VDI) of 36% and 68% on d 10 and d 14 respectively. They also found that O_2 supply significantly influenced the VDI. Strict *et al.* (1991), incubated chicken eggs in 12, 16, 21, 45 and 70% O_2 from d 7 to d 14 and showed that the graded exposure to O_2 produced a dose-dependent change in VDI. Hypoxia increased and hyperoxia decreased VDI. The CAM attains its maximal growth at d 14 – 16 and corresponds to the time of the maximal growth rate of the embryo (Romanoff, 1967; Dietz *et al.*, 1998). From this period, 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 (Dunker, 1978), enhance oxygen delivery rate. Several studies (Paganelli *et al.*, 1988; Visschedijk, 1968a; Seymour and Visschedijk, 1988; Reizis *et al.*, 2005) have shown that respiratory gas exchange is higher in the portion of the CAM under the air cell. This has been related to the greater porosity and conductance of the shell in the blunt end of the egg where the air cell is formed.

The role of oxygen

OXYGEN REQUIREMENT AND ITS EFFECTS DURING PRE-INCUBATION PERIOD

Oxygen is the gas that drives the metabolic machinery of the embryonic cells in order to execute the complex manoevers of development (Rahn and Ar, 1979). Before incubation, hatching eggs are usually stored for some days after lay. Few studies have actually demonstrated, directly, the effects of O_2 on the egg during the storage period. Proudfoot (1964, 1965) demonstrated that storing eggs in high concentrations of air is detrimental to hatchability. In other studies where eggs were stored in closed environments such as plastic bags of different permeability, it was shown that the gaseous environment of the egg during storage influenced egg quality and the hatchability of eggs (Becker, 1964; Becker *et al.*, 1964; Proudfoot, 1963; Bose and Stewart, 1948 : Rutherford and Murray, 1963; Goodwin *et al.*, 1962; Fletcher *et al.*, 1958).

In another experiment where eggs were stored unpackaged or packaged in cryovac bags before incubation, Becker *et al.* (1968) concluded that there may be an optimal level of CO_2 and O_2 for the embryos prior to incubation and that these levels are different from that in air. These studies suggest that egg storage in air before incubation increases the loss of CO_2 from the egg, increases albumen pH, decreases Haugh units, increases water loss and inevitably lowers hatchability of eggs and prolongs hatching time. However, it is not absolutely clear whether these effects are directly the effect of oxygen in air or the loss of water from the egg during storage. The reports of Hinton (1968) and Walsh *et al.* (1995) concluded that a requirement for long-term storage of eggs was the prevention of water loss from the egg to avert a delay in embryonic development, early embryonic mortality, extensions of incubation duration (Mather and Laughlin, 1976; 1979) and a decline in hatchability. The studies of Becker *et al.* (1964; 1968) using chickens and turkey eggs reported that the effects of water loss are only apparent if eggs are stored for over 8 days. Thus the effect observed on egg quality parameters and hatchability when eggs are stored in air unpackaged for up to 7 days is most likely to be related to gas exchanges in the environment of the egg.

OXYGEN REQUIREMENT AND ITS EFFECTS DURING INCUBATION PERIOD

During incubation, the oxygen uptake of the avian egg increases exponentially as the embryo grows rapidly during the first two weeks (Tazawa, 1980; Vleck *et al.*, 1979; Gefen and Ar, 2001). In practice, chicken eggs are currently incubated in a gaseous environment of 21% O_2 in the presence of CO_2 that may be up to 0.5% at varying periods during incubation to achieve optimum embryo development and hatchability. Incubator ventilation is designed to provide adequate O_2 to the embryo and eliminate excessive CO_2 from the incubator. In this part of the review, the effects of hypoxia and hyperoxia will be discussed.

In nature, O_2 supply varies with altitude and thus the possibility for the occurrence of a condition of hypoxia is a reality. Oxygen supply decreases with high altitudes and influences incubation duration and hatchability (Visschedijk, 1985; Hassanzadeh *et al.* 2002). Smith *et al.* (1969) showed that incubating eggs at high altitude retards embryo growth. As the oxygen level decreases with altitude, its requirement and consumption by the embryos might decline as a result of adaptation leading usually to shorter incubation duration and early hatch (Julian, 2000; Hassanzadeh *et al.*, 2004). The aetiology of ascites in broiler chickens has been linked to differences in the oxygen requirements of fast and slow growing lines of chickens during the incubation period (Peacock *et al.*, 1990; Julian, 1993, 2000; Hassanzadeh *et al.*, 2004).

Effects on hatching process and hatchability

Several studies have investigated the requirements for O_2 at different stages of incubation and their effects on embryo development, hatching process and hatchability (Cruz and Romanoff, 1944; Taylor *et al.*, 1956; Dzialowski *et al.*, 2002; Altimiras and Phu, 2000; Chan and Burggren, 2005; McCutcheon *et al.*, 1962; Stock and Metcalfe, 1987; Stock *et al.*, 1983; Strick *et al.*, 1991). Chronic and acute hypoxia or hyperoxia have been reported to influence the development of chick embryo and hatchability differentially and their effect may depend on the timing of their application during incubation.

The early studies of Cruz and Romanoff (1944) and Taylor *et al.* (1956), show that chick embryos are very sensitive to O_2 deprivation during early incubation. O_2 levels below 18%, applied during the first 5 days of incubation depressed hatchability relative to the level of O_2 deprivation. At levels below 10% (corresponding to acute hypoxia) significant embryo mortality was recorded. The authors also reported that chick embryos can tolerate O_2 levels up to 60-79% beyond which hatchability becomes depressed. In contrast to short-term exposure, Barott (1937) reported that continuous exposure to O_2 levels of 30-50% throughout incubation decreased hatchability. These two contrasting results suggest that high O_2 levels may be beneficial or detrimental at certain developmental windows during incubation.

Oxygen requirements during the mid-incubation period (5-8 days and 9-12 days) have been studied by Taylor and Kreutziger (1965, 1966). O_2 levels below 15% depressed hatchability similar to that at day 1-5, the effect becoming acute at about 12.5%. This suggests a better tolerance to acute hypoxia with increasing age of the embryo. At day 5 - 8, O_2 levels above 45% were detrimental to hatchability of embryos. Tolerance level increased to 60-65% at day 9-12 suggesting that the high O_2 tolerance of the embryos shifts as the embryo grows during mid incubation. Similar shifts in O_2 tolerance had earlier been reported by other researchers (Barrot, 1937; Saddler *et al.*, 1954; Riddle, 1924; Remotti, 1933).

The tolerance of embryos to hyperoxia increases further between d 13 and d 16 of incubation when tolerance level can be as high as 85% (Taylor and Kreutziger, 1969) with no effect on hatchability. Stock *et al.* (1983, 1985) reported enhancing effect of hyperoxia of up to 70% on embryo growth at d 13 – d 16. Between d16 and d18, the tolerance of the

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embryo to hyperoxia shifts again to a lower level. Taylor *et al.* (1971) reported a tolerance level of up 50% with higher levels resulting in significant reduction in hatchability. Similarly, Stock *et al.* (1983, 1985) reported an enhanced embryo growth but that the effect of 70% O_2 was less than that obtained with 40 or 60% O_2. As in the earlier stages of development, O_2 levels lower than 16% reduced hatchability and embryo growth (Taylor *et al.*, 1971; Stock and Metcalfe, 1987).

Physiological, morphological and metabolic effects

Early indications as to how O_2 might influence the physiological processes involved in embryo development came from the studies of Remoti (1933) in which he observed that exposure to varying levels of O_2 had effect on the vascularity and the density of the allantoic membrane. More recent studies have also affirmed that O_2 levels during incubation influence the morphological and physiological aspects of embryo development (Chan and Burggren, 2005; Altimiras and Phu, 2000; Dzialowski *et al.*, 2002; Burton and Palmer, 1992; Hopes and Jahn, 1995; Strick *et al.*, 1991).

Embryo growth

Several studies have shown reduced survivability during early development due to the effect of hypoxia on metabolism and growth of the embryo (Sharma *et al.*, 2006; Ruijtenbeek *et al.*, 2003; Altimiras and Phu, 2000) suggesting that a threshold of O_2 availability is required to initiate and sustain early embryo development. Some studies suggest that chronic hypoxia reduces the body mass of the embryo whether it is applied during early, mid or late in development (McCutcheon *et al.*, 1982; Stock and Metcalfe, 1987; Burton and Palmer, 1992; Rouwet *et al.*, 2002; Dzialowski *et al.*, 2002; Sharma *et al.*, 2006; Villamor *et al.*, 2004) whereas others found no changes (Chan and Burggren, 2005; Altimiras and Phu, 2000). The studies of Stock *et al.* (1983, 1985), Stock and Metcalfe (1987), clearly showed that hyperoxia during the second half of incubation is beneficial to the growth of the embryo. O_2 demand increases during the latter part of incubation due to increased metabolic activities. The findings of Stock *et al.* tend to support a hypothesis that O_2 availability at this period limits growth and metabolism of chick embryos incubated under normoxic conditions as currently practiced. Physiological parameters such as embryo metabolism show a decrease under hypoxia (Dzialowski *et al.*, 2002; Stock and Metcalfe, 1987), without a change in T_3 but higher corticosterone level (Blacker *et al.*, 2004) suggesting that embryos under hypoxic conditions may be under stress.

CAM development

Strick *et al.* (1991) reported that CAM development was inversely related to the level of O_2 supplied during mid-incubation (7-14 days). Data from the reports of Chan and Burggren (2005), Richards *et al.* (1991), Hoper and Jahn (1995) show that chronic hypoxia enhances the vascularity and the weight of the area vasculosa when applied early or late during incubation but depresses its growth if applied throughout the duration of incubation (Burton and Palmer, 1992). Conversely, hyperoxia decreases the vascularity of the area vasculosa and weight of the CAM (Strick *et al.*, 1991; Hoper and Jahn, 1995). This seems rather incompatible with the growth promoting effects of hyperoxia as growth requires adequate vascular distribution of nutrients.

Cardiovascular system

After 4 days of incubation, the chick embryo reaches a critical mass that requires cardiac function and intravascular blood flow for nutrient delivery for metabolic activities. Many studies have shown that hypoxia impairs cardiovascular development and function.

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Reports are conflicting about heart mass. It has been variously reported to increase (Stock and Metcalfe, 1987; Rouwet *et al.*, 2002; McCutcheon *et al.*, 1982), remain unchanged (Dzialowski *et al.*, 2002, Altimiras and Phu, 2000; Chan and Burggren, 2005) or decrease (Richards *et al.*, 1991) when embryos were exposed to hypoxia. However, heart defects that alter heart rates and detrimental vascular remodeling that impair arterial pressure have consistently been reported (Sharma *et al.*, 2006; Tobita and Keller, 2000; Ruckman *et al.*, 1985; Villamor *et al.*, 2004; Crossley *et al.*, 2003; Haring *et al.*, 1970).

Brain and lungs

Other organs have also been reported to be affected by hypoxic incubation. Brain weight decreased (Stock and Metcalfe, 1987; Richards *et al.*, 1991) or remained unchanged (Assom-Batres *et al.*, 1989; Chan and Burggren, 2005; Ruijtenbeek *et al.*, 2003) when applied in the last third of incubation. Lung to body mass ratio increased (Xu and Mortola, 1989) and the development of lung surfactant system is advanced (Blacker *et al.*, 2004) when applied towards the last days of incubation. It can also advance the switch from embryonic to adult haemoglobin (Hb) (Baumann *et al.*, 1983) or alter haematocrit levels (Xy and Mortola, 1989). These changes in lung functions under hypoxic conditions enhance the transition to pulmonary ventilation and early hatching.

Ascites in broiler chickens

In broilers, hypoxia during the last stages of incubation is associated with the incidence of ascites (Decuypere, 2002). Structural changes in the cardiovascular and pulmonary systems have been observed during late embryonic development (Decuypere *et al.*, 2005). Julian (1989) reported that insufficient lung volume, or oxygen exchange area, in meat-type chickens leads to ascites. Broiler embryos incubated at high altitude hatch early, have higher T_3, T_4, corticosterone and embryonic mortality but lower incidence of ascites at high altitude compared to those incubated at low altitude and transported to high altitude (Hasssanzadeh *et al.*, 2004). This suggests that the incidence of ascites is not only related to oxygen insufficiency at high altitude but can be alleviated by acclimation of the embryos to oxygen insufficiency during incubation.

When incubated under normal conditions, ascites sensitive lines show lower metabolism, lower thyroid activity at the end of incubation and lower embryonic body weight early in incubation compared to ascites resistant lines (Dewil *et al.*, 1996; De Smit *et al.*, 2005) suggesting that 21% O_2 may not be sufficient for ascites sensitive lines of broilers. This agrees with the findings reported for the Leghorn egg type lines. This suggests that more studies are required to establish the requirements of the different lines of chickens especially in view of recent studies that have shown that metabolic rates differ between the embryos of layers and broilers and also between different lines of broilers (Janke *et al.*, 2004; Tona *et al.*, 2004; De Smit *et al.*, 2005; Chwalibog *et al.*, 2006).

The role of carbon dioxide

The advent of large capacity incubators has made the storage of eggs before incubation a necessity in order to make up the numbers that are required to fill the incubators. Storage of eggs before incubation has consequences on the quality of the eggs and their hatchability. At lay, the egg contains a copious amount of bicarbonate and CO_2 stored in the albumen (Healy and Peter, 1925; Brook and Pace, 1938).

The pH of the albumen has variously been recorded to range from 7.6 to 8.2 at lay but increases to about 9.2-9.7 after storage in air (Becket *et al.*, 1968; Tona *et al.*, 2001). This indicates that the egg loses CO_2 during storage (Smith, 1933) and this has been shown to

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alter the Haugh units (a measure of the internal quality of the albumen). Paradoxically, the egg must lose some of the CO_2 to improve its hatchability but the loss must come at the right time. Decuypere *et al.* (2001) stated that excessive loss of CO_2 leads to high albumen pH and may have a negative effect on the initiation of embryo development.

Gillespie and McHanwell (1987) showed that optimum gastrulation occurs at pH 8.2. Thus the alkalinity of the albumen needs to be preserved. A few reports have shown that the supply of CO_2 during incubation may be beneficial to the growth of embryos and the hatchability of eggs. In nature, hypercapnia is common occurrence under the incubating hen (Walsberg, 1980) suggesting that CO_2 may have a role in incubation.

CARBON DIOXIDE REQUIREMENT AND ITS EFFECTS DURING PRE-INCUBATION PERIOD

During the pre-incubation period, hatching eggs must be stored such that it retains its original pH, appropriate CO_2 content in the albumen and Haugh Units to maintain good quality before setting for incubation. Thus storage must ensure inexcessive loss of CO_2 from the egg. Cotterill *et al.* (1958) concluded that the total amount of CO_2 given off during storage is a function of the partial pressure of CO_2 in the atmosphere. Smith *et al.* (1931) demonstrated that in order to maintain the pH of egg albumen near its original value at lay, the storage atmosphere should contain 2-3% CO_2 at 32°F and 3-4.5% CO_2 at room temperature. Thus Swanson *et al.* (1954), Cotterill *et al.* (1957), Fletcher *et al.* (1959) and Becker *et al.* (1968) showed that storing eggs in moisture proof packages in a CO_2 atmosphere reduced the loss of albumen quality and maintained pH at the level of fresh eggs.

The presence of ambient CO_2 during storage decreases the rate of CO_2 loss such that the total amount lost is minimized over a given period of storage depending on the environmental temperature. Romanoff (1960) reported that an increase in albumen pH is associated with a decrease in albumen quality and an increase in early embryonic mortality. An increase in early embryonic mortality has also been associated with good albumen quality in eggs incubated without storage (Brake *et al.*, 1993).

Meuer and Baumann (1988) reported that the albumen of a fresh egg presents great resistance to gaseous diffusion during early incubation and ultimately lead to embryonic mortality. Walsh *et al.* (1995) reported that eggs stored in CO_2 environment for 7 days exhibited higher albumen height but higher embryonic mortality than the controls whereas those stored for 14 days also had higher albumen height and lower pH but lower embryonic mortality than the controls during incubation. Becker *et al.* (1964) recorded higher hatchability results in chicken eggs stored in CO_2 for 8-15 days compared to the controls.

Sauveur *et al.* (1967) reported three times higher hatchability of the eggs of a broiler type chicken when eggs were stored in 2% CO_2 atmosphere for 3 weeks and that hatchability decreased as the duration of storage decreased. The reports of Sauveur *et al.* (1967) and Walsh *et al.* (1995) stressed a greater benefit of storage in CO_2 for eggs from older breeder hens than those for young breeder hens, probably because of the higher conductivity of shells of older breeders, hence a quicker loss of CO_2.

CARBON DIOXIDE REQUIREMENT AND ITS EFFECTS DURING INCUBATION PERIOD

During incubation, CO_2 is released by eggs, first from the natural reservoir in the albumen, combined with the limited metabolic production by the early embryo, and later as the metabolic byproduct of the developing embryo and limited release by the albumen. Burke (1925) reported that in the naturally incubating hen, the CO_2 in the air around the clutch of chicken eggs increased from 0.05% to 0.9% by the end of incubation. Freeman

and Vince (1974) also showed that as incubation progresses, the embryo produces several times the CO_2 produced during earlier stages of development.

Lundy (1969), Taylor and Kreutziger (1965, 1966, 1969), Taylor *et al.* (1971) have shown that chicken embryos become less sensitive to elevated nest or incubator CO_2 concentrations with increasing age. Although higher incubator CO_2 levels have previously been regarded as detrimental to embryo development, recent reports have shown that hypercapnia may be beneficial to the developing embryo depending on the timing of its occurrence. In practice, CO_2 levels in the range of 0.1-0.5% are used during incubation of poultry eggs. Recent research studies have shown that higher levels change the course of embryo development and the hatchability of the eggs.

Effects on hatching process and hatchability

The early studies of Taylor *et al.* (1956), Taylor and Kreutziger (1965, 1966) showed that CO_2 concentrations more than 1% during the first 4 days of incubation, 3% from 3 to 5 days of incubation, 6% between 9 and 12 days, 8% during d13 – d16 or more than 7% from d 17 to d 20 of incubation depressed hatchability. The presence of CO_2 levels higher than 6–7% has been shown to decrease O_2 levels in the incubator significantly and exacerbates the detrimental effects of these high CO_2 levels (Taylor and Kreutziger, 1989; Taylor *et al.*, 1971). However, in the second half of the incubation period, the restoration of O_2 levels to normoxic levels in the presence of high CO_2 has been reported to restore optimum hatchability while restoration to hyperoxic levels caused an increase in hatchability compared with control incubations (Taylor and Kreutziger, 1969). This suggests a synergistic effect of CO_2 and O_2 at high levels which may be of benefit to the developing embryo. Romanoff and Romanoff (1933), Barrott (1937) also reported negative impact on the hatchability of chicken eggs when incubator CO_2 levels exceeded 1% during very early incubation.

More recent studies have shown that a gradual increase in CO_2 levels up to 1.5% in the first 10 days of incubation enhanced embryo growth, stimulated early hatching and increased hatchability of chicken or turkey eggs (Gildersleeve and Boeschen, 1983; Hogg, 1997; De Smit *et al.*, 2006; Tona *et al.*, 2006). Saddler *et al.* (1954) reported that 4% CO_2 stimulated embryo growth by as much as 20% in the first 48 hours of incubation. Haring *et al.* (1970) also recorded a 10% increase in embryo body weight when eggs were incubated for just 24 h on any one day in the first 10 d of incubation under a CO_2 level that has been shown to be teratogenic (6%). In the experiment, however, high embryo mortality was recorded.

Bruggeman *et al.* (2006) have also recently reported that hypercapnic embryos hatched earlier when CO_2 level in the incubator was increased to 1.5% at the 96th hr of incubation and maintained at that level until the 10th day of incubation. De Smit *et al.* (2007) demonstrated that increasing the incubator CO_2 level to 0.7% during the first 10 days of incubation accelerates the hatching process in ascites resistant as well as in ascites sensitive broiler lines. Everaert *et al.* (2007) found that exposure of the embryos to high CO_2 (4%) during the second half of the incubation period (d10 – d18) had no effect on hatchability or hatching time but increased embryo weights. These studies indicate clearly that the sensitivity of the chick embryo to environmental CO_2 changes with age as it does with O_2.

Embryo buffering and sub-embryonic fluid

The role of incubation CO_2 during early incubation has been shown to influence the rapid acidification of the albumen, liquefaction of the albumen and the formation of the sub-embryonic fluid (Benton and Brake, 1996; Bruggeman *et al.*, 2006). As CO_2 is highly soluble in egg albumen (Visschedijk, 1968), protons and bicarbonates are rapidly formed

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for albumen buffering effects around the developing embryo. The early liquefaction of the albumen also facilitates the movement of various nutrients from the albumen towards the embryo (Burley and Vadehra, 1989) and also reduces any physical barrier to O_2 diffusion to the embryo (Meuer and Baumann, 1988).

Latter and Baggott (2002) have demonstrated in vitro using quail embryos that CO_2 has a role in the formation of the sub-embryonic fluid (SEF) during early incubation. Deeming (1989) reported that the formation of the SEF has a pivotal role in the survival of the embryo. De Smit *et al.* (2006) have argued that with the early and enhanced occurrence of these processes in the presence of higher CO_2 environment, embryonic development is enhanced and thus explains the higher body weights recorded for embryos under tolerable hypercapnic conditions during early incubation (De Smit *et al.*, 2006; Tona *et al.*, 2006; Saddler *et al.*, 1954).

Meuer *et al.* (1989) reported that exposure to high CO_2 (3%) can lead to decreases in blood and tissue pH of the embryo which may impact on cellular processes. Dawes and Simkiss (1969, 1971), Boutilier *et al.* (1977) have shown that the chick embryo is capable of maintaining blood pH with little changes when incubated under hypercapnic conditions either in the first half or the second half of incubation periods. CO_2 levels up to 9% CO_2 did not affect acid/base balance in the blood during the 12th-17th day of incubation (Dawes and Simkiss, 1971). This has been shown to be due to the capacity of the embryo to generate or increase bicarbonate from shell reabsorption or renal/allantoic compensations.

In recent studies, Everaert *et al.* (2007) affirmed that the chick embryo has several adaptive mechanisms for coping with high environmental CO_2 during the second half of the incubation period. They observed that blood pCO_2 did not differ between the control embryos and those exposed to incubator CO_2 level of 4% between the 10th and 18th day of incubation. Blood bicarbonate concentration was higher in the high CO_2 group but was not accompanied by a more acid pH suggesting that protons were taken up from the blood and were buffered elsewhere. The buffering caused a temporary increase in blood calcium suggesting a reaction of the protons with calcium carbonate from the shell. There was also an increase in potassium suggesting an exchange of H^+ with K^+ at the cellular level. The authors did not observe an increase in allantoic fluid acidity or ammonia level suggesting that renal contribution for buffering at this stage may be minor.

Bruggeman *et al.* (2006) measured albumen pH, CAM weight, air cell pCO_2, blood pCO_2 and bicarbonates in a CO_2-controlled incubator that increased CO_2 gradually between 25th and 96th hour to reach 1.5% at 96 hours and continued incubation until day 10 at 1.5%. The authors observed no difference in CAM weight compared with control incubation. However, albumen pH, air cell pCO_2, blood pCO_2 and bicarbonates were higher in hypercapnic embryos but blood pH was not different. The study suggests that CO_2 levels of 1.5% are tolerable to the chick embryo beyond 96th hour of incubation. Thus the increasing tolerance of the embryo to CO_2 during incubation may be ascribed partly to the several buffering mechanisms.

Embryo growth

Studies on the hypercapnic effects (0.4% at d14-d19) during the second half of incubation have shown that CO_2 also increased embryonic weight, reduced the incidence of ascites, initiated early hatch and increased chick hatching weight but some of these effects were more obvious in an ascites sensitive line of broilers than in an ascites resistant line (Buys *et al.*, 1998). De Smit *et al.* (2007) observed increased embryonic growth in broiler lines selected for ascites sensitivity or ascites resistance when eggs were incubated under 0.7% CO_2. Other reports have also confirmed the growth promoting effect of increased incubator CO_2 level either during early incubation or during the second half of

incubation (Saddler *et al.* 1954; Haring *et al.* 1970; De Smit *et al.*, 2006; Tona *et al.*, 2006; Everaert *et al.* 2007).

Post-hatch growth of chicks that hatch from eggs that were incubated under higher CO_2 levels (1.5%) has been reported to be higher during the first two weeks of rearing compared to those from control incubation (De Smit *et al.* 2006). In another study, De Smit *et al.* (2007) observed higher post hatch growth in ascites resistant and ascites sensitive lines that were incubated under higher CO_2 levels (0.7%) during embryonic development. At slaughter age, both lines had higher body weights but when exposed to stressful conditions they showed higher incidence of ascites and mortality.

Metabolic effects, organ development and embryo mortality

A decrease in hatchability often results from high embryonic mortality or morphological /physiological abnormalities in the presence of high CO_2 depending on the timing of application. The implications of higher CO_2 level on morphological/physiological aspects of development have not been studied in depth. It can only be speculated that the effect of CO_2 must have been an inhibition of those parameters that enhance embryonic survival.

Saddler *et al.* (1954) reported that CO_2 enhanced amnion closure during early incubation but high levels beyond 1% retarded its closure at a specific window of the 48-72 hours of incubation. The authors concluded that although the chick embryo is tolerant to high CO_2 up to 4% during the first ten days, relatively low tolerance periods may exist between the 2nd and 10th day of incubation. They established that 0.9% seems to be the highest tolerance level at these sensitive periods. Haring *et al.* (1970) reported that the high level of embryonic mortality after exposure to 6% CO_2 for 24 hours at any time during the first 10 d of incubation resulted from non-cardiac and cardiac malformations.

Incubation under hypercapnic conditions during the last half of incubation has also been reported to influence hatching process (Buys *et al.*, 1998) and the morphological and physiological parameters of the embryo (Mortola, 2004; Gonya and Stoke, 1998; Dawes and Simkiss, 1991; Buys *et al.*, 1998). Buys *et al.* (1998) associated early hatching in hypercapnic embryos with increases in plasma T_3 levels at day 20 of incubation. De Smit *et al.* (2006) and Tona *et al.* (2006) also reported increased T_3 and higher pCO_2 in the air cell at day 20 when embryos were incubated under hypercapnic conditions early in incubation.

Dawes (1975), Menna and Mortola (2003) and Mortola (2004) reported that short-term hypercapnia affects lung function during the transition to pulmonary ventilation at external pipping. The authors demonstrated the stimulatory effect of CO_2 on the chemoreceptors that enhance breathing efficiency and that hyperoxia at this period diminishes the effect of hypercapnia. Thus hypercapnia can achieve a similar effect as hypoxia on lung function during pipping and hatching.

Decuypere *et al.* (1991) and Visschedijk (1968) have linked the level of blood T_3, air cell pCO_2 to the process of early hatching. Thus the positive effects of hypercapnic incubation suggest an augmentation of T_3 and air cell pCO_2 to effect early hatch and enhanced hatchability. Visschedijk (1968b) demonstrated that covering the air space to increase pCO_2 accelerates pipping. However, more studies are required to establish the limit of beneficial levels.

Gonya and Stokes (1978) showed that progressive hypercapnia during the 14th-19th day of incubation is associated with a gradual decline in motor activity in the spinal cord with an effect on limb muscles of the chick embryo.

Conclusion

Taken together, these reports suggest that hypoxia at any level below 15%, no matter when applied but more especially during early development, alters the developmental profile of the embryonic chicken which may compromise the survivability of the chick, thus lowering hatchability. If the chick survives until hatch, it might be disposed to cardiovascular diseases as often seen in ascites chickens. There are indications however, that hypoxia during the pipping period may be beneficial for early hatching.

From hatchery management point of view, higher O_2 levels than are currently used at mid incubation period may be of benefit for embryo development and hatchability since any detrimental effects are not apparent below the 50% level. Taylor and Kreutziger (1966) actually reported higher hatchability than the control (21% O_2) in 8 of 9 experiments with O_2 above 21% at day 4-8 suggesting the possibility of increasing hatchability depending on the metabolic rate of the embryo.

With regards to the roles of CO_2, it would seem that the chicken embryo requires CO_2 at specific windows of development to enhance growth, early hatch and optimum hatchability. CO_2 tends to achieve similar effects as hypoxia as it also promotes the development and functioning of certain embryonic organs. Thus it could be substituted for O_2 at the effective level. For example, as hypoxia is detrimental at early incubation but has been reported to enhance CAM development, increasing the level of CO_2 at this time period may be practiced without detrimental effects on embryo development. And for the purpose of stimulating early hatch and shortening incubation period, CO_2 seems to achieve this when applied at the appropriate level early or late in incubation.

Further fine tuning and determining the critical windows for hypercapnia as well as for hypoxia or hyperoxia, and the optimal levels of CO_2 and O_2 during other periods seem to be a challenge for further research. These studies are also necessary for optimizing the gaseous conditions for practical incubation in order to obtain the best chick quality for later performance and robustness. These conditions may vary as a function of what is determined as best chick quality with respect to the strains of the chicks and the rearing conditions.

Acknowledgement

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