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Review

Poultry Egg Incubation: Integrating and Optimizing Production Efficiency

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ABSTRACT

Due to its central position in the production chain, *in-ovo* development is influenced by pre-incubation factors that affect the quality of embryonated eggs and incubation conditions themselves, and both may influence egg hatchability and chick quality, as well as bird survival, growth performance, and phenotype in the field. The evolution of the incubation process over the years is characterized by significant scientific and technological development. Presently, the main current focuses of research are the manipulation of thermal incubation conditions, eggshell temperature, and the integrated effects of factors that influence incubation. In this context, one of the questions that needs to be asked is how effective are the current physical conditions of incubation to promote greater hatchability and better quality chicks, and higher survival and better performance in the field under adverse conditions or not. What are the new and future prospects for incubation? The purpose of this paper was to review the role of the physical agents of incubation, such as temperature, relative humidity, O_2 and CO_2 concentration, and egg turning and position from an integrated perspective, considering egg incubation as the transitional link between egg and poultry production.

INTRODUCTION

Over the past 50 years, global annual meat production has almost quadrupled from 78 million tons in 1963 to 308 million tons in 2015, achieving an impressive growth from about 205 million tons to 319 million tons between 1995 and 2015. During these last two decades, the production of poultry meat increased almost 108% increase (from 54 to 112 million tons), corresponding to a 36% growth of its share in total meat production (Avisite, 2015; SNA News, 2015). Meat production is estimated to double by 2020-2022 due to the growth of the global population and of meat consumption *per capita*. According to this trend, global consumption of poultry meat is estimated in 128 million tons by 2022 (OACD / FAO Agricultural Outlook, 2015).

In order to meet this high demand for poultry meat, hatcheries need to maximize chick production, and this entails not only the incubation of more fertile eggs. Today, hatcheries need to achieve high production efficiency in a sustainable manner, which, in our view, includes maximizing the hatchability of healthy chicks with high survival rates and the maximum expression of their genetic growth potential under any conditions in the field.

Scientific knowledge on incubation acquired over the years shows that the physical factors to which the eggs are subjected before and during incubation determine the production efficiency of hatcheries and poultry farms. Nevertheless, little is known about the effective

participation of the integrated effects of physical factors during ontogenetic development on the phenotype of poultry during the different stages of production.

INCUBATION REVOLUTION

In the last few years, artificial egg incubation systems have experienced a technological, economic, and social revolution. Remarkable technological and scientific developments allowed the transition from manual incubation to large incubation machines and hatcheries, which incubate a much greater number of eggs using less labor, increasing chick production throughout the year. On the other hand, this incubation revolution generated costs related to the construction of more sophisticated facilities, as well as operational costs, such as energy and water expenses to maintain adequate incubation conditions. It also influenced social relations, creating two classes: producers and consumers.

The principles of artificial egg incubation were established centuries ago. At that time, heat, moisture and air renewal of the incubation environment, well as the egg turning, were already taken into consideration. Based in historical records, Paniago (2005) and van den Sluis (2011) mention that, in ancient Egypt, eggs were incubated in mud-brick buildings (an "incubation house") divided in incubation chambers similar to ovens separated by a central hallway and accessible through manholes. In the upper part of the egg chambers, there were shelves for burning, straw, dung, or charcoal to heat the eggs below. Vents in the roof allowed the smoke from the fires to escape and provided some light. In this primitive incubation system, the temperature within the incubation chambers was managed by controlling fire intensity and opening the manholes, vents, and the hallway. Humidity was controlled by placing moistened jute on eggs, which were manually turned twice per day. Mechanical incubating was not invented until the year of 1749 by Reamur in Paris, France, and the first commercial incubator was manufactured by Hearson in 1881.

An incubator should to be able to regulate factors, such as temperature and humidity, and to allow air renewal and egg turning, providing the perfect environmental conditions for embryonic development, aiming at achieving high hatchability of healthy chicks, which is directly correlated with the survival and performance of individual chicks in the field. Currently, incubators capable of incubating different numbers of eggs of different species of birds are commercially available, with more or less sophisticated of temperature, humidity, ventilation, and egg turning control systems. Modern state-of-the-art commercial hatcheries are provided with automatic systems controlling all the physical factors of incubation: egg turning; environmental temperature set according to eggshell temperature determined by thermosensors; air relative humidity and egg water loss determined by egg tray weight using weight sensors; and air quality (O_2 and CO_2 levels). However, as already pointed out by Paniago (2005), despite the technological advances of the modern incubation machines, the success of incubation still depends on the quality of labor both inside and outside the hatcheries, which requires training.

PHYSICS OF EGG INCUBATION: AN INTEGRATED PROCESS

From a macroscopic point of view, although the external and egg internal environments seem to be completely isolated, the eggshell participates in and allows exchanges between those two environments, as determined by the interaction among temperature, relative humidity, ventilation (air quality) and egg turning during incubation, which are essential for the success of embryonic and fetal development. The physical exchanges between the egg and the external environment (egg and air of the incubator) include heat transfer and the exchange of O_2 CO_2 and water. Egg characteristics (size, composition, and shape, and eggshell thickness, porosity, and heat and water vapor conductance), embryo metabolism rate and physical incubation conditions, as well as pre-incubation conditions, may cause deviations from the optimal values of these physical agents (Figure 1). Such deviations may can interfere with, or even hinder, *in-ovo* development, resulting in negative effects on hatchability and on the quality of the hatchlings and their subsequent performance, phenotype, and survival. In contrast, optimal physical incubation conditions benefit egg hatchability and chick quality, with possible survival and performance benefits.

Egg heat transfer

Heat transfer occurs when there is a temperature difference between two regions or media, and always on the thermal gradient. Eggs present four mechanisms for heat transfer: conduction, radiation, convection, and evaporation (Meijerhof & van Beek, 1993; French, 1997). However, eggs gain or lose heat only

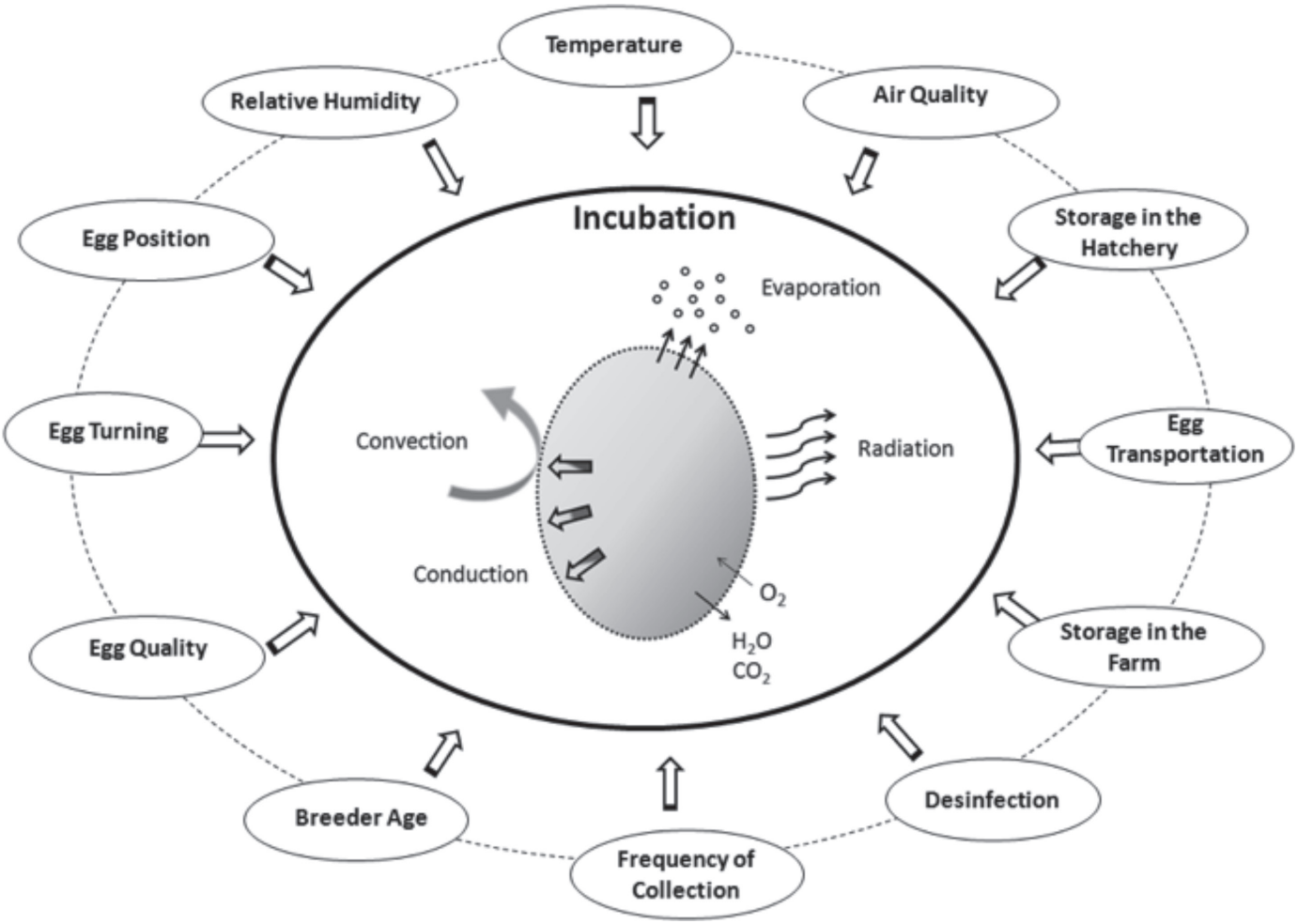

Figure 1 – Physical exchanges of the eggs with the environment during incubation (heat transfer, water loss and gases exchanges) depend of the egg characteristics (size, composition, form, and eggshell thickness, porosity and heat and water vapor conductance), embryo metabolism rate and physical incubation conditions, but also of the pre-incubation conditions.

when there is a temperature difference between the environment and the eggshell, and this is influenced by several factors associated with egg quality (breeder age; egg size, composition, and shape; and eggshell characteristics), water loss, and incubation conditions.

Conduction: Heat transfer by conduction occurs between regions or media that are in contact with each other, from the warmer to the colder regions. Heat transfer rate by conduction depends on the temperature difference and the thermal conductivity of the regions involved. Therefore, in the egg, heat is transferred by conduction from the embryo to the eggshell, provided their temperatures are different, as well as to the air layer in direct contact with the eggshell. Eggs gain or lose heat when air temperature is warmer or colder than the eggshell, respectively. However, heat transfer by conduction inside the eggs is faster than that from the eggs to the air, because the water present in the eggs has higher thermal conductivity compared with air. Therefore, conduction accounts only for a small portion of heat transfer from the egg to the environment. This also means that the heat transfer by conduction between the embryo and the egg surface depends of the egg water content and may be influenced by egg water loss. Other factors influence heat transfer by conduction, such as egg size, eggshell thickness, and embryo metabolic heat production rate, which is determined by egg size and thermal incubation conditions.

Convection: Convective heat transfer refers to heat transfer by air currents, and occurs when a body loses heat by conduction. Therefore, when eggs lose heat by conduction to the surrounding air, the air near the eggshell is warmed and rises, moving cooler air moves near the eggshell in replacement of the warm air, generating convection currents, which help to remove heat from the egg. It should be emphasized that convection currents are essential for the egg to continue to lose heat by conduction, because conductive heat loss does not take place when the air temperature near the

eggshell is similar to that of the eggshell. In this context, air movements across the eggshell surface in the setter and the egg turning program (tilt angle, velocity, and frequency) should be taken into consideration, because they influence conductive-convective heat dissipation from the eggshell. More or less intense movements of the air or of the eggs in the setter increase or decrease, respectively, egg heat loss by conduction and convection and may influence embryo and/or fetal development and, consequently, incubation production efficiency. The rate of heat transfer by conduction and convection from the eggs to the environment are required for optimal *in-ovo* development. However, this rate changes during incubation according to the physical incubation conditions, determined by temperature, relative humidity, air movement, and egg turning, as well as to eggshell characteristics, such as total surface area, thickness, and conductivity.

Radiation: Radiant heat transfer occurs from the surface of a warm body by emission of heat waves that propagate through air. On the other hand, when radiant energy is absorbed by a body, it is transformed into heat. According to this thermodynamic principle, egg heat loss or gain by radiation depends on the temperature difference between egg surface and the surfaces in the incubation environment. Therefore, radiant heat transfer allows heating the eggs. The sources of radiant heat used throughout the history of artificial incubation, were the sun, burning of coal, manure, or gases, electricity, etc. However, the exposure of eggs to environmental temperatures higher than the eggshell temperature (very high incubation temperature) may increase conductive and radiant heat gain by the eggs, which in turn experience hyperthermia and may compromise embryonic and fetal development due to the lack of compensatory heat loss. According to these principles, radiant heat loss occurs when the eggs are exposed to temperatures lower than the eggshell temperature. The exposure of eggs to low environmental temperature causes hypothermia, reducing or precluding embryonic and fetal development, and may lead to both embryonic or fetal death.

Evaporation: According to laws of thermodynamics, evaporative heat loss occurs when water is changed from liquid into gas, because this reaction requires heat. In addition, the flow of water vapor occurs from high vapor pressure or high humidity to low vapor pressure or low humidity, respectively. For this reason, eggs lose heat by evaporation by the diffusion of water molecules through the eggshell pores as a result of the higher water vapor pressure inside the eggs relative to the outside. This means that evaporative heat loss is determined by eggshell conductance, which depends on eggshell pore number, size, and shape; and on the incubation physical conditions established by temperature, air relative humidity, air movement, and egg turning. Evaporative heat loss from the egg is of 2,26KJ per gram of water loss, and causes the cooling of the eggshell (Meijerhof, 2013), which is important for the establishment of heat loss by conduction.

Egg Water transfer

Water diffusion is a physical process that, according to Fick's laws, is the movement of water molecules down a concentration gradient. The diffusion flow is given by concentration gradient and by temperature: the higher the gradient and the temperature, the faster is the water diffusion. Eggs lose water by diffusion through the eggshell as a result of the water pressure differences between the inside and the outside of the egg, as determined by the temperature and relative humidity of both sides. Egg water loss depends of eggshell porosity (Deeming, 2002), given by its pore number, diameter, length, and shape. Therefore, it is higher in eggs laid older breeders or larger eggs from a same breeder age incubated at high temperatures and/or low relative humidity levels (Morita *et al.*, 2009, 2010; Sgavioli *et al*., 2015). Although the velocity of the air on the egg surface has no direct effect on water loss (Meijerhof & van Beek, 1993), as previously mentioned in this review, it allows continuous conductive-convective heat dissipation, and therefore, indirectly influences egg water loss.

Egg water content is a finite quantity of water deposited in the yolk and in the albumen during the ovarian folliculogenesis and egg production in the lateral oviduct of the birds. Egg water content corresponds to just over 70% of its initial weight (74.3% in chickens, 71.8% in guinea fowls, 71.9% in turkeys, 70.3% in geese and ducks, and 73.4% in quails (Romanoff & Romanoff, 1949; Panda & Singh, 1990). During incubation, egg water content is absorbed by the embryo and fetus from embryonic annexes (amniotic cavity, allantois, yolk sac), (Davis, 1988; Ar, 2004), whereas a small portion is lost to the external environment (Drent, 1970; Ar & Rahn, 1980). In addition, metabolic water is also produced inside the eggs by embryo lipid metabolism, and particularly by fetal lipid metabolism during the last week of incubation (Boerjan, 2006), accounting for 8 to 13%of the fetal water content (Ar, 2004).

As previously mentioned in this paper, water loss during incubation is associated with egg heat loss by evaporation and conduction. However, water loss is important not only for egg heat loss, but also for the formation of the air chamber. The volume of the water lost is replaced by an equivalent volume of gas, determining the size of the air chamber (Visschedijk, 1968). When the egg air chamber is small (<1.8 inches), the chicks are not able to perform internal pipping; they pip below the inner membrane and die by drowning in the fluid still present in the amniotic and allantoic cavities. On the other hand, large air chambers (> 3.16 inches), indicate that the fluid has dried, and the chicks are born weak and adhered to the eggshell. Egg water loss is also important by gas exchange through the eggshell, as they occur through the same pores.

Egg O_2 and CO_2 exchanges

In-ovo development requires that all embryonic, fetal, and embryonic annex cells are supplied with the energy required for their survival, proliferation, migration, and differentiation. Energy utilization demands aerobic respiration, efficient oxygen supply, and carbon dioxide elimination. The demand for gas exchange increases during incubation as a result of the increasing metabolic rate of the embryo, according to its different developmental stages, i.e., embryo morphogenesis, fetal growth, and hatching. In order to ensure efficient gas exchange during *in-ovo* development, different surfaces are used. During the first three days of incubation, gas exchanges are carried out directly by the embryonic cells. As the embryo develops, the amniotic cavity is formed, which makes the direct gas diffusion from the embryonic cells inefficient, demanding the establishment of gas transport systems. From the third day of incubation, with the emergence of the embryonic circulatory system and yolk vascularization, gas exchange is exchanged through the vitelline vessels. Yolk vascularization continues as the embryo develops, increasing the surface area of gas exchange. However, fetal development requires greater O_2 supply than that provided by vitelline gas exchange. Furthermore, vitelline circulation is disappears as the yolk sac is incorporated in the fetal abdominal cavity, and its contents start to be absorbed exclusively by the intestinal route. Therefore, by days10-12 of incubation, gases start to be exchanged by the allantoic vessels, which supply the high oxygen requirements for increased fetal metabolism thereafter (Hamburger & Hamilton, 1951; Tazawa, 1980; Deeming, 2002; Mortola, 2009). After internal pipping, gases gradually begin to be exchanged via the pulmonary respiratory system (Decuypere & Bruggeman, 2006; Mortola, 2009), andO_2deficit and CO_2 saturation inside the egg air chamber are the main factors that induce external pipping and hatching *per se* (Mortola, 2009). In addition to changes in the gas exchange surfaces, hematological adjustments also occur, increasing gas exchange capacity during incubation: red blood cell counts (RBC), hematocrit (Ht) values, hemoglobin (Hb) levels increase, while mean corpuscular volume decreases (Morita *et al*., 2009; Tazawa *et al*., 2011, 2012).

Nevertheless, all those morphological and physiological adaptations do not ensure the efficiency of gas exchange. The embryonic and fetal development of the birds inside the eggs requires gas exchange primarily between the egg and the incubation environment. Therefore, gas exchange through the eggshell requires gas concentration differences between internal and external egg environments. If the O_2 concentration outside the eggs is higher than that present inside the egg, oxygen is diffused into the egg. If the CO_2 concentration is higher inside than outside the eggs, CO_2 diffuses in the opposite direction, i.e., out of the egg. Therefore, the concentration of these gases in the setter are essential for embryonic and fetal O_2 supply and CO_2 elimination.

INCUBATION PHYSICAL CONDITIONS

Air relative humidity

As mentioned above, egg water loss during incubation is essential for adequate *in-ovo* development; however, water losses outside a normal range may result in chick abnormalities or death *in ovo*. Low air relative humidity during incubation may cause excessive egg water loss, resulting in embryo dehydration and death (Reinhart & Hurnik, 1984) or the hatching of small and dehydrated chicks (van der Pol *et al*., 2013), due to fluid deficit in the amniotic and allantoic cavities, which impairs embryonic development and hatching. However, hatchlings with low body weight, as a result of skin and muscle dehydration, may present compensatory growth between 7 and 10 post-hatch, and normal development thereafter (Davis *et al*., 1988). On the other hand, if the air relative humidity is too high, the incubation period is shortened, and the chicks are wet at hatch and residual albumen may be present (Taylor, 1999; Decuypere *et al*., 2002; Tona *et al*., 2003).

Air relative humidity (RH) also influences evaporative egg heat loss, and consequently, embryonic or fetal temperature (Decuypere *et al*., 2002; Molenaar *et al*., 2010). As the amount of energy required to evaporate water is 2.26kJ, eggs lose 2.26kJ energy as heat per gram of evaporated water. Thus, the lower is the relative humidity inside the setter room, the greater is the loss of water by the egg and, therefore, its heat loss. This indicates that eggs incubated at low or high RH conditions may require different incubation temperatures to maintain the same embryo temperature (van der Pol *et al*., 2013), as both incubation relative humidity and temperature affect water vapor diffusion through the eggshell (Figure 2). Van der Pol *et al.* (2013) obtained higher hatchability in broiler eggs incubated at 55-60% RH when eggshell temperature was maintained at 37.8ºC. Boleli & Aidar (2013) determined that 36ºC and 60% RH are the optimal conditions for the incubation of red-winged tinamou (*Rhynchotus rufescens*) eggs.

Evaporative water loss around 12-14% relative to initial egg weight provide optimal hatchability (Tullett, 1981; Peebles, 1986). There is a consensus among studies that egg water loss should be approximately 12-14% of the initial egg weight. The following optimal air relative humidity in the setter room were determined: 40-70% for domestic chicken eggs (*Gallus gallus*) incubated at 37.8ºC (Lundy, 1969); 55% for turkey (*Meleagris gallopavo*) eggs (89.1g) incubated at 37.5ºC (Meir *et al*., 1983.); 61-65% until the 20th day and 70-73% after the 21st day of incubation for bobwhite quail (*Colinus virgianus*) eggs weighing approximately 12g and incubated at 37.5ºC (Kealy, 1969; Krueger, 1972; Wilson *et al*., 1975); and 20 to 25% until the 38th day and 40 to 50% from 39th day at 36.6ºC for ostrich (*Struthio camellus*) eggs weighing 1.406 to 1.525g (Horbanczuk *et al*., 1999; Kontecka *et al*., 2011). The majority of bird eggs loses 15% to 18% of its initial mass during incubation (Ar & Rahn 1980; Drent, 1970, Ar *et al*., 1974).

The current commercial setter models allow controlling egg weight loss during incubation by monitoring egg tray weight loss. However, the efficacy of this control depends on the knowledge of egg water loss during pre-incubation period (especially during the storage period) and on the homogeneous distribution (weight or size) of the eggs in the setter trays.

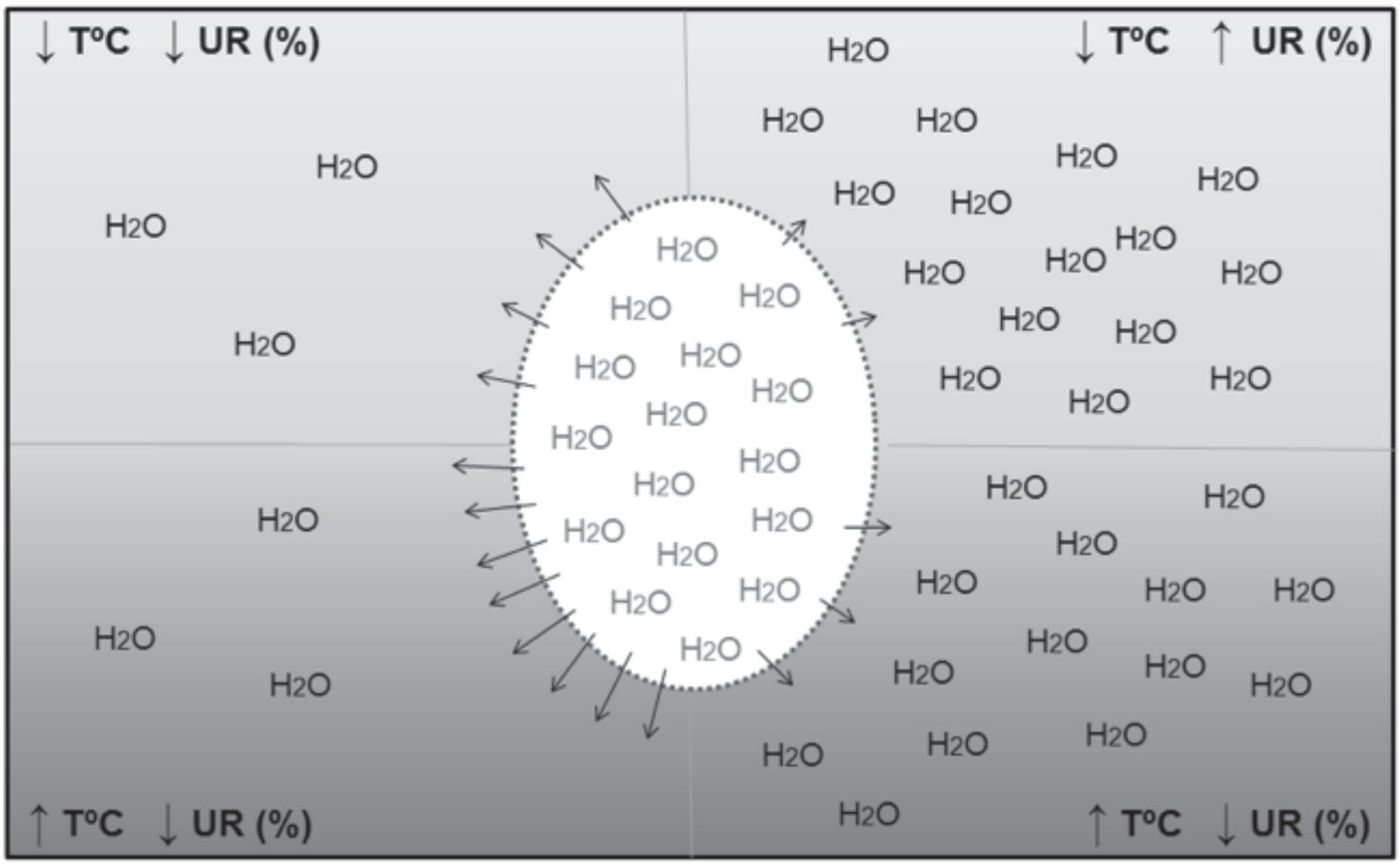

Figure 2 – Changes in the egg water loss during incubation with temperature (T) and relative humidity (RH).

Egg turning, egg position, and ventilation

The associated effects of egg turning, egg position, and ventilation influence several processes, including gas exchange and heat transfer between the eggs and the external environment, egg water loss, adhesion of the embryo to the extra-embryonic membrane structures (corium, amnion and allantois), and nutrient availability.

Egg turning is a natural behavior of birds during incubation, and therefore, this practice was included in the artificial incubation process. Egg turning allows the diffusion of gases inside the eggs and between the eggs and the external environment. It is critical particularly during the first week of incubation, due to the long distance between the embryo and the shell, and to the high albumen density. During this period, the embryo depends on the diffusion of gases through the eggshell and the albumen to obtain O_2 and eliminate CO_2, because the embryo develops on the yolk surface and gases are exchanged directly by the embryonic cells. After the emergence of the circulatory system (~36 h of incubation) and the formation of the amniotic cavity and yolk vascularization, gases are exchanged through the vitelline vessels. The allantois begins to protrude out of the embryo body around days 3-4 of incubation, and continues to grow in size, surrounding the amniotic cavity, where the fetus develops. As both fetus and allantois grow, the allantois comes closer to the eggshell, reducing the distance traveled by the gases and allowing gas exchange via allantoic vessels by diffusion through the eggshell pores. Gas exchange through the allantoic arteries and veins begins on days 11-12 of incubation. By days 13-14 of incubation, fetal metabolic heat production increases, and egg turning aids the circulation of air in the inner surface of the egg (external shell membrane) and air chamber, and allows heat loss by conduction, convection, and evaporation. However, egg turning is also important to prevent dehydration and incorrect embryo development (Wilson, 1991). Moreover, egg turning moves nutrients, facilitating their absorption (Brinsea, 2006).

Egg turning is not as simple as it may seem. Egg turning frequency, axis of setting, angle, and plane of rotation influence *in-ovo* development, which may affect hatchability and chick quality (Wilson, 1991). Landauer (1967) demonstrated that chicken hens turned eggs approximately 96 times daily during natural incubation. Kaltofen & Ubbels (1954) and Kaltofen (1956) showed that eggs turned 24 times per day (every hour) presented higher hatchability compared with eggs turned less frequently. Years later, Wilson (1990) observed that high egg-turning frequency (96 times/day or turning every 15 minutes) improves *in-ovo* development and hatchability. However, turning every 15 minutes is operationally difficult and increases equipment maintenance costs, and therefore, it is not applied in commercial settings.

The effect of egg turning rate on *in-ovo* development is also related to the tilt angle of the eggs. According to French (1997), normal embryo development requires eggs to be rotated 90 degrees every hour. This is achieved by horizontally tilting the eggs (horizontal setters) or the trays (vertical setters) over a 45° angle from side to side (Figure 3). This is the angle that best fits the operational conditions of commercial setters, according Elibol & Braket (2006) and Tona *et al.* (2005). Neves (2005), however, recommends that eggs should be horizontally turned 24 times daily at 20°-45° angles. In commercial hatcheries, broiler breeder eggs are turned 45° ± 5° per hour until day 18 of incubation. Eggs are submitted to circular movements because the chorioallantoic membrane may break, causing embryonic mortality (Brito, 2006). Chicks hatched from eggs that turned at 45° angle were heavier and presented lighter dry residual yolk (Cutchin *et al.*, 2009). Egg-turning failures may reduce the formation of embryonic fluids, as well as the formation and growth of embryonic annexes, hindering embryonic and fetal development (Robinson, 2013).

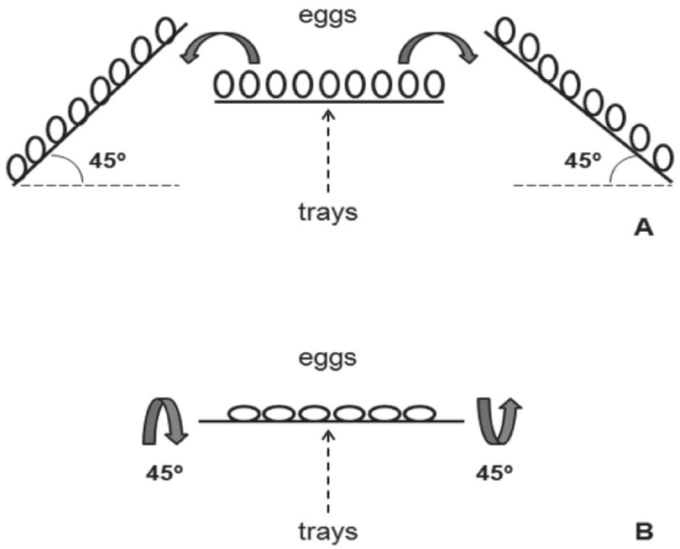

Figure 3 – Egg turning in vertical (A) and horizontal (B) incubators (adapted from Alvarado Mora, 2008)

Similar to natural conditions, eggs incubated in horizontal setters are set in a horizontal position. However, differences in egg size (jumbo, extra-large, medium, small) and egg shape (pointed, normal,

or round) may change its position, impairing *in-ovo* development. The position of the eggs in the setter is critical for the formation of the air chamber, which irregular position and size may prevent internal pipping (Deeming, 1989), and result in *in-ovo* mortality when the chicks do not perform direct eggshell pipping. In addition, the correct position of the egg in the setter allows adequate gas exchange through the shell during incubation (Rondon & Murakami, 1998). In vertical setters, eggs should be set with the large end up, i.e., where the air chamber is located, thereby allowing gas exchange between the egg and the environment. According to North & Bell (1990), up to 4% of eggs are set in the wrong position, *i.e.*, with the large end down, resulting in embryonic oxygen deficit and delayed metabolism. Wrong positioning of the eggs at setting may be related to slightly more rounded shape of the eggs, impairing the formation of the air chamber, and consequently, internal pipping.

Air renewal in the setter room is also essential in artificial egg incubation (Kornfeld *et al*., 2004). The fans in the setter room have two main functions: (i) to allow the intake of fresh air and removal of already circulated air, and (ii) to provide uniform air flow over the eggs, creating a homogeneous microclimate inside the setter that promotes adequate heat transfer, gas exchange, and water loss between the eggs and the incubation environment. The levels of CO_2 inside the setter room should not exceed 0.4% (Cobb, 2008). Sotherland *et al.* (1987) state that setter room ventilation should be able to reduce the heat transfer coefficient in the beginning of incubation to heat the eggs, and to increase it at the end to allow egg heat loss. Therefore, air renewal in the setter room is essential for the removal of excessive CO_2 and heat produced by the eggs, and for the restoration of O_2 levels (Calil, 2007). On the other hand, air speed within the setter room may influence *in-ovo* development: an air velocity of 2 m/s results in a 0.5-1°C difference between air and egg temperatures, while 0.5 m/s promotes a difference of 1-2.5ºC, which has strong effects the embryo development, resulting in weak hatchlings that may be unable to get out of the eggshell (Meijerhof and van Beek, 1993).

Air quality: O_2 and CO_2 concentrations

Maintaining of adequate O_2 and CO_2concentrations in the setter room is essential for efficient gas exchange between the eggs and the incubation environment. As previously mentioned, the diffusion of O_2 into the eggs and diffusion of CO_2 and H_2O out of eggs depends on the presence of pores in the eggshell and the gas concentration gradient between the internal and external environment of the eggs. Moreover, gas diffusion rate can be influenced by the physical characteristics of the eggs (surface area, pore number and geometry) and incubation conditions. In this context, larger egg surface areas, higher pore number and diameter, and thinner eggshells increase the rate of gas exchange between the eggs and the environment. This indicates that large eggs (typically laid by older hens) present greater gas exchange potential than small eggs (laid by young hens) (Morita *et al*., 2009). Gas diffusion increases with increasing temperature, according to the principles of physics. This means that the diffusivity of water vapor and gases through the eggshell increases as the incubation temperature increases (Booth & Seymour, 1987; Morita *et al*., 2009).

Determining the optimal concentration of gases for egg hatching eggs is not an easy task, because all factors that affect gas diffusion must be considered. Furthermore, it is essential to take into account embryonic and fetal growth rate is different between large and small eggs, and therefore, have different metabolic rates. In addition, the increasing energy requirements of the embryos as they develop mean that the optimal concentration of gases inside the setter room also changes during incubation. This indicates that, although it is possible to establish optimal O_2 and CO_2concentrations for egg incubation, from a practical perspective these concentrations can only be achieved in single-stage incubation. When establishing the optimal gas concentrations in the setter room required to optimize the incubation process, the effects of hypoxia and hypercapnia on embryonic and fetal development need to be considered.

Although setter rooms are provided with air renewal systems, the concentration of gases is determined by the quality of the atmospheric air where the hatchery is geographically located. The atmospheric concentrations of O_2 and CO_2 is the same at all altitudes (21% and 0.03% at sea level, respectively). However, at higher altitudes, the air has fewer oxygen molecules and lower partial pressure of O_2 (1.0% less for each 500m increase) due to the decrease in barometric partial pressure. Egg incubation in high altitude areas (over 600 meters above sea level) increases late embryonic mortality and reduces hatchability and hatchling weight due to reduced O_2 partial pressure (Schmidt-Nielsen, 2010; Sahan *et al*., 2011), which creates a hypoxic or a hypercarpnic environment (Coleman, 1986; Bagley & Christensen 1989; Mauldin & Buhr, 1991; Sahan *et al*., 2011.). Although the lower partial pressure

of O_2 leads to compensatory physiological responses (Tazawa; *et al.,* 1971; Ruijtenbeek *et al.,* 2000), these are not sufficient to restore normal embryonic and fetal development.

Hypoxia during incubation has different effects on poultry embryonic and fetal development, depending on the period of embryo development, and on the duration and level of hypoxia (Azzam *et al*., 2007; Ferner & Mortola, 2009;Zhang & Burggren, 2012). Interestingly, Bahadoran *et al.* (2010) found that early hypoxia (incubation at 1800m above sea level until embryonic day 10), followed by normoxia (incubation at sea level) reduces the duration of incubation, reduces the incidence of ascites, and improves the feed conversion ratio and body weight of 42-d-old broilers reared in normoxia. All these results indicate that the first half of incubation (1-11 days of incubation) is the critical window for the adverse effects of hypoxia on *in-ovo* development, while the second half (from day 12 until hatching) is the critical window for the compensatory response of organs to hypoxia. The effects of early hypoxia are related with the increase in the gas exchange surface, particularly to the increase in chorioallantoic mass and to angiogenesis (Chan & Burggren, 2005; Azzam & Mortola, 2007; Zhang & Burgreen, 2012), as well as with increased pulmonary angiogenesis and vascularization (Lewallen & Burggren, 2015).

Relative to hypercapnia, CO_2 levels in the setter equal to 1% or higher on days 1-4 (Taylor *et al*., 1956), 3% on days 5-8, and higher than 6% on days 9-12 of incubation (Taylor *et al*., 1965, 1966) decrease hatchability. However, other studies showed that the gradual increase of CO_2 concentration until day 10 of incubation promoted embryonic growth, reduced incubation time, increased hatchability (Gildersleeve *et al*., 1983; De Smit *et al*., 2006; Bruggeman *et al*., 2008), and improved chick quality (Tona *et al*., 2007; De Smit *et al*., 2006, 2008). These results were associated with the development of the circulatory system and increased production of red blood cells, allowing greater O_2 uptake and energy conservation (Tazawa *et al*., 2002; Decuypere *et al.,* 2006; Habermann *et al*., 2008; Verhoelst *et al*., 2011).

Despite the considerable significant scientific knowledge on the effects of hypoxia and hypercapnia on *in-ovo* development acquired in the last few years, from the practical perspective, the optimal O_2 and CO_2 levels in the setter rooms during commercial incubation still need to be determined.

Incubation temperature

Although the interaction among several physical agents during incubation influences *in-ovo* development, temperature has the strongest influence (Freeman & Vince, 1974; Decuypere & Michels, 1992; Meijerhof, 2009), because it can hinder, promote, or maintain embryonic and fetal development, as well as determine its rate and duration. Eggs are submitted to different temperatures from reception of the eggs at the hatchery until hatching. During storage, temperature is reduced to delay embryonic development. Subsequently, eggs are heated to reactivate embryonic development immediately before setting. During incubation, the temperature must be maintained to ensure the production of healthy chicks. In addition, the manipulation of incubation temperature allows anticipating or delaying hatch according to the demand for chicks.

Optimal incubation temperatures result in high hatchability of healthy chicks with good post-hatch performance. In this context, deviations need to be prevented because they can impair embryonic development (Romanoff, 1960), hatchability (Deeming & Ferguson, 1991; Wilson, 1991; Decuypere & Michels, 1992), hatchling quality (Lourens *et al*., 2005, 2007, Hulet *et al.,* 2007) and post-hatch performance (Lundy, 1969; Decuypere, 1984; Wilson, 1991). The optimal temperature for poultry egg incubation is closer to the maximum temperature tolerated by the birds (high LT100) than of the minimum temperature (low LT100). Therefore, slight deviations above the recommended incubation temperatures are more detrimental to *in-ovo* development than similar deviations below those temperatures. High incubation temperatures have negative impacts on chick heart weight (Wineland *et al*., 2000; Leksrisompong *et al*., 2007; Lourens *et al*., 2007), on bone development (Oviedo-Rondon *et al*., 2009a, b; van der Pool *et al*., 2014), and on the immune system (DuRant *et al*., 2012), predisposing chicks to ascites, leg problems, and immune deficiencies, respectively.

During natural incubation, eggs are often exposed to temperature fluctuations in the nest, which may be caused by environmental temperature variations or changes in the attention hens direct to the eggs. Before the complete development of the chorioallantoic membrane, around day 12 of incubation (Tullett & Deeming, 1987), the embryo responds to the temperature gradient between the egg region in contact with the hen and the part of the egg in contact with the nest material, directing the blood flow to the colder

region, regulating its internal temperature (Tzschentke & Nichelmann, 1997). After the complete development of the chorioallantoic membrane, the embryo is able to redistribute heat through its bloodstream (Turner, 1997), which allows regulating its temperature within certain limits. Both the direction of the heat flow and heat distribution through the bloodstream make the embryo less dependent of climate conditions around the egg. This shows that the embryo is able to react to minor temperature fluctuations inside the egg, and that it attempts to regulate its internal temperature within a very narrow range (Tzschentke & Nichelmann, 1997). Based on these findings, studies were carried out to assess the effects of incubation temperature on embryo temperature, thermal manipulation during incubation, and *in-ovo* injection of the anti-stress nutrients, such as vitamin C, as means to induce possible thermal adaptation of poultry during rearing or to promote phenotypic changes to meet specific objectives (Sgavioli *et al*., 2013, 2015, 2016; Ferreira *et al*., 2015; Almeida *et al*., 2016; Morita *et al*., 2016 a,b).

Eggshell temperature

Although embryonic and/or fetal temperature inside the egg is not identical to that of the egg surface, for practical reasons, eggshell temperature (EST) has been used as an indicator of embryo/fetus temperature (French, 1997; Lourens *et al*., 2005; Joseph *et al*., 2006; Hulet *et al*., 2007, Molenaar *et al*., 2010; Walstra *et al*., 2010), due to the small differences between those temperatures, usually of 0.1-0.2 ° C (Meijerhof & Van Beek, 1993; French, 1997).

In chicken eggs, eggshell temperature remains low during the first week of incubation and increases during the second week, reaching a temperature plateau around day 14-15 of incubation (French, 2007). It was demonstrated that eggshell temperature increases from the second third of incubation at 36-39°C incubation temperature (Sgavioli *et al*., 2015; Almeida *et al.*, 2016; Morita *et al*., 2016). This increase is related with the greater metabolic heat production by the fetus during this high growth rate phase (Lourens *et al*., 2006, 2007). After internal pipping, around day 19 of incubation, chorioallantoic gas exchange is gradually replaced by pulmonary respiration and, consequently, heat production by the fetuses of broiler chickens nearly doubles (Rahn, 1981; Janke *et al*., 2004). As mentioned above, according to the laws of thermodynamics, heat is transferred between the eggs and the incubation environment down a thermal gradient, i.e., always from the warmer to colder region. Under optimal incubation temperature (around 37.5-37.8ºC), eggshell temperature remains lower than the setter room temperature during the first week, starts to increase in the second week, and it is higher than the setter room temperature during the last week of incubation (French, 2007; Sgavioli *et al*., 2015). This indicates that eggs incubated under those temperatures need to gain heat in the beginning and to lose it during the last week of incubation, respectively. Therefore, incubation temperature needs to be set according to requirements of the embryo and fetus to optimize incubation efficiency.

Constant eggshell temperature during incubation results from a balance between embryo or fetus heat production and heat transfer between the egg and the environment (Meijerhof & van Beek, 1993). In order to maintain an optimal eggshell temperature of 37.5-38.0 °C during the entire incubation period, setter room air temperature must be higher than 37.5 to 38.0 °C during the first days of incubation and reduced from day 9 of incubation onwards (French, 1997; Lourens *et al*., 2005, 2006; Yahav *et al*., 2009). Maintaining constant eggshell temperature at 37.5-38.0 °C throughout incubation period promotes high hatchability and good chick quality (Lourens *et al*., 2005, 2007; Joseph *et al*., 2006; Leksrisompong *et al*., 2007). These results led companies to develop egg setter with temperature control based on eggshell temperature. From a practical point of view, however, it should be noted that only single-stage incubators allow reducing incubation temperature to maintain constant eggshell temperature.

Constant eggshell temperature during incubation may be achieved by reducing of setter room air temperature during the second half of *in-ovo* development due to the greater eggshell heat loss to the setter room air. However, recent studies (data not published) suggest that eggshell temperature may not be the most important factor for the optimization of *in-ovo* development, and consequently, of incubation results, and may change the current concepts of optimal incubation conditions.

Thermal manipulation during incubation

Embryo thermal manipulation or changes in incubation temperature have been studied by several research groups to determine an embryonic development window during which thermal manipulation may promote the acquisition of specific characteristics. The main objectives are to increase chicken thermo tolerance during rearing or to modulate commercially important characteristics, such as immune response, fat deposition, etc. (Lourens *et*

al., 2005; Joseph *et al*., 2006; Molenaar *et al*., 2010; Walstra *et al*. 2010; Ipek *et al*., 2014; Almeida *et al*., 2015; Ferreira *et al*., 2015; Sgavioli *et al*., 2015; Morita *et al*., 2016). Researchers have applied different types of thermal manipulation, in terms of intensity, frequency, duration, and embryonic development period (Tables 1-3). The results obtained until now have shown that fetal development (from days 8-9 of incubation) seem to be the most promising period for the application thermal manipulation, and that are other possible windows within this period or not, according to the target system, organ, or tissue. In addition, those studies have generated data that provide new insights about optimal incubation conditions and about the potential responses of poultry to variations in these conditions. Our research group analyzed the effects of moderate thermal manipulation (36ºC and 39ºC) from day 13 of incubation until hatch, and found that egg incubation at 39ºC reduced hatchling adiposity (Almeida *et al*., 2015). Thermal manipulation during incubation changes thermal preference of chickens. Broiler chicks incubated at high temperature (1 °C above the optimal temperature) preferred higher rearing temperature during the first three weeks of age, and presented greater tolerance to heat stress until the fourth week than those incubated at optimal (37.5ºC) or low (36°C) temperatures (Morita *et al*., 2016). These authors also found that high incubation temperature (1.5°C higher than the optimal temperature) from day 13 until hatch induces changes in the skin characteristics related with heat loss, such reduced thickness and greater irrigation (Morita *et al*., 2016 b), which may account for the preference of these birds for warmer environments (Morita *et al.,* 2016). In our view, these results demonstrate that, opposite to what it was previously known, birds physiologically respond to environmental responses to temperature variations already during the fetal stage.

In recent years, *in-ovo* nutrition has also been applied to manipulate phenotypic and growth characteristics birds submitted to heat stress during rearing. Ferreira *et al.* (2015) showed that association of *in-ovo* injection of vitamin C (anti-stressor) with high incubation temperature (39 °C) reduces the deleterious effects of high environmental temperature on the meat quality of broilers. Sgavioli *et al.* (2015), on the other hand, showed that high incubation temperature, with or without *in-ovo* injection of vitamin C, induces

Table 1 – Overview of the effects of incubation temperature manipulation onhatchability, incubation length, and chickcharacteristics.

Author	Incubation Treatments			Hatchability	Incubation duration	Chick characteristics			
	Temperature	Period	Duration			Body temperature	Body weight	Yolk sac weight	Quality
Yalçin & Siegel (2003)	36.9°C	E0-E8	6 h/day	x	x	x	=	x	x
	39.6°C			x	x	x	=	x	x
	36.9°C	E10-E18		x	x	x	=	x	x
	39.6°C			x	x	x	=	x	x
Yahav *et al.* (2004b)	39.5°C	E8-E10	3 h/day	=	x	x	=	x	x
		E16-E18		↑	x	x	=	x	x
	41.0°C	E8-E10	3 h/day	=	x	x	=	x	x
		E16-E18		=	x	x	=	x	x
Collin *et al.* (2005)	39.5°C	E16-E18	3 h/day	=	x	=	=	x	x
			6 h/day	=	x	=	=	x	x
			12 h/day	=	x	=	=	x	x
			24 h/day	=	x	=	=	x	x
Collin *et al*. (2007)	39.5°C	E8-E10	3 h/day	↑	x	↓	=	x	x
		E16-E18		↑	x	↓	=	x	x
		Both E		↓	x	=	=	x	x
Piestun *et al.* (2008)	39.5°C	E7-E16	12 h/day	=	x	↓	=	x	x
			24 h/day	↓	x	↓	↓	x	x
Tona *et al.* (2008)	39.5°C	E16-E18	3 h/day	=	↑	↓	=	x	x
Shinder *et al.* (2011)	15°C	E18-E19	2x30 min	=	x	=	=	x	x
			2x60 min	=	x	↓	=	x	x
Loyau *et al.* (2013)	39.5°C	E7-E16	12 h/day	=	x	↓	=	x	x
Almeida *et al.* (2015)	36°C	From E13	24 h/day	=	↑	=	=	=	=
	39°C			↑	=	=	=	=	↓

E; embryonic day. =; no significant difference between the control and the thermally-manipulated groups. ↑ and ↓: higher and lower values determined in thermally-manipulated groups compared with the control group, respectively. X: not reported.

Table 2 – Overview of the effects of eggshell temperature manipulation on hatchability, incubation length, and chick characteristics.

Author	Incubation Treatment			Hatchability	Incubation duration	Chick characteristics			
	EST	Period	Duration			Body temperature	Body weight	Yolk sac weight	Quality
Lourens *et al.* (2005)	36.7°C	E0-E7	24 h/day	↓	x	x	↓	x	x
	38.9°C	E14-E21		↓	x	x	=	x	x
	36.7°C	Both E		↓	x	x	↓	x	x
	38.9°C	Both E		↓	x	x	↓	x	x
Joseph *et al*. (2006)	36.6°C	E0-E10	24 h/day	↓	x	x	↑	=	x
	39.5°C	From E19		↑	x	x	↓	=	x
	36.6°C	Both E		=	x	x	=	=	x
	39.9°C	Both E		=	x	x	=	=	x
Molenaar *et al.* (2010)	38.9°C	E14-E18	24 h/day	=	↓	x	=	↑	x
Wasltra *et al.* (2010)	40.0°C	E14-E18	4 h/day	=	↓	↓	=	x	x
Ipek *et al.* (2014)	33.7°C-36.7°C	E10-E18	24 h/day	↓	x	=	↓	↓	x
	38.9°C-40.0°C			↓	x	=	↓	↑	x

EST; eggshell temperature. E; embryonic day. =; no significant difference between the control and the thermally-manipulated groups. ↑ and ↓; higher and lower values determined in thermally-manipulated groups compared with the control group, respectively. X; not reported.

Table 3 – Performance and thermotolerance of broilers submitted to thermal manipulation or not during incubation.

Author	Incubation Treatments			Post-hatch				
	Temperature	Period	Duration	Thermal challenge	Improved heat tolerance?	Final Body weight	Feed conversion	Meat quality
Collin *et al.* (2005)	39.5°C	E16-E18	3 h/day	d3, 41°C, 6h	Yes	x	x	x
			6 h/day		No	x	x	x
			12 h/day		No	x	x	x
			24 h/day		No	x	x	x
Collin *et al*. (2007)	39.5°C	E8-E10	3 h/day	d42, 35°C, 6h	No	=	=	↓
		E16-E18	3 h/day		No	=	=	↓
		Both E	3 h/day		No	=	=	=
Piestun *et al*. 2008)	39.5°C	E7-E16	12 h/day	d35, 35°C, 5h	Yes	=	x	x
	39.5°C	E7-E16	24 h/day		Yes	↓	x	x
Tona *et al.* (2008)	39.5°C	E16-E18	3 h/day	d42, 35°C, 6h	No	=	x	x
Wasltra *et al*. (2010)	40.0°C	E14-E18	4 h/day	d35, 34°C, 4h	No	=	x	x
Halle & Tzschentke (2011)	38.2-38.4°C	From E18	2 h/day	d3-d35, 32°C, 24h	x	=	=	x
			24/ day		x	↓	=	x
Shinder *et al.* (2011)	15°C	E18-E19	2x30 min	d14-d21, 20°C and d21-d35, 15°C	Yes	↑	x	x
			2x60 min			=	x	x
Loyau *et al*. (2013)	39.5°C	E7-E16	12 h/day	d34, 32°C, 5h	No	↓	x	=

E; embryonic day. d: days of age.=; no significant difference between the control and the thermally-manipulated groups. ↑ and ↓; higher and lower values determined in thermally--manipulated groups compared with the control group, respectively. X; not reported.

epigenetic adaptations of the electrolyte balance of broilers reared under hot temperatures, reducing their susceptibility to respiratory alkalosis.

CONCLUSIONS

Physical exchanges between eggs and environment are required for *in-ovo* development. Deficient exchanges negatively affect the incubation process, while excessive exchanges may improve incubation efficiency. Physical exchanges depend firstly on eggshell porosity and conductance and on temperature and relative humidity differences between the eggs and the environment. These factors are maternally influenced by egg quality (weight, size, chemical composition, and eggshell porosity, surface area and

conductance), egg storage conditions (temperature, relative humidity, air velocity) and duration, and the incubation conditions (temperature, relative humidity, egg turning and position at setting, and air velocity and gas concentrations). The maternal effects on the physical exchanges show that the optimal storage and incubation conditions vary with breeder age and egg weight. However, optimal storage and incubation conditions as a function of egg weight or weight range still need to be established. Although this proposal seems to be unrealistic at first sight, it may be feasible as the control systems of incubation physical conditions (e.g., controlling egg weight loss, setting incubation temperature according to the eggshell temperature) are further technologically developed, allowing their easier and quicker determination. In addition, incubation conditions, which are essential for maximizing the production efficiency of hatcheries, may be optimized.

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