Perinatal development and nutrient utilization in chickens

Effects of incubation conditions

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Thesis

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Abstract

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Suboptimal incubation conditions can negatively affect survival and development of chicken embryos. However, physiological mechanisms that may explain these effects, and the long-lasting consequences are largely unknown. Therefore, the first aim of this thesis was to investigate effects of eggshell temperature (EST) and O_2 availability during incubation on survival, development, physiology, and nutrient utilization of chicken embryos. The second aim was to investigate long-lasting effects of suboptimal EST on survival and subsequent performance of broiler chickens. The first study investigated effects of a high (38.9°C) or a normal (37.8°C) EST combined with a low (17%), normal (21%), or high (25%) O_2 concentration from day 7 until 19 of incubation on the survival rate, nutrient utilization, and the developmental and physiological status of broiler embryos. The second study investigated effects of high EST on glucose metabolism in broiler embryos using [U-^{13}C]glucose. The third study investigated effects of high EST on growth performance and the incidence of ascites in broiler chickens. Finally, effects of a high EST and a hole in the air cell on the developmental and physiological status of layer hatchlings were investigated. Results showed that a high EST or low O_2 availability from the first week of incubation onward negatively affected survival and development of broiler chickens from their perinatal period until slaughter age. Body development of broiler hatchlings was reduced after high EST incubation because of a lower efficiency in protein utilization for growth. This was possibly due to the use of glucogenic amino acids as a glucogenic energy source because high EST increased the glucose oxidation in broiler embryos during the second half of incubation and resulted in lower hepatic glycogen. Body development was proportional to the O_2 availability during incubation. In addition, differences in O_2 concentration during incubation seem to affect the development of adaptive mechanisms, and these mechanisms might possible influence nutrient utilization and body development. High EST in the last week of incubation in layer embryos negatively affected hatchling development, but the effect of a hole in the air cell was minimal. Effects of high EST were long-lasting in broiler chickens expressed by a lower body weight and a higher ascites incidence during the growout period. In conclusion, negative effects of suboptimal incubation conditions can be partly explained by changes in nutrient utilization and metabolite levels in the perinatal period and can have long-lasting effects on the survival and performance of broiler chickens.

Voorwoord

De afgelopen vijf jaar waren een feest! Er zijn heel wat mensen die de slingers hebben opgehangen en het feest tot een succes hebben gemaakt. Er waren echter niet alleen hoogtepunten, maar ook dieptepunten en dat maakte deze vijf jaar een leerzame periode voor mij.

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Contents

General Introduction

Nutrient Utilization during Incubation

The chicken embryo grows without direct maternal influences and uses the nutrients that are stored in the egg for growth and development during the 21 days of incubation (Wilson, 1997; Giussani et al., 2007). At oviposition, an egg contains on average 65.6% water, 12.1% protein, 10.5% lipids, 0.9% carbohydrates, and 10.9% minerals (Romanoff and Romanoff, 1949). Proteins are mainly used for body development during incubation (Romanoff, 1967). From the initial total amount of protein in the egg, approximately 48% is found in the chicken at hatch, 47% is left in the residual yolk, 2.5% is found in the allantois, and only 2.5% is lost, likely as a result of catabolic processes (Romanoff, 1967). Most lipids in the egg are found in the yolk, and they are the main energy source during incubation (~90%; Noble and Cocchi, 1990). Lipid oxidation increases from day 9 until the end of incubation concurrently with the increase in embryo development (Romanoff, 1967). From the initial total amount of fat in the egg, approximately 20% is found in the chicken at hatch, 40% is left in the residual yolk, and 40% is burned (Romanoff, 1967). Carbohydrates are present at very low levels in the egg at oviposition (<1%), and the total amount decreases even further throughout incubation (Romanoff and Romanoff, 1949). A major portion of the carbohydrate glucose is used in the first week of incubation, as the chorioallantoic membrane (CAM) is not sufficiently developed to provide enough O_2 for complete fatty acid oxidation (Kučera et al., 1984; Moran, 2007). Glucose oxidation increases at the end of incubation again because of the energy-demanding hatching process and the limited O_2 availability during this period (Freeman, 1969; Tazawa et al., 1983). Therefore, enough glucose must be built up and stored as glycogen before the hatching process begins (Beattie, 1964; Foye et al., 2007). To synthesize glucose, the activity of gluconeogenic enzymes increases throughout incubation with glucogenic amino acids, glycerol, or lactate as potential precursors (Evans and Scholz, 1973; Watford et al., 1981; Dickson, 1983; Foye et al., 2007). Glucose is stored as glycogen in the heart, liver, muscles, and yolk sac membrane (Beattie, 1964; García et al., 1986; Foye et al., 2007). When the embryo starts to emerge from the eggshell, hepatic glycogen is preferably mobilized (Freeman, 1965, 1969; García et al., 1986; Foye et al., 2007). Because muscle activity is high and O_2 availability is limited during the hatching process, anaerobic glycolysis occurs; this results in increased plasma lactate concentrations (Freeman, 1965; John et al., 1987, 1988; Moran, 2007; De Oliveira et al., 2008).

Above mentioned physiological and metabolic processes in the chicken embryos are likely affected by temperature and availability of O_2 during incubation. This may explain the decrease in survival and body development that is found after suboptimal incubation conditions, such as a high temperature or a low O_2 availability (Freeman and Vince, 1974; Stock and Metcalfe, 1984; Lourens et al., 2005, 2007; Leksrisompong et al., 2007).

Temperature

During the incubation of chicken embryos, the temperature is the most important environmental condition to start development (Freeman and Vince, 1974; Decuypere and Michels, 1992; Meijerhof,

2009). In natural incubation, a temperature gradient is present within an egg because the egg is in contact with the bottom of the nest on one side and is covered by the broodpatch of the hen or surrounded by air during a recess on the other side (Freeman and Vince, 1974; Turner, 1997). After the complete development of the CAM around day 12 of incubation (Tullett and Deeming, 1987), the embryo may be able to regulate its temperature within certain limits by redistributing heat via its circulation (Turner, 1997). An indication for this temperature regulation is that the blood flow in the CAM redistributes because of temperature changes in the last week of incubation (Holland et al., 1998; Nichelmann et al., 2001).

In artificial incubation, no temperature gradient is present within the egg and the incubator temperature is controlled between narrow limits. The air temperature is often maintained between 36 and 38°C throughout incubation (Lundy, 1969; Decuypere et al., 2001). The temperature that the embryo experiences differs from the air temperature during incubation (Lourens et al., 2005, 2006), and this is related to the heat production of the embryo and the heat transfer between the egg and the surroundings (Meijerhof and Van Beek, 1993). Because the embryo temperature is difficult to measure without killing the embryo, the eggshell temperature (EST) is often used as an indicator of the embryo temperature as it deviates less than 0.1 to 0.2°C from the embryo temperature (Meijerhof and Van Beek, 1993; French, 1997). In the last 6 years, different studies have shown that a continuous EST of 37.5 to 38.0°C during incubation results in the highest hatchability and chick development (Yildirim and Yetisir, 2004; Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007; Piestun et al., 2009a). As a result, the incubator temperature needs to be adjusted to obtain this narrow EST range.

In practice, a high EST is often found at the end of incubation; this is a result of the higher heat production and an insufficient heat loss of the developing embryos that can be due to a poor air velocity or cooling capacity in the incubator (French, 1997; Hulet, 2007; Elibol and Brake, 2008). In addition, temperature setpoints can be too high in the incubator. Different studies have shown that a high EST (≥38.9°C) in the last week of incubation can negatively affect hatchability and chick development (Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007; Piestun et al., 2008a, 2009a). The reduced chick development at a high EST compared with normal EST is expressed by a lower yolk-free body mass, smaller organs, larger residual yolk, and a poorer navel condition (Wineland et al., 2000; Givisiez et al., 2001; Lourens et al., 2005, 2007; Leksrisompong et al., 2007; Piestun et al., 2008a). This reduction in body development from incubation at a high EST may negatively affect the performance or health status later in life (Hulet et al., 2007; Leksrisompong et al., 2009) and reduce the economic return.

O_2 concentration

The O_2 availability is the second most important environmental factor during incubation that affects survival and chick development (Metcalfe et al., 1981; Lourens et al., 2007). Oxygen availability depends on the gas exchange across the shell and shell membranes which occurs by diffusion (Wangensteen and Rahn, 1970/1971; Rahn et al., 1979; Burton and Tullett, 1985). The

rate of diffusion through the eggshell depends on the partial pressure gradient between the inside and the outside of the egg, the surface area of the egg, and the eggshell conductance (Rahn et al., 1987). Embryonic O_2 consumption increases exponentially after day 9 of incubation and shows a plateau phase from day 15 until 19 of incubation (Lourens et al., 2005, 2007; Piestun et al., 2009a). This plateau phase is the result of an increased metabolic rate in the developing embryos and a restriction in the O_2 supply by the eggshell and shell membranes (Dietz et al., 1998; Janke et al., 2004; Mortola and Cooney, 2008). A lower O_2 availability, for example because of a high altitude, can decrease survival rate and body development during incubation (Stephens and Ploog, 1967; Visschedijk, 1985; Hassanzadeh et al., 2004; Giussani et al., 2007).

Temperature × O_2 concentration

The effects of a high EST on survival and development may interact with embryonic O_2 availability. A high EST increases the metabolic rate, which likely increases the O_2 requirement as well (Lourens et al., 2007; Oznurlu et al., 2010). Lourens et al. (2007) found no interaction between EST and O_2 concentration for body development, but their results showed that body development at hatch increased with higher O_2 concentrations from day 7 until 19 of incubation in both the normal and the high EST. Although the effects of EST and O_2 concentrations during incubation on survival and development have been investigated (Stephens and Ploog, 1967; Visschedijk, 1985; Hassanzadeh et al., 2004; Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007, 2009; Piestun et al., 2009a), the mechanisms that may explain these effects are largely unknown. Suboptimal incubation conditions, such as a high EST or a low O_2 availability, probably change metabolism, physiology, and nutrient utilization during incubation and this may explain the negative effects on survival and development that are found. The changes in metabolism, physiology, and nutrient utilization may be expressed by differences in energy partitioning, energy stores, hormones, and blood metabolites in the perinatal period (day 18 of incubation until 48 hours after emergence from the eggshell), but this is largely unknown in both layer and broiler embryos. Furthermore, the negative effects of suboptimal incubation conditions may also have long-lasting effects on development, morbidity, and mortality throughout the production cycle of the chickens, but this has not been intensively investigated (Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2009).

Aim and Outline of the Thesis

The first aim of this thesis was to investigate the effects of EST and O_2 availability during incubation on survival, development, physiology, and nutrient utilization in chicken embryos. The second aim was to investigate the long-lasting effects of EST on survival and subsequent performance in broiler chickens. At first, the effect of EST and O_2 concentration during incubation on survival, development, physiology, and nutrient utilization was investigated in broiler chicken embryos (Chapter 1, 2). A high (38.9°C) or normal (37.8°C) EST was applied from day 7 of incubation

onward in combination with a low (17%), normal (21%), or high (25%) O_2 concentration from day 7 until 19 of incubation. Embryonic mortality was evaluated, and hatchlings were measured at 12 and 48 hours after emergence from the eggshell to evaluate body and organ weights, body composition, efficiency of nutrient utilization for growth, blood metabolites, and hepatic glycogen stores. The results of the first experiment showed that hepatic glycogen stores were negatively affected by high EST incubation, which may be related to a higher glucose oxidation at the end of incubation. Therefore, the effect of a high EST (38.9°C) from day 10.5 of incubation onward on glucose metabolism was investigated using a tracer study (Chapter 3). Tracer studies are not intensively used during chicken embryo incubation and two small-scale studies were performed to develop the technique and to evaluate glucose metabolism at a high (38.9°C) or a normal (37.8°C) EST in the last week of incubation of broiler embryos. To investigate the long-lasting effects of different EST, broiler embryos were incubated at a normal (37.8°C) or high (38.9°C) EST from day 7 of incubation onward and raised at a normal or cold growout temperature; the latter was used to induce ascites (Chapter 4). Chick quality at hatch, growth performance, slaughter characteristics, and mortality during the growout period, with a focus on mortality associated with ascites, were evaluated. Because studies on the effect of incubation conditions on layer eggs are very limited, the effect of a high (38.9°C) EST and increased O_2 availability by puncturing a hole in the air cell was investigated in layer embryos in the last week of incubation (Chapter 5). Finally, the effects of suboptimal incubation conditions, such as a high EST or low O_2 availability, on the survival and body development from the perinatal period until the slaughter age are discussed in the general discussion. Furthermore, the physiological and metabolic mechanisms that may explain the negative effects on survival and body development from the perinatal period until the slaughter age are discussed.

1

Effect of Eggshell Temperature and Oxygen Concentration on Survival Rate and Nutrient Utilization in Chicken Embryos

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Abstract

Environmental conditions during incubation such as temperature and O_2 concentration affect embryo development that may be associated with modifications in nutrient partitioning. Additionally, prenatal conditions may affect postnatal nutrient utilization. Using broiler chicken embryos, we studied the effects of eggshell temperature (EST; 37.8 or 38.9°C) and O_2 (17, 21, or 25%) applied from day 7 until 19 of incubation in a 2 × 3 factorial design. Effects of these factors on embryonic survival, development, and nutrient utilization were assessed in the pre- and posthatch period. High EST reduced yolk-free body mass compared with normal EST (36.1 vs. 37.7 g), possibly through reduced incubation duration (479 vs. 487 hours) and lower efficiency of protein utilization for growth (83.6 vs. 86.8%). Increasing O_2 increased yolk-free body mass (from 35.7 to 38.3 g) at 12 hours after emergence from the eggshell, but differences were larger between the low and normal O_2 than between the normal and high O_2. This might be due to the lower efficiency of nutrient utilization for growth at low O_2. However, the effects of O_2 that were found at 12 hours were less pronounced at 48 hours posthatch. When O_2 was shifted to 21% for all treatments at day 19 of incubation, embryos incubated at low O_2 used nutrients more efficiently than those incubated at normal or high O_2. An additional negative effect on survival and chick development occurred when embryos were exposed to a combination of high EST and low O_2. Possible explanations include reduced nutrient availability for hatching, decreased body development to fulfill the energy-demanding hatching process, and higher incidence of malpositions. In conclusion, EST and O_2 during incubation affect nutrient utilization for growth, which may explain differences in survival and development. Embryos raised under suboptimal environmental conditions in the prenatal period may develop adaptive mechanisms that still continue in the posthatch period.

Key words: incubation conditions, survival rate, body composition, nutrient utilization, chicken embryo

Introduction

Environmental conditions during embryonic development are crucial for optimal prenatal growth and development (Dziuk, 1992; Fleming et al., 2004) and may influence performance and health in later life (Fleming et al., 2004; Dickinson and Wintour, 2007). The chicken embryo can be used as a model to investigate the effects of environmental conditions on development and physiology because it grows independently from the mother and consequently without direct maternal influences (Giussani et al., 2007). The chicken embryo can use only nutrients stored in the egg (Wilson, 1997), which contains a large amount of protein and fat but only a small amount (<1%) of carbohydrates (Romanoff and Romanoff, 1949). During the first week of egg incubation, glucose is the predominant energy source because the chorioallantois is not sufficiently developed to provide the required O_2 for complete fatty acid oxidation (Kučera et al., 1984; Moran, 2007). During the second half of incubation, fatty acids are the predominant substrate for adenosine triphosphate (ATP) production (Noble and Cocchi, 1990; Sato et al., 2006) and proteins are presumably deposited (Freeman and Vince, 1974; Sato et al., 2006). At the end of incubation, around day 19 of incubation, the embryo begins the energy-demanding hatching process (Tazawa et al., 1983) and glucose acts as the main energy source (Freeman, 1965; Dickson, 1983). Because low concentrations of carbohydrates are initially available in the egg, gluconeogenesis is indispensable with amino acids, glycerol, or lactate as potential precursors (Evans and Scholz, 1973; Watford et al., 1981).

During embryonic growth, nutrient utilization for growth and ATP production may be influenced by environmental conditions, especially in avian eggs with limited resources. Consequently, survival rate and embryonic development may be influenced. Indeed, suboptimal environmental conditions during incubation have been found to negatively affect survival rate and embryonic development (Wineland et al., 2000; Lourens et al., 2005, 2007). Two of the most important environmental conditions during incubation that are known to influence embryonic development are temperature and O_2 (Stock and Metcalfe, 1984; Lourens, 2004; Meijerhof, 2009). A continuous eggshell temperature (EST) of 37.8°C throughout incubation has given the highest development (Lourens et al., 2005, 2007) and the O_2 concentration in normal air is 21%. High eggshell temperatures (≥38.9°C) and low O_2 concentrations (≤17%) negatively affect embryonic development (Stock and Metcalfe, 1984; Leksrisompong et al., 2007; Lourens et al., 2007), and these conditions may interact with each other. Negative effects of high temperatures during incubation may be counteracted by higher O_2 concentrations, which can improve development (Lourens et al., 2007) and survival rate (Christensen and Donaldson, 1992; Altan et al., 2006). Although the consequences of suboptimal incubation on survival rate and embryonic development are evident, the underlying effects on nutrient utilization and body composition have not been assessed. Nutrient utilization for embryonic growth under different incubation conditions during the prenatal and early postnatal period may explain differences in hatchability, embryonic development, and metabolism (Christensen et al., 1993; Leksrisompong et al., 2007; Lourens et

al., 2007). Therefore, this study assessed the effects of two EST and three O_2 concentrations on the survival rate and nutrient utilization for growth in chicken embryos.

Materials and Methods

Experimental Design

The experiment was designed as a 2 × 3 factorial arrangement with two EST (37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) applied from day 7 to 19 of incubation. Each treatment was repeated twice. Six consecutive batches of eggs were incubated with two treatments per batch. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University.

Hatching Eggs

Hatching eggs of a commercial Hybro grandparent female line were used (n = 1,320). Eggs weighed between 60 and 65 g and the age of the parent stock ranged from 45 to 51 weeks.

Egg Storage and Incubation until day 7

Eggs were stored for 3 to 5 days at 18°C at Torsius Breeder Hatchery (Putten, the Netherlands). Thereafter, eggs were transported to the experimental accommodation of Wageningen University and placed in a HT-combi incubator with a maximum capacity of 4,800 eggs (HatchTech Incubation Technology B.V., Veenendaal, the Netherlands). In each batch, eggs were equally divided among four egg trays (n = 150 eggs). Egg trays were half-filled with eggs; every other egg space remained empty to ensure uniform air flow around the eggs. Eggshell temperature was measured by temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) placed halfway between the blunt end and the pointed end of five individual eggs. Temperature sensors were attached to the eggshell using heat-conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and tape. Incubator temperature was adjusted manually twice a day to maintain an EST of 37.8°C. Relative humidity was set at 55%, and eggs were turned each hour over 90°. The CO_2 concentration was between 0.05 and 0.07% at day 7 of incubation before the eggs were transferred.

Incubation from day 7 to 19

After candling at day 7 of incubation, 220 fertile eggs per batch were randomly divided between two identical small open-circuit climate respiration chambers (CRC; Lourens et al., 2006). Eggshell temperature was maintained at 37.8°C (normal) or 38.9°C (high), and O_2 level was maintained at 17% (low), 21% (normal), or 25% (high). The EST of the two chambers was identical within each batch. Eggshell temperature and O_2 concentrations in the CRC were regulated as described by Lourens et al. (2007). Because of the fixed ventilation rate, O_2 decreased on average from day 7 to 19 of incubation from 17.1% to 16.3% in the low O_2 treatment, from 20.9% to 20.0% in the normal O_2 treatment, and from 25.0% to 23.9% in the high O_2 treatment. In all treatments, CO_2

concentration was roughly 0.1% at day 7 of incubation and increased to 0.6 to 0.8% by day 18 of incubation due to the CO_2 production of the embryos. Relative humidity was maintained at 50% in all treatments, and eggs were turned each hour over 90°.

Incubation from day 19 until 48 hours after emergence from the Eggshell

At day 19 of incubation, all eggs were transferred from the egg trays to individual hatching baskets (120 × 135 mm) in one large open-circuit CRC (1.5 × 3.5 m; Verstegen et al., 1987). The previous EST treatment, which was identical between the two treatments per batch, was maintained for half a day. After this period, the CRC temperature was fixed at the last EST setpoint, and the EST was allowed to increase during the hatching process. Oxygen was not regulated after day 19 of incubation and remained at approximately 21%. Relative humidity was maintained at 55%. The number of hatchlings emerging from eggshells was recorded every 2 hours to calculate the average incubation duration per treatment. Hatchlings were selected for measurements either 12 or 48 hours after emergence from the eggshell. To distribute hatchlings per treatment equally across the hatching period, sequential hatchlings were alternately allocated to the 12- or 48-hour measurement groups. From the time of emergence until 12 hours afterward, hatchlings were kept in hatching baskets in the large open-circuit CRC. At 12 hours after emergence from the eggshell, hatchlings that were measured at 48 hours were transferred to individual hatching baskets (120 × 135 mm) in another, identical large CRC until 48 hours after emergence from the eggshell. Environmental temperature in this CRC was maintained at 33°C, and relative humidity was maintained at 55%. No feed or water was provided, and hatchlings were continuously exposed to light.

Embryo Mortality and Hatch of Fertile

Eggs were candled at day 19 of incubation to identify nonviable embryos. At day 23 of incubation, nonhatched eggs were opened to classify embryo mortality per day as described previously (Lourens et al., 2006). Dead embryos after day 18 of incubation that exhibited head between legs (HBL) or head over wing (HOW) positions were classified as malpositioned (Dove, 1935). Embryos that died during the first week of incubation were excluded from the analyses. Hatch of fertile eggs was expressed as the percentage of chicks that hatched from the fertile eggs and was calculated per treatment and batch.

Heat Production and Hatchling Measurements

Oxygen consumption and CO_2 production in the CRC was measured every 9 minutes from day 7 to 18 of incubation. After correction for dead embryos, heat production was calculated in milliwatts per egg per day (Brouwer, 1965). At day 18 of incubation, 20 fertile eggs per treatment were randomly selected and weighed. Embryos were killed by decapitation and the embryo and residual yolk were weighed. Chick development was measured at 12 and 48 hours after emergence from the eggshell. Body weight including the residual yolk, chick length, and navel condition of all hatchlings was determined. Chick length was measured from the top of the beak to the tip of the middle-toe excluding the nail (Hill, 2001). Navel condition was scored as 1 (closed and clean navel

area), 2 (black button up to 2 mm or black string), or 3 (black button exceeding 2 mm or open navel area). Hatchlings were decapitated and bled, and the residual yolk was removed and weighed. Residual yolk and the yolk-free body (YFB) were stored at -20°C for further analysis.

Dry matter (ISO 6496, 1998), CP (ISO 5983, 2005), crude fat (ISO 6492, 1999) and ash (ash content in albumen and yolk was determined at a temperature of 800°C instead of 550°C; ISO 5984, 2002) were determined in ten random samples of albumen and egg yolk per batch and in ten samples of YFB and residual yolk per treatment and hatchling age. Yolk-free body masses and residual yolks that were selected for analysis were equally distributed across the hatching period. Dry matter, fat, and ash concentrations were determined once and CP concentration was determined twice per sample.

At day 0 of incubation, 20 eggs per batch were boiled and their albumens and yolks were separated. Ten yolks and 20 albumens were weighed and frozen at -20°C and then dried in a freeze dryer. Albumens of two eggs were combined to obtain sufficient DM for all analyses (n = 10). One frozen YFB was placed in approximately 150 mL of water and autoclaved for 10 hours at 2 bar and 130°C. The YFB in water was homogenized using an Ultra-Turrax disperser (IKA Werke GmbH & Co. KG, Staufen, Germany) for 10 minutes, and the suspension was used for CP analysis. Aliquots of the suspension were frozen at -20°C and then dried in a freeze dryer to determine DM, fat and ash concentrations. Residual yolk samples were dried in a freeze dryer before analysis of DM, fat, protein, and ash concentrations.

Calculations

The rates of change in YFB mass and residual yolk weight during the period between day 18 of incubation and 12 hours after emergence from the eggshell or between 12 and 48 hours after emergence from the eggshell were determined by calculating the difference in YFB or residual yolk weight between the two time points and dividing this value by the number of days. The period between day 18 and 12 hours after emergence from the eggshell was calculated because incubation durations differed among treatments. The period between 12 and 48 hours was always fixed at 1.5 days.

Carbohydrate concentration (g/kg DM) was calculated as 1,000 – fat – protein – ash (g/kg DM). The energy contents of albumen, egg yolk, YFB, and residual yolk were calculated from the energy densities of protein (23.7 kJ/g of DM; Znaniecka, 1967), fat (39.2 kJ/g of DM; Znaniecka, 1967) and carbohydrate (17.2 kJ/g of DM; Davidson et al., 1979). The efficiency with which energy from the initial egg was converted to YFB mass (E_{YFB}) was calculated using the formula described previously (Lourens et al., 2006):

$$E_{YFB} = \frac{\text{YFB (kJ)} \times 100\%}{\text{Albumen (kJ) + Egg yolk (kJ) - Residual yolk (kJ)},}$$

where energy in YFB and residual yolk was measured at 12 and 48 hours posthatch and energy in the albumen and egg yolk was measured at day 0 of incubation. This efficiency was calculated for

protein energy, protein-free energy (i.e., energy in fat and carbohydrates), and total energy at 12 and 48 hours after emergence from the eggshell.

Statistical Analysis

The CRC was treated as the experimental unit in all statistical analyses. Distributions of the means and residuals were examined to verify model assumptions. Hatch of fertile, embryo mortality, and frequencies of HBL and HOW malpositioned embryos were expressed as percentages of the number of fertile eggs. These percentages and incubation duration were analyzed using the GLM procedure in SAS (Version 9.1, SAS Institute 2004). The model was:

$$Y_{ij} = \mu + EST_i + Batch(EST_i) + O_{2j} + Interactions + \varepsilon_{ij} \quad [1]$$

where Y_{ij} was the dependent variable, μ was the overall mean, EST_i was EST (i = 37.8°C or 38.9°C), and O_{2j} was O_2 concentration (j = 17%, 21%, or 25%). Batch(EST_i) was the EST nested within the six batches, and this term was used as an error term to test for effects of EST. Interactions between EST_i and O_{2j} were tested against ε_{ij}, which was the residual error term. For analyses of chick length, YFB mass, residual yolk weight, navel condition, energy content of YFB and residual yolk, energy loss from the beginning of incubation, and efficiency of energy transfer from the egg to the YFB, model [1] was extended with the age of the bird (age_k; k = 12 or 48 hours after emergence from the eggshell) and interactions of the other factors with age. The increase in YFB mass and the decrease in residual yolk weight per 24 hours in the periods between day 18 of incubation and 12 hours after emergence from the eggshell and between 12 and 48 hours after emergence from the eggshell were analyzed using the GLM procedure where model [1] was extended with period ($period_l$; l = day 18 of incubation to 12 hours after emergence from the eggshell or 12 to 48 hours after emergence from the eggshell) and interactions of the other factors with period. Mean heat production per 24 hours from day 7 to 18 of incubation was analyzed using the MIXED procedure for repeated measurements. Model [1] was extended with the repeated factor day of incubation (day_m; m = day 7 to 18 of incubation), and interactions of other factors with day and an auto-regressive covariance structure was used. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as means ± SE. In all cases, differences were considered significant at $P \leq 0.05$.

Results

EST

Eggshell temperature did not affect embryo mortality in the second week of incubation (Table 1). Incubation duration, from the start of incubation until emergence from the eggshell, decreased by 8 hours ($P = 0.04$; Table 1), chick length decreased by 0.2 cm ($P = 0.05$; Table 2), and residual yolk weight increased by 1.2 g ($P < 0.001$; Table 2) at high EST compared with normal EST. The increase in YFB mass from day 18 of incubation until 48 hours after emergence from the eggshell was 0.2

g/24 hours less ($P = 0.01$) in embryos at high compared with normal EST (Table 3). The decrease in residual yolk per day during the same period did not differ between EST treatments (Table 3). Hatchlings at high EST had lower protein ($P = 0.006$) and total energy ($P = 0.007$) content in the YFB and had higher protein ($P = 0.007$), carbohydrate ($P = 0.02$), and total energy ($P = 0.008$) content in the residual yolk compared with hatchlings at normal EST (Table 4). The efficiency in transferring protein from the egg to the YFB was 3.2% lower in the high EST treatment (83.6%) than in the normal EST treatment (86.8%; $P = 0.05$; Table 4). Interactions were found between EST and age of the hatchling for fat content in the YFB ($P = 0.04$) and protein-free energy loss ($P = 0.05$) (Table 5). At both 12 and 48 hours after emergence from the eggshell, the fat content in the YFB was higher in the normal than in the high EST treatment ($P < 0.05$; Table 5). Differences between EST treatments were less pronounced at 48 than at 12 hours after emergence. Protein-free energy loss did not differ between EST treatments at 12 hours after emergence but was higher in the normal than in the high EST treatment at 48 hours after emergence ($P < 0.05$; Table 5).

O_2 concentration

Oxygen concentration did not affect embryo mortality in the second week of incubation (Table 1). Head between legs, HOW, and incubation duration were also not affected by O_2 level (Table 1). Chick length increased with higher O_2 ($P < 0.001$; Table 2). The increase in YFB mass during the period from day 18 of incubation until 48 hours after emergence from the eggshell was 0.6 g/24 hours higher at low than at high O_2 concentration ($P < 0.05$; Table 3). The normal O_2 concentration was intermediate (Table 3). The decrease in residual yolk per day during the same period did not differ among O_2 treatments (Table 3). Protein, carbohydrates, and total energy in the residual yolk were higher (all $P < 0.05$) at low O_2 than at normal and high O_2 (Table 4). Protein-free and total energy loss showed the opposite result; low O_2 resulted in lower loss ($P < 0.05$) than the normal and high O_2 treatments (Table 4). Interactions were found between O_2 and age of the hatchling for YFB mass ($P = 0.01$) and residual yolk weight ($P = 0.02$) (Table 2). At 12 hours after emergence from the eggshell, YFB mass increased and residual yolk weight decreased with increasing O_2 (Table 2). At 48 hours after emergence from the eggshell, YFB mass increased and residual yolk weight decreased between low and normal O_2 ($P < 0.05$), but there was no longer any difference in YFB mass and residual yolk weight between normal and high O_2 (Table 2). Interactions were found between O_2 and age of the hatchling for protein ($P = 0.05$), fat ($P = 0.002$), and total energy ($P = 0.002$) content in the YFB and on fat energy ($P = 0.05$) in the residual yolk (Table 5). At 12 hours after emergence from the eggshell, these energy contents were lower in the low than in the normal and high O_2 treatments ($P < 0.05$; Table 5). At 48 hours after emergence from the eggshell, the differences among O_2 concentrations were less pronounced. At 12 and 48 hours after emergence, fat energy in the residual yolk was higher in the low than in the normal and high O_2 treatments ($P < 0.05$; Table 5). Interactions were found between O_2 and age of the hatchling for protein loss ($P = 0.02$), protein ($P = 0.03$), and total efficiency ($P = 0.05$). At 12 hours after emergence, protein loss decreased and efficiency of protein and total energy utilization for growth increased with increasing O_2. At 48 hours after emergence, responses to O_2 were reversed (Table 5).

EST × O_2 concentration

High EST increased the number of HBL embryos by 1.7% ($P = 0.05$) and the number of HOW embryos by 3.1% ($P = 0.005$) compared with normal EST (Table 1). These effects were primarily caused by the combination of high EST and low O_2, which showed the highest percentage of malpositioned embryos (Table 1). Interactions were found between EST and O_2 concentration for hatch of fertile ($P = 0.02$) and embryo mortality in the third week of incubation ($P = 0.006$) (Table 1). High EST and low O_2 decreased ($P < 0.05$) hatch of fertile by 23% and increased ($P < 0.05$) embryo mortality in the third week of incubation by 22% compared with all other treatments (Table 1). Interactions between EST and O_2 were also found for YFB mass ($P = 0.01$) and navel condition ($P = 0.01$) (Table 2). Yolk-free body mass was lower ($P < 0.05$) at the high EST than at normal EST. YFB mass increased ($P < 0.05$) with increasing O_2 in both EST treatments, but this increase was more pronounced between low and normal O_2 than between normal and high O_2. The high EST and low O_2 combination exhibited the lowest YFB mass and the poorest navel condition. Energy content of the initial egg and partitioning of this energy over YFB, residual yolk, and ATP production at 12 or 48 hours after emergence from the eggshell are presented in Table 6.

Age

Chick length increased by 0.6 cm ($P < 0.001$) between 12 and 48 hours after emergence from the eggshell (Table 2). Yolk-free body mass increased by 2.8 g/24 hours between day 18 of incubation and 12 hours after emergence from the eggshell and YFB mass decreased by 0.3 g/24 hours between 12 and 48 hours after emergence from the eggshell ($P < 0.001$; Table 3). Protein, carbohydrate, and total energy in the residual yolk and the efficiency in transferring protein-free energy from the egg to the YFB (all $P < 0.001$) decreased between 12 and 48 hours after emergence from the eggshell (Table 4). Total energy loss increased with age ($P < 0.001$).

Heat production

Eggshell temperature, O_2 and day of incubation interacted on heat production ($P = 0.02$; Figure 1). Heat production increased between day 8 and 18 of incubation for all six treatments ($P < 0.05$). Heat production at high EST was 4.8 mW/egg (6%) higher compared with normal EST at day 13 of incubation and 5.1 mW/egg (5%) higher at day 14 of incubation (EST × day of incubation; $P < 0.001$). Embryos at low O_2 exhibited a lower heat production after day 13 of incubation than those at high O_2 (O_2 × day of incubation; $P < 0.001$). Heat production was lower in the low compared with the normal O_2 treatment after day 14 of incubation and lower in the normal compared with the high O_2 treatment after day 15 of incubation.

Discussion

EST

High EST reduced hatchling development compared with normal EST, as demonstrated by lower YFB mass, shorter chick length, greater residual yolk weight, and lower protein and total

energy content at both 12 and 48 hours after emergence. These results are consistent with other studies (Romanoff, 1936; Leksrisompong et al., 2007; Lourens et al., 2007; Piestun et al., 2008a) and emphasize how important EST is during incubation. The reduced hatchling development at high EST may be due to the 8 hour decrease in incubation duration, which resulted in a shorter time to use nutrients of the yolk and develop. In addition, the 3.2% decrease in protein utilization for growth at high EST may have contributed to the reduced hatchling development. This lower efficiency corresponds with a tendency toward higher plasma uric acid concentrations at high EST (R. Molenaar, unpublished data). Embryos at high EST may have an obligatory need for glucose to fuel anaerobic metabolism, especially during the energy-demanding hatching process (Tazawa et al., 1983; Moran, 2007). Similarly, carbohydrate oxidation increases in exercising human subjects at high environmental temperatures (Febbraio, 2001; Mündel, 2008). In the initial egg, little glucose is present (Romanoff and Romanoff, 1949), but glucose can be synthesized from glucogenic amino acids, which may explain the lower efficiency of protein utilization at high EST. On the other hand, it is found that utilization of the amino acids glutamate and glutamine for endogenous glucose production is negligible at the end of incubation in chicken embryos (Sunny et al., 2007), which may suggest that processes other than gluconeogenesis drive deamination of amino acids at high EST. Alternative fates for the carbon skeletons of amino acids are fatty acid synthesis or ATP production. However, fatty acid synthesis from amino acids is probably low in growing embryos because fatty acids are abundantly available in the egg (Romanoff and Romanoff, 1949) and inhibit de novo fatty acid synthesis (Goodridge, 1968).

Apart from the effect of EST, the efficiency of protein utilization for growth was high during embryonic development (85.2% ± 0.6) in the current study. This is comparable with results of Lourens et al. (2006) and higher than in growing humans, pigs, rats, calves, and sheep (45 to 81%; Waterlow, 1999; Van den Borne et al., 2006). In general, the efficiency of protein utilization tends to be higher in young animals and decreases with age (Reeds and Garlick, 2003; Van den Borne et al., 2006). High efficiency during embryonic development fits within this pattern and indicates that the amino acid profile of egg proteins matches the amino acid requirements for embryonic growth. Heat production was measured until day 18 of incubation in the current study and was higher at high EST than at normal EST only on day 13 and 14 of incubation. Total energy loss, as derived from body composition at 12 and 48 hours posthatch, did not differ between EST treatments. These results indicate that although energy losses were comparable between EST, efficiencies of protein utilization for growth and incubation duration differed among EST, which may have contributed to the differences in body development at hatch. In summary, high EST impaired chicken development through a shorter incubation period and less efficient utilization of egg protein for embryonic growth.

O_2 concentration

An increase in O_2 increased hatchling development at 12 hours after emergence from the eggshell, as demonstrated by higher heat production and YFB mass, a longer chick length, and lower residual yolk weight. These findings are consistent with those of a previous study (Lourens et al.,

2007). The increased development at higher O_2 was further expressed in the body composition of hatchlings at 12 hours after emergence from the eggshell. These hatchlings exhibited higher amounts of protein and fat in the YFB and lower amounts of protein, fat, and carbohydrates in the residual yolk. However, hatchling development and body composition in the YFB did not respond linearly to increasing O_2. The increase in YFB mass between low and normal O_2 was larger than the increase between normal and high O_2 in the current study as well as in a previous study (Lourens et al., 2007). This may be related to the efficiencies of nutrient utilization for growth. Embryos at low O_2 converted protein and total energy less efficiently into body tissue than those at normal and high O_2 at 12 hours after emergence. This may explain the larger difference in development between embryos at low and normal O_2 than between those at normal and high O_2 at 12 hours after emergence.

Nonetheless, our data suggest that embryos incubated at low O_2 converted more residual yolk into YFB than those at high O_2 after the shift to 21% O_2. At day 19 of incubation, all embryos were exposed to 21% O_2. Hence, the low O_2 (17%) embryos were enriched in O_2 and the high O_2 (25%) was reduced in O_2 at day 19 of incubation. The rate of increase in YFB mass per day was higher at low than at high O_2 from day 18 of incubation onward, without any difference in the rate of decrease in residual yolk mass per day. Consequently, the efficiency of nutrient utilization for growth may have increased at low O_2 compared with normal and high O_2 after day 19 of incubation. This may explain the increase in protein and total energy efficiencies between 12 and 48 hours after emergence at low O_2, whereas birds incubated at normal or high O_2 decreased their protein and total energy utilization for growth. Embryos incubated at low O_2 may have adapted to the low O_2 availability and possibly enhanced their O_2-carrying capacity through increases in red blood cells, hemoglobin mass, or blood volume (Baumann et al., 1983; Xu and Mortola, 1989; Azzam and Mortola, 2007; Ramirez et al., 2007). Such adaptations to low O_2 may have improved O_2 availability at the tissue level and increased the conversion of residual yolk to YFB after day 19 of incubation. However, this improved conversion did not increase YFB mass at low O_2 from 12 to 48 hours after emergence from the eggshell.

In summary, a higher O_2 concentration until day 19 of incubation was related to increased embryonic development at 12 hours after emergence. Efficiencies of nutrient utilization for growth improved in the posthatch period only for animals incubated at low O_2. These findings suggest that body composition and efficiencies of nutrient utilization for growth in the posthatch period are influenced by the O_2 concentration that embryos experience during incubation.

EST × O_2 concentration

An additional negative effect on survival rate and hatchling development was found when embryos were exposed to a combination of high EST and low O_2. Embryos at high EST and low O_2 had the lowest hatch of fertile (72.4%) compared with the other treatments (95.8% on average), and more embryos died, especially in the third week of incubation (24.7% vs. 3.1% on average). Various studies have shown that high EST (Romanoff, 1936; French, 2000; Lourens et al., 2005) or low O_2 (Onagbesan et al., 2007) during incubation reduces hatchability, but the interaction between these two factors has not been previously assessed. First, the reduced hatchability at high EST and low

O_2 might be related to a difference in nutrient mobilization and utilization during incubation, resulting in decreased availability of nutrients (e.g., glycogen) for the energy-demanding hatching process (Beattie, 1964; Christensen et al., 1993). Second, embryos at high EST and low O_2 had the lowest YFB mass and tended to have the highest residual yolk weight. This poor development might be the combined result of decreased incubation duration due to high EST (Romanoff, 1936; Lourens et al., 2007) and decreased growth rate due to low O_2 (Metcalfe et al., 1981). Under these conditions, the hatchling may not be sufficiently developed to cope with the energy-demanding hatching process. Third, the higher incidence of malpositioned embryos may have negatively affected hatchability and the ability to emerge from the egg. High EST, especially high EST combined with low O_2, increased the number of HBL and HOW embryos compared with the normal EST. The reason for these malpositions is not known (French, 2000), but HBL might be a consequence of retarded development (Robertson, 1961) or weakness of the embryo as observed in heat-stressed hatchlings (Leksrisompong et al., 2007). The large size of the residual yolk at the high EST and low O_2 combination may have resulted in the poorest navel condition because embryos were not able to insert the yolk properly into the body cavity, consistent with a previous study (Piestun et al., 2008a). The causes of poor navel conditions have not been extensively investigated, but the consequences of poor navel condition for subsequent performance are negative (Fasenko and O'Dea, 2008).

In summary, EST and O_2 interacted negatively on hatchability and development. The highest embryo mortality in the last week of incubation and the poorest body development were found at the high EST and low O_2 combination. Possible explanations include reduced nutrient availability for hatching, decreased body development to fulfill the energy-demanding hatching process, and higher incidence of malpositions.

In conclusion, prenatal incubation conditions such as EST and O_2 concentration influence survival rate and body development due to changes in nutrient utilization for growth and body composition. High EST reduced hatchling development compared with normal EST, due to decreased incubation duration and lower efficiency of protein utilization for growth. Embryonic development improved with increasing O_2 at 12 hours after emergence, but the difference between low and normal O_2 was larger than that between normal and high O_2. When O_2 was shifted to 21% for all treatments at day 19 of incubation, embryos incubated at low O_2 utilized nutrients more efficiently than those incubated at normal or high O_2. The combination of high EST and low O_2 resulted in the lowest hatchability and poorest development. This study indicates that embryos raised under suboptimal environmental conditions may develop adaptive mechanisms that continue to operate in the posthatch period.

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Table 1. Hatchability of fertile eggs, embryo mortality, malpositioned embryos, and incubation duration of embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	Hatch of fertile eggs	Embryo mortality		Malpositioned embryos		Incubation duration
		Second week	Third week	Head between legs	Head over wing	
	(%[1])	(%[1])	(%[1])	(%[1])	(%[1])	(hours)
EST (°C)						
37.8	96.8 ± 1.2	1.3 ± 0.6	1.9 ± 0.7	0.2[b] ± 0.2	1.1[b] ± 0.4	487[a] ± 0.8
38.9	86.9 ± 4.7	1.7 ± 1.0	11.6 ± 4.2	1.9[a] ± 1.1	4.2[a] ± 1.3	479[b] ± 0.5
O_2 (%)						
17	84.6 ± 7.1	2.0 ± 1.3	13.5 ± 6.5	2.3 ± 1.6	4.9 ± 1.9	483 ± 2.2
21	97.5 ± 1.8	1.1 ± 0.8	1.7 ± 1.1	0.3 ± 0.3	0.8 ± 0.5	482 ± 2.7
25	93.6 ± 0.7	1.4 ± 0.8	5.0 ± 1.1	0.6 ± 0.3	2.2 ± 0.5	483 ± 2.9
EST (°C) × O_2 (%)						
37.8 × 17	96.7[a] ± 1.1	1.1 ± 1.1	2.2[b] ± 0.0	0.0 ± 0.0	1.7 ± 0.6	486 ± 2.9
37.8 × 21	100.0[a] ± 0.0	0.0 ± 0.0	0.0[b] ± 0.0	0.0 ± 0.0	0.0 ± 0.0	487 ± 0.4
37.8 × 25	93.9[a] ± 1.7	2.8 ± 0.6	3.3[b] ± 1.1	0.6 ± 0.6	1.7 ± 0.6	488 ± 0.7
38.9 × 17	72.4[b] ± 3.1	2.8 ± 2.8	24.7[a] ± 0.3	4.6 ± 2.4	8.0 ± 1.2	480 ± 0.7
38.9 × 21	95.0[a] ± 2.7	2.2 ± 1.1	3.3[b] ± 1.1	0.5 ± 0.6	1.7 ± 0.5	478 ± 0.3
38.9 × 25	93.3[a] ± 0.0	0.0 ± 0.0	6.7[b] ± 0.0	0.6 ± 0.6	2.8 ± 0.6	478 ± 0.8
Source of variation						
EST	0.005	0.77	<0.001	0.05	0.005	0.04
O_2	0.02	0.67	0.006	0.54	0.06	0.90
EST × O_2	0.02	0.13	0.006	0.43	0.10	0.67

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Expressed as a percentage of fertile eggs.

Table 2. Development of hatchlings at 12 and 48 hours after emergence from the eggshell, incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

Treatment	Chick length (cm)	YFB mass[1] (g)	Residual yolk (g)	Navel condition[2]
EST (°C)				
37.8	19.5^{a} ± 0.1	37.7 ± 0.2	4.1^{b} ± 0.6	1.4 ± 0.0
38.9	19.3^{b} ± 0.2	36.1 ± 0.4	5.3^{a} ± 0.6	1.8 ± 0.1
O_2 (%)				
17	18.9^{c} ± 0.1	35.7 ± 0.5	5.9 ± 0.8	1.8 ± 0.1
21	19.5^{b} ± 0.1	37.2 ± 0.3	4.5 ± 0.7	1.5 ± 0.1
25	19.8^{a} ± 0.1	37.8 ± 0.3	3.8 ± 0.7	1.5 ± 0.1
Age (hours)				
12	19.1^{b} ± 0.1	37.1 ± 0.4	6.5 ± 0.3	1.6 ± 0.1
48	19.7^{a} ± 0.1	36.7 ± 0.4	2.9 ± 0.3	1.6 ± 0.1
EST (°C) × O_2 (%)				
37.8 × 17	19.1 ± 0.2	36.8^{b} ± 0.2	5.1 ± 1.2	1.4^{bc} ± 0.0
37.8 × 21	19.6 ± 0.2	37.8^{ac} ± 0.1	4.0 ± 1.1	1.4^{c} ± 0.0
37.8 × 25	19.8 ± 0.2	38.5^{a} ± 0.3	3.3 ± 1.0	1.4^{bc} ± 0.1
38.9 × 17	18.7 ± 0.2	34.5^{d} ± 0.2	6.7 ± 1.1	2.1^{a} ± 0.0
38.9 × 21	19.4 ± 0.2	36.6^{b} ± 0.2	5.0 ± 1.0	1.6^{b} ± 0.1
38.9 × 25	19.7 ± 0.2	37.2^{bc} ± 0.3	4.2 ± 1.0	1.7^{b} ± 0.0
O_2 (%) × Age (hours)				
17 × 12	18.6 ± 0.1	35.7^{c} ± 0.5	7.8^{a} ± 0.4	1.8 ± 0.2
21 × 12	19.2 ± 0.1	37.4^{b} ± 0.3	6.3^{b} ± 0.3	1.5 ± 0.1
25 × 12	19.4 ± 0.0	38.3^{a} ± 0.4	5.4^{c} ± 0.3	1.5 ± 0.1
17 × 48	19.2 ± 0.1	35.7^{c} ± 0.8	3.9^{d} ± 0.5	1.8 ± 0.2
21 × 48	19.8 ± 0.1	37.0^{b} ± 0.5	2.6^{e} ± 0.4	1.5 ± 0.1
25 × 48	20.1 ± 0.1	37.3^{b} ± 0.4	2.1^{e} ± 0.3	1.6 ± 0.1
Source of variation[3]				
EST	0.05	<0.001	<0.001	0.002
O_2	<0.001	<0.001	<0.001	<0.001
Age	<0.001	<0.001	<0.001	0.60
EST × O_2	0.09	0.01	0.06	0.01
O_2 × Age	1.00	0.01	0.02	0.71

[a-e]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Yolk-free body mass.

[2]Average navel condition of chickens per treatment group scored with a 1, 2, or 3, where 1 = good, 2 = moderate, and 3 = poor.

[3]No two-way interaction between EST and Age or three-way interaction between EST, O_2 concentration, and Age was found ($P > 0.05$).

Table 3. Increase in yolk-free body (YFB) mass and decrease in residual yolk weight per day between day 18 of incubation and 12 hours after emergence from the eggshell and between 12 and 48 hours after emergence from the eggshell for hatchlings incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

Treatment	YFB increase (g/24 hours)	Residual yolk decrease (g/24 hours)
EST (°C)		
37.8	1.3[a] ± 0.5	2.5 ± 0.1
38.9	1.1[b] ± 0.5	2.3 ± 0.1
O_2 (%)		
17	1.5[a] ± 0.6	2.4 ± 0.2
21	1.3[ab] ± 0.6	2.5 ± 0.1
25	0.9[b] ± 0.6	2.3 ± 0.1
Period		
day 18 to 12 hours[1]	2.8[a] ± 0.1	2.4 ± 0.1
12 to 48 hours[2]	-0.3[b] ± 0.1	2.4 ± 0.1
Source of variation[3]		
EST	0.01	0.24
O_2	0.001	0.26
Period	<0.001	0.72

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Day 18 of incubation to 12 hours after emergence from the eggshell.

[2]Period 12 to 48 hours after emergence from the eggshell.

[3]No two-way or three-way interaction between EST, O_2 concentration, and Period was found ($P > 0.05$).

Table 4. Body composition, energy loss and efficiency of energy transfer from egg to yolk-free body (YFB) of embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	EST		O_2			Age		Source of variation[1]				
	37.8°C	37.8°C	17%	21%	25%	12 hours	48 hours	EST	O_2	Age	EST × Age	O_2 × Age
YFB (kJ)												
Protein	127.7^{a} ± 2.8	116.6^{b} ± 3.4	116.1 ± 5.2	123.2 ± 3.9	127.2 ± 2.9	113.6 ± 2.8	130.7 ± 2.1	0.006	0.002	<0.001	0.70	0.05
Fat	79.5 ± 1.8	71.5 ± 2.1	69.8 ± 2.9	76.9 ± 2.0	80.0 ± 2.1	76.7 ± 2.8	74.4 ± 1.5	0.02	<0.001	0.06	0.04	0.002
Carbohydrate	2.1 ± 1.0	3.9 ± 1.0	3.5 ± 1.6	2.5 ± 1.1	3.1 ± 1.3	3.6 ± 1.2	2.5 ± 0.9	0.49	0.31	0.36	0.99	0.69
Total	209.4^{a} ± 3.5	192.0^{b} ± 4.3	189.4 ± 6.7	202.5 ± 3.8	210.2 ± 3.6	193.9 ± 5.1	207.5 ± 3.0	0.007	<0.001	<0.001	0.09	0.002
Residual yolk (kJ)												
Protein	22.2^{b} ± 3.3	32.0^{a} ± 3.7	33.4^{a} ± 4.7	25.7^{b} ± 4.4	22.1^{b} ± 4.2	37.4^{a} ± 2.3	16.8^{b} ± 2.1	0.007	<0.001	<0.001	0.70	0.96
Fat	32.1 ± 6.2	39.9 ± 5.8	48.5 ± 8.0	32.8 ± 6.4	26.6 ± 5.9	53.0 ± 3.9	18.9 ± 2.5	0.06	<0.001	<0.001	0.50	0.05
Carbohydrate	3.0^{b} ± 0.5	4.1^{a} ± 0.5	4.5^{a} ± 0.6	3.4^{b} ± 0.6	2.9^{b} ± 0.6	5.0^{a} ± 0.3	2.1^{b} ± 0.3	0.02	<0.001	<0.001	0.11	0.68
Total	57.3^{b} ± 10.0	75.9^{a} ± 9.8	86.4^{a} ± 13.1	61.8^{b} ± 11.1	51.6^{b} ± 10.6	95.5^{a} ± 6.0	37.7^{b} ± 4.9	0.008	<0.001	<0.001	0.62	0.11
Energy loss[2] (kJ)												
Protein	19.6 ± 1.3	22.8 ± 1.0	20.5 ± 1.6	21.3 ± 1.7	21.8 ± 1.4	19.4 ± 1.1	23.0 ±1.2	0.09	0.77	0.009	0.88	0.02
Protein-free	142.3 ± 7.1	132.2 ± 6.7	129.4^{b} ± 8.9	137.9^{a} ± 8.5	144.4^{a} ± 8.3	117.0 ± 3.4	157.5 ± 3.7	0.27	0.002	<0.001	0.05	0.94
Total	161.9 ± 8.0	155.1 ± 6.8	150.0^{b} ± 8.4	159.2^{a} ± 9.2	166.3^{a} ± 9.5	136.4^{b} ± 3.0	180.5^{a} ± 3.8	0.40	0.006	<0.001	0.09	0.20
E_{YFB}[3] (%)												
Protein	86.8^{a} ± 0.7	83.6^{b} ± 0.8	84.8 ± 1.5	85.2 ± 1.1	85.5 ± 0.6	85.3 ± 1.0	85.0 ± 0.7	0.05	0.72	0.76	0.63	0.03
Protein-free	36.8 ± 1.5	36.7 ± 1.3	36.6 ± 1.7	36.9 ± 1.8	36.8 ± 2.0	40.7^{a} ± 0.9	32.9^{b} ± 0.7	0.96	0.06	<0.001	0.14	0.27
Total	56.7 ± 1.1	55.5 ± 0.8	56.0 ± 1.1	56.2 ± 1.2	56.1 ± 1.4	58.7 ± 0.6	53.5 ± 0.5	0.43	0.16	<0.001	0.09	0.05

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Interactions between EST and O_2 or EST, O_2, and Age were not significant ($P > 0.10$).

[2]Energy loss was calculated as egg energy – YFB energy – residual yolk energy.

[3]Efficiency of energy transfer from egg to YFB.

Table 5. Interactions between eggshell temperature (EST) and age of the hatchling or between O_2 concentration and age of the hatchling on energy content of the yolk-free body (YFB) and residual yolk, energy loss from the start of incubation and efficiency of energy transfer from egg to YFB in embryos incubated at two EST (37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	EST (°C) × Age (hours)						*P*-value
	37.8 × 12	38.9 × 12	37.8 × 48	38.9 × 48			
YFB (kJ)							
Fat	82.0^{a} ± 3.0	71.4^{b} ± 3.8	77.1^{a} ± 1.6	71.7^{b} ± 2.2			0.04
Energy loss[1] (kJ)							
Protein-free	120.1^{c} ± 3.8	113.9^{c} ± 5.8	164.5^{a} ± 3.6	150.5^{b} ± 5.3			0.05
	O_2 (%) × Age (hours)						
	17 × 12	21 ×12	25 × 12	17 × 48	21 × 48	25 × 48	*P*-value
YFB (kJ)							
Protein	105.0^{d} ± 4.6	115.6^{c} ± 4.3	120.3^{bc} ± 2.5	127.2^{ab} ± 4.9	130.7^{a} ± 3.8	134.1^{a} ± 0.9	0.05
Fat	66.8^{c} ± 4.2	79.5^{ab} ± 3.3	83.7^{a} ± 2.8	72.7^{bc} ± 4.1	74.2^{b} ± 1.7	76.2^{b} ± 2.1	0.002
Total	176.5^{c} ± 6.6	197.6^{b} ± 6.1	207.6^{ab} ± 6.5	202.2^{ab} ± 7.5	207.5^{ab} ± 3.7	212.9^{a} ± 3.5	0.002
Residual yolk (kJ)							
Fat	68.8^{a} ± 3.7	48.9^{b} ± 3.4	41.5^{b} ± 3.5	28.2^{c}± 3.7	16.7^{d} ± 2.3	11.7^{d} ± 2.1	0.05
Energy loss[1] (kJ)							
Protein	21.3^{a} ± 2.9	18.4^{a} ± 1.7	18.5^{a} ± 0.9	19.7^{a} ± 1.9	24.2^{a} ± 2.1	25.0^{a} ± 1.0	0.02
E_{YFB}[2] (%)							
Protein	83.0^{a} ± 2.5	86.2^{a} ± 1.6	86.7^{a} ± 0.6	86.5^{a} ± 1.6	84.3^{a} ± 1.5	84.3^{a} ± 0.5	0.03
Total	57.5^{ab} ± 1.7	59.2^{a} ± 0.5	59.3^{a} ± 0.9	54.4^{bc} ± 1.0	53.3^{c} ± 0.7	52.8^{bc} ± 0.9	0.05

[a-d]Means followed by different superscripts within a row are significantly different ($P \leq 0.05$).

[1]Energy loss was calculated as egg energy – YFB energy – residual yolk energy.

[2]Efficiency of energy transfer from egg to YFB.

Table 6. Initial energy content of the egg and energy content percentages of the yolk-free body (YFB) and residual yolk and energy loss at 12 and 48 hours after emergence from the eggshell relative to initial energy content of the egg for embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

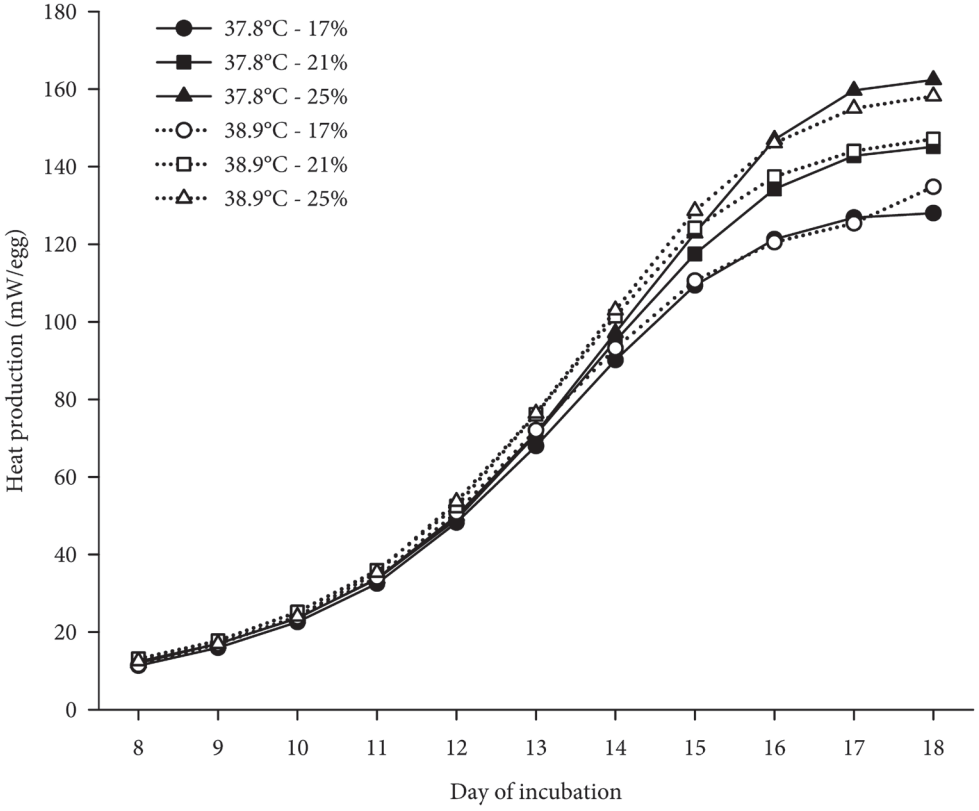
	EST (°C) × O_2 concentration (%)					
	37.8 × 17	37.8 × 21	37.8 × 25	38.9 × 17	38.9 × 21	38.9 × 25
Egg						
Initial energy content (kJ)						
Protein	168	170	171	172	171	171
Protein-free[2]	260	258	258	251	248	256
Total	428	428	429	423	419	427
Hatchlings						
Protein (%)						
YFB, 12 h	66	72	73	57	64	68
Residual yolk, 12 h	23	19	16	29	24	22
Loss[1], 12 h	11	9	11	14	12	11
YFB, 48 h	81	81	79	69	73	77
Residual yolk, 48 h	10	7	5	17	11	9
Loss[1], 48 h	10	12	16	13	16	14
Protein-free[2] (%)						
YFB, 12 h	29	33	36	27	31	32
Residual yolk, 12 h	28	19	16	30	24	20
Loss[1], 12 h	43	48	48	43	45	48
YFB, 48 h	31	29	31	28	31	30
Residual yolk, 48 h	9	5	4	15	9	7
Loss[1], 48 h	60	65	65	57	59	63
Total (%)						
YFB, 12 h	44	49	51	39	45	46
Residual yolk, 12	26	19	16	30	24	21
Loss[1], 12 h	30	33	33	31	32	33
YFB, 48 h	50	50	50	45	48	49
Residual yolk, 48 h	10	6	4	16	10	8
Loss[1], 48 h	40	44	46	39	42	43

[1]Energy loss was calculated as egg energy – YFB energy – residual yolk energy.

[2]Fat energy and carbohydrate energy.

Figure 1. Heat production (mW/egg) of embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation. The legend indicates the level of significance (* = $P < 0.05$).

Day	8	9	10	11	12	13	14	15	16	17	18
EST	-	-	-	-	-	*	*	-	-	-	-
O_2	-	-	-	-	-	*	*	*	*	*	*
EST*O_2	-	-	-	-	-	-	-	*	*	*	*



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Effect of Eggshell temperature and Oxygen concentration during Incubation on the Developmental and Physiological Status of Broiler Hatchlings in the Perinatal Period

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Abstract

This study evaluated the influence of incubation conditions on the developmental and physiological status of birds in the perinatal period, which spans the end of incubation until the early posthatch period. Embryos were incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) and a low (17%), normal (21%), or high (25%) O_2 concentration from day 7 until 19 of incubation. After day 19 of incubation, EST was maintained, but O_2 concentrations were 21% for all embryos. Body and organ weights, and hepatic glycogen levels were measured at day 18 of incubation, and 12 and 48 hours after emergence from the eggshell. In addition, blood metabolites were measured at 12 and 48 hours after emergence from the eggshell. High EST compared with normal EST decreased yolk-free body mass (YFBM). This may be due to the shorter incubation duration of 8 hours, the lower weight of supply organs (i.e., heart and lung), or a lack of glucose precursors. Because of this lack of glucose precursors, embryos incubated at high EST may have used proteins for energy production instead of body development at the end of incubation. Yolk-free body mass at day 18 of incubation increased with an increase in O_2 concentration. However, differences between the normal and high O_2 concentration disappeared at 12 and 48 hours after emergence, possibly because the high O_2 concentration had difficulties to adapt to lower O_2 concentrations in the perinatal period. Blood metabolites and hepatic glycogen were comparable among O_2 concentrations, indicating that the physiological status at hatch may be related to the environment that the embryo experienced during the hatching process. Embryos incubated at a high EST and low O_2 concentration had the highest mortality in the last week of incubation, which may be related with their low YFBM or a reduced nutrient availability for hatching (i.e., hepatic glycogen). In conclusion, EST and O_2 concentration differentially influence the developmental and physiological status of broilers during the perinatal period.

Key words: eggshell temperature, O_2 concentration, hepatic glycogen, blood metabolites, broiler embryo

Introduction

Broiler chicks increase their body weight approximately 50-fold within 40 days of age. Because of this short life cycle, optimal body development during incubation and in the posthatch period is important (Nir and Levanon, 1993; Wolanski et al., 2006). Incubation conditions influence embryonic development, as demonstrated by changes in yolk-free body mass, organ weights, residual yolk size, and chick length at hatch (Metcalfe et al., 1981; Van Golde et al., 1998; Leksrisompong et al., 2007; Lourens et al., 2007; Piestun et al., 2008a). Two of the most important incubation conditions that are known to influence embryonic development are temperature and O_2 (Stock and Metcalfe, 1984; Lourens, 2004; Meijerhof, 2009). Changes found in development due to different incubation conditions (Lourens et al., 2007) may be related to the physiological status of hatchlings. This physiological status can be reflected by plasma metabolites, which are products of intermediary metabolism (Artacho et al., 2007). In addition, plasma metabolites may indicate the nutritional status of the bird (Artacho et al., 2007).

At the end of incubation of chicken embryos, plasma metabolite concentrations may reflect which nutrients were used during the energy-demanding hatching process. Glycogen stores are largely mobilized during this period (Freeman, 1965, 1969) and indicate that glucose metabolism has an important role during the hatching process. Because glucose is hardly available in the egg at oviposition (Romanoff and Romanoff, 1949), glucose synthesis is important during incubation. Gluconeogenesis is indispensable in the embryo during incubation (Delphia and Elliott, 1965) with lactate, glycerol, or glucogenic amino acids as potential precursors (Evans and Scholz, 1973; Watford et al., 1981). Different incubation conditions may influence nutrient availability and utilization of for example glucose and thereby change the physiological status of broiler hatchlings (De Oliveira et al., 2008).

Furthermore, changes in developmental and physiological processes due to different incubation conditions are probably not restricted to the period of embryogenesis alone but also influences the period after hatch. Few studies investigated the effects of incubation conditions on developmental and physiological status of hatchlings in the early posthatch period. Hatchlings have often no access to feed or water up to 24 to 72 hours after hatch in practice due to variation in hatch time, hatchery management, and transport time (Dibner et al., 1998; Noy and Sklan, 1998; Careghi et al., 2005). Therefore, this study was performed to investigate the effect of different incubation conditions on the developmental and physiological status of broiler embryos in the perinatal period, which spans the end of incubation until the early posthatch period. Broiler embryos were incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) and a low (17%), normal (21%), or high (25%) O_2 concentration.

Materials and methods

Experimental Design

The experiment was designed a 2 × 3 factorial arrangement with two EST (37.8°C and 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) applied from day 7 until 19 of incubation. Each treatment was repeated twice. Six consecutive batches of eggs were incubated with two EST and O_2 concentration combinations per batch. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University.

Hatching Eggs

Hatching eggs of a commercial Hybro grandparent stock line were used (n = 1,320). Eggs weighed between 60 and 65 g and were not significant different between the EST and O_2 combinations and batches of eggs ($P > 0.10$). The age of the parent stock ranged from 45 to 51 weeks.

Egg Storage and Incubation until day 7

Eggs were stored for 3 to 5 days at 18°C at Torsius Breeder Hatchery (Putten, the Netherlands). Thereafter, eggs were transported to the experimental accommodation of Wageningen University and placed in a HT-combi incubator with a maximum capacity of 4,800 eggs (HatchTech B.V., Veenendaal, the Netherlands). In each of the six batches of eggs, eggs were equally divided among four egg trays (n = 150 eggs). Egg trays were half-filled with eggs; every other egg space remained empty to ensure uniform air speed around the eggs. Eggshell temperature was measured by temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK) placed halfway between the blunt and pointed end of five individual eggs. Temperature sensors were attached to the eggshell using heat-conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and tape. Incubator temperature was adjusted manually twice a day to maintain an EST of 37.8°C. Relative humidity was set at 55%, and eggs were turned each hour over 90°. The CO_2 concentration was between 0.05 and 0.07% at day 7 of incubation before the eggs were transferred.

Incubation from day 7 to 19

After candling at day 7 of incubation, 220 fertile eggs were randomly divided between two identical small open circuit Climate Respiration Chambers (CRC; Lourens et al., 2006). Eggshell temperature was maintained at 37.8°C (normal) or 38.9°C (high), and the O_2 level was maintained at 17% (low), 21% (normal), or 25% (high). The EST of the two chambers was identical within each batch of eggs. Eggshell temperature and O_2 concentration in the CRC were regulated as described by Lourens et al. (2007). Because of the fixed ventilation rate, O_2 concentration decreased on average from day 7 until 19 of incubation from 17.1% to 16.3% in the low O_2 concentration, from 20.9% to 20.0% in the normal O_2 concentration, and from 25.0% to 23.9% in the high O_2 concentration. In all the six batches of eggs, CO_2 concentration was roughly 0.1% at day 7 of incubation and

increased to 0.6 to 0.8% by day 18 of incubation due to the CO_2 production of the embryos. Relative humidity was maintained at 50%, and eggs were turned each hour over 90°.

Incubation from day 19 until 48 hours after emergence from the Eggshell

At day 19 of incubation, eggs were transferred from the egg trays to individual hatching baskets (120 × 135 mm) in one large open circuit CRC (1.5 × 3.5 m; Verstegen et al., 1987). The previous EST, which was identical between the two treatments per batch of eggs, was maintained for half a day. After this period, the CRC temperature was fixed at the last EST setpoint, and the EST was allowed to increase during the hatching process. Oxygen was not regulated after day 19 of incubation and remained at approximately 21%. Relative humidity was maintained at 55%. The number of hatchlings emerging from eggshells was recorded every 2 hours to calculate the average incubation duration per treatment. Hatchlings were selected for body and organ development, and hepatic glycogen and blood metabolite measurements either 12 or 48 hours after emergence from the eggshell. To distribute hatchlings per EST and O_2 combination equally across the hatching period, sequential hatchlings were alternately allocated to the 12- or 48-hour measurement groups. From the time of emergence until 12 hours afterward, hatchlings were kept in hatching baskets in the large open-circuit CRC. At 12 hours after emergence from the eggshell, hatchlings that were measured at 48 hours were transferred to individual hatching baskets (120 × 135 mm) in another, identical large CRC until 48 hours after emergence from the eggshell. Environmental temperature in this CRC was maintained at 33°C, and relative humidity was maintained at 55%. No feed or water was provided, and hatchlings were continuously exposed to light.

Embryo Mortality and Hatch of Fertile

Eggs were candled at day 19 of incubation to identify nonviable embryos. At day 23 of incubation, nonhatched eggs were opened to classify embryo mortality per day as described previously (Lourens et al., 2006). Embryos that died during the first week of incubation were excluded from the analyses. Hatch of fertile (HOF) was expressed as the percentage of chicks that hatched from the fertile eggs and was calculated per EST and O_2 combination and batch of eggs.

Body and Organ Weights

At day 18 of incubation, 20 fertile eggs per EST and O_2 combination were randomly selected. Embryos were killed by decapitation, the residual yolk was removed and the embryos were weighed. Hatchlings at 12 or 48 hours after emergence from the eggshell were weighed and decapitated and the residual yolk was removed and weighed. Yolk-free body mass (YFBM) was calculated as body weight minus residual yolk weight.

Livers were immediately weighed after decapitation at day 18 of incubation or at 12 or 48 hours after emergence from the eggshell. Due to time limitations, the YFBM was frozen in liquid nitrogen and stored at -20°C for further analysis of organ weights. Heart, lung, stomach, intestines, liver, spleen, and bursa of Fabricius from all embryos and hatchlings were weighed after defrosting the YFBM in a plastic bag in a water bath at 37°C for 15 minutes.

Blood Metabolites Determination

Hatchlings were decapitated at 12 or 48 hours after emergence from the eggshell. Blood was collected in a 4-mL blood tube containing 10 mg of sodium fluoride and 8 mg of potassium oxalate (BD Vacutainer, New Jersey, USA). An extra droplet (0.02 mL) of 10% heparin was added and mixed into the tube before sampling. Blood was centrifuged (2,900 × *g*) for 15 minutes and plasma was decanted and stored at -20°C until further analysis. Plasma glucose, lactate, and uric acid concentrations were determined with a commercially available kit (DiaSys Diagnostic Systems International, Holzheim, Germany).

Hepatic Glycogen Determination

Embryos at day 18 of incubation and hatchlings at 12 or 48 hours after emergence from the eggshell were weighed and decapitated. After bleeding, livers were immediately dissected, weighed, and frozen in liquid nitrogen. Livers were stored at -80°C until further analysis. All procedures for hepatic glycogen determination were carried out on ice. The whole liver was homogenized with a glass stirring spoon after the addition of 1 µL of 7% $HCLO_4$/g of wet tissue. The suspension was centrifuged (2,900 × *g*) at 4°C for 15 minutes. The supernatant was decanted, cleaned with 1 mL of petroleumether, and frozen at -80°C until further analysis. The supernatant was defrosted, centrifuged, and decanted again. Hepatic glycogen was determined by the iodine binding assay (Dreiling et al., 1987) and hepatic bovine glycogen (Type IX; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a standard.

Statistical Analysis

The CRC was the experimental unit in all statistical analyses. Distributions of the means and residuals were examined to verify model assumptions. An arcsine transformation was used for HOF and embryo mortality. Hatch of fertile, embryo mortality, and incubation duration were analyzed using the GLM procedure in SAS (Version 9.1, SAS Institute 2004). The model was

$$Y_{ij} = \mu + EST_i + Batch(EST_i) + O_{2j} + interactions + \varepsilon_{ij} \quad [1]$$

where Y_{ij} is the dependent variable, μ is the overall mean, EST_i is eggshell temperature (i = 37.8°C or 38.9°C), and O_{2j} is oxygen concentration (j = 17%, 21%, or 25%). Batch(EST_i) is the eggshell temperature nested within the six batches, and this term was used as an error term to test for effects of eggshell temperature. Interactions between EST_i and O_{2j} were tested against ε_{ij}, which was the residual error term. For analyses of plasma glucose, lactate, and uric acid concentrations, model [1] was extended with the age of the bird (age_k; k = 12 or 48 hours after emergence from the eggshell) and interactions of the other factors with age. Organ weights were expressed as percentages of the YFBM. Yolk-free body masses, organ weights, and total hepatic glycogen levels were analyzed using model [1], extended with the age of the bird (age_k; k = day 18 of incubation, 12 or 48 hours after emergence from the eggshell) and interactions of all other factors with age. Least squares

means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as means ± SE. In all cases, differences were considered significant at $P \leq 0.05$.

Results

EST

An interaction was found between EST and age of the bird for YFBM ($P < 0.001$; Table 2). At day 18 of incubation, YFBM was comparable between EST (Table 4). At 12 and 48 hours after emergence from the eggshell, YFBM was lower in the high EST compared with the normal EST. An interaction was found between EST and age of the bird for relative heart ($P = 0.02$), stomach ($P < 0.001$), and intestinal weights ($P = 0.01$) (Table 2). Relative heart weight was lower in the high EST compared with the normal EST, but differences between EST were more pronounced at 12 hours after emergence than at day 18 of incubation or 48 hours after emergence from the eggshell (Table 4). Relative stomach and intestinal weights were higher in the high EST than in the normal EST at day 18 of incubation. The opposite was found at 12 hours after emergence from the eggshell; relative stomach and intestinal weight were lower in the high EST than in the normal EST. No effect of EST was found on relative stomach and intestinal weight at 48 hours after emergence from the eggshell. An interaction was found between EST and age of the bird for hepatic glycogen ($P < 0.001$; Table 3). At day 18 of incubation, total hepatic glycogen was 5.3 mg (30%) lower in the high EST than in the normal EST ($P < 0.001$; Table 4). At 12 or 48 hours after emergence from the eggshell, total hepatic glycogen did not differ between EST.

EST did not affect embryo mortality in the second week of incubation ($P = 0.89$; Table 1). Incubation duration was 8 hours shorter in the high compared with the normal EST ($P = 0.04$; Table 1). High EST compared with normal EST resulted in a 2% lower relative lung weight (0.92% for the high EST vs. 0.95% for the normal EST; $P = 0.04$) and 20% lower relative bursa of Fabricius weight (0.08% for the high EST vs. 0.10% for the normal EST; $P = 0.02$) (Table 2). Relative spleen weight did not differ between EST. Plasma glucose, lactate, and uric acid concentrations did not differ between EST at 12 and 48 hours after emergence from the eggshell (all $P > 0.05$; Table 3).

O_2 concentration

An interaction between O_2 concentration and age of the birds was found for YFBM ($P < 0.001$; Table 2). At day 18 of incubation, YFBM was higher for increased O_2 concentrations (Figure 1). At 12 and 48 hours after emergence from the eggshell, YFBM was lower in the low than in the normal O_2 concentration, but YFBM did not differ between the normal and the high O_2 concentration.

Oxygen concentration did not affect embryo mortality in the second week of incubation ($P = 0.87$; Table 1). Relative heart, lung, spleen, and bursa of Fabricius weights did not differ among O_2 concentrations (all $P > 0.10$; Table 2). In addition, plasma glucose, lactate and uric acid concentrations, and total hepatic glycogen did not differ among O_2 concentrations (all $P > 0.05$; Table 3).

EST × O_2 concentration

An interaction between EST and O_2 concentration was found for HOF ($P = 0.04$) and embryo mortality in the third week of incubation ($P = 0.03$) (Table 1). High EST and low O_2 decreased HOF by 23% on average and increased embryo mortality in the third week of incubation by 22% on average compared with all other EST and O_2 combinations. An interaction between EST and O_2 concentration was found for YFBM ($P = 0.003$; Table 2). High EST compared with normal EST had a lower YFBM, but this difference was more pronounced at low O_2 concentrations than at normal or high O_2 concentrations (Table 5). An interaction between EST and O_2 concentration was found for relative stomach ($P = 0.05$) and intestinal weight ($P = 0.01$) (Table 2). The normal EST and low O_2 concentration had a higher relative stomach weight than the normal EST and high O_2 concentration and the high EST and high O_2 concentration (Table 5). Relative intestinal weight differed between the high and normal EST when this was combined with a high O_2 concentration. The normal EST and high O_2 concentration had a higher intestinal weight than the high EST and high O_2 concentration.

A three-way interaction was found between EST, O_2 concentration, and age of the hatchling for relative liver weight ($P = 0.02$; data not shown). Relative liver weight increased with age in all the EST and O_2 concentration combinations. Differences in relative liver weight among EST and O_2 concentration combinations were only found at 48 hours after emergence from the eggshell. The normal EST and low O_2 concentration had a higher relative liver weight than the normal EST and high O_2 concentration, the high EST and normal O_2 concentration, and the high EST and high O_2 concentration at 48 hours after emergence from the eggshell.

Age

Regardless of EST and O_2 concentration, all relative organ weights increased between day 18 of incubation and 12 hours after emergence from the eggshell ($P < 0.05$; Table 2, 5). Between 12 and 48 hours after emergence from the eggshell, an increase in relative organ weights was found for all EST and O_2 concentration combinations ($P < 0.05$), except for relative heart weight of the normal EST ($P > 0.05$; Table 4) and relative spleen weight ($P > 0.05$; Table 2). Between 12 and 48 hours after emergence from the eggshell, plasma glucose concentrations decreased by 6.4 mg/dL (3%; $P = 0.01$), plasma lactate concentrations did not differ ($P = 0.45$), and plasma uric acid concentrations increased by 0.9 mg/dL (23%; $P < 0.001$) (Table 3).

Discussion

EST

Embryo development was comparable between EST at day 18 of incubation and embryo development was lower in the high EST compared with the normal EST at 12 and 48 hours after emergence from the eggshell. This seems to be largely comparable with data of Romanoff et al. (1938), who showed that a high incubation temperature of 40.5°C from day 7 of incubation onward increased embryo development in the second week of incubation, but finally decreased

body development in the last week of incubation. There are three possible reasons that may explain the lower body development at hatch in the high EST compared with the normal EST, which is consistently found in different studies (Romanoff, 1935; Romanoff, 1936; Wineland et al., 2000; Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007).

The first reason might be the reduction in incubation duration (Lourens et al., 2007); embryos in the high EST had 8 hours less to develop in the current study. The second reason for the reduced development at high EST may be related with nutrient utilization during incubation. Residual yolk absorption per 24 hours from day 18 of incubation until hatch did not differ between EST, but the YFBM increase was lower in the high compared with the normal EST during the same period (Molenaar et al., 2010). This suggests that embryos in the high EST had more difficulties to convert yolk into YFBM than embryos in the normal EST at the end of incubation. The impaired conversion from yolk into YFBM may be caused by a lack of glucose precursors in the high EST. Glycogen stores are largely mobilized at the end of incubation (Freeman, 1965, 1969), when embryos start the energy-demanding hatching process. Embryos incubated at the high EST had less hepatic glycogen available before the hatching process started, at day 18 of incubation, than embryos incubated at the normal EST. This reduction in hepatic glycogen might be due to a lower glycogen synthesis rate or a greater glycogen use before day 18 of incubation. No differences in hepatic glycogen were found between EST at 12 or 48 hours after emergence and this may indicate that animals in the high EST used less hepatic glycogen from day 18 of incubation onward than animals in the normal EST. Assuming that energy requirements for the hatching processes are the same between EST, energy may have originated in the high EST from other sources than hepatic glycogen as well. This energy may originate from glucogenic amino acids because plasma uric acid concentrations tended to increase by 14% in the high EST compared with the normal EST (0.5 mg/dL; $P = 0.06$; Table 3). After deamination or transamination of glucogenic amino acids, the carbon skeleton might be immediately oxidized for adenosine triphosphate (ATP) production (McArdle et al., 1981) or first converted to glucose by gluconeogenesis (Hazelwood and Lorenz, 1959). If the glucogenic amino acids were used for energy instead of body development; this may have resulted in a lower YFBM in the high EST. The third reason that may explain the reduction in body development in the high EST may be related with organ development. Relative heart and lung weights were negatively affected by the high EST. A decrease in these supply organs may reduce body development.

The reduction in relative heart weights with high incubation temperatures is consistent with other studies (Wineland et al., 2000; Givisiez et al., 2001; Leksrisompong et al., 2007; Lourens et al., 2007). After day 9 of incubation, high incubation temperatures negatively affect cardiac cell divisions (Romanoff, 1960; Leksrisompong et al., 2007) and this may have also occured in lung and bursa of Fabricius tissue in the current study. However, not all organs were negatively affected by the high EST, which is consistent with other studies (Zhang and Whittow, 1992; Givisiez et al., 2001; Leksrisompong et al., 2007; Lourens et al., 2007). Tissues may be sensitive for high temperatures during certain parts of embryogenesis (Decuypere and Michels, 1992).

O_2 concentration

Embryos were exposed to different O_2 concentrations from day 7 until 19 of incubation but all embryos were exposed to 21% O_2 after day 19 of incubation. At day 18 of incubation, YFBM was higher for increased O_2 concentrations and this showed that embryonic development occurred in a manner proportional to the O_2 concentration. At 12 and 48 hours after emergence from the eggshell, YFBM increased between the low and normal O_2 concentrations, but YFBM was comparable between the normal and high O_2 concentrations. Lourens et al. (2007) found numerical differences between the three O_2 concentrations comparable to those in the current study at day 21 of incubation. The discrepancy between the low and normal and normal and high O_2 concentration after emergence from the eggshell in both the current study and the study of Lourens et al. (2007) might be due to the change in O_2 concentration after day 19 of incubation. Embryos in the high O_2 (25%) concentration experienced a reduction in O_2 concentration (to 21%) after day 19 of incubation. The metabolism of embryos in the high O_2 concentration may not have been adapted to the lower O_2 concentrations provided after day 19 of incubation. Subsequently, O_2 availability and the related growth of the embryo may have decreased more in the high O_2 than in the normal O_2 concentration. On the other hand, embryos in the low O_2 (17%) concentration experienced an enrichment in O_2 concentration (to 21%) after day 19 of incubation, but no increase in YFBM was found between 12 and 48 hours after emergence.

No differences among O_2 concentrations were found for relative organ weights, blood metabolite or hepatic glycogen levels, which is not consistent with other studies (McCutcheon et al., 1982; Van Golde et al., 1998). However, O_2 concentrations were more extreme and applied until the end of the incubation process in the study of McCutcheon et al. (1982) and Van Golde et al. (1998). In the current study, all embryos experienced the same O_2 concentration after day 19 of incubation and the physiological status of the hatchling may be related to the environment experienced by the embryo at the end of incubation, during the hatching process.

EST × O_2 concentration

Hatchability was lowest and embryo mortality in the third week of incubation was highest in the high EST and low O_2 concentration and this indicates that the embryos in this treatment had more difficulties with hatching. These difficulties may be related to their low YFBM or a poorly developed pipping muscle (Delphia and Elliott, 1965; Piestun et al., 2009a). On the other hand, the lower survival rate might be related to impaired nutrient availability or utilization before or during the hatching process (Bjønnes et al., 1987; Decuypere and Michels, 1992; Christensen et al., 1999). No interactions between EST and O_2 concentration were found for hepatic glycogen or blood metabolites in the current study, but these factors were analyzed in embryos that did survive. High EST decreased the amount of hepatic glycogen and when this was combined with a low O_2 concentration from day 7 until 19 of incubation, this may have been fatal for some embryos.

Age

Although the negative effect of delayed food and water excess in broiler chickens on subsequent performance are investigated (Noy and Sklan, 1999; Batal and Parsons, 2002; Juul-Madsenet al., 2004; Henderson et al., 2008), the developmental and physiological changes during this early posthatch period are not intensively investigated. In the current study, all treated animals were deprived of food and water and exposed to the same environmental temperature and O_2 concentration from 12 to 48 hours after emergence from the eggshell. Such periods of feed and water deprivation are common in practice (Dibner et al., 1998; Henderson et al., 2008). Relative organ weights increased during the perinatal period, indicating that the birds developed and matured in this period (Sotherland and Rahn, 1987; Zhang and Whittow, 1992). Hepatic glycogen and plasma glucose concentrations decreased, and plasma uric acid concentrations increased in the perinatal period. These results are consistent with another study (Hazelwood and Lorenz, 1959) investigating the effects of fasting on carbohydrate metabolism in layer chickens 5 to 11 weeks old. Carbohydrate metabolism is particularly influenced by fasting (Miova et al., 2008); hepatic glycogen is converted to glucose (Miova et al., 2008) and used as a metabolic fuel. The higher uric acid concentration in the hatchlings at 48 hours after emergence may suggest that additional glucose was made by gluconeogenesis of glucogenic amino acids (Hazelwood and Lorenz, 1959) or that the amino acid skeleton was used for immediate ATP production (McArdle et al., 1981).

In conclusion, EST and O_2 concentrations during incubation differentially influence the developmental and physiological status of broilers in the perinatal period. The high EST and low O_2 concentration produced the highest embryo mortality in the third week of incubation, which may be related to the low YFBM of these hatchlings. High EST compared with normal EST decreased development at hatch, potentially due to the shorter incubation duration, a lack of glucose precursors, or the lower weight of the supply organs (i.e., heart and lung) at the end of incubation. Embryonic development at day 18 of incubation was proportional to O_2 concentration exposure ranging from 17% to 25%. Differences between the high and normal O_2 concentration were smaller than those between the low and normal O_2 concentration after hatch. Embryos and hatchlings may have had difficulties to adapt to lower O_2 concentrations after day 19 of incubation. The physiological status at hatching seems to be related to the environment that the embryo experienced at the end of incubation.

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Table 1. Hatchability of fertile eggs, embryo mortality, and incubation duration of embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

Treatment	Hatch of fertile (%[1])	Embryo mortality		Incubation duration (hours)
		Second week (%[1])	Third week (%[1])	
EST (°C)				
37.8	96.8 ± 1.2	1.3 ± 0.6	1.9 ± 0.7	487[a] ± 0.8
38.9	86.9 ± 4.7	1.7 ± 1.0	11.6 ± 4.2	479[b] ± 0.5
O_2 (%)				
17	84.6 ± 7.1	2.0 ± 1.3	13.5 ± 6.5	483 ± 2.2
21	97.5 ± 1.8	1.1 ± 0.8	1.7 ± 1.1	482 ± 2.7
25	93.6 ± 0.7	1.4 ± 0.8	5.0 ± 1.1	483 ± 2.9
EST (°C) × O_2 (%)				
37.8 × 17	96.7[a] ± 1.1	1.1 ± 1.1	2.2[b] ± 0.0	486 ± 2.9
37.8 × 21	100.0[a] ± 0.0	0.0 ± 0.0	0.0[b] ± 0.0	487 ± 0.4
37.8 × 25	93.9[a] ± 1.7	2.8 ± 0.6	3.3[b] ± 1.1	488 ± 0.7
38.9 × 17	72.4[b] ± 3.1	2.8 ± 2.8	24.7[a] ± 0.3	480 ± 0.7
38.9 × 21	95.0[a] ± 2.7	2.2 ± 1.1	3.3[b] ± 1.1	478 ± 0.3
38.9 × 25	93.3[a] ± 0.0	0.0 ± 0.0	6.7[b] ± 0.0	478 ± 0.8
Source of variation				
EST	0.003	0.89	<0.001	0.04
O_2	0.04	0.87	0.01	0.90
EST × O_2	0.04	0.12	0.03	0.67

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Expressed as a percentage of fertile eggs and the data is first transformed to arcsine before analysis.

Table 2. Yolk-free body mass (YFBM) and relative organ weights of embryos and hatchlings incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	YFBM (g)	Heart (% of YFBM)	Lung (% of YFBM)	Stomach (% of YFBM)	Liver (% of YFBM)	Spleen (% of YFBM)	Intestines (% of YFBM)	Bursa of Fabricius (% of YFBM)
EST (°C)								
37.8	35.3 ± 0.9	0.71 ± 0.03	0.95[a] ± 0.03	6.45 ± 0.44	2.57 ± 0.12	0.04 ± 0.00	4.08 ± 0.50	0.10[a] ± 0.01
38.9	34.1 ± 0.8	0.59 ± 0.02	0.92[b] ± 0.04	6.43 ± 0.39	2.48 ± 0.11	0.04 ± 0.00	3.98 ± 0.47	0.08[b] ± 0.01
O_2 (%)								
17	33.2 ± 1.1	0.65 ± 0.04	0.91 ± 0.04	6.52 ± 0.53	2.56 ± 0.17	0.04 ± 0.00	4.02 ± 0.62	0.09 ± 0.01
21	34.9 ± 1.0	0.64 ± 0.04	0.95 ± 0.05	6.43 ± 0.52	2.51 ± 0.14	0.04 ± 0.00	4.04 ± 0.60	0.09 ± 0.01
25	36.0 ± 0.8	0.66 ± 0.03	0.93 ± 0.05	6.37 ± 0.49	2.50 ± 0.13	0.04 ± 0.00	4.03 ± 0.59	0.09 ± 0.01
Age (d[1] or h[2])								
18[1]	30.3 ± 0.5	0.51 ± 0.02	0.75[c] ± 0.02	4.39 ± 0.09	1.94 ± 0.02	0.03[b] ± 0.00	1.37 ± 0.05	0.07[c] ± 0.00
12[2]	37.1 ± 0.4	0.69 ± 0.02	0.95[b] ± 0.02	6.41 ± 0.07	2.54 ± 0.03	0.04[ab] ± 0.00	4.54 ± 0.05	0.09[b] ± 0.00
48[2]	36.7 ± 0.4	0.75 ± 0.02	1.10[a] ± 0.01	8.53 ± 0.08	3.10 ± 0.04	0.04[a] ± 0.00	6.19 ± 0.06	0.11[a] ± 0.00
Source of variation								
EST	0.003	<0.001	0.04	0.81	0.02	0.63	0.15	0.02
O_2	<0.001	0.37	0.25	0.004	0.02	0.76	0.50	0.78
Age	<0.001	<0.001	<0.001	<0.001	<0.001	0.03	<0.001	<0.001
EST × O_2	0.003	0.48	0.77	0.05	0.77	0.09	0.01	0.63
EST × Age	<0.001	0.02	0.65	<0.001	0.07	0.96	0.01	0.90
O_2 × Age	<0.001	0.09	0.21	0.28	0.001	0.32	0.27	0.80
EST × O_2 × Age	0.24	0.21	0.50	0.27	0.02	0.22	0.07	0.64

[a-c]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Day 18 of incubation.

[2]12 or 48 hours after emergence from the eggshell.

Table 3. Plasma glucose, lactate and uric acid concentrations, and total hepatic glycogen levels for embryos and hatchlings incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	Glucose (mg/dL)	Lactate (mg/dL)	Uric acid (mg/dL)	Total hepatic glycogen (mg)
EST (°C)				
37.8	211.4 ± 1.7	29.3 ± 1.1	3.2 ± 0.2	10.0 ± 1.7
38.9	210.0 ± 2.1	28.0 ± 0.8	3.7 ± 0.2	7.9 ± 1.1
O_2 (%)				
17	208.6 ± 2.1	26.0 ± 0.8	3.6 ± 0.2	8.5 ± 1.6
21	209.8 ± 1.8	30.7 ± 1.2	3.4 ± 0.2	9.2 ± 1.8
25	213.8 ± 2.8	29.1 ± 1.0	3.5 ± 0.3	9.2 ± 2.0
Age (d[1] or h[2])				
18[1]				15.0 ± 0.9
12[2]	213.9[a] ± 1.6	28.2 ± 0.9	3.0[b] ± 0.2	10.4 ± 0.5
48[2]	207.5[b] ± 1.8	29.0 ± 1.1	3.9[a] ± 0.1	1.5 ± 0.2
Source of variation[3]				
EST	0.69	0.46	0.06	0.01
O_2	0.06	0.06	0.79	0.45
Age	0.01	0.45	<0.001	<0.001
EST × Age	0.08	0.12	0.21	<0.001

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Day 18 of incubation.

[2]12 or 48 hours after emergence from the eggshell.

[3]No two-way interactions between EST × O_2 or O_2 × Age or three-way interactions between EST × O_2 × Age were found ($P > 0.10$).

Table 4. Two-way interaction between eggshell temperature (EST) and age for yolk-free body mass (YFBM), relative stomach, heart and intestinal weight, and total hepatic glycogen of embryos and hatchlings incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	EST (°C) × Age (d[1] or h[2])					
	37.8 × 18[1]	37.8 × 12[2]	37.8 × 48[2]	38.9 × 18[1]	38.9 × 12[2]	38.9 × 48[2]
YFBM (g)	$30.4^{d} \pm 0.6$	$37.8^{a} \pm 0.5$	$37.6^{a} \pm 0.2$	$30.1^{d} \pm 0.9$	$36.5^{b} \pm 0.5$	$35.7^{c} \pm 0.5$
Heart (% of YFBM)	$0.56^{d} \pm 0.02$	$0.77^{a} \pm 0.01$	$0.81^{a} \pm 0.01$	$0.46^{e} \pm 0.01$	$0.61^{c} \pm 0.01$	$0.70^{b} \pm 0.01$
Stomach (% of YFBM)	$4.19^{e} \pm 0.11$	$6.60^{b} \pm 0.04$	$8.57^{a} \pm 0.13$	$4.59^{d} \pm 0.09$	$6.21^{c} \pm 0.05$	$8.48^{a} \pm 0.09$
Intestines (% of YFBM)	$1.32^{e} \pm 0.08$	$4.67^{b} \pm 0.02$	$6.26^{a} \pm 0.05$	$1.42^{d} \pm 0.07$	$4.42^{c} \pm 0.05$	$6.11^{a} \pm 0.10$
Total hepatic glycogen (mg)	$17.7^{a} \pm 0.8$	$11.4^{bc} \pm 0.6$	$0.9^{d} \pm 0.2$	$12.4^{b} \pm 0.6$	$9.3^{c} \pm 0.7$	$2.1^{d} \pm 0.2$

[a-e]Means followed by different superscripts within a row are significantly different ($P \leq 0.05$).

[1]Day 18 of incubation.

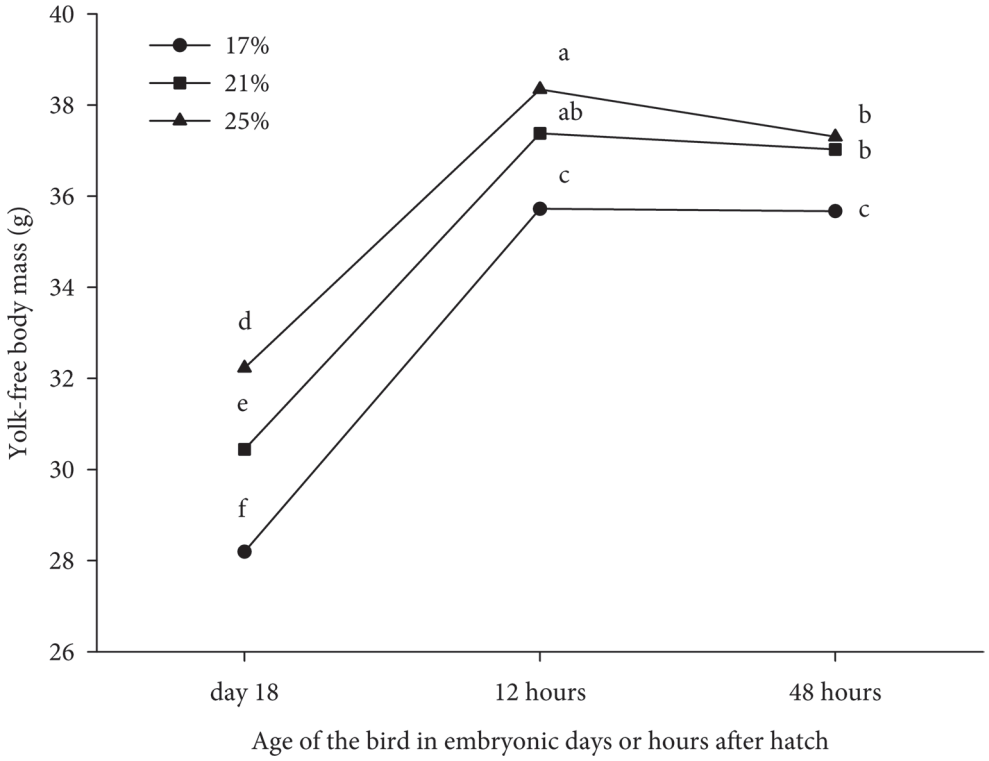
[2]12 or 48 hours after emergence from the eggshell.

Table 5. Two-way interaction between eggshell temperature (EST) and O_2 concentration for yolk-free body mass (YFBM) as well as relative stomach and intestinal weight of embryos incubated at two eggshell temperatures (EST; 37.8°C or 38.9°C) and three O_2 concentrations (17%, 21%, or 25%) from day 7 to 19 of incubation.

	EST (°C) × O_2 (%)					
	37.8 × 17	37.8 × 21	37.8 × 25	38.9 × 17	38.9 × 21	38.9 × 25
YFBM (g)	$34.2^{b} \pm 1.7$	$35.3^{a} \pm 1.6$	$36.3^{a} \pm 1.4$	$32.2^{c} \pm 1.5$	$34.6^{b} \pm 1.3$	$35.6^{a} \pm 1.0$
Stomach (% of YFBM)	$6.65^{a} \pm 0.84$	$6.36^{ab} \pm 0.83$	$6.35^{b} \pm 0.74$	$6.39^{ab} \pm 0.72$	$6.51^{ab} \pm 0.71$	$6.38^{b} \pm 0.72$
Intestines (% of YFBM)	$4.01^{ab} \pm 0.93$	$4.06^{ab} \pm 0.93$	$4.19^{a} \pm 0.90$	$4.04^{ab} \pm 0.91$	$4.03^{ab} \pm 0.85$	$3.87^{b} \pm 0.85$

[a-c]Means followed by different superscripts within a row are significantly different ($P \leq 0.05$).

Figure 1. Yolk-free body mass (YFBM) of embryos and hatchlings incubated at different O_2 concentrations from day 7 to 19 of incubation.



[a-f]Different letters indicate a significant difference ($P \leq 0.05$).

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Effect of Temperature on Glucose Metabolism in the Developing Chicken Embryo

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Abstract

This study investigated the effect of high eggshell temperature (EST) on glucose metabolism using [U-^{13}C]glucose and measuring ^{13}C enrichment in intermediate pools and end-products of glucose metabolism in chicken embryos. Embryos were incubated at a high (38.9°C) or normal (37.8°C) EST from day 10.5 of incubation and injected with a bolus of [U-^{13}C]glucose at day 17.5 of incubation in the chorio-allantoic fluid. ^{13}C enrichment in expired CO_2 was measured from day 17.5 until 21.6 of incubation. Embryo and chick characteristics, ^{13}C enrichment in plasma glucose and lactate, and ^{13}C in hepatic glycogen were measured at eight time points between day 17.5 and 21.6 of incubation. The high EST treatment resulted in a higher ^{13}C enrichment in expired CO_2 from day 17.6 until 17.8 of incubation, a tendency for a higher total recovery of ^{13}C enrichment in expired CO_2, a higher ^{13}C enrichment in plasma lactate at day 17.8 of incubation, and a lower ^{13}C in hepatic glycogen at day 18.8 of incubation after the [U-^{13}C]glucose injection compared with the normal EST treatment. These results indicated that glucose oxidation was higher at high EST and may explain the lower hepatic glycogen concentrations that were found at day 18.2 and 18.8 of incubation in the high EST treatment. Plasma uric acid concentrations increased in the high EST compared with the normal EST by 43% at day 21.6 of incubation. This result may indicate that when glycogen stores become limited at the end of incubation at high EST, amino acids are used for gluconeogenesis or immediately for ATP production, which may explain the lower body development at hatch that was found with high EST incubation. In conclusion, embryos incubated at high EST compared with normal EST have a higher glucose oxidation at the end of incubation. When glycogen stores become limited during incubation at high EST because of the higher glucose oxidation, embryos may use amino acids for gluconeogenesis or immediately for ATP production, which impairs development at hatch.

Key words: chicken embryo, eggshell temperature, glucose metabolism, tracer study

Introduction

In practice, eggshell temperature (EST) often increases at the end of incubation due to the increased heat production of the developing embryo and a lack of cooling capacity or air velocity in the incubator (French, 1997; Hulet, 2007; Elibol and Brake, 2008). Different studies have shown that a high EST (~38.9°C) in the second half of incubation decreases hatchability, body development, and subsequent performance compared with a normal EST (~37.8°C) during incubation (Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007, 2009). It is largely unknown what mechanisms may explain the decrease in survival and body development and the decrease may be related to changes in nutrient utilization during incubation (Christensen et al., 1993).

Fatty acids are the predominant energy source during incubation, but glucose becomes an indispensable substrate for ATP production at the end of incubation when the hatching process occurs (Freeman, 1965; De Oliveira et al., 2008). The hatching process is energy-demanding and occurs during a period when the supply of O_2 is restricted by the gas exchange across the eggshell and chorio-allantoic membranes (Tazawa et al., 1983; Rahn et al., 1979). Because muscle activity is high and O_2 availability is low during the hatching process, glucose is used in the muscles for anaerobic glycolysis and this results in an increased plasma lactate concentration (Freeman, 1965; John et al., 1987, 1988; Moran, 2007; De Oliveira et al., 2008). Furthermore, plasma glucose concentrations increase at the end of incubation probably to ensure normal activity of the central nervous system (Freeman, 1969). The large use of glucose at the end of incubation indicates that glucose is required for successful hatching, but glucose is hardly available in the egg at the start of incubation (<1%; Romanoff and Romanoff, 1949). Therefore, glycogen stores are built up during incubation in the heart, liver, muscle, and yolk sac membrane (Beattie, 1964; García et al., 1986; Foye et al., 2007). Several precursors such as amino acids, glycerol, or lactate may be used for this gluconeogenesis (Evans and Scholz, 1973; Watford et al., 1981).

Hepatic glycogen is to a great extent mobilized when the embryo starts to emerge from the eggshell (Freeman, 1965, 1969; García et al., 1986; Foye et al., 2007). Molenaar et al. (2009b) showed that hepatic glycogen stores at day 18 of incubation, just before the hatching process started, were lower in embryos incubated at high EST (38.9°C) compared with normal EST (37.8°C) from day 7 of incubation onward. This reduction in energy stores at high EST that are required for completing the hatching process (Christensen et al., 1993; Molenaar et al., 2009b) may increase embryo mortality in the last week of incubation (Lourens et al., 2005). The lower hepatic glycogen levels further suggest that hepatic glycogen synthesis was lower or that glucose oxidation was higher at high EST compared with normal EST. Exercising humans exhibit increased carbohydrate oxidation at high environmental temperatures (Febbraio, 2001; Mündel, 2008). To test the hypothesis that glucose oxidation is also increased at high EST in chicken embryos, the effect of EST on glucose metabolism was investigated using a labeled [U-^{13}C]glucose tracer. Embryos incubated at a normal or high EST were injected with [U-^{13}C]glucose in the chorio-allantoic fluid at day 17.5 of incubation and ^{13}C enrichments in expired CO_2, plasma glucose and lactate, and hepatic glycogen concentrations were evaluated. In addition, plasma metabolites and hepatic glycogen

concentrations were evaluated to investigate possible changes in the nutritional status of chicken embryos during high EST incubation.

Materials and Methods

Design and Measurements Study I and II

Two small-scale studies were conducted to develop the technique to measure oxidation of [U-^{13}C]glucose that was injected into the chorio-allantoic fluid of chicken embryos. In addition, a contrast in EST was created in each of the two studies to provide general insight into the effects of EST on glucose oxidation in chicken embryos in the last week of incubation. For both studies, 150 fertile broiler eggs at day 13 of incubation were obtained from a Ross flock of 59 weeks of age (Morren B.V., Lunteren, the Netherlands). Eggs were randomly divided between two identical open-circuit climate respiration chambers (CRC; Lourens et al., 2006) and incubated at a normal (37.8°C; n = 75) or a high (38.9°C; n = 75) EST. The normal and high EST values were based on studies by Lourens et al. (2005, 2007) because these studies have shown that an EST of 38.9°C negatively influenced hatchability and body development. Eggshell temperature was regulated in the CRC as described by Lourens et al. (2006, 2007) and relative humidity was maintained at 55%. In study I, a solution containing [U-^{13}C]glucose (99 atom% 13C, Sigma-aldrich Chemie B.V., Zwijndrecht, the Netherlands; 1.0 mg in 250 µL of sterile water) was injected as a single bolus into the chorio-allantoic fluid in each of the 150 eggs at day 14.5 of incubation. In study II, a solution containing [U-^{13}C]glucose (Sigma-aldrich Chemie B.V.; 0.73 mg in 250 µL of sterile water) was repeatedly injected as a bolus into the chorio-allantoic fluid in each of the 150 eggs for four consecutive days from day 14.5 of incubation onward.

For *in ovo* [U-^{13}C]glucose injection, the air cell was located by candling the egg. The blunt end of each egg was sterilized with 70% ethanol. A 20-gauge needle was punctured through the eggshell at 2 to 3 mm above the chorio-allantoic membrane. A 25-gauge needle was inserted through the hole into the chorio-allantoic fluid to inject the [U-^{13}C]glucose solution. From day 13 until 18 of incubation, $^{12}CO_2$ and $^{13}CO_2$ production were measured every 6 minutes in both studies (Alferink et al., 2003). ^{13}C enrichment in expired CO_2 was expressed as a percentage of total CO_2 production (atom% ^{13}C). This ^{13}C enrichment was corrected for the natural background of ^{13}C in expired CO_2, which was measured during 1.5 days prior to the [U-^{13}C]glucose injection and was expressed as atom percentage excess (APE).

Design Study III

The main study was designed to investigate effects of EST on glucose metabolism in chicken embryos during the perinatal period. A normal (37.8°C) or high (38.9°C) EST treatment was applied from day 10.5 until 21.6 of incubation in four replicates of 160 eggs each. Fertile broiler eggs at day 10.5 of incubation were obtained from a commercial Ross breeder flock between 42 and 44 weeks of age (Lagerwey B.V., the Netherlands). On day 10.5 of incubation, eggs weighing between 60 and 64 g were selected. Per replicate, eggs were randomly distributed between two

identical open-circuit CRC and incubated at a normal (37.8°C; n = 80) or a high (38.9°C; n = 80) EST as described for the first two studies.

At day 17.5 of incubation, all eggs were injected with a single bolus of [U-^{13}C]glucose (1.0 mg in 250 µL of sterile water) into the chorio-allantoic fluid with the same technique as described in the first two studies. After the injection, eggs were transferred from the egg tray to a hatching basket (560 × 360 mm) in the first three replicates and to individual hatching baskets (120 × 135 mm) for the fourth replicate. The hatching baskets were placed in the same CRC again and eggs were exposed to the same EST treatment as in the setter; this EST was maintained in the CRC until day 18.5 of incubation. At day 18.5 of incubation, the environmental temperature in the CRC was fixed at the last EST setpoint, and the EST was allowed to increase during the remainder of the hatching process. Eggs in the CRC in the first three replicates were recorded by a camera to determine individual hatch times. The study was approved by the Institutional Animal Care and Use Committee of Wageningen University.

Measurements Study III

In three replicates, ^{13}C enrichment in expired CO_2 was determined after injection of a single bolus of [U-^{13}C]glucose (99 atom% 13C, Sigma-aldrich Chemie B.V., Zwijndrecht, the Netherlands; 1.0 mg in 250 µL of sterile water) into the chorio-allantoic fluid at day 17.5 of incubation. In the fourth replicate, chick quality characteristics, blood metabolites, and ^{13}C enrichment in plasma glucose and hepatic glycogen were determined at eight time points between day 17.5 and 21.6 of incubation.

In the first three replicates, $^{12}CO_2$ and $^{13}CO_2$ production in the CRC were measured at 6 minute intervals (Alferink et al., 2003) from day 10.5 until 21.6 of incubation. The ^{13}C enrichment in excess of the natural background of ^{13}C enrichment in CO_2 was calculated from these measurements, as described for the first two studies. The background enrichment was determined for each EST and replicate by calculating the mean ^{13}C enrichment from day 10.5 until 17.5 of incubation. ^{13}C enrichment in expired CO_2 was calculated per hour but expressed in days of incubation. All chickens were euthanized with CO_2 at day 21.6 of incubation.

In replicate four, ten animals per EST were randomly chosen at eight different time points during incubation to determine chick development, blood metabolites and ^{13}C enrichment in plasma glucose and lactate, and hepatic glycogen. The time points were selected to cover the peak in $^{13}CO_2$ expiration, estimated from the first three replicates, and were at day 17.5, 17.8, 18.2, 18.8, 19.7, 20.4, 20.9, and 21.6 of incubation. Embryos or hatchlings were weighed before blood collection. Blood samples were taken from the jugular vein in embryos and after decapitation in hatchlings. All blood samples were collected in a 4-mL blood tube containing 10 mg of sodium fluoride and 8 mg of potassium oxalate (BD Vacutainer, Franklin Lakes, NJ). An extra droplet (0.02 mL) of 10% heparin was added and mixed into the blood tube before sampling. Blood was centrifuged (2,900 × *g*) for 15 minutes and plasma was decanted and stored at -20°C until further analysis. Plasma glucose, lactate, and uric acid concentrations were determined with commercial kits (DiaSys Diagnostic Systems International, Holzheim, Germany). After bleeding, the liver of

embryos and hatchlings was immediately dissected, weighed and frozen in liquid nitrogen. Livers were stored at -80°C until further analysis. The yolk was removed and weighed. Yolk-free body mass (YFBM) was calculated as body weight minus yolk weight.

To determine ^{13}C enrichment in plasma glucose, 1 mL ice-cold ethanol (96%) was added to 100 µL of plasma and this mixture was stored overnight at -20°C before centrifugation (15,339 × *g*) at 4°C for 20 minutes. After evaporating the supernatant to dryness, 75 µL acetic acid anhydride-pyridine (10:5 vol/vol) was added for 1.5 hours at room temperature to convert glucose to its penta-acetate derivate. The reagent was evaporated to dryness again and dissolved in 500 µL chloroform. The $^{13}C/^{12}C$ ratio was then determined by gas chromatography-combustion isotope ratio mass spectrometry (GC-C-IRMS) with a Delta S/GC instrument (Finnigan MAT, Bremen, Germany). The measured ^{13}C enrichment was corrected for the dilution of C atoms during the derivatization.

^{13}C enrichment in plasma lactate was determined by GC-C-IRMS analysis (Delta V Plus; Thermo Scientific, Bremen, Germany) of the 2-O-ethoxycarbonyl ethyl ester of lactic acid. Atomic composition of lactic acid was obtained by correction for carbon added in derivatization (n = 5) using a mass balance equation as previously described (Tetens et al., 1995).

To determine hepatic glycogen enrichment, the whole liver was homogenized with a glass-stirring spoon after the addition of 1 µL of 7% $HCLO_4$/g of wet tissue. The suspension was centrifuged (2,900 × *g*) at 4°C for 15 minutes. The supernatant was decanted and cleaned with 1 mL of petroleumether. To extract hepatic glycogen, 2.5 mL ice-cold ethanol (96%) was added to 400 µL supernatant for 120 minutes at -20°C. Then, the solution was centrifuged (2,900 × *g*) at 0°C for 15 minutes, the fluid was removed and a glycogen pellet was left on the bottom of the tube. The glycogen was dissolved in 1.025 mL water, and the glucose units were released by incubating the solution with a mixture of 25 µL amyloglucosidase (3200 U/mL; Omnilabo, Breda, the Netherlands), 0.875 mL distilled water and 125 µL 2M acetate buffer (2M acetic acid:2M acetate, 4:6 vol/vol; pH = 4.8) at 60°C for 1 hour. The reaction was finished by placing the tubes on ice, and 100 µL of the mixture was used to determine the $^{13}C/^{12}C$ ratio in glucose by GC-C-IRMS using the procedure described for plasma glucose enrichment. The measured ^{13}C enrichment was corrected for the dilution of C atoms during the derivatization.

The remaining mixture (1.905 mL) was used to determine the hepatic glycogen concentration. Two hundred µL of Carrez I (106 g potassium hexacyanoferrate (II) trihydrate dissolved in 1 L water) plus 200 Carrez II (219.5 g zinc acetate dihydrate and 30 g acetic acid dissolved in 1 L water) solution was added to precipitate proteins. After centrifugation (15,339 × *g*) at 4°C for 10 minutes, the supernatant was used to determine glucose concentration with a commercial kit (DiaSys Diagnostic Systems International, Holzheim, Germany).

The ^{13}C enrichment in plasma glucose and lactate, and hepatic glycogen was corrected for the natural background that was determined in embryos sampled at day 17.5 of incubation that did not receive [U-^{13}C]glucose, and was expressed as APE. The total amount of hepatic glycogen was calculated in mmol from the hepatic glycogen concentrations and liver weights and multiplied by the ^{13}C enrichment in hepatic glycogen to obtain ^{13}C in hepatic glycogen in mmol.

Calculation Recovery Study III

Recovery of ^{13}C in expired CO_2 was calculated by expressing $^{13}CO_2$ production in excess of the background relative to the dose injected at day 17.5 of incubation. The dose was corrected for the embryos that died during incubation and this correction was proportional to the excretion curve of ^{13}C in expired CO_2.

Statistical Analysis

Data from study I and II were not statistically analyzed because these studies were designed to develop the technique to measure [U-^{13}C]glucose oxidation and to provide an indication of the interaction with EST. Repeated measurements on the same treatment combination were therefore lacking. In the first three replicates of study III, ^{13}C enrichment in expired CO_2 was calculated per hour but expressed in days of incubation and analyzed using the MIXED procedure for repeated measurements in SAS (Version 9.1, SAS Institute 2004) with EST treatment, day of incubation, and the interaction as class variables. Replicate was the repeated factor, and an auto-regressive covariance structure was used. Results of YFBM, yolk weight, plasma glucose, lactate and uric acid concentrations, hepatic glycogen concentration, ^{13}C enrichment in plasma glucose and lactate, and ^{13}C in hepatic glycogen were analyzed using a GLM procedure with EST treatment, day of incubation, and their interaction as class variables. Recovery of ^{13}C in expired air was analyzed using a GLM procedure with EST treatment as a class variable and replicate as a block. CRC was the experimental unit in replicates one to three and embryo or hatchling was the experimental unit in replicate four. Distributions of the means and residuals were examined to check model assumptions. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as least squares means ± SEM. In all cases, a significant difference was considered at $P \leq 0.05$.

Results

Study I and II: ^{13}C enrichment in Expired CO_2

In study I, a single bolus of [U-^{13}C]glucose was injected at day 14.5 of incubation and ^{13}C enrichment in expired CO_2 showed a peak in both EST treatments within 8 hours after the injection (Figure 1A). Eggs at the normal EST were injected on average 1.5 hours earlier than eggs at the high EST. The peak in ^{13}C enrichment was 13% higher at the high EST treatment compared with the normal EST treatment (0.05630 APE vs. 0.04906 APE, respectively). From day 14.8 of incubation onward, ^{13}C enrichment decreased in both EST treatments but did not return to the baseline at day 17.6 of incubation (APE ~0.00590). In study II, a bolus of [U-^{13}C]glucose was injected on four consecutive days from day 14.5 of incubation onward; this resulted in a peak of ^{13}C enrichment in expired CO_2 after the injection, similar to the result found in study I (Figure 1B). However, with each subsequent injection the ^{13}C enrichment peak in expired CO_2 started higher and declined faster. The ^{13}C enrichment in expired CO_2 was numerically higher by 0.003542 APE (20%) in the normal compared with the high EST treatment approximately one day after the first

injection at day 14.5 of incubation. The ^{13}C enrichment in expired CO_2 was numerically higher by 0.01819 APE (49%) in the high EST compared with the normal EST treatment approximately one day after the fourth injection at day 17.5 of incubation.

Study III: Embryo and Chick Characteristics (Replicate 4)

An interaction between EST and day of incubation was found for YFBM ($P < 0.001$; Figure 2A). Eggshell temperature did not affect YFBM from day 17.5 until 20.4 of incubation, but YFBM was lower ($P < 0.05$) at the high EST than at the normal EST treatment at day 20.9 ($\Delta = 2.74$ g) and at day 21.6 ($\Delta = 3.81$ g) of incubation. Yolk weight decreased with time ($P < 0.001$), and no difference between EST treatments was found ($P = 0.20$; Figure 2B).

Study III: Blood Metabolites (Replicate 4)

Plasma glucose ($P = 0.68$; Figure 3A) and lactate ($P = 0.53$; Figure 3B) concentrations were not affected by EST, but both increased with time ($P < 0.001$). An interaction between EST and the day of incubation was found for plasma uric acid concentrations ($P = 0.009$; Figure 3C). Hatchlings incubated at high EST had a higher plasma uric acid concentration (2.8 mg/dL, +43%) at day 21.6 of incubation than those incubated at normal EST. Within EST, plasma uric acid concentrations were highest in the high EST treatment at day 21.6 of incubation (6.50 mg/dL) and in the normal EST treatment at day 18.8 of incubation (4.75 mg/dL). On average, hepatic glycogen concentrations decreased from 11.16 to 1.50 mg/g between day 17.5 and 21.6 of incubation in both EST treatments. An interaction between EST and day of incubation was found for hepatic glycogen concentrations ($P = 0.02$; Figure 3D). Hepatic glycogen concentration was lower in the high EST compared with the normal EST treatment at day 18.2 of incubation ($\Delta = 4.37$ mg/g) and at day 18.8 of incubation ($\Delta = 4.59$ mg/g) ($P < 0.05$).

Study III: ^{13}C enrichment in Expired CO_2 (Replicate 1 to 3)

Eggs in the normal EST treatment were injected first and this occurred approximately 1.5 hours earlier than for eggs in the high EST treatment. In both treatments, the ^{13}C enrichment in expired CO_2 showed two peaks after the injection (Figure 4A). The first peak was within 3 hours after the injection and this peak was approximately 36% higher in the high EST than in the normal EST treatment ($\Delta = 0.02512$; $P < 0.05$). The ^{13}C enrichment in expired CO_2 was higher in the high EST than in the normal EST from day 17.6 until 17.8 of incubation ($P < 0.05$). In the high EST treatment, the second peak in ^{13}C enrichment in expired CO_2 was lower than the first peak ($P < 0.05$), whereas the height of both peaks in the normal EST treatment was similar ($P > 0.05$). The heights of the second peaks did not differ between EST treatments ($P > 0.05$). The second peak in ^{13}C enrichment in expired CO_2 occurred 7 hours earlier in the high EST treatment (day 18.5 of incubation) than in the normal EST treatment (day 18.8 of incubation). The ^{13}C enrichment in expired CO_2 decreased with time after the second peak and returned to baseline at day 19.5 of incubation for the high EST treatment and at day 19.8 of incubation for the normal EST treatment ($P > 0.05$). Recovery of ^{13}C in expired CO_2 relative

to the dose of ^{13}C that was injected as glucose in the eggs tended to be higher ($P = 0.07$) in the high (46.7 ± 0.96%) than in the normal (42.0 ± 0.96%) EST treatment.

Study III: ^{13}C enrichment in Plasma Glucose and Lactate, and ^{13}C in Hepatic Glycogen (Replicate 4)

^{13}C enrichment in plasma glucose (Figure 4B) was not affected ($P = 0.35$) by EST and showed a similar pattern as the ^{13}C enrichment in expired CO_2 with the highest peak at day 17.8 of incubation. At day 18.8 of incubation, ^{13}C enrichment in expired CO_2 showed a second peak, but ^{13}C enrichment in plasma glucose did not differ ($P > 0.05$) from the background. An interaction was found between EST treatment and day of incubation for ^{13}C enrichment in plasma lactate ($P < 0.001$; Figure 4C). At day 17.8 of incubation, ^{13}C enrichment in plasma lactate was 82% higher in the high EST than in the normal EST treatment. For both treatments, the ^{13}C enrichment in plasma lactate was higher than the background (day 17.5 of incubation) until day 18.8 of incubation. An interaction ($P = 0.03$) was found between EST treatment and day of incubation for ^{13}C in hepatic glycogen (Figure 4D). At day 18.8 of incubation, ^{13}C in hepatic glycogen was lower ($P < 0.05$) in the high EST than in the normal EST treatment. Within EST treatments, ^{13}C in hepatic glycogen showed a peak at day 17.8 and 18.2 of incubation in the high EST treatment and at day 18.8 of incubation in the normal EST treatment.

Discussion

Study I and II

In the first two studies, [U-^{13}C]glucose was injected into the chorio-allantoic fluid and $^{13}CO_2$ production was measured for estimating oxidation rates of [U-^{13}C]glucose. The tracer was assumed to be absorbed by the embryo through the chorio-allantoic capillary network (Sunny and Bequette, 2010). The chorio-allantoic sac is a respiratory organ and was first thought to mainly collect waste products from the kidneys (Romanoff, 1960). However, Ten Busch et al. (1997) and Epple et al. (1997) showed that the allantois is a highly regulated depot for metabolites and hormones and that both are secreted in and absorbed from the chorio-allantoic sac during incubation.

The small amounts of [U-^{13}C]glucose that were injected in the current study were assumed to have no effect on embryonic metabolism or nutrient fluxes (Sunny and Bequette, 2010). The total amount of exogenous glucose was 1.0 mg (in one bolus) in study I and III, and 0.73 mg (in each of the four boluses) in study II, which accounts for respectively 0.24 and 0.18% of the average glucose pool (~415 mg) in a 60-g egg from day 14 to 17 of incubation (Romanoff, 1967). The period during which $^{13}CO_2$ was expired in excess of the background values was approximately 3 days in the current study, which is much longer than is commonly found in studies with humans, calves, goats, mice, and rats (<20 hours; Junghans et al., 1997; Christian et al., 2002; Ishihara et al., 2002; Robertson et al., 2002; Van den Borne et al., 2007; Luengo et al., 2009). This is likely due to the higher glucose entry and greater heat production of these species that is driven by food intake.

In study II, the height of the peaks in ^{13}C enrichment in expired CO_2 increased with each consecutive injection. This suggests that glucose oxidation increased during embryonic development and supports findings that glycolytic activity in muscle and liver increase during incubation (Pearce, 1977). Gluconeogenesis and glycogenesis have also been found to increase during the second half of incubation (Kilsheimer et al., 1960; Rinaudo, 1962; Ballard and Oliver, 1963; Nelson et al., 1966), indicating that total carbohydrate metabolism is upregulated. In addition, study II indicated that differences in glucose oxidation between EST treatments became more pronounced over time; with a higher ^{13}C enrichment in expired CO_2 in the high EST compared with the normal EST treatment. The larger difference in ^{13}C enrichment in expired CO_2 between EST treatments after the [U-^{13}C]glucose bolus was just before the period that the energy-demanding hatching process started (~day 19 of incubation) and when glucose metabolism has an important role as an energy substrate (Pearce, 1971; Wittman and Weiss, 1981; De Oliveira et al., 2008). Therefore, we decided to investigate the effect of EST on glucose metabolism at the end of incubation in study III. Eggshell temperature treatments were applied from day 10.5 of incubation onward and [U-^{13}C] glucose was injected once at day 17.5 of incubation to evaluate differences between EST treatments over time.

Study III

Results of the current study showed that a high EST (38.9°C) decreased body development, which was indicated by the reduced YFBM at hatch. This is consistent with other studies (Romanoff, 1936; Lourens et al., 2005, 2007; Leksrisompong et al., 2007; Molenaar et al., 2010). Our hypothesis was that glucose oxidation would increase at the high EST compared with the normal EST treatment. The high EST treatment had a higher ^{13}C enrichment in expired CO_2 from day 17.6 to 17.8 of incubation, and ^{13}C enrichment in plasma lactate was substantially higher (82%) at day 17.8 of incubation, compared with the normal EST treatment. Furthermore, total recovery of ^{13}C enrichment in expired CO_2 tended to be 4.7% higher in the high EST than in the normal EST treatment, whereas ^{13}C enrichment in hepatic glycogen was lower in the high EST than in the normal EST treatment at day 18.8 of incubation. These results indicate that embryos incubated at high EST compared with normal EST oxidize more glucose after day 17.5 of incubation. The increased glucose oxidation at the high EST may also occur prior to day 17.5 of incubation because study II showed that ^{13}C enrichment in expired CO_2 was numerically higher after the [U-^{13}C] glucose bolus at day 16.5 of incubation.

Increased glucose oxidation at a high EST probably explain the lower hepatic glycogen stores that were found at day 18.2 and 18.8 of incubation in the high EST. The lower hepatic glycogen stores may be related to the earlier start of the hatching process at high EST (Romanoff, 1936; Freeman, 1969; Lourens et al., 2007). However, only two out of the ten embryos were internally pipped in the high EST treatment and none at the normal EST treatment at day 18.2 of incubation, which indicates that embryos in the high EST treatment had a lower amount of hepatic glycogen before the hatching process started, consistent with studies by Clawson (1975) and Molenaar et al. (2009b). A lower amount of readily available energy (i.e., hepatic glycogen) may explain the higher

embryonic mortality at high EST (Christensen et al., 1993) that was found in studies by Yildirim and Yetisir (2004) and Lourens et al. (2005).

Hepatic glycogen amounts were comparable between EST treatments from day 19.7 of incubation onward in the current study, and this may suggest that hepatic glycogen use at the end of incubation was lower in the high EST compared with the normal EST treatment. At high EST incubation, carbohydrate stores such as hepatic glycogen were smaller before hatching and may become limited earlier during hatching. Embryos incubated at a high EST may therefore need to obtain additional energy to survive. In the current study, plasma uric acid concentrations were higher in the high EST than in the normal EST treatment at day 21.6 of incubation. This is consistent with data reported by Molenaar et al. (2010), who found a higher trend for plasma uric acid concentrations and a lower efficiency in protein utilization for growth in hatchlings incubated at high (38.9°C) compared with normal (37.8°C) EST from day 7 of incubation. This may indicate that glucogenic amino acids are deaminated and the carbon skeleton is immediately oxidized for ATP production (McArdle et al., 1981) or first converted to glucose by gluconeogenesis (Hazelwood and Lorenz, 1959). Because these amino acids were not used for development, YFBM was probably decreased at day 20.8 and 21.6 of incubation in the current study.

High EST was expected to increase the metabolic rate (Oznurlu et al., 2010) while O_2 availability is limited at the end of incubation by the eggshell and the chorio-allantoic membrane (Whittow and Tazawa, 1991; Janke et al., 2002). As a consequence, anaerobic glycolysis may increase to fulfill the higher energy requirement at high EST compared with normal EST. Although anaerobic ATP production from glucose can not be quantitatively assessed with the methodology used in the current study, plasma lactate concentration was measured to obtain an indicator of glycolysis. Plasma lactate concentrations were similar for both EST treatments, indicating that the current study does not suggest effects of EST on anaerobic metabolism in chicken embryos. However, plasma lactate concentrations do not have to be increased with higher anaerobic glycolysis (Péronnet, 2010). The plasma lactate concentration is the result of the production and clearance of lactate (Péronnet, 2010), and these may both increase at high EST. For instance, Christensen et al. (2007) showed that lactate recycling increased at high temperatures at the pipping stage in turkey embryos.

In both EST treatments, plasma lactate concentrations increased to approximately 46 mg/dL at day 20.8 of incubation; this is twice as high as concentrations found in studies with layer chickens (~20.0 to 27.3 mg/dL; Freeman, 1965; Tazawa et al., 1983; García et al., 1986). This discrepancy may be due to differences in growth potential because the current study included broiler embryos which have been selected for a high daily gain instead of layers selected for egg production. If this implies that the requirements for anaerobic ATP production have been increased in modern broiler embryos, then glucose precursors can be considered key nutrients during the perinatal period.

In conclusion, embryos incubated at high EST compared with normal EST had a higher glucose oxidation after day 17.5 of incubation. Consequently, hepatic glycogen concentrations were lower in the high EST treatment before the start of the hatching process (~day 18 of incubation). During the hatching process, glycogen stores are depleted and may become limited at high EST. Amino

acids may be deaminated and used for gluconeogenesis or immediately for ATP production as indicated by the higher plasma uric acid concentrations found after hatch at high EST. Because of the use of these amino acids for energy instead of growth, body development may be impaired at high EST.

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Figure 1A. Study I: ^{13}C enrichment in expired CO_2 after injecting a single bolus of [U-^{13}C]glucose (1.0 mg in 250 µl sterile water) in the chorio-allantoic fluid at day 14.5 of incubation of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 13 of incubation onward (n = 1).

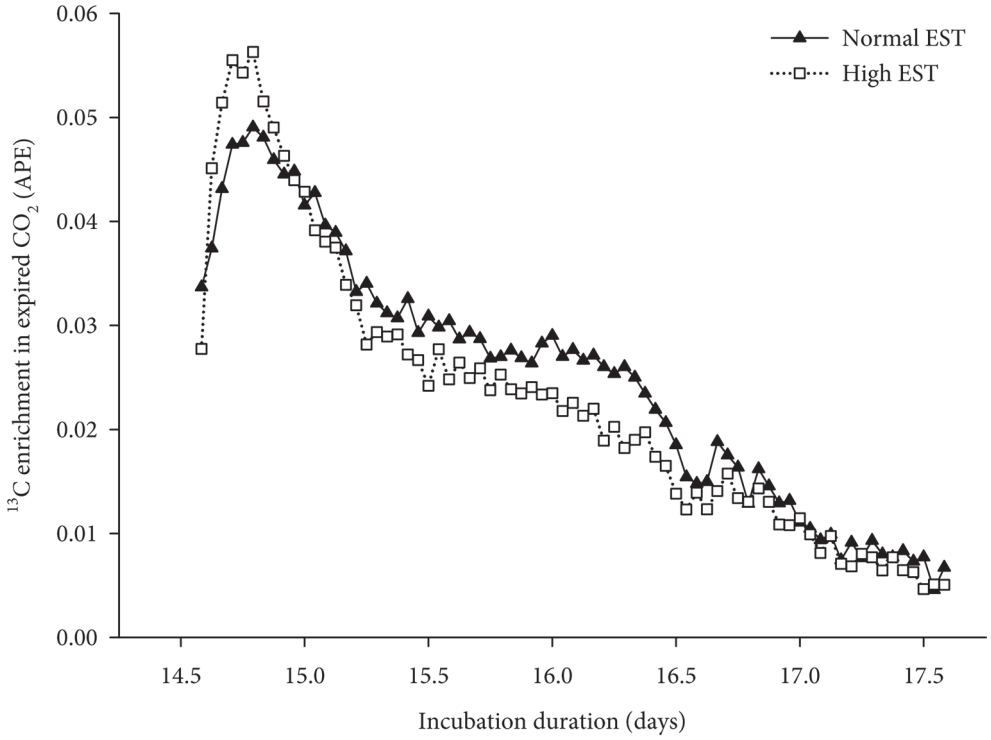


Figure 1B. Study II: ^{13}C enrichment in expired CO_2, after injecting a single bolus of [U-^{13}C] glucose (0.73 mg in 250 µl sterile water) for four consecutives days from day 14.5 of incubation in the chorio-allantoic fluid of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 13 of incubation onward (n = 1).

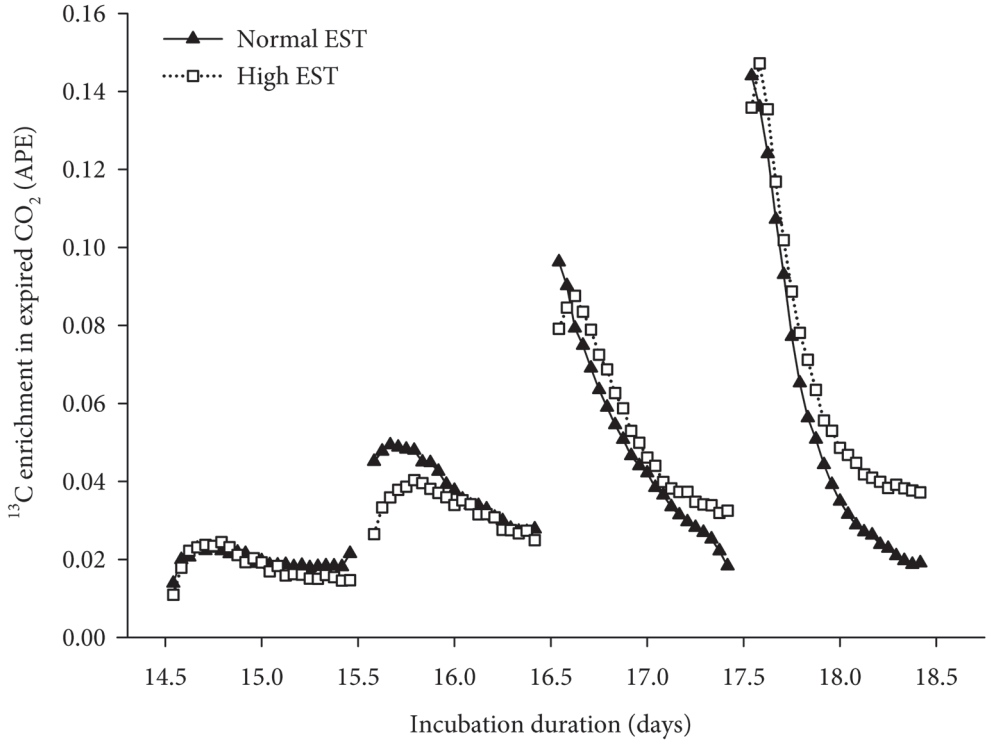
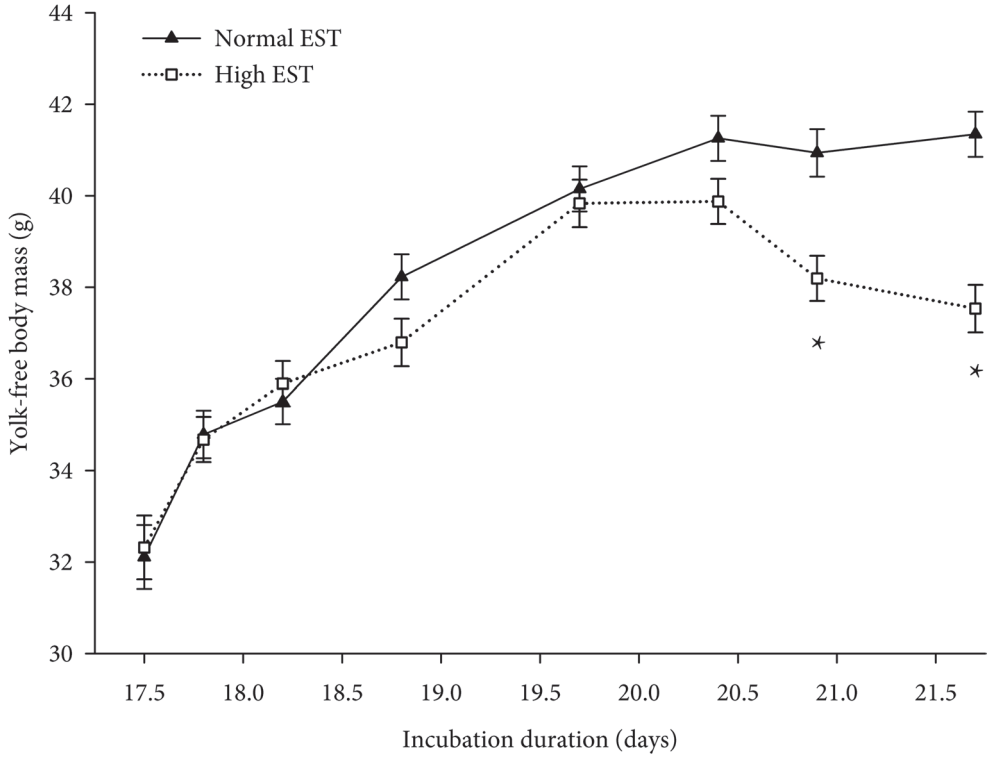


Figure 2A. Study III: Yolk-free body mass of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



*Significant difference between EST treatments ($P < 0.05$).

Figure 2B. Study III: Yolk weight of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.

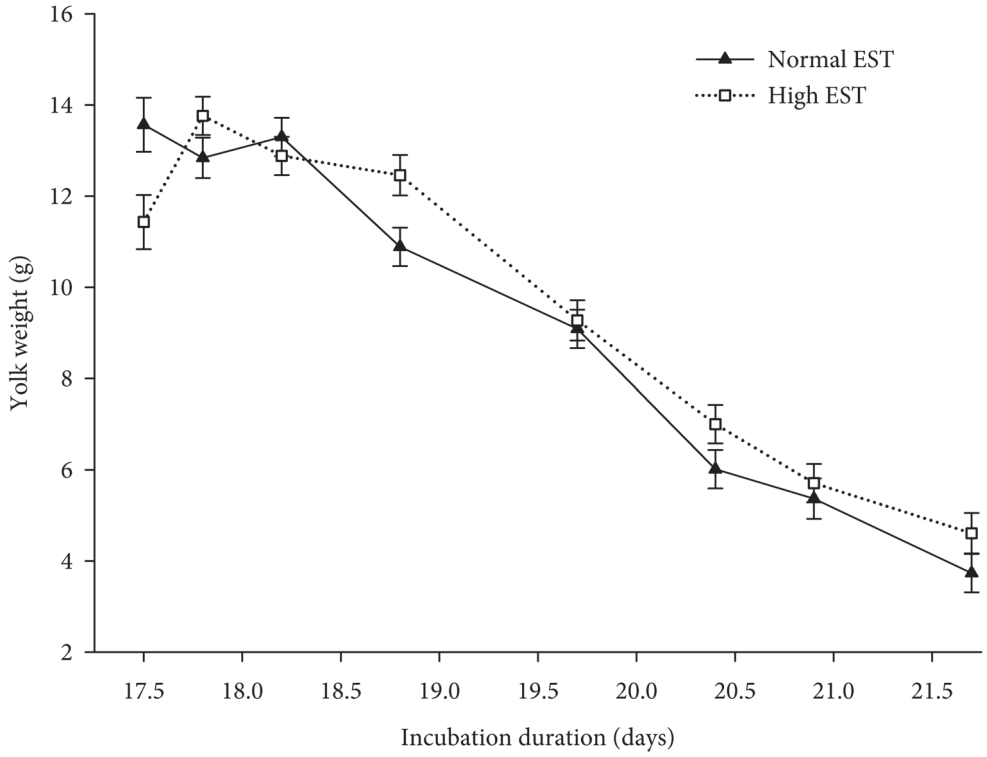


Figure 3A. Study III: Plasma glucose concentration of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.

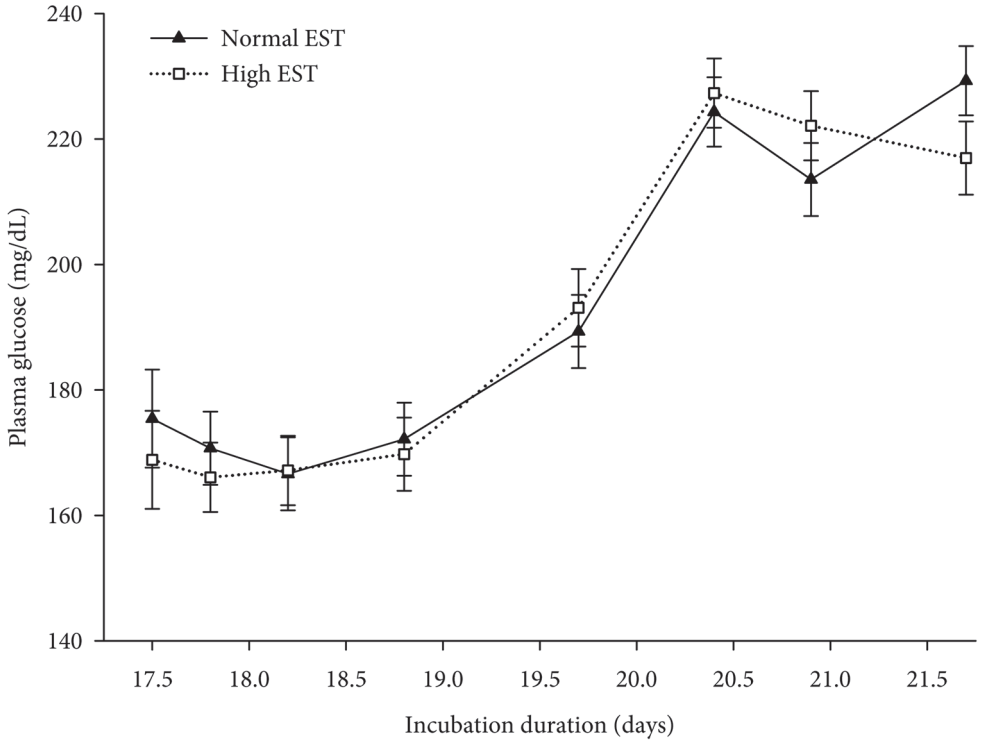


Figure 3B. Study III: Plasma lactate concentration of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.

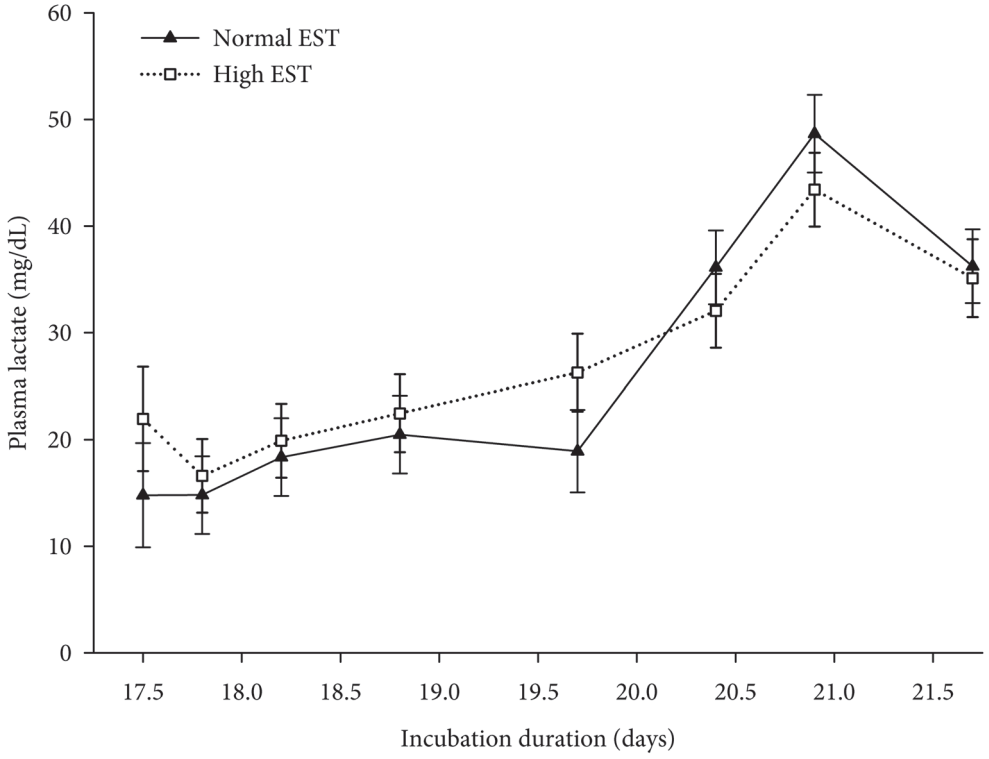
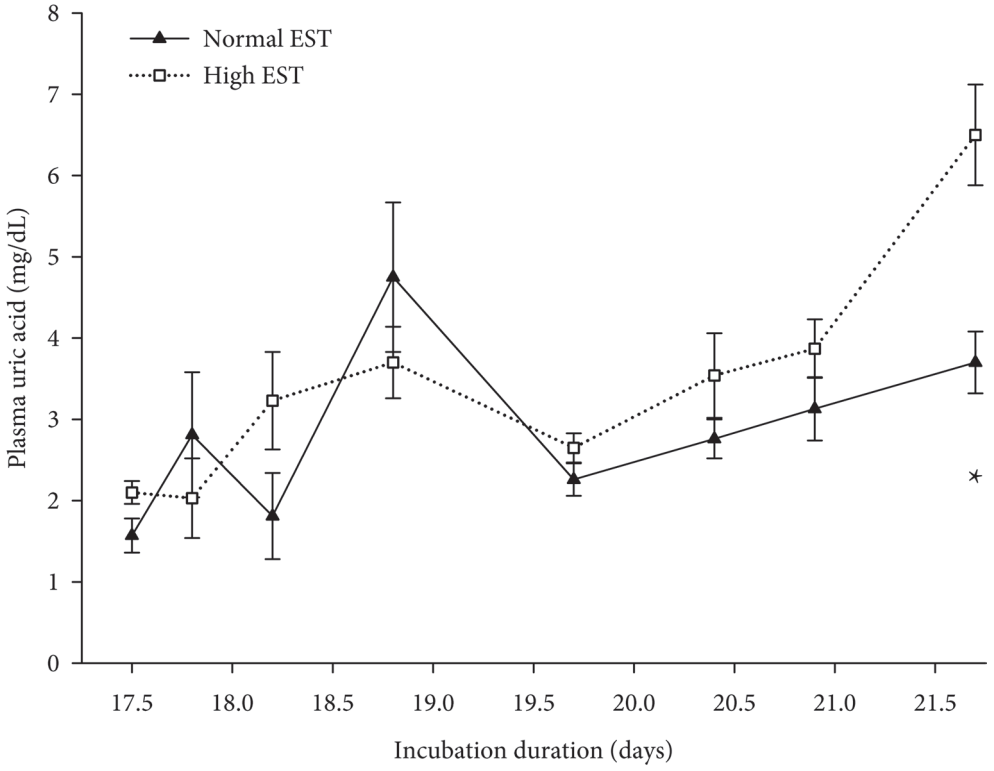
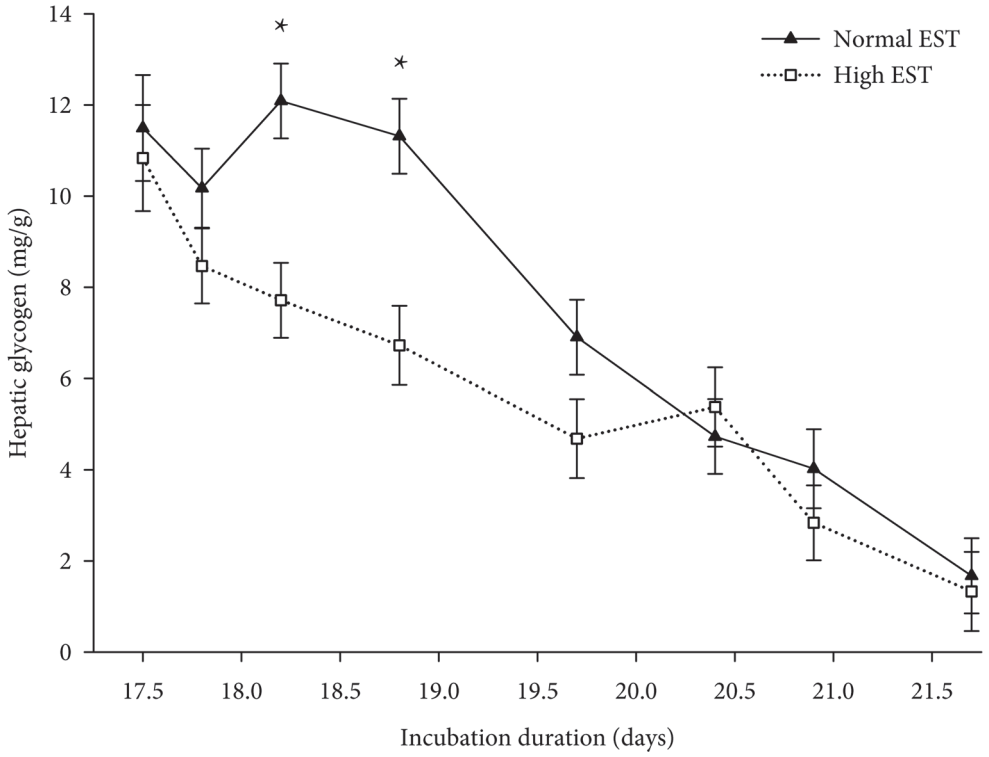


Figure 3C. Study III: Plasma uric acid concentration of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



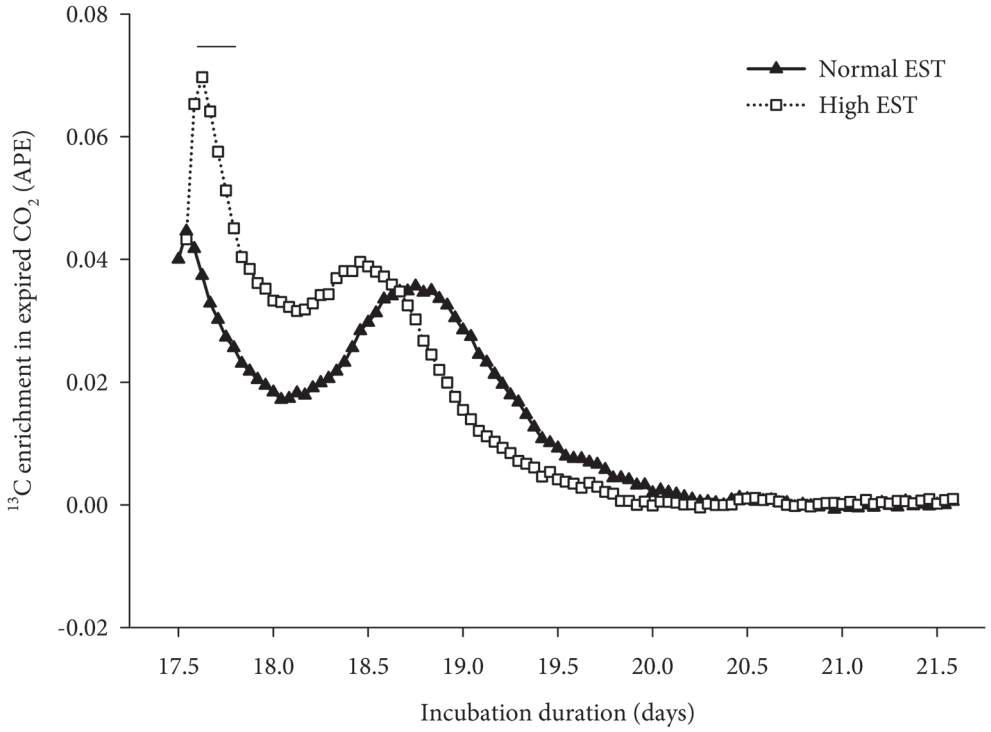
*Significant difference between EST treatments ($P < 0.05$).

Figure 3D. Study III: Hepatic glycogen concentration of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



*Significant difference between EST treatments ($P < 0.05$).

Figure 4A. Study III: ^{13}C enrichment in expired CO_2 after injecting a single bolus of [U-^{13}C]glucose (1.0 mg in 250 µl sterile water) in the chorio-allantoic fluid of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



The straight line indicates a significant difference between EST treatments ($P < 0.05$).

Figure 4B. Study III: ^{13}C enrichment in plasma glucose after injecting a single bolus of [U-^{13}C] glucose (1.0 mg in 250 µl sterile water) at day 17.5 of incubation in the chorio-allantoic fluid of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.

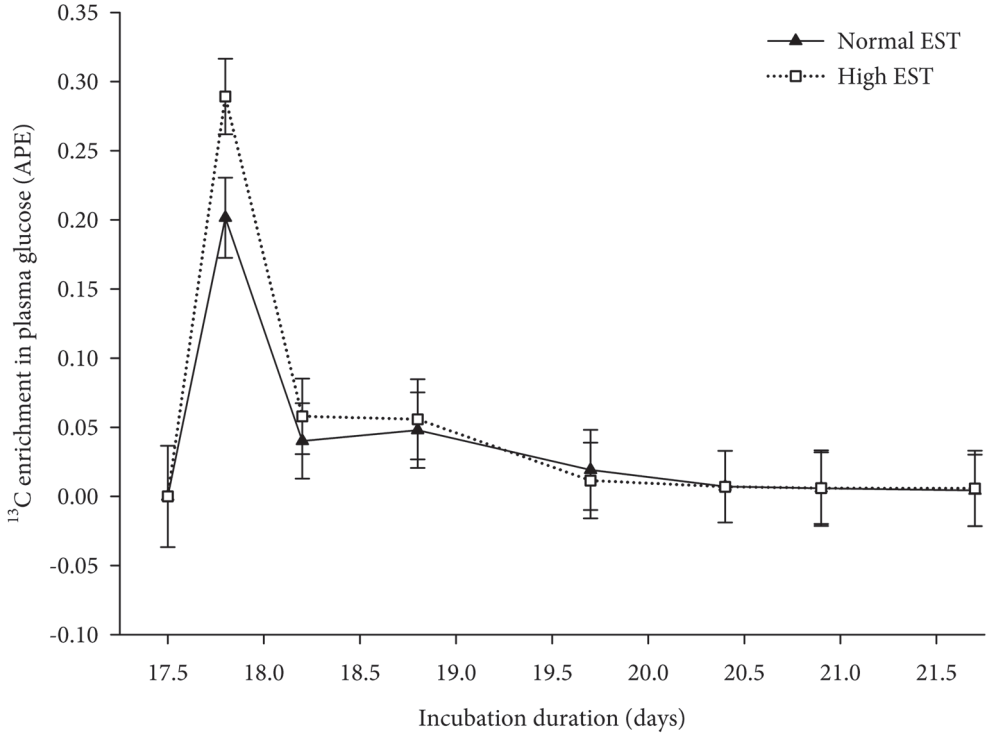
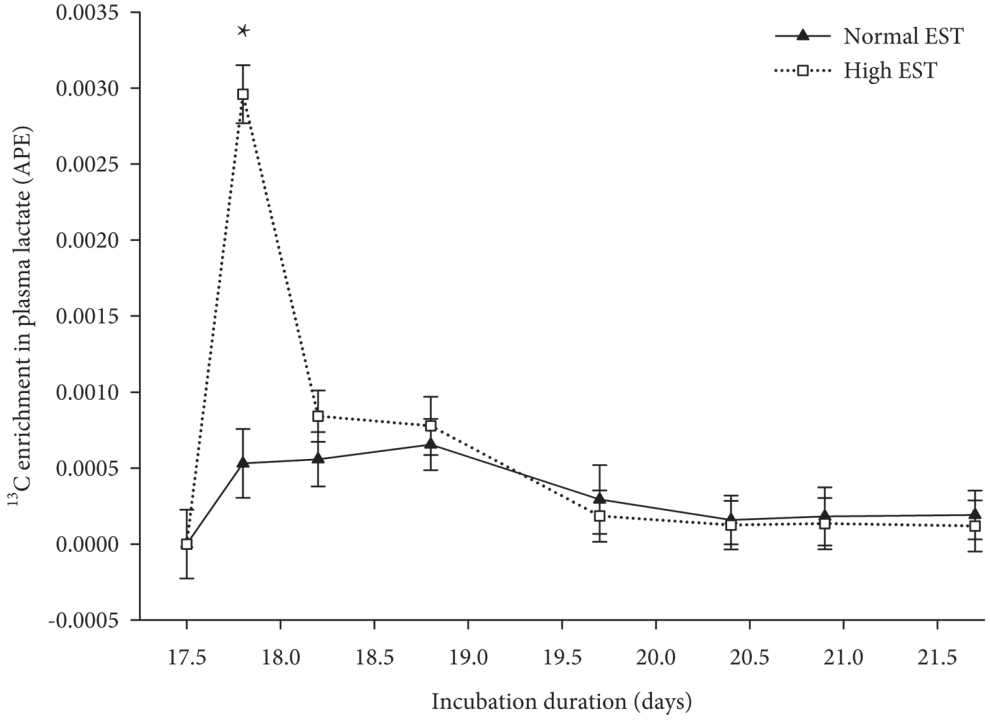
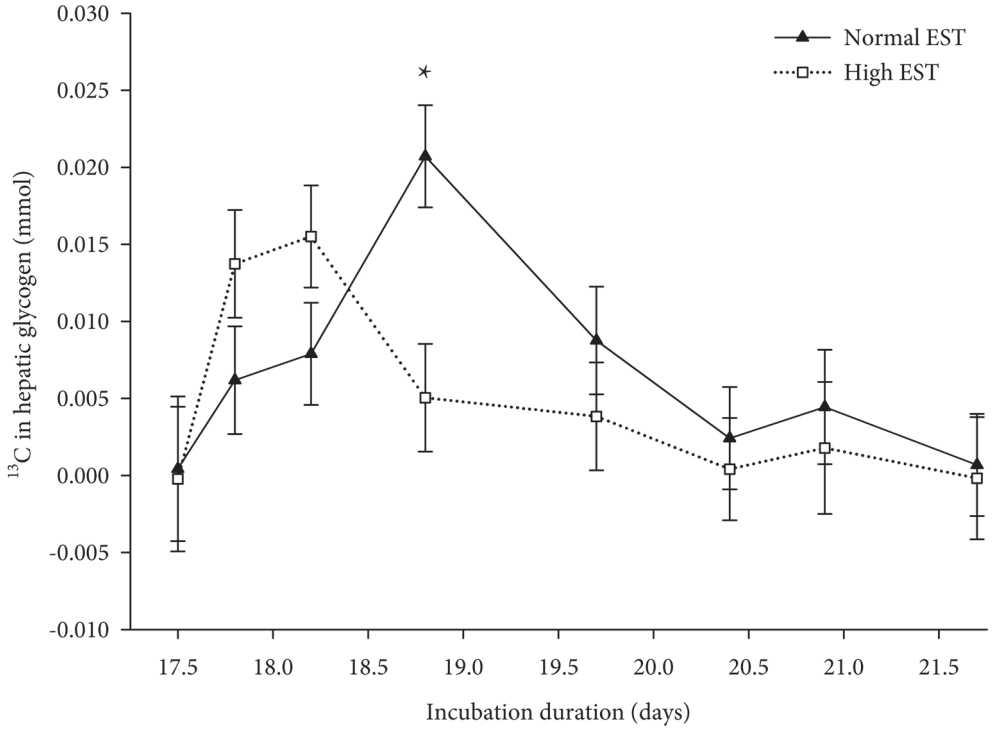


Figure 4C. Study III: ^{13}C enrichment in plasma lactate after injecting a single bolus of [U-^{13}C] glucose (1.0 mg in 250 µl sterile water) at day 17.5 of incubation in the chorio-allantoic fluid of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



*Significant difference between EST treatments ($P < 0.05$).

Figure 4D. Study III: ^{13}C in hepatic glycogen after injecting a single bolus of [U-^{13}C] glucose (1.0 mg in 250 µl sterile water) at day 17.5 of incubation in the chorio-allantoic fluid of embryos incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from day 10.5 of incubation onward.



*Significant difference between EST treatments ($P < 0.05$).

4

High Eggshell Temperatures during Incubation Decrease Growth Performance and Increase the Incidence of Ascites in Broiler Chickens

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Abstract

High eggshell temperatures (EST; ≥ 38.9°C) during the second half of incubation are known to decrease body and organ development of broiler hatchlings. In particular, relative heart weights are decreased by a high EST, and this may increase the incidence of metabolic disorders that are associated with cardiovascular development such as ascites. The current study investigated the effects of a high EST on chick quality, subsequent performance, and the incidence of ascites later in life. Eggs were incubated at a normal (37.8°C) or high (38.9°C) EST from day 7 of incubation onward. After hatching, the chickens were housed per EST in pens, and a normal or cold temperature schedule was applied during the growout period. Hatchability, hatchling quality, body weight, feed conversion ratio (FCR), total mortality, mortality associated with ascites, slaughter characteristics, and ascites susceptibility at 6 weeks of age were evaluated. Except for total ventricle weight, no interaction was found between EST and the growout temperature. Hatchability was comparable between the EST treatments, but the percentage of second-grade chickens was 0.7% higher at the high EST. Yolk-free body mass was 3.0 g lower, and heart weights were 26% lower at hatch in the high EST compared with the normal EST treatment. Body weight continued to be less during the growout period after high EST incubation. However, breast meat yield was 1.0% higher in the high EST than in the normal EST. Feed conversion ratios did not differ between EST treatments. Total mortality was 4.1% higher and mortality associated with ascites was 3.8% higher in the high EST compared with the normal EST treatment. The ratio between right and total ventricle was 1.1% higher in the high EST compared with the normal EST treatment at slaughter age. In conclusion, a high EST from day 7 of incubation onward decreased hatchling quality and growth performance, but increased breast meat yield. Furthermore, high EST incubation increased the incidence of ascites, which may be related to the reduced heart development at hatch.

Key words: eggshell temperature, chick quality, growth performance, broiler chickens, ascites

Introduction

Due to genetic selection, growth rates of broiler chickens have been increased, and this has decreased the production cycle time by 60% within 40 years (Wolanksi et al., 2004; Hulet, 2007; Baghbanzadeh and Decuypere, 2008). As a consequence, the incubation period has become a larger part of the total life span of a broiler chicken (Havenstein et al., 2003; Wolanski et al., 2004). Optimizing development and maturation during incubation is therefore important and will in turn optimize development during the growout period (Hulet, 2007). Several studies have shown that incubation conditions influence chick development (Freeman and Vince, 1974; Lourens et al., 2005, 2007) and that temperature is one of the most important environmental factors during incubation (Decuypere and Michels, 1992). Eggshell temperature (EST) often increases at the end of incubation due to the higher heat production of the embryos (Lourens et al., 2005) and problems with cooling and air velocity in the incubators (French, 1997; Elibol and Brake, 2008). High EST (≥ 38.9°C), compared with normal EST (37.8°C), during the second half of incubation reduces hatchling quality expressed by a lower yolk-free body mass (YFBM), a shorter chick length, and a poorer navel condition (Lourens et al., 2005, 2007; Hulet et al., 2007; Leksrisompong et al., 2007). In addition, several studies have shown that organ weights of hatchlings, and especially heart weights, are reduced because of a high EST (Wineland et al., 2000; Leksrisompong et al., 2007; Lourens et al., 2007).

The reduced heart weights at hatch that are due to a high EST may increase the susceptibility to and the incidence of metabolic disorders related to cardiovascular development later in life such as ascites (Leksrisompong et al., 2007), but the relationship between EST and ascites was never investigated. Chickens that are incubated at a high EST may have an insufficient pulmonary vascular capacity, which may increase their metabolic demands for O_2 and result in the development of ascites (Lubritz and McPherson, 1994). As a result of the increased O_2 requirement, the O_2 carrying capacity is enhanced by an increase in the number of red blood cells (Decuypere et al., 2000). As a consequence, the viscosity of the blood increases; this can lead to an increased cardiac output, pulmonary hypertension, and right ventricle hypertrophy, which are signs of the development of ascites (Scheele et al., 1991; Julian, 1993; Lubritz and McPherson, 1994; Decuypere et al., 2000). An increase in the intravascular pressure results in fluid accumulation in the abdominal cavity and pericardium (Julian, 1993; Decuypere et al., 2000), and birds will eventually die from these lesions.

Ascites has become a major cause of mortality in modern broiler production affecting around 5.0 to 8.0% of all broilers worldwide (Balog, 2003; Pavlidis et al., 2007). Modern broiler chickens are more sensitive to metabolic disorders such as ascites because of the genetic selection for rapid growth, low feed conversion ratio (FCR), and high meat yield (Scheele et al., 1991; Decuypere et al., 2000; Balog, 2003; Arce-Menocal et al., 2009) that has resulted in decreased visceral organ development (Havenstein et al., 2003). Although genetic selection against ascites is effective and broiler breeder companies have implemented selection programs to reduce ascites (Wideman and French, 2000; Pakdel et al., 2005; Pavlidis et al., 2007; Arce-Menocal et al., 2009; Hassanzadeh et

al., 2010), broilers susceptible for ascites are still present in commercial flocks and may account for nearly 50% of the total mortality (Arce-Menocal et al., 2009). Therefore, the current study investigated the effect of high EST on chick quality, performance, and mortality, with particular attention given to the incidence of ascites. To induce ascites, a cold growout temperature was used (Balog, 2003).

Materials and Methods

Experimental Design

The experiment was designed as a 2 × 2 factorial arrangement with two EST treatments (normal or high) during incubation and two temperature schedules (regular or cold) during the growout period. Chick quality was evaluated at hatch, and performance and ascites susceptibility and incidence was evaluated during the growout period and at slaughter age. The experimental protocol was approved by the PSU Animal Care and Use Committee (IACUC).

Hatching Eggs

Twenty trays were used, each containing 132 first-grade hatching eggs (n = 2,640) from commercial Ross × Cobb (500) breeders that were 33 weeks of age. The average egg weight when the eggs were set was 60.1 g.

Storage and Incubation

Eggs were stored for 5 days at 18°C at the animal experiment facilities of Penn State University (State College, USA). The eggs were then set in a Buckeye incubator (Chickmaster Inc., Medina, OH). Eggshell temperature was measured twice a day from ten eggs with a Thermoscan (Braun, Kronberg, Germany) and maintained at 37.8°C. Eggs were turned hourly over 90° between day 0 and 18 of incubation. On day 7 of incubation, eggs were randomly divided between two Buckeye incubators (Chickmaster Inc., Medina, OH). The empty trays in the incubator were filled with unfertilized eggs to ensure an uniform air speed around the eggs. The EST was again measured twice a day from ten eggs with a Thermoscan (Braun, Kronberg, Germany) and maintained at either the normal (37.8°C) or high (38.9°C) EST treatment. On day 18 of incubation, the eggs were transferred to hatching boxes in two separate Buckeye hatchers (Chickmaster Inc., Medina, OH), and the previous normal or high EST was maintained. From day 19 of incubation, the incubator temperature was fixed at the last EST setpoint, and the EST was allowed to increase during the hatching process. Relative humidity was set at 50% during the entire incubation process.

Hatch until Slaughter Age

At hatch, all chickens were taken from both hatchers and vaccinated for Marek's disease. The hatchlings were classified as first- or second-grade chickens. A chick was classified as first-grade when it was clean, dry, and without deformities or lesions (Tona et al., 2004a). The other chickens were classified as second-grade chickens, and this also included the chickens that died in

the hatching basket after emergence from the eggshell. The percentage of first- and second-grade chickens was expressed as percentage of fertile eggs. Fifty first-grade chickens per EST treatment were randomly selected and their body weights, including the residual yolk, chick length, and navel condition, were determined. Chick length was measured from the top of the beak to the tip of the middle-toe, excluding the nail (Hill, 2001). Navel condition was scored as 1 (closed and clean navel area), 2 (black button up to 2 mm or black string), or 3 (black button exceeding 2 mm or open navel area). The hatchlings were decapitated and bled, and the residual yolk and heart were removed and weighed. The yolk-free body mass (YFBM) was calculated as body weight minus residual yolk.

Nonhatched eggs were opened, and infertile eggs or embryo mortality were classified as described by Lourens et al. (2006). The embryos in nonhatched eggs that were older than 18 days and not located with their head under the right wing were classified as malpositioned (Dove, 1935). The number of infertile eggs was expressed as a percentage of the total eggs. The embryonic mortality per week and malpositioned embryos were expressed as a percentage of the fertile eggs.

Per EST treatment, 36 first-grade chickens were housed in a pen, with 24 replicates per EST treatment (n = 1,728). A cold or regular temperature schedule was applied to half of the pens per treatment during the growout period (Figure 1). Pens were prepared as described by Hulet et al. (2007). The chickens were fed a crumbled starter diet (3,086 kcal of ME/kg, 22.06% CP) until 14 days of age, a pelleted grower diet (3,152 kcal of ME/kg, 19.89% CP) until 28 days of age, and a pelleted finishing diet (3,219 kcal of ME/kg, 18.18% CP) until 42 days of age. Feed and water were provided ad libitum throughout the experiment by round pan hanging feeders and nipple drinkers, respectively. The light schedule was 20L:4D throughout the growout period. The chickens were weighed and their feed was weighed back per pen at 0, 7, 14, 21, 28, 35 and 42 days of age. Feed conversion ratio was calculated by dividing the total feed consumption per pen by the total growth per pen, where the growth of the animals that died was included as well. Dead chickens were removed and recorded daily per pen. Body weight, cause of death, and fluid in the pericardium and abdominal cavity were recorded, and the right ventricle (RV) and total ventricle (TV) were weighed. Pericardial and abdominal fluid was scored as either a 0 if no fluid was present, or a 1 if accumulation of fluid was observed. Dead chickens were classified as having ascites when fluid was found in the pericardium and abdomen or when the calculated RV:TV ratio was above 27% (Wideman, 2001). At the slaughter age on day 42 or 43, 20 males and 20 females per EST × growout treatment were processed; the body weight, carcass weight, breast meat yield, and abdominal fat were weighed (n = 160). Furthermore, hematocrit, fluid in the pericardium and abdomen, and RV and TV weight were measured. Chickens were deprived of feed approximately 6 hours before slaughter.

Statistical Analysis

Percentage of infertile eggs, first- and second-grade chickens, embryonic mortality per week, malpositioned embryos, and weight loss were analyzed using the GLM procedure in SAS (Version 9.1, SAS Institute 2004), with EST as the class variable and egg tray as the experimental unit. Body

weight at hatch, YFBM, chick length, and heart and residual yolk weight were analyzed using the GLM procedure, with EST as the class variable. Navel condition was analyzed using the LOGISTIC procedure with EST as the class variable. For all hatchling traits, the chicken was the experimental unit. Body weight and FCR during the growout period were analyzed using the MIXED procedure for repeated measurements with EST, growout temperature, and their interaction as class variables and the percentage of males per pen as a co-variable. Pen was the repeated factor. The measurements taken at slaughter age (i.e., body weight, carcass weight, breast meat yield, abdominal fat, and hematocrit) were analyzed using the GLM procedure with EST, growout temperature, sex and their interactions as the class variables, and day of processing as a block. Breast meat yield was calculated as a percentage of carcass weight. Total mortality and mortality that was associated with ascites was calculated per pen and analyzed using the GLM procedure with EST, growout temperature, sex and their interactions as the class variables, and the percentage of males per pen as a co-variable. Distributions of the means and residuals were examined to check the model assumptions. In all analyses, non significant interactions ($P > 0.05$) were excluded from the model. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as means ± SE. In all cases, a difference was considered significant at $P \leq 0.05$.

Results

EST × Growout Temperature

An interaction was found between EST and growout temperature for TV weight ($P = 0.02$; Table 5). At the cold growout temperature, TV weight did not differ between EST and was on average 10.0 g. At the normal growout temperature, TV weight was less in the high EST treatment (8.4 ± 0.21 g) than in the normal EST treatment (9.0 ± 0.23 g).

EST

The percentage of infertile eggs was on average 3.0% and did not differ between EST treatments ($P = 0.26$; Table 1). Hatch of fertile eggs did not differ between EST treatments ($P = 0.12$), but percentage of second-grade chickens was 0.7% higher ($P = 0.02$) in the high EST than in the normal EST treatment. Embryonic mortality in the second and third week of incubation and the percentage of malpositioned embryos did not differ between EST treatments (all $P > 0.05$). Body weight at hatch was 3.4 g and YFBM was 3.0 g less in the high EST treatment compared with the normal EST treatment (both $P < 0.001$). Chick length did not differ between EST treatments ($P = 0.14$) and was on average 19.6 cm. Residual yolk weight was 0.5 g lower in the high EST than in the normal EST treatment ($P = 0.05$). Navel condition was poorer in the high EST than in the normal EST treatment ($P = 0.002$). Heart weight at hatch was reduced by 26% in the high compared with the normal EST treatment ($P < 0.001$).

Body weight during the growout period was lower in the high EST than in the normal EST treatment ($P < 0.001$; Table 2). At 42 days of age, the body weight difference was 41 g between the high and normal EST treatment. The FCR between 0 and 42 days of age did not differ between the

EST treatments and was on average 1.92 ($P = 0.37$; Table 3). Total mortality was 4.1% greater in the high EST than in the normal EST treatment ($P = 0.008$). Mortality associated with ascites was 3.8% greater in the high EST than in the normal EST treatment ($P = 0.001$).

At slaughter age, body weight, carcass weight, abdominal fat, hematocrit, pericardial and abdominal fluid, RV and TV weight, and relative heart weight did not differ between the EST treatments (all $P > 0.05$; Tables 4, 5). The percentage of breast meat yield was 1.0% and the RV:TV ratio was 1.1% greater in the high EST than in the normal EST treatment ($P < 0.001$ and $P = 0.04$, respectively).

Growout Temperature

An interaction was found between growout temperature and age of the bird for body weight ($P < 0.001$; Table 2). From 21 days of age onward, body weight was lower in the cold compared with the regular growout temperature treatment. At 42 days of age, the body weight difference was 148 g between the cold and regular growout temperature. The FCR between 0 and 42 days of age was poorer in the cold (1.98) than in the regular (1.86) growout temperature ($P < 0.001$; Table 3). Total mortality was 4.7% greater in the cold than in the regular growout temperature ($P = 0.02$). Mortality associated with ascites was 3.8% greater in the cold than in the regular growout temperature ($P = 0.004$).

Body weight at slaughter age and percentage of breast meat yield were not influenced by growout temperature (both $P > 0.05$; Table 4). Carcass weight was 92 g and abdominal fat was 6 g less in the cold than in the regular growout temperature (both $P < 0.01$). Hematocrit was 3% greater in the cold than in the regular growout temperature ($P < 0.001$). Pericardial and abdominal fluid were not affected by the growout temperature, neither was RV weight (all $P > 0.05$; Table 5). The cold growout treatment had a lower RV:TV ratio and a higher relative heart weight than did the normal growout treatment (both $P < 0.01$).

At slaughter age, body weight, carcass weight, RV weight, TV weight, and the RV:TV ratio were higher in males than in females (all $P < 0.001$; Table 4, 5). In addition, more males than females were scored with heart fluid at slaughter age ($P = 0.004$).

Discussion

EST × Growout Temperature

Except for TV weight at slaughter age, no interaction was found between EST and growout temperature. A higher mortality associated with ascites could be expected in the high EST treatment and cold rearing treatment, as both factors might be predisposing factors for ascites. However, TV weights were comparable between EST treatments at the cold growout temperature and lower at the high EST than the normal EST at the normal growout temperature. Cold growout temperatures increase relative heart weights of broiler chickens, which may partly counteract the differences in relative heart weights resulting from the high EST treatment and depress the mortality related with ascites.

EST

Eggshell temperature did not affect hatchability in the current study, but a high EST increased the percentage of second-grade chickens by 0.7%. A higher percentage of second-grade chickens is consistent with other studies regarding high incubation temperatures; second-grade chickens are often characterized by a small, pale appearance, and a poor navel quality (Romanoff, 1936; Byerly, 1938; Thompson et al., 1976; Leksrisompong et al., 2007; Piestun et al., 2009a). Second-grade chickens are often culled in practice; the current results therefore show that a high EST can decrease the number of salable hatchlings. Embryonic mortality in the third week was only numerically higher (1.2%) in the high EST compared with the normal EST. Other studies have shown that a high EST in the second half of incubation can increase embryonic mortality in the last week of incubation (Byerly, 1938; French, 1994; Lourens et al., 2005). A higher third week embryonic mortality can be related to an increased number of malpositioned embryos (Byerly, 1938; French, 1994), but no indications of this problem were found in the current study, and this phenomenon may only occur at a higher EST.

Hatchling quality of first-grade chickens was reduced in the high compared with the normal EST treatment, expressed by a lower body weight and YFBM and a poorer navel quality, which is consistent with other studies (Romanoff, 1936; Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007; Piestun et al., 2009a). Furthermore, high EST decreased subsequent performance, expressed by an average lower body weight during the growout period. In the current study, measurements at slaughter age evaluated a smaller number of birds (n = 160), but they showed the same trend. Relative breast meat yield was 1.0% greater in the high EST than in the normal EST treatment, and this may be related to increased muscle cell proliferation and accelerated differentiation after high EST incubation (Piestun et al., 2009b). Different studies have shown that body weight up to 21 days was lower in chickens incubated at a high EST (38.9 to 39.5°C) compared with a normal EST (~37.8°C) at the end of incubation (Lourens et al., 2005; Joseph et al., 2006; Leksrisompong et al., 2009). This difference disappeared at slaughter age in the study of Joseph et al. (2006). Hulet et al. (2007) found a lower body weight of 48.1 g at 44 days of age in birds incubated from day 16 of incubation onward at an EST of 39.7°C compared with an EST of 37.5°C. However, the same study showed that an EST of 38.6°C from day 16 of incubation onward increased body weight at 44 days of age with 49.5 g compared with the normal EST of 37.5°C. The differences between studies on the effect of high EST on subsequent performance might be explained by compensatory growth or the temperatures that the birds experienced during their growout period. Piestun et al. (2008b) and Yalçin et al. (2010) have shown that a continuous high temperature during incubation can improve the thermotolerance of broiler chickens. When birds incubated at a high temperature experience relatively high temperatures during the growout period, they may have an improved ability to cope with these temperatures and are better able to maintain growth compared with birds incubated at a normal incubation temperature (Yalçin et al., 2010).

FCR was not affected by EST treatment in the current study. However, body weight was greater in the normal EST treatments at 42 days of age, and this increases the FCR. When the FCR was

adjusted to a 2 kg body weight (-0.01 FCR with every 25 g above 2 kg of body weight), total FCR was 1.59 for the high EST treatment and 1.55 for the normal EST treatment. The higher FCR may be related with the lower intestinal development and maturation at hatch that is found with incubation temperatures above 37°C (Wineland et al., 2006), which may negatively affect nutrient utilization in later life.

Different factors are known to be involved in the development of ascites; examples include genetics, diet composition, and environmental conditions during the growout period (Lubritz and McPherson, 1994; Acar et al., 2001; Aftab and Khan, 2005; Druyan et al., 2008; Baghbanzadeh and Decuypere, 2008; Izadinia et al., 2010). A relationship between high incubation temperatures and ascites was never investigated (Leksrisompong et al., 2007). The current study showed that high EST can also be a predisposing factor for ascites. The difference that was found in total mortality between EST treatments was almost completely explained by the difference in mortality associated with ascites. Birds that resulted from eggs incubated at a high EST had a two-fold higher mortality associated with ascites than did the birds resulting from eggs incubated at a normal EST. The higher ascites incidence in the high EST treatment may be related to the decreased heart development at hatch, which decreased the ability to supply the body with O_2 for its respiratory demands: the first step in the development of ascites.

Dewil et al. (1996) found a significantly lower relative heart weight in embryos of a fast growing line that is susceptible for ascites compared with embryos from a slow growing line that is resistant for ascites. This reduction in heart weight may be related to a line difference, or it could be caused by a difference in EST that the eggs of the different lines experienced during incubation. Egg weight was higher for the ascites-susceptible line than for the ascites-resistant line, and both lines were incubated together in the same incubator. Lourens et al. (2006) showed that heat production of larger eggs is higher than that of smaller eggs in the second half of incubation. Therefore, the larger eggs of the ascites-susceptible line may have had higher EST despite identical incubation temperatures. As shown in the current study, the heart weights at hatch were lower at a high EST compared with a normal EST and may be a predisposing factor for ascites.

The decrease in heart weight in the high EST compared with the normal EST was 26% in the current study; this value was within the range found by other studies (17-31%; Wineland et al., 2000; Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2009a). This reduction in heart weight is suggested to be caused by a decrease in cell division at high temperatures during the second half of incubation (Romanoff, 1960; Leksrisompong et al., 2007).

Furthermore, a higher susceptibility for ascites after high EST incubation may be indicated by the higher RV:TV ratio at slaughter age, although the average RV:TV ratio was still within the normal range (Wideman, 2001). Five animals from the high EST and two animals from the normal EST had a RV:TV ratio that was above 27% which is an indicator for ascites (Wideman, 2001); these birds may have died due to ascites in the future. No further indications were found at slaughter age for ascites susceptibility, such as a higher hematocrit value or a lower relative heart weight.

Growout temperature

A low growout temperature combined with a fast growth rate is one of the main triggers that induce ascites because of the higher metabolic rate and the increased O_2 demand (Scheele et al., 1991; Acar et al., 2001; Wideman, 2001). The current study also showed that chickens in the cold growout treatment had a more than two-fold increase in mortality associated with ascites compared with the normal growout temperature. The birds tried to adapt to the low growout temperature and higher metabolic rate by increasing the hematocrit value and relative heart weight, which is consistent with other studies (Shlosberg et al., 1992; Lubritz and McPherson, 1994; Buys et al., 1999; Blahová et al., 2007). However, the RV:TV ratio was lower in the cold compared with the regular growout temperature in the current study, and this is in contrast with studies of Shlosberg et al. (1992) and Lubritz and McPhershon (1994). Higher hematocrit values increase cardiac output and can eventually result in a higher RV:TV ratio. The reason that no higher RV:TV ratio was found in the cold growout temperature might be that these chickens were not yet developing ascites.

Further consequences of the cold growout temperature were that the birds showed decreased body and carcass weight, consistent with other studies (Lubritz and McPherson, 1994; Buys et al., 1999; Pakdel et al., 2005). To maintain body temperature at the cold growout temperatures, the birds consumed more feed; this resulted in a higher FCR, as shown by Buys et al. (1999) and Akşit et al. (2008). The abdominal fat content was lower in the cold than in the normal growout temperature, likely because more energy was used for heat production and the maintenance of body temperature instead of for fat deposition (Yunianto et al., 1997; Blahová et al., 2007).

Different studies have shown that male birds are more susceptible than female birds for ascites (Bendheim et al., 1992; Balog, 2003; Arce-Menocal et al., 2009) because of their high metabolic rate and growth. Although the current study found that male birds had a lower hematocrit value than female birds, the higher RV:TV ratio that was found in male birds indicated that male birds were more susceptible for ascites than female birds.

In conclusion, a high EST reduced body development both at hatch and in the growout period and increased the incidence of mortality associated with ascites, which may be related to the decreased cardiovascular development that was found at hatch. A cold growout temperature reduced subsequent performance and was, together with high EST incubation, predisposing factors for ascites in broiler chickens.

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Table 1. Percentage of infertile eggs, hatchability of fertile eggs, second-grade chickens, embryonic mortality, and malpositioned embryos of eggs incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from 7 through 21 days of incubation.

	n	Normal EST	High EST	*P*-value
Incubation[1]				
Infertile eggs (%[2])	20	2.6 ± 0.45	3.4 ± 0.56	0.26
Hatchability of fertile eggs (%[3])	20	94.5 ± 0.57	92.5 ± 1.04	0.12
Second-grade chickens (%[4])	20	0.2[b] ± 0.11	0.9[a] ± 0.30	0.02
Embryo mortality (%[3])				
Second week	20	1.5 ± 0.27	1.1 ± 0.29	0.34
Third week	20	1.9 ± 0.48	3.1 ± 0.68	0.17
Malpositioned embryos (%[3])		1.4 ± 0.41	2.4 ± 0.67	0.24
Hatchling[5]				
Body weight (g)	100	40.6[a] ± 0.39	37.2[b] ± 0.41	<0.001
YFBM[6] (g)	100	36.9[a] ± 0.33	33.9[b] ± 0.33	<0.001
Chick length (cm)	100	19.5 ± 0.07	19.7 ± 0.07	0.14
Residual yolk (g)	100	3.7[a] ± 0.15	3.2[b] ± 0.18	0.05
Navel condition[7]	100			
1		50	18	
2		48	80	0.002
3		2	2	
Heart weight (g)	100	0.38[a] ± 0.00	0.28[b] ± 0.00	<0.001

[a,b]Means followed by different letters within a row are significantly different ($P \leq 0.05$).

[1]Tray was the experimental unit.

[2]Expressed as a percentage of the total number of eggs.

[3]Expressed as a percentage of fertile eggs.

[4]Expressed as a percentage of hatched chickens.

[5]Chick was the experimental unit.

[6]Yolk-free body mass.

[7]Percentage of chickens per treatment group that were scored with a navel condition of 1, 2, or 3, where 1 = good, 2 = moderate, and 3 = poor.

Table 2. Body weight of birds incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from 7 through 21 days of incubation and grown at a regular or cold temperature treatment until 42 days of age.

	Body weight (g)					
	EST			Growout temperature		
	Normal	High	Delta (Normal - High)	Regular	Cold	Delta (Regular - Cold)
Day						
0	41[a] ± 0.1	38[b] ± 0.1	3	40[a] ± 0.4	40[a] ± 0.3	0
7	165[c] ± 0.9	164[d] ± 1.1	2	163[b] ± 1.0	167[b] ± 0.9	-4
14	443[e] ± 2.3	437[f] ± 2.4	5	436[c] ± 1.8	443[c] ± 2.7	-7
21	925[g] ± 6.9	891[h] ± 12.8	34	930[d] ± 12.2	886[e] ± 6.7	44
28	1,520[i] ± 14.1	1,470[j] ± 16.2	50	1,545[f] ± 10.2	1,445[g] ± 14.0	100
35	2,208[k] ± 25.4	2,161[l] ± 25.7	47	2,272[h] ± 19.3	2,097[i] ± 18.1	175
42	2,895[m] ± 23.9	2,854[n] ± 20.4	41	2,948[j] ± 14.0	2,800[k] ± 18.7	148
Source of variation[1]						
EST			<0.001			
Growout temperature			<0.001			
Day			<0.001			
Growout temp × Day			<0.001			

[a-n]Means followed by different letters within factor and row are significantly different ($P \leq 0.05$).

[1]Interactions between EST × Day, EST × Growout temperature, and EST × Growout temperature × Day were not significant ($P > 0.05$).

Table 3. Feed conversion ratio (FCR), total mortality, and mortality associated with ascites of birds incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from 7 through 21 days of incubation and grown at a regular or cold temperature treatment until 42 days of age.

Treatment	n[1]	FCR	Total mortality	Mortality associated with ascites
		0 through 42 days	(%)	(%)
EST				
Normal	24	1.91 ± 0.02	8.4[b] ± 1.28	2.8[b] ± 0.65
High	24	1.93 ± 0.02	12.5[a] ± 1.16	6.6[a] ± 1.02
Growout temperature				
Regular	24	1.86[b] ± 0.01	8.1[b] ± 1.00	2.8[b] ± 0.69
Cold	24	1.98[a] ± 0.02	12.8[a] ± 1.37	6.6[a] ± 1.00
Source of variation[2]				
EST		0.37	0.008	0.001
Growout temperature		<0.001	0.02	0.004

[a,b]Means followed by different letters within a column and factor are significantly different ($P \leq 0.05$).

[1]Pen was the experimental unit.

[2]Interaction between EST × Growout temperature was not significant ($P > 0.05$).

Table 4. Slaughter and ascites characteristics of male and female birds incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from 7 through 21 days of incubation and grown at a regular or cold temperature treatment until 42 days of age.

Treatment	n[1]	Body weight (g)	Carcass weight (g)	Breast meat yield (%[2])	Abdominal fat (g)	Hematocrit (%)
EST						
Normal	80	2,953 ± 0.0	2,188 ± 20.9	29.7[b] ± 0.2	39 ± 1.6	34 ± 0.5
High	80	2,909 ± 0.0	2,166 ± 18.0	30.7[a] ± 0.2	39 ± 1.3	34 ± 0.6
Growout temperature						
Regular	80	2,962 ± 0.0	2,223[a] ± 19.4	30.0 ± 0.2	42[a] ± 1.3	32[b] ± 0.6
Cold	80	2,899 ± 0.0	2,131[b] ± 19.2	30.4 ± 0.2	36[b] ± 1.4	35[a] ± 0.5
Sex						
Male	80	3,161[a] ± 0.0	2,333[a] ± 16.8	30.1 ± 0.2	38 ± 1.34	33[b] ± 0.6
Female	80	2,700[b] ± 0.0	2,022[b] ± 12.9	30.4 ± 0.2	40 ± 1.47	35[a] ± 0.6
Source of variation[3]						
EST		0.27	0.46	<0.001	0.75	0.67
Growout temperature		0.11	0.003	0.16	0.002	<0.001
Sex		<0.001	<0.001	0.36	0.24	0.002

[a,b]Means followed by different letters within a column and factor are significantly different ($P \leq 0.05$).

[1]Animal was the experimental unit.

[2]As a percentage of carcass weight.

[3]Interactions between EST × Growout temperature × Sex were not significant ($P > 0.05$).

Table 5. Ascites characteristics of male and female birds at slaughter age that had been incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) from 7 through 21 days of incubation and grown at a regular or cold temperature treatment until 42 days of age.

Treatment	n	Pericardial fluid[1]		Abdominal fluid[1]		RV[2]	TV[3]	RV:TV[4]	Heart
		0 (%)	1 (%)	0 (%)	1 (%)	(g)	(g)	(%)	(% of body weight)
EST									
Normal	80	34	66	98	2	1.8 ± 0.05	9.5 ± 0.17	19.2^{b} ± 0.40	0.32 ± 0.00
High	80	32	68	98	2	1.9 ± 0.06	9.3 ± 0.18	20.3^{a} ± 0.43	0.32 ± 0.01
Growout temperature									
Regular	80	39	61	96	4	1.8 ± 0.06	8.7 ± 0.16	20.7^{a} ± 0.47	0.29^{b} ± 0.00
Cold	80	27	73	100	0	1.9 ± 0.05	10.0 ± 0.16	18.9^{b} ± 0.34	0.35^{a} ± 0.00
Sex									
Male	80	22	78	98	2	2.1^{a} ± 0.05	10.2^{a} ± 0.15	20.4^{a} ± 0.41	0.32 ± 0.00
Female	80	44	56	99	1	1.6^{b} ± 0.05	8.5^{b} ± 0.15	19.1^{b} ± 0.42	0.32 ± 0.01
Source of variation[5]									
EST		0.68		0.56		0.21	0.31	0.04	0.75
Growout temperature		0.07		0.95		0.20	<0.001	0.002	<0.001
Sex		0.004		0.56		<0.001	<0.001	0.02	0.17
EST × Growout temp		NS		NS		NS	0.02	NS	NS

[a,b]Means followed by different letters within a column and factor are significantly different ($P \leq 0.05$).

[1]Percentage of chickens per treatment group that were scored with a 0 or 1, where 0 = no fluid, and 1 = fluid accumulation.

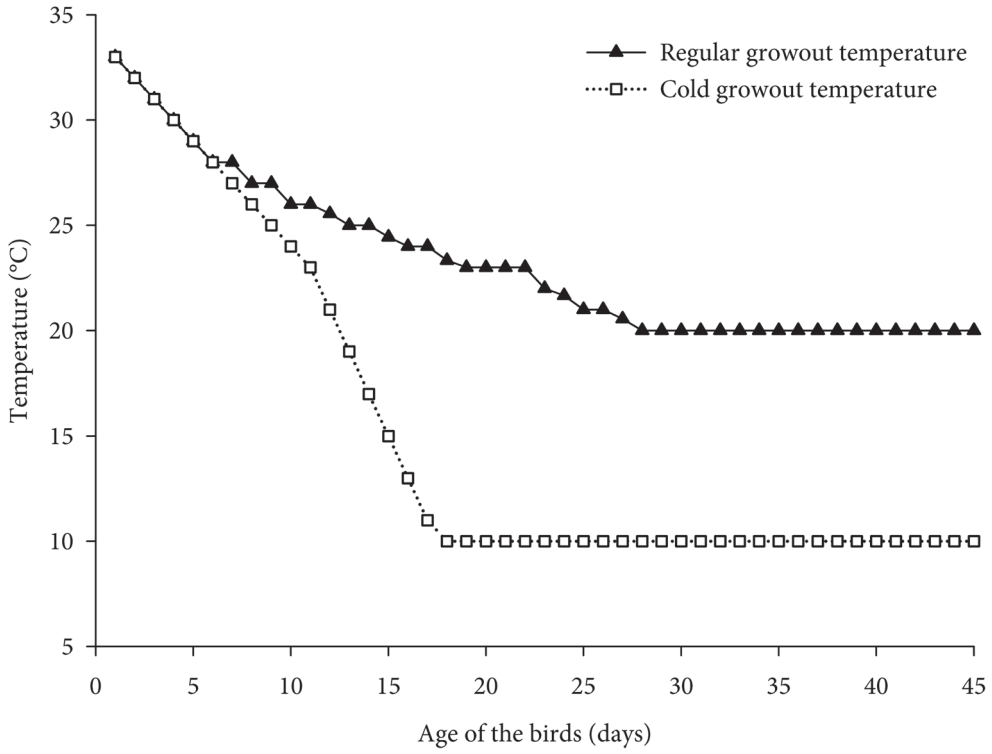
[2]Right ventricle.

[3]Total ventricle.

[4]Ratio between right and total ventricle.

[5]Interactions between EST × Sex, Growout temperature × Sex, and EST × Growout temperature × Sex were not significant ($P > 0.05$).

Figure 1. Regular and cold temperature schedule during the growout period of broiler chickens.



5

Effect of Eggshell Temperature and a Hole in the Air Cell on the Perinatal Development and Physiology of Layer Hatchlings

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Abstract

To investigate the effect of incubation conditions on layer hatchlings, an experiment was performed in which layer eggs were incubated at a normal (37.8°C) or high (38.9°C) eggshell temperature (EST) and a hole was punctured in the air cell of half of the eggs in both EST treatments from day 14 of incubation onward. Chick development, plasma metabolites, and hepatic glycogen were measured at 12 hours after emergence from the eggshell. Embryo mortality was not affected by the EST or hole treatment. At the high EST, yolk-free body mass (YFBM) was 0.7 g lower and residual yolk weight was 0.7 g higher than at the normal EST. This may be related to the shorter incubation duration at the high EST. Relative heart, lung, stomach, liver, spleen, and intestinal weights were lower in the high EST than in the normal EST. Yolk-free body mass did not differ between eggs with or without a hole, but residual yolk weight was slightly lower in eggs with a hole (0.3 g). Relative lung weights were higher in eggs with than without a hole, whereas no effect on other organs was found. Plasma glucose, lactate, and uric acid concentrations did not differ between the EST or hole treatment. Hepatic glycogen was lower in the high EST (7.3 mg) than in the normal EST (11.2 mg) at 12 hours after emergence from the eggshell, and this effect may be related to the shorter hatching process at the high EST. Hepatic glycogen levels were lower in eggs with a hole (8.6 mg) compared with eggs without a hole (10.0 mg), and this may be related to the longer period between external pipping and hatching in eggs with a hole. In conclusion, the EST and hole treatment did not interact, and neither treatments affected embryonic survival. High EST negatively affected hatchling development and seemed to change the carbohydrate metabolism in layer embryos. The effect of a hole in the air cell was limited.

Key words: eggshell temperature, gas exchange, development, physiology, layer hatchling

Introduction

Several authors have found that incubation conditions, such as temperature and O_2 concentration, can affect hatchability, embryo development, chick quality, and subsequent chick performance (Metcalfe et al., 1981; Hulet et al., 2007; Leksrisompong et al., 2007; Lourens et al., 2007; Piestun et al., 2008a). Most of these studies are performed in broilers because the incubation period is a major part of their lifespan (up to 33%; Wolanski et al., 2004; Hulet, 2007). Variables that affect broiler chick development can easily affect the short production period of broilers and can lead to decreases in economic return (Lourens and Van Middelkoop, 2000; Joseph et al., 2006; Hulet, 2007; Hulet et al., 2007; Leksrisompong et al., 2007). Although Romanoff (1936) already found an effect of incubation temperature on hatchling development in layer chickens, the influences of incubation conditions on layer development have not been extensively investigated (Pal et al., 2002; Janke et al., 2004; Ohta et al., 2004; Sato et al., 2006; Everaert et al., 2008).

In practice, it is suggested that greater embryo mortality is found at the end of incubation in layers than in broilers (Everaert et al., 2008) and this may be related to a difference in eggshell characteristics. Layer breeders produce eggs with a higher eggshell strength than broiler breeders (Johansson et al., 1996; Kemps et al., 2006) and this may complicate external pipping (EP) and negatively affect gas exchange by pulmonary respiration. Puncturing a hole in the air cell at the end of incubation in layer eggs may increase gas exchange (Okuda and Tazawa, 1988; Meir and Ar, 1996) and improve the survival rate (Meir et al., 1999). One of the factors that might interact with gas exchange is eggshell temperature (EST). High (38.9°C) EST increases the metabolic rate and O_2 requirement of embryos during incubation compared with normal (37.8°C) EST (Lourens et al., 2007). However, the O_2 requirement also increases at the end of incubation due to the increased development of the embryo. Gas exchange is limited by the eggshell, and O_2 tension decreases in the air cell at the end of incubation (Wangensteen, 1972; Khandoker et al., 2003). Therefore, embryos incubated at high compared with normal EST may have more metabolic difficulties at the end of incubation due to their higher O_2 requirement. As a consequence, their development, physiology, and survival rate may be negatively affected. Improving gas exchange by puncturing a hole in the air cell may be especially beneficial at high EST in eggs from layer strains. This study was performed to investigate the influence of EST and a hole in the air cell from day 14 of incubation onward on the embryo mortality, development and physiology of layer hatchlings at 12 hours after emergence from the eggshell.

Materials and methods

Experimental Design

The experiment was designed with a 2 × 2 factorial arrangement with two EST (37.8°C and 38.9°C) and the presence or absence of a hole in the air cell from day 14 of incubation onward. Hatchling traits were assessed at 12 hours after emergence from the eggshell. The experimental protocol was approved by the Animal Care and Use Committee of Wageningen University.

Hatching Eggs

First-grade hatching eggs from a commercial Dekalb White parent flock with an age of 40 weeks were used (n = 240). Eggs weighed between 60 and 63 g, and all eggs were stored at a temperature of 18°C and a relative humidity of 75% for 7 days at Ter Heerdt hatchery (Babberich, the Netherlands).

Incubation from day 0 until 14

Eggs were transported to the experimental facilities of Wageningen University and set in a HT-Combi incubator with a capacity of 4,800 eggs (HatchTech Incubation Technology, B.V., Veenendaal, the Netherlands). Eggs were evenly distributed between two eggs trays (n = 150/tray), and open spaces were filled with infertile eggs. The two trays were placed in the middle of the trolley. Four egg trays with infertile eggs were placed above and under the trays with the experimental eggs to ensure uniform air flow around the eggs. Eggshell temperature was measured by temperature sensors (NTC Thermistors: type DC 95, Thermometrics, Somerset, UK) placed halfway between the blunt and pointed ends of five individual eggs. Temperature sensors were attached to the eggshell by heat-conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and tape. The incubator's temperature was adjusted manually twice a day to maintain an EST of 37.8°C. Relative humidity was set at 50%, and eggs were turned each hour at an angle of 90°. Carbon dioxide concentrations in the incubator increased gradually from 0.04% to 0.08% at day 14 of incubation.

Incubation from day 14 until 18

After candling at day 14 of incubation, fertile eggs were randomly divided between two identical HatchTech Picoclimer setters with a capacity of 1,408 eggs (HatchTech Incubation Technology B.V.). In each setter, eggs were evenly distributed between two egg trays (n = 88/tray); open spaces were filled with infertile eggs. In half of the eggs, a hole was punctured in the blunt end of the egg. Before puncturing the hole, the eggs were disinfected with 70% ethanol, dried, and again disinfected with a betadine solution (Meda Pharma B.V., Amstelveen, the Netherlands). A hole was made though the eggshell into the air cell with an 18-gauge needle (B Braun Medical B.V., Oss, the Netherlands). Trays with experimental eggs were placed in the middle of the trolley. The remainder of the trays in the trolley were filled with infertile eggs to ensure uniform air flow around the eggs. Candling the eggs and puncturing the holes in the eggshells were performed at room temperature and took approximately 2 hours. In each setter, EST was measured by temperature sensors attached to four eggs, as described earlier. The setter temperature was adjusted automatically to maintain an EST of either 37.8°C (normal) or 38.9°C (high). The normal and high EST was based on studies of Lourens et al. (2005, 2007). Relative humidity was maintained at 50%, and eggs were turned each hour at an angle of 90° in both setters. Carbon dioxide concentrations in the incubators were approximately 0.04% between day 14 and 18 of incubation.

Incubation from day 18 until 12 hours after emergence from the Eggshell

At day 18 of incubation, eggs were transferred to individual hatching baskets (120 × 135 mm) and placed in two large open climate respiration chambers (CRC; Verstegen et al., 1987). Eggs were exposed to the same EST treatment as in the setter, and this EST was maintained in the CRC for 12 hours. Then, the CRC temperature was fixed at the last EST setpoint, and the EST was allowed to increase during the hatching process. Relative humidity was maintained at 50% and the CO_2 concentration was approximately 0.05% in both CRC.

Egg and Hatchling Measurements

All eggs were weighed at day 0, 14, and 18 of incubation to calculate egg weight loss. Eggs were candled at day 14 of incubation, and clear eggs and eggs with dead embryos were opened. Nonhatched eggs were opened at day 22 of incubation as well. Embryo mortality was classified per week as described by Lourens et al. (2006). The total number of infertile eggs was calculated as a percentage of the total number of eggs set. Embryo mortality from day 14 of incubation until hatching was expressed as a percentage of the number of fertile eggs at day 14 of incubation. Eggs were candled every 2 hours from day 18 until 22 of incubation to establish the time of internal pipping (IP), EP, and hatching. Overall hatchability was calculated as a percentage of the total number of fertile eggs. Hatchlings were kept in hatching baskets until 12 hours after emergence from the eggshell, and no food or water was provided.

Body weight, chick length, and navel condition were measured at 12 hours after emergence from the eggshell. Chick length was measured from the top of the beak to the tip of the middle-toe, excluding the nail (Hill, 2001). Navel condition was scored as 1 (closed and clean navel area), 2 (black button up to 2 mm or black string), or 3 (black button that exceeds 2 mm or open navel area). After these chick quality measurements, hatchlings were killed by decapitation and bled. Residual yolk, liver, and heart were immediately dissected and weighed. The yolk-free body mass (YFBM) was frozen at -20°C for further analysis of organ weights. Lung, stomach, spleen, intestine, and bursa of Fabricius were weighed after defrosting the YFBM in a plastic bag in a water bath at 37°C for 15 minutes.

Plasma Metabolite Determination

After decapitation, blood was collected in a 4-mL blood tube containing 10 mg of sodium fluoride and 8 mg of potassium oxalate (BD Vacutainer, Franklin Lakes, NJ). Two extra droplets (0.04 mL in total) of 10% heparin were added and mixed into the tube before sampling. Blood was centrifuged (2,900 × *g*) for 15 min, and plasma was decanted and stored at -20°C until further analysis. Plasma glucose, lactate, and uric acid concentrations were determined with commercially available enzymatic photometric kits (DiaSys Diagnostic Systems International, Holzheim, Germany).

Hepatic Glycogen Determination

After bleeding, livers were immediately dissected, weighed, and frozen in liquid nitrogen. Livers were stored at -80°C until further analysis. All procedures for hepatic glycogen determination were carried out on ice. The whole liver was homogenized with a glass stirring spoon after the

addition of 1 µL of 7% $HCLO_4$/g of wet tissue. The suspension was centrifuged (2,900 × *g*) at 4°C for 15 minutes. The supernatant was decanted, cleaned with 1 mL of petroleum ether, and frozen at -80°C until further analysis. The supernatant was defrosted, centrifuged, and decanted again. Hepatic glycogen was determined by the iodine binding assay described by Dreiling et al. (1987), and hepatic bovine glycogen (Type IX, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a standard.

Statistical Analysis

The egg and chick were the experimental units in all statistical analyses. Distributions of the means and residuals were examined to verify model assumptions. All data, except embryo mortality and navel condition, were analyzed with the GLM procedure using SAS (Version 9.1, SAS Institute 2004); EST, the hole treatment, and their interaction were included as class variables. Embryo mortality between day 14 and hatching as well as navel condition were analyzed with the logistic procedure of SAS Institute (Version 9.1, SAS Institute 2004); EST, hole treatment, and their interaction were included as class variables. In all analyses, non significant interactions ($P > 0.05$) were excluded from the model. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Data are presented as means ± SE. In all cases, differences were considered significant at $P \leq 0.05$.

Results

EST

Except for the bursa of Fabricius weight ($P < 0.001$), no interaction between EST and hole treatment was found for any of the determined variables ($P > 0.05$). Bursa of Fabricius weights were highest in eggs incubated at the normal EST with a hole in the air cell (0.16% of the YFBM). The other treatments had similar bursa weights (0.11 to 0.12% of the YFBM). Embryo mortality in the last week of incubation did not differ between EST treatments ($P = 0.69$; Table 1). Overall, the percentage of infertile eggs was 2.9% and hatch of fertile was 93.6%. Egg weight loss from day 14 until 18 of incubation was 0.2% higher in the high EST than in the normal EST treatment ($P < 0.001$). In the high EST compared with the normal EST treatment, IP occurred 7 hours earlier, EP 8 hours earlier, and hatch time 9 hours earlier (all $P < 0.001$; Table 2). The time between EP and hatching was 2 hours shorter ($P = 0.05$) and time between IP and hatching 3 hours shorter ($P < 0.001$) in the high EST than in the normal EST treatment. Body weight, chick length, and navel condition did not differ between EST treatments (all $P > 0.10$; Table 3). The YFBM was 0.7 g lower and residual yolk weight 0.7 g higher in the high EST than normal EST treatment (both $P < 0.001$). Relative heart, lung, stomach, liver, spleen, and intestinal weights were lower in the high EST than in the normal EST treatment (all $P < 0.05$; Table 4). Plasma concentrations of glucose, lactate, and uric acid did not differ between EST treatments (all $P > 0.05$; Table 5). Total hepatic glycogen was 3.9 mg (= 35%) lower in the high EST than in the normal EST treatment at 12 hours after emergence from the eggshell ($P < 0.001$).

Presence or Absence of a Hole in the Air Cell

Embryo mortality did not differ between eggs with or without a hole ($P = 0.73$; Table 1). In eggs with compared with eggs without a hole in the air cell, egg weight loss between day 14 and 18 of incubation was 0.5% higher ($P < 0.001$) and egg weight loss between day 0 and 18 of incubation was 0.6% higher ($P = 0.002$). Compared with eggs without a hole, the IP and hatching times did not differ (both $P > 0.10$), but EP occurred 3 hours later in eggs with a hole in the air cell ($P = 0.001$; Table 2). As a consequence, the time between IP and EP was 3 hours longer and the time between EP and hatching 4 hours shorter in eggs with than without a hole in the air cell (both $P < 0.01$). Body weight, YFBM, chick length, and navel condition did not differ between eggs with or without a hole (all $P > 0.10$; Table 3). The residual yolk weight was 0.3 g lower in eggs with than without a hole ($P < 0.001$). Relative heart, stomach, liver, spleen, and intestinal weights did not differ between the hole treatment groups ($P > 0.05$; Table 4). Relative lung weight was higher in eggs with than without a hole ($P = 0.05$). Plasma concentrations of glucose, lactate, and uric acid did not differ between the hole treatment groups ($P > 0.10$), but total hepatic glycogen was 1.4 mg (= 14%) lower in eggs with than without a hole ($P = 0.005$; Table 5).

Discussion

EST

Embryo mortality in the last week of incubation did not differ between EST treatments and was on average 3.1%. Compared with other studies in layer embryos (5.0%, Everaert et al., 2008; 7.4%, Shafey, 2002), the embryo mortality in the current study was relatively low and the overall hatch of fertile relatively high with 93.6%. Egg weight loss between day 14 and 18 of incubation was 0.2% higher at the high compared with the normal EST treatment, which is consistent with other studies (Pringle and Barott, 1937; Nakage et al., 2003; Eiby and Booth, 2009) and can be explained by a difference in the water vapor pressure deficit (Lundy, 1969; Meijerhof and Van Beek, 1993).

The hatching process (i.e., the period between IP and hatching) was 3 hours shorter in the high EST than in the normal EST treatment. Assuming that the energy requirement for the hatching process was the same for both EST treatments, more energy was required per time unit in the high EST treatment condition due to the shorter hatching process. The high energy requirement per time unit may especially come from carbohydrate sources, which are used for energy during the hatching process (Freeman, 1965; Dickson, 1983); this may explain the lower hepatic glycogen amount of 3.9 mg (35%) in the high EST treatment.

Apart from the effect of EST, hatching time in the normal EST treatment (505 hours) was 16 hours later in the current study than that observed in a layer study (489 hours) by Everaert et al. (2008). This difference may be related to the temperature or the CO_2 concentration that the embryos experienced in the two studies. Embryos in the current study were incubated at an EST of 37.8°C, and embryos in the study by Everaert et al. (2008) were incubated at a machine temperature of 37.8°C. Due the heat production of the embryos at the end of incubation, the EST may be higher in the study by Everaert et al. (2008) than in the current study. This higher EST may have reduced the

incubation duration, as shown in the current study and in a broiler study by Lourens et al. (2007). Second, a high CO_2 concentration at the end of incubation may increase the CO_2 and decrease the O_2 tension in the air cell of the eggs, which is known to induce pipping (Wangensteen, 1972). The CO_2 percentage was low (~0.05%) in the incubator and CRC throughout incubation in the current study. The CO_2 percentage in the study by Everaert et al. (2008) was not measured, but it could have contributed to the difference in hatching time between the two studies.

High EST reduced body development in the current study, as demonstrated by the lower YFBM and the higher residual yolk weight of the layer hatchlings; this finding is consistent with studies in broiler hatchlings (Romanoff, 1935; Wineland et al., 2000; Lourens et al., 2005, 2007; Leksrisompong et al., 2007). Leksrisompong et al. (2007) found a difference of 5.2 g in the YFBM of broiler eggs (67 to 71 g) incubated at a normal or high EST in the last week of incubation. A difference of 0.7 g was found in the YFBM between the EST treatments in the current study. The large difference between the two studies in YFBM may suggest that broiler embryos are more sensitive to high EST than layer embryos. The decreased body development in the high EST treatment may be caused by the reduced incubation duration in the current study, which is consistent with results from a broiler study (Lourens et al., 2007). Embryos in the high EST treatment had, on average, 9 hours less time to develop than the embryos in the normal EST treatment in the current study. However, when the YFBM was corrected for hatching time, the difference in the YFBM between the EST treatments was still significant ($P < 0.001$). The reason for the decreased body development in the high EST treatment may be related to an increase in metabolic rate (Lourens et al., 2007) that resulted from a higher yolk use or a lower efficiency for growth of the used nutrients. Studies of Lourens et al. (2005, 2007) seem to show that residual yolk size is not influenced by EST. The authors know of no studies investigating the relationship between hatchling development and egg production in layer strains.

In comparison to the normal EST condition, high EST reduced the relative and absolute organ weights of the heart (0.25 vs. 0.33 g), lung (0.25 vs. 0.28 g), stomach (2.04 vs. 2.26 g), liver (0.85 vs. 1.00 g), spleen (0.012 vs. 0.014 g), intestines (1.26 vs. 1.42 g), and bursa of Fabricius (0.044 vs. 0.052 g). Differences in absolute organ weights between the normal and high EST treatment ranged from 9.7% (stomach weight) to 24.2% (heart weight). Studies in broilers found consistently that relative heart weight was affected by high EST (Wineland et al., 2000; Givisiez et al., 2001; Leksrisompong et al., 2007; Lourens et al., 2007). The relative weights of the liver, spleen, gizzard, and intestinal tract were not always largely affected by high EST in broilers, however, this differed between and even within studies (Zhang and Whittow, 1992; Givisiez et al., 2001; Leksrisompong et al., 2007; Yalçin et al., 2008a). The authors know of no studies investigating the consequences of lower relative organ weights in layer hatchlings on subsequent egg production or health status.

In contrast to the lower hepatic glycogen amount 12 hours after emergence from the eggshell, plasma glucose, lactate, and uric acid concentrations were not affected by the EST treatment. Plasma metabolites are products of intermediary metabolism and reflect the physiological status of the hatchling (Artacho et al., 2007). Glucose and lactic acid concentrations are indicators of carbohydrate metabolism (Freeman, 1965, 1969), and uric acid concentration is an indicator of protein catabolism (Artacho et al., 2007). None of these plasma metabolites were affected by

the EST treatment. The reduced hepatic glycogen level in the high EST treatment indicates that carbohydrate metabolism may differ between EST treatments. Compared with the normal EST treatment, hepatic glycogen synthesis may be lower or hepatic glycogen utilization higher in the high EST treatment. Lower hepatic glycogen stores may negatively affect survival rate (Christensen et al., 1993), but no indication was found in the current study.

Presence or Absence of a Hole in the Air Cell

Embryo mortality did not differ between eggs with or without a hole, consistent with a study by Visschedijk (1968). Our hypothesis was that embryo mortality would decrease in eggs with a hole in the air cell due to an improvement in gas exchange. Meir and Ar (1996) and Meir et al. (1999) found that puncturing a hole in the air cell increased hatchability in goose eggs with a low or medium conductance. However, they concluded that this was related to the improvement in water loss rather than in O_2 availability. Results from the current study showed a normal amount of egg weight loss at day 18 of incubation (Meir et al., 1984; Hulet et al., 1987), which may indicate that gas exchange was sufficient and that the size of the air cell was large enough for successful hatching. Several studies have shown that eggshell strength is higher in layer eggs than in broiler eggs (Johansson et al., 1996; Everaert et al., 2008), but egg conductance is comparable or higher in layer eggs than broiler eggs (Shafey, 2002; Everaert et al., 2008). The higher embryo mortality at the end of incubation in layer compared with broiler eggs that is suggested in practice (Everaert et al., 2008) may not be related to the eggshell characteristics or a limited exchange of O_2 and CO_2 in layer eggs. This may explain why puncturing a hole in the air cell did not reduce embryo mortality in the current study. On the other hand, the increase in diffusive capacity and O_2 due to a single hole may be low as well (Meir et al., 1999; Hottman and Pelletier, 2008).

The time between IP and hatching did not differ between embryos with or without a hole, but the period between IP and EP was longer in embryos with a hole in the air cell. This might be due to a higher partial pressure of O_2 in the air cell and consequent lower stimulus for EP. The period between EP and hatching was shorter in eggs with than without a hole and the reason for this is unclear. No differences between IP, EP, and hatching time were found in turkeys after puncturing a hole in the air cell at day 24 or 25 of incubation (Hottman and Pelletier, 2008).

Chick quality was not largely influenced by the presence or absence of a hole in the air cell. Compared with hatchlings without a hole, residual yolk weight was 0.3 g lower and YFBM 0.3 g higher (not significant) in hatchlings with a hole in the air cell. Visschedijk (1968) showed that the gas exchange of the air cell encompasses about 30% of the total gas exchange until pipping. A normal air cell covers only 25% of the area of the chorioallantoic membrane at the end of incubation, and O_2 is probably only slightly increased in the air cell due to the presence of a hole in the air cell (< 1%; Meir and Ar, 1996; Meir et al., 1999); this may not have had a major impact on development in the current study. Compared with hatchlings without a hole, the hepatic glycogen level was lower in hatchlings with a hole in the air cell. The period between EP and hatching was shorter in eggs with a hole, and this may have increased the rate of hepatic glycogen utilization instead of the utilization of other energy sources.

In conclusion, high EST and the presence of a hole in the air cell from day 14 of incubation onward did not interact with each other, probably because the effect of a hole in the air cell was limited. The EST and hole treatment did not affect embryonic survival in the last week of incubation. High EST negatively affected hatchling development and seemed to change carbohydrate metabolism in layer embryos. The hole in the air cell delayed EP and increased hepatic glycogen levels at 12 hours after emergence from the eggshell, but it did not affect embryonic survival, body development or plasma metabolites.

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Table 1. Mortality and egg weight loss of hatchlings incubated under two eggshell temperature (EST) treatment conditions with or without a hole in the air cell from day 14 of incubation onward.

Treatment	n	Mortality day 14 - hatch (%)	Egg weight loss day 14 - 18 (%)	Egg weight loss day 0 - 18 (%)
EST (°C)				
37.8	112	3.6	$3.2^{b} \pm 0.05$	11.1 ± 0.12
38.9	113	2.7	$3.4^{a} \pm 0.05$	11.3 ± 0.14
Hole				
Yes	111	2.7	$3.5^{a} \pm 0.05$	$11.5^{a} \pm 0.13$
No	114	3.5	$3.0^{b} \pm 0.04$	$10.9^{b} \pm 0.12$
Source of variation[1]				
EST		0.69	<0.001	0.12
Hole		0.73	<0.001	0.002

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]No interaction between EST and the hole treatment was found ($P > 0.05$).

Table 2. Internal pipping (IP), external pipping (EP), and hatching time along with the intervals between IP and EP, EP and hatching time, and IP and hatching time for hatchlings incubated under two eggshell temperature (EST) treatment conditions with or without a hole in the air cell from day 14 of incubation onward. in the air cell from day 14 of incubation onward.

Treatment	n	IP (hours)	EP (hours)	Hatch (hours)	IP - EP (hours)	EP - Hatch (hours)	IP - Hatch (hours)
EST (°C)							
37.8	108	$477^{a} \pm 0.8$	$493^{a} \pm 0.8$	$505^{a} \pm 0.7$	16 ± 0.7	$12^{a} \pm 0.5$	$28^{a} \pm 0.5$
38.9	109	$470^{b} \pm 0.6$	$485^{b} \pm 0.8$	$496^{b} \pm 0.7$	15 ± 0.6	$10^{b} \pm 0.5$	$25^{b} \pm 0.5$
Hole							
Yes	108	474 ± 0.7	$491^{a} \pm 0.8$	501 ± 0.7	$17^{a} \pm 0.7$	$9^{b} \pm 0.5$	26 ± 0.5
No	109	473 ± 0.8	$487^{b} \pm 0.9$	500 ± 0.9	$14^{b} \pm 0.6$	$13^{a} \pm 0.6$	27 ± 0.6
Source of variation[1]							
EST		<0.001	<0.001	<0.001	0.17	0.05	<0.001
Hole		0.32	0.001	0.62	0.006	<0.001	0.74

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]No interaction between EST and the hole treatment was found ($P > 0.05$).

Table 3. Chick quality characteristics of embryos incubated under two eggshell temperature (EST) treatment conditions with or without a hole in the air cell from day 14 of incubation onward.

Treatment	n	Body weight (g)	YFBM[1] (g)	Chick length (cm)	Residual yolk (g)	Navel condition[2] 1	2	3
EST (°C)								
37.8	107	42.7 ± 0.13	37.7[a] ± 0.12	18.7 ± 0.04	5.0[b] ± 0.09	21	54	25
38.9	105	42.7 ± 0.14	37.0[b] ± 0.13	18.8 ± 0.04	5.7[a] ± 0.08	18	73	9
Hole								
Yes	105	42.7 ± 0.14	37.5 ± 0.14	18.7 ± 0.04	5.2[b] ± 0.08	20	63	17
No	107	42.7 ± 0.13	37.2 ± 0.13	18.7 ± 0.04	5.5[a] ± 0.10	18	65	17
Source of variation[3]								
EST		0.97	<0.001	0.25	<0.001		0.12	
Hole		0.64	0.12	0.61	<0.001		0.65	

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Yolk-free body mass.

[2]Percentage of chickens per treatment group scored with a navel condition of 1, 2, or 3, where 1 = good, 2 = moderate, and 3 = poor.

[3]No interaction between EST and the hole treatment was found ($P > 0.05$).

Table 4. Relative organ weights (% of yolk-free body mass) of embryos incubated under two eggshell temperature (EST) treatment conditions with or without a hole in the air cell from day 14 of incubation onward.

Treatment	n	Heart	Lung	Stomach	Liver	Spleen	Intestines	Bursa of Fabricius
EST (°C)								
37.8	108	0.86[a] ± 0.01	0.75[a] ± 0.01	5.99[a] ± 0.05	2.66[a] ± 0.02	0.04[a] ± 0.00	3.77[a] ± 0.04	0.14 ± 0.00
38.9	107	0.67[b] ± 0.01	0.67[b] ± 0.02	5.51[b] ± 0.05	2.30[b] ± 0.02	0.03[b]± 0.00	3.43[b] ± 0.04	0.12 ± 0.00
Hole								
Yes	107	0.76 ± 0.01	0.73[a] ± 0.01	5.81 ± 0.06	2.48 ± 0.02	0.03 ± 0.00	3.61 ± 0.04	0.14 ± 0.00
No	108	0.78 ± 0.01	0.69[b] ± 0.02	5.69 ± 0.05	2.49 ± 0.02	0.03 ± 0.00	3.59 ± 0.04	0.12 ± 0.00
Source of variation[1]								
EST		<0.001	<0.001	<0.001	<0.001	0.02	<0.001	<0.001
Hole		0.20	0.05	0.09	0.46	0.79	0.64	<0.001

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]No interaction between EST and the hole treatment was found ($P > 0.05$), except for the bursa of Fabricius ($P < 0.001$).

Table 5. Plasma metabolites and hepatic glycogen levels of embryos incubated under two eggshell temperatures (EST) treatment conditions with or without a hole in the air cell from day 14 of incubation onward.

Treatment	n	Glucose (mg/dL)	Lactate (mg/dL)	Uric acid (mg/dL)	Hepatic glycogen (mg)
EST (°C)					
37.8	106	222.4 ± 1.56	27.6 ± 0.58	2.7 ± 0.11	11.2^{a} ± 0.40
38.9	107	222.4 ± 2.02	29.0 ± 0.66	2.7 ± 0.15	7.3^{b} ± 0.36
Hole					
Yes	105	221.5 ± 1.70	28.9 ± 0.63	2.5 ± 0.13	8.6^{b} ± 0.39
No	108	223.4 ± 1.89	27.8 ± 0.62	2.8 ± 0.13	10.0^{a} ± 0.44
Source of variation[1]					
EST		1.00	0.10	1.00	<0.001
Hole		0.45	0.24	0.19	0.005

[a,b]Means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]No interaction between EST and the hole treatment was found ($P > 0.05$).

General Discussion

Introduction

Nutrient Utilization

The chicken embryo grows without direct maternal influences and uses the nutrients that are stored in the egg for growth and development (Wilson, 1997; Giussani et al., 2007). At oviposition, the egg contains a large amount of water (65.6%), fat (10.5%), and protein (12.1%), but carbohydrates are present at very low levels (<1%; Romanoff and Romanoff, 1949). Proteins are mainly used for body development in the second half of incubation, when the major growth of the embryo occurs (Romanoff, 1967). Fat and carbohydrates are the two main energy sources during incubation, and their use is largely dependent on the availability of O_2 (Romanoff, 1967; Sato et al., 2006; De Oliveira et al., 2008).

In the first week of incubation, glucose is the predominant energy source because the chorioallantoic membrane (CAM) is not sufficiently developed to provide enough O_2 for complete fatty acid oxidation (Kučera et al., 1984; Moran, 2007). After the first week of incubation, however, fat is the predominant energy source because O_2 availability becomes large enough (Romanoff, 1967; De Oliveira et al., 2008). Glucose becomes an important substrate again during the energy-demanding hatching process that starts around day 19 of incubation for broiler embryos (Freeman, 1969; Tazawa et al., 1983). The trigger for the embryo to internally and externally pip as well as emerge from the eggshell is a decrease in O_2 tension in the air cell from 21% to 14% and an increase in the CO_2 tension to 6% during the course of incubation (Rahn, 1981). The reason that the gas tensions in the air cell change is due to the increase in the metabolic rate, and therefore O_2 consumption, throughout the development of the embryo, but the O_2 availability is limited by the rate of O_2 diffusion through the eggshell and the CAM (Whittow and Tazawa, 1991; Janke et al., 2002). The complete hatching process (i.e., from internal pipping until hatching) takes about 24 hours (Freeman and Vince, 1974; De Smit et al., 2008) and then another 6 to 10 hours before the chicken is completely dry and able to stand on its feet.

Glucose seems to be important during the end of incubation for two reasons. First, because muscle activity of the chicken is high, but O_2 is limited during the hatching process, glucose is used as an energy source (John et al., 1987, 1988; Moran, 2007). Due to anaerobic glycolysis in the muscles, plasma lactate concentration increases (Freeman, 1965; De Oliveira et al., 2008). Second, plasma glucose concentrations increase at the end of incubation, which may be required to ensure normal activity of the central nervous system (Freeman, 1969). To obtain enough glucose at the end of incubation, glucose is synthesized and stored as glycogen during incubation (Foye et al., 2007). To synthesize glucose, the activity of gluconeogenic enzymes increases throughout incubation using glucogenic amino acids, glycerol, or lactate as potential precursors for glucose (Evans and Scholz, 1973; Watford et al., 1981; Dickson, 1983; Foye et al., 2007). Glucose is stored as glycogen in the heart, liver, muscle, and yolk sac membrane (Beattie, 1964; García et al., 1986; Foye et al., 2007). Hepatic glycogen is preferably mobilized when the embryo starts to emerge from the eggshell (Freeman, 1965, 1969; García et al., 1986; Foye et al., 2007).

Above mentioned physiological and metabolic processes are likely affected by suboptimal environmental conditions during incubation (Christensen et al., 1993), and this may negatively affect survival and embryonic development (Romanoff, 1935, 1936; Lundy, 1969; Wineland et al., 2000; Lourens et al., 2005, 2007; Leksrisompong et al., 2007). The two most important environmental conditions known to affect survival and development during incubation are temperature and O_2 availability (Freeman and Vince, 1974; Decuypere and Michels, 1992).

Temperature

Historically, air temperature in the incubator was maintained between 36 and 38°C throughout incubation (Lundy, 1969; Decuypere et al., 2001). The air temperature in the incubator differs from the temperature experienced by the embryo (French, 1997). Due to the heat production of the developing embryo, the temperature of the embryo increases when the incubation has a constant air temperature and air velocity (Meijerhof and Van Beek, 1993). Because embryo temperature is difficult to measure without killing the embryo, eggshell temperature (EST) is often used as an indicator. Eggshell temperature deviates less than 0.1 to 0.2°C from the embryo temperature (Meijerhof and Van Beek, 1993; French, 1997). Studies by Lourens et al. (2005, 2007) and Leksrisompong et al. (2007) showed that a continuous EST between 37.5 and 38.0°C resulted in the highest hatchability and best chick quality at hatch. To maintain this constant EST, air temperature needs to decrease during incubation because of the heat produced by the developing embryos (Lourens et al., 2006). High EST (≥38.5°C) at the end of incubation is commonly found and can negatively affect survival and development (Romanoff, 1935, 1936; Lourens et al., 2005, 2007; Hulet, 2007; Leksrisompong et al., 2007). When air velocity is poor or cooling capacity insufficient in the incubator, EST can easily increase at the end of incubation (French, 1997; Hulet, 2007; Elibol and Brake, 2008). In addition, the temperature setpoints can be too high because the EST in the incubator is not monitored closely.

O_2 concentration

Oxygen availability is the second important environmental condition during incubation that affects survival and chick development (Metcalfe et al., 1981; McCutcheon et al., 1982; Lourens et al., 2007). Low O_2 availability, for example due to incubation at a high altitude, can negatively affect survival and body development during incubation (Stephens and Ploog, 1967; Visschedijk, 1985; Hassanzadeh et al., 2004; Giussani et al., 2007). Embryos experience severe hypoxia when the O_2 concentration falls below 15% (Chan and Burggren, 2005), which probably only occurs at high altitudes. High EST may affect O_2 availability because high EST increases the metabolic rate of chicken embryos, which increases their O_2 requirement (Lourens et al., 2007; Oznurlu et al., 2010). Increasing the O_2 availability during incubation may positively affect survival and development, in particular when the EST is high (Lundy, 1969; Metcalfe and Stock, 1984; Lourens et al., 2005, 2007).

Aim of the General Discussion

Although the negative effects of suboptimal environmental conditions such as high EST or low O_2 availability on survival and development have been studied (Yildirim and Yetisir, 2004; Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al. 2007, 2009; Piestun et al., 2009a), the mechanisms that explain these negative effects are largely unknown. Suboptimal incubation may change metabolism, physiology, nutrient utilization or nutrient availability during incubation. These changes are probably reflected in differences in energy partitioning, energy stores, hormones, or blood metabolites in the perinatal period (day 18 of incubation until 48 hours after emergence from the eggshell). Furthermore, the effects of suboptimal environmental conditions may have long-lasting effects on morbidity and mortality throughout the production cycle of chickens, although this has not been intensively investigated (Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2009). Therefore, the first aim of this thesis was to investigate the effects of EST and O_2 availability during incubation on the survival, development, physiology, and nutrient utilization in chicken embryos. The second aim was to investigate the long-lasting effects of EST on survival and subsequent performance in broiler chickens. In this discussion, effects of EST and O_2 availability on the survival and on body development during the perinatal and growout period are discussed. The physiological and metabolic changes that may explain effects on survival and development from the perinatal period until the age of slaughter are discussed based on the results of previous chapters in this thesis. Finally, the conclusions of the current thesis are presented.

Survival

Perinatal Period

The effects of high (38.9 to 39.9°C) EST compared with normal (37.5 to 38.0°C) EST on the hatchability of broiler embryos are variable (Yildirim and Yetisir, 2004; Lourens et al., 2005; Joseph et al., 2006). Lourens et al. (2005) showed a large decrease of up to 9.8% in hatchability after high EST (38.9°C) during the last week of incubation of broiler embryos. Yildirim and Yetisir (2004) showed that an EST of ~39.5°C from day 17 until 21 of incubation did not decrease the hatchability of broiler embryos, but an EST of ~39.9°C in the same period decreased hatchability by 4.9% compared with the control EST of ~37.7°C. This finding suggests that the effects of high EST on hatchability are influenced by the duration and extent of the EST. The number of second-grade chickens, which are often unsalable chickens, can also be increased by high EST incubation. Lourens et al. (2005), Joseph et al. (2006), and the results presented in Chapter 4 showed that the number of second-grade chickens can increase up to 6% after high EST compared with normal EST incubation. Second-grade chickens often have a small, pale appearance, a relative large residual yolk, and/ or poor navel quality (Tona et al., 2004b; Piestun et al., 2009a). Hatchability may therefore not always be negatively affected by high EST, but the number of salable chickens can be reduced due to a high percentage of second-grade chickens (Joseph et al., 2006; Chapter 4).

The results presented in Chapter 1 showed that high EST (38.9°C) from day 7 of incubation onward decreased hatch of fertile by 26% when it was combined with a low O_2 concentration from day 7 until 19 of incubation. The lower hatch of fertile could be predominantly explained by a high embryo mortality in the third week of incubation. The lowest YFBM was found in the high EST and low O_2 concentration treatment, and some embryos may not have developed enough to cope with the energy-demanding hatching process and, thus, died. Furthermore, a high incidence of the malpositions 'head over wing' and 'head between legs' was found in embryos that died in the high EST and low O_2 concentration treatment. These malpositions deviate from the correct hatching position, in which the head is under the right wing and the embryo is able to create a circle at the blunt end of the egg with its beak and rotates in the egg by moving its right wing to emerge (Dove, 1935). Malpositions are more often found after high EST incubation (French, 2000), but the reason that the malpositions occur is largely unknown. Poor development (Robertson, 1961) or a decrease in readily available nutrients (e.g., glycogen) before the hatching process (Beattie, 1964; Christensen et al., 1993) may be two possible explanations. High EST compared with normal EST reduced the hepatic glycogen amount before the start of the hatching process around day 18 of incubation (Chapter 2, 3). No interaction was found between EST and O_2 concentration for the hepatic glycogen amount in Chapter 2, but this was in the broiler embryos that did survive. Hepatic glycogen amounts may have been too low in the embryos incubated in the high EST and low O_2 concentration that did not survive.

The lower amount of hepatic glycogen at day 18 of incubation after high EST compared with normal EST incubation is the result of either a decrease in glycogen synthesis or an increase in glucose utilization. The results presented in Chapter 4 show that embryos incubated at a high EST seem to have a higher glucose demand than embryos incubated at a normal EST. After an injection with [U-^{13}C]glucose at day 17.5 of incubation, a higher ^{13}C enrichment in expired CO_2 and plasma lactate was found almost immediately after injection in the high EST compared with the normal EST treatment. Furthermore, a tendency for higher recovery of ^{13}C enrichment in expired CO_2 and lower ^{13}C enrichment in hepatic glycogen at day 18.8 of incubation was found in the high EST compared with the normal EST. These results indicate that more glucose was used for oxidation at high EST compared with normal EST incubation, and this may explain why glucose stores such as hepatic glycogen were lower at day 18 of incubation in the high EST.

Possible explanations for the increased glucose utilization at high EST compared with normal EST incubation may be related to a higher metabolic rate, higher enzyme reaction rate (Q_{10} effect), or redistribution of the blood flow. High EST incubation may increase the O_2 demand and energy requirement as a result of the higher metabolic rate (Oznurlu et al., 2010). The higher metabolic rate at high EST compared with normal EST is shown by the higher heat production on day 13 and 14 of incubation in Chapter 1, and the higher heat production from day 9 until 15 of incubation in the study by Lourens et al. (2007). However, O_2 availability is restricted by gas exchange across the shell and shell membranes, which results in a plateau phase from day 15 to 19 of incubation (Lourens et al., 2006; 2007). To fulfill the energy requirement in embryos incubated at a high EST during the plateau phase, carbohydrate metabolism may be increased. The reason that carbohydrates are

used for energy production instead of fat or proteins might be because the lowest amount of O_2 is required for ATP production with carbohydrate oxidation (McArdle et al, 1981; Schreurs et al., 2007). A second reason for the higher glucose utilization at high EST compared with normal EST might be that enzyme reactions increase as temperature increases. With every 10°C increase in temperature, a two- to three-fold increase in enzyme reaction is generally observed (Febbraio, 2001; Kotov et al., 2007). The high EST used in this thesis was 1.1°C higher than the normal EST, which may have increased enzyme reactions up to 30%, resulting in an increased carbohydrate metabolism. Finally, high EST may redistribute the blood flow within the egg. Nichelmann et al. (2001) showed that the blood flow increases in the CAM in the last days before hatching in chicken embryos incubated at high temperatures. This increase in blood flow in the CAM may reduce the blood flow and O_2 availability to other tissues, favoring carbohydrate metabolism (McArdle et al, 1981; Schreurs et al., 2007).

Growout Period

The effects of suboptimal incubation conditions on mortality in the posthatch period have not been extensively investigated in broiler chickens. The current thesis investigated the effect of high EST on posthatch mortality in broilers. The results presented in Chapter 4 showed that the mortality was 4.2% higher in 6-week-old broiler chickens that were incubated at high EST (38.9°C) compared with normal EST (37.8°C) and maintained at a regular growout temperature. This increase in mortality was related to a more than three-fold increase in mortality associated with ascites in chickens incubated in the high EST compared with normal EST (4.4% vs. 1.2%, respectively). Ascites is a metabolic disorder related to pulmonary hypertension (Wideman, 2001) and develops when birds are unable to keep up with increased metabolic demands (Lubritz and McPherson, 1994). The decreased heart and lung weights at hatch after high EST incubation (Chapter 1, 4) may have caused the high mortality associated with ascites. Chickens incubated at a high EST may have a lower pulmonary vascular capacity and be more susceptible to develop ascites later in life than those incubated at a normal EST. Therefore, EST can be included in the list of other predisposing factors for ascites such as genetics, diet composition, and environmental conditions during the growout period (Lubritz and McPherson, 1994; Maxwell and Robertson, 1998; Acar et al., 2001; Aftab and Khan, 2005; Baghbanzadeh and Decuypere, 2008; Druyan et al., 2008; Izadinia et al., 2010). Part of the genetic effects on ascites that have previously been described in literature (Dewil et al., 1996; Buys et al., 1999; Pakdel et al., 2002; Pavlidis et al., 2007) may be explained by differences in EST during incubation. If, for example, genetic lines susceptible to ascites with different egg sizes are incubated at the same incubation temperature, large eggs may experience a higher EST due to their higher heat production compared with small eggs (Lourens et al., 2006). This higher EST may negatively affect the pulmonary vascular development at hatch and may increase mortality associated with ascites in later life.

Furthermore, high EST incubation may reduce the humoral immune response of broiler chickens, making them more vulnerable to infectious diseases and increasing mortality later in life. This hypothesis was not specifically investigated in the current thesis, but the decrease in the bursa

of Fabricius weights at hatch after high EST (38.9°C) from day 7 of incubation onward (Chapter 1) supports this idea. The bursa of Fabricius is a lymphoid organ and plays an important role in antibody production in the chicken (Glick et al., 1956; Dibner et al., 1998). Oznurlu et al. (2010) showed that high (~40.3°C) EST compared with control (~39.2°C) EST from day 10 of incubation onward reduced the weight and changed the morphology of the thymus and bursa of Fabricius, as well as decreased the number of lymphocytes in the blood in the peri- and postnatal period. These effects may lead to immunosuppression later in life. The control treatment in the study by Oznurlu et al. (2010) was incubated at an EST of 39.2°C which is slightly higher than the high EST of 38.9°C used in this thesis. Incubation at a normal EST of 37.8°C may, therefore, further improve the development and functionality of the bursa of Fabricius. However, the hypothesis that high EST suppresses the immune system of broiler chickens requires further investigation.

Body Development

Perinatal Period in Broiler Chickens

Several studies have shown that body development at hatch was decreased in broiler chickens after high EST incubation (Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007; Piestun et al., 2009a). The negative effect of high EST on body development at hatch was expressed by a lower yolk-free body mass (YFBM = body weight – residual yolk). The decrease in YFBM ranged from 0.3 to 3.0 g (0.7 to 8.0% of the YFBM) (Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007; Piestun et al., 2009a; Chapter 1, 3, 4). The decrease in development at hatch was further evidenced by lower relative organ weights in the high EST compared with the normal EST (Lourens et al., 2007; Lekrisompong et al., 2009; Chapter 2, 4). Relative heart weights were consistently found to be reduced at hatch after high EST incubation and these reductions ranged between 17 and 31% (Lourens et al., 2007; Lekrisompong et al., 2009; Chapter 2, 4). Relative lung, liver, and intestinal weights at hatch were sometimes reduced by high EST incubation, but this was not consistent between, or even within, studies (Lourens et al., 2007; Lekrisompong et al., 2009; Chapter 2, 4).

Although the negative effects of high EST on the body development of broiler chickens were found at hatch, it was unknown when these negative effects on body development occurred during incubation. Results presented in Chapter 2 and 3 showed that body weights at day 18 of incubation were comparable between the high (38.9°C) and normal (37.8°C) EST. However, the relative heart, lung, and bursa of Fabricius weights were already negatively affected by high EST incubation at day 18 of incubation (Chapter 2). Relative spleen and liver weights were not affected and relative stomach and intestinal weights were increased at day 18 of incubation in embryos incubated at a high EST compared with normal EST (Chapter 2). The reduced heart weight may be caused by a decreased amount of cell divisions at high temperatures (Romanoff, 1960; Leksrisompong et al., 2007). High temperatures have a positive effect on cell divisions in the heart during the first week of incubation, but this is reversed after approximately 9 days of incubation (Romanoff, 1960; Leksrisompong et al., 2007). The reason why not all organs were affected in the same way by high EST at day 18 of

incubation is unclear. However, it may be due to specific periods during embryogenesis that tissues are sensitive to high EST (Leksrisompong et al., 2007), and these sensitive periods may be related to the growth and maturation of tissues (Decuypere and Michels, 1992).

The decrease in YFBM in broiler embryos incubated at high EST compared with normal EST was found after the chickens hatched (Chapter 3, see Figure 2A). The low YFBM may not be explained by the early hatching time at high EST. Results presented in Chapter 5 showed that the YFBM of layer hatchlings incubated at high EST was still lower after correction for incubation time. The lower YFBM at hatch after high EST incubation may be explained by a the higher utilization of protein for energy instead of growth to compensate for limited glycogen stores at day 18 of incubation (Chapter 1). There are several indications for this hypothesis in the current thesis. First, the efficiency of protein utilization for growth was 3.2% less in embryos incubated in the high (38.9°C) EST compared with the normal (37.8°C) EST from day 7 of incubation (Chapter 1). Second, a tendency for a higher plasma uric acid concentration in the high (38.9°C) EST compared with the normal (37.8°C) EST from day 7 of incubation onward was found at hatch in Chapter 2. A much higher plasma uric acid concentration of 43% was found at day 21.6 of incubation in the high (38.9°C) EST compared with the normal (37.8°C) EST from day 10.5 of incubation onward in Chapter 3. These higher plasma uric acid concentrations indicated that amino acids were deaminated. Third, broiler embryos that hatched after high EST as compared with normal EST incubation had less hepatic glycogen on day 18 of incubation but comparable hepatic glycogen concentrations at hatch (Chapter 1, 3). This result suggested that less hepatic glycogen was made available at high EST incubation during the hatching process and that amino acids were used as an additional glucogenic energy source. Glucogenic amino acids may be used for gluconeogenesis (Hazelwood and Lorenz, 1959) or for immediate ATP production (McArdle et al., 1981).

An additional indication that glucose availability is important during incubation and that this may limit the development and viability of chicken embryos may be found in the effects of *in ovo* feeding (Uni and Ferket, 2004). During *in ovo* feeding, an isotonic solution containing carbohydrates, proteins, or a mixture of both is injected in the amnion of the embryo around day 18 of incubation (Uni and Ferket, 2004). This technique is used to improve the nutritional status of the embryo and was found to increase hatchling weight, immune system development, muscle development, and breast meat yield in later life (Uni and Ferket, 2004; Uni et al., 2005; Foye et al., 2006). Embryos incubated at high EST who seem to have a high glucose demand and low protein efficiency for growth may especially benefit from *in ovo* feeding.

The effect of O_2 concentration during incubation on body development in broiler chickens was investigated together with EST treatments in Chapters 1 and 2. Embryos were exposed to low (17%), normal (21%), or high (25%) O_2 concentrations from day 7 until 19 of incubation. After day 19 of incubation, all eggs were exposed to 21% O_2. This experiment showed that body development increased with an increase in O_2 concentration at day 18 of incubation and that organ development was proportional to body development. The lowest YFBM was found when a low O_2 concentration was combined with a high (38.9°C) EST. The results presented in Chapters 1 and 2 and the study by Lourens et al. (2007) showed that the increase in YFBM was not linearly related to the increase in O_2

concentration after hatch. The difference in YFBM between the low and normal O_2 concentration was higher than that between the normal and high O_2 concentration, which may be related to the efficiency of nutrient utilization for growth. The protein and total energy utilization for growth 12 hours after emergence from the eggshell was less efficient in the low O_2 concentration treatment compared with the normal and high O_2 concentration treatment (Chapter 1).

However, this result may have been interfered with the change in O_2 concentration to 21% for all treatment after day 19 of incubation. Consequently, embryos in the low O_2 concentration were enriched in O_2, and embryos in the high O_2 concentration had reduced O_2 after day 19 of incubation. The low O_2 concentration embryos may have adapted to the low O_2 and enhanced their O_2-carrying capacity and O_2 availability through increases in red blood cells, hemoglobin mass, or blood volume (Baumann et al., 1983; Xu and Mortola, 1989; Azzam and Mortola, 2007; Ramirez et al., 2007). These adaptations probably increased the conversion of residual yolk to YFB after day 19 of incubation, which was shown in Chapter 1. Furthermore, these adaptations may have increased the protein and total energy efficiencies between 12 and 48 hours after emergence at low O_2, whereas birds incubated at normal or high O_2 decreased their protein and total energy utilization for growth in the same period (Chapter 1). However, the YFBM at low O_2 concentration were still lower than the normal O_2 concentrations after hatch.

In contrast to the low O_2 concentration, embryos incubated at a high O_2 concentration may not have been adapted to the lower O_2 concentrations after day 19 of incubation and they may have had difficulties with the lower O_2 availability. This result was evidenced by the lower conversion of residual yolk to YFBM after day 19 of incubation in the high O_2 concentration and may have resulted in comparable YFBM between the normal and high O_2 concentration at hatch (Chapter 1). These results suggest that body and organ development is proportional to the O_2 availability that the embryo experiences during incubation. In addition, differences in O_2 concentration during incubation seem to affect the development of adaptive mechanisms, and these mechanisms might possible influence nutrient utilization and body development.

Perinatal Period in Layer Chickens

The effect of high EST (38.9°C) in the last week of incubation was also investigated in layer embryos in Chapter 5. Results from Chapters 1 and 5 seems to show that the effects of EST on YFBM were comparable between layer and broiler chickens 12 hours after emergence from the eggshell. This finding is in contrast with other studies that compared layer and broiler chickens at hatch and found that body development was lower in layer compared with broiler chickens at hatch (Pal et al., 2002; Ohta et al., 2004; Sato et al., 2006; Everaert et al., 2008; Druyan, 2010). However, the layer and broiler eggs were incubated in the same incubator in all studies, and EST was not maintained at the same level, which may have interfered with the results.

In the current thesis, egg sizes in the layer and broiler study were largely comparable (Chapter 1, 5) and ranged between 60 and 65 g. The high EST had a YFBM of 36.9 ± 0.07 g in the broiler study and 37.0 ± 0.13 g in the layer study 12 hours after emergence from the eggshell. The normal EST had a YFBM of 37.9 ± 0.14 g in the broiler study and 37.7 ± 0.12 g in the layer study 12 hours

after emergence from the eggshell. Residual yolks were larger in embryos incubated at high EST compared with normal EST in both the broiler and layer study. Hepatic glycogen amounts were comparable between the high and normal EST in the broiler chickens 12 hours after emergence from the eggshell (9.6 ± 0.80 mg and 11.7 ± 0.94 mg, respectively), but hepatic glycogen amounts were lower in the high EST compared with normal EST in the layer chickens 12 hours after emergence from the eggshell (7.3 ± 0.36 mg and 11.2 ± 0.40 mg, respectively). In addition, a tendency for higher plasma uric acid concentration in the high EST compared with the normal EST was found in the broiler chickens and no difference in plasma uric acid concentrations between EST treatments were found in layer chickens.

These results may indicate that layer embryos may be better able to mobilize hepatic glycogen than broiler embryos at the end of incubation at high EST and that amino acids may not be required to obtain additional energy. The reduction in development in the layer embryos after high EST incubation may therefore be explained by other changes in nutrient absorption or nutrient utilization that may differ from broiler embryos. Broilers are selected for high daily body weight gain and layers are selected for egg production, which may affect the protein and fat deposition in their YFBM (Pal et al., 2002). A difference in body composition may also change nutrient utilization for growth and ATP production between broiler and layer embryos during incubation in both normal and high EST. However, high EST was only applied for one week in the layer study (Chapter 5), whereas it was applied for 2 weeks in the broiler study (Chapter 1). The shorter period of high EST in the layer embryos may have decreased the glucose requirement in the layer embryos compared with the broiler embryos during incubation. In summary, the effects of high EST reduced body development in both layer and broiler hatchlings, but the underlying mechanisms behind reduced body development may differ between layer and broiler hatchlings and requires further investigation.

Growout Period

The effect of suboptimal conditions on body development in later life has been investigated in several studies using high EST incubation (Lourens et al., 2005; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2009). In general, a negative effect of high EST on body weight in later life was found (Lourens et al., 2005; Joseph et al., 2006; Leksrisompong et al., 2009). However, the extent of the effect of the high EST on body weight in broiler chickens was variable between studies (Lourens et al., 2005; Joseph et al., 2006; Leksrisompong et al., 2009), which may be explained by the duration and extent of the EST. The longer and higher EST was applied during incubation, the larger the decrease in body development at hatch and probably throughout the growout period (Leksrisompong et al., 2007). A larger decrease in body development may also result in a larger decrease in important supply organs such as the heart and gastro-intestinal tract (Chapter 2, 5), which may impair growth performance due to a possible decrease in the capacity of these organs. The duration that chickens after hatch are exposed to high temperatures in the incubator seems to influence the decrease in body development as well. Results presented in Chapter 3 showed that

the difference in YFBM become larger in the posthatch period when chickens were still exposed to a high or normal temperature in the incubator.

The differences in body weight during the growout period between chickens incubated in a high EST compared with a normal EST seemed to diminish over time, as shown by studies of Lourens et al. (2005) and Leksrisompong et al. (2009), and even disappeared in the study of Joseph et al. (2006) from day 21 of age. Results presented in Chapter 4 also showed that the difference in body weight between EST treatments became smaller throughout the growout period. The difference in bird weight between the high (38.9°C) EST and normal (37.8°C) EST was 8.4% (3.4 g) at hatch and this diminished to 1.6% (48.2 g) at day 42 of age when the chickens were maintained in the normal growout temperatures. Furthermore, Hulet et al. (2007) found that body weights were on average 48.1 g lower at 44 days of age in birds incubated from day 16 of incubation onward at an EST of 39.7°C compared with an EST of 37.5°C. However, the same study showed that an EST of 38.6°C in the same period increased body weight at 44 days of age by 49.5 g compared with an EST of 37.5°C. The reduction in the body weight difference in later life and the improvement in body weight with the relatively high EST of 38.6°C in the study of Hulet et al. (2007) may be related to compensatory growth or the temperature that birds experience during the growout period. Different studies have shown that broiler chickens incubated at high temperatures during the last 2 weeks of incubation can better cope with high temperatures during their growout period and maintain their growth (Yahav et al., 2004; Piestun et al., 2008a; Yalçin et al., 2008b, 2010). This finding raises the question of how much broiler chickens experience high temperatures at the end of their growout period. Normal growout temperatures are maintained around 20°C in the last week of the growout period (Lubritz and McPherson, 1994; Ipek and Sahan, 2006; Hulet et al., 2007; Akşit et al., 2008; Özkan et al., 2010), but this temperature may be too high for chickens after 5 weeks of age because they grow around 600 g a week (Lubritz and McPherson, 1994; Özkan et al., 2010). An indication that a temperature of 20°C is relatively high for broilers at the end of their growout period may be found in the results of Chapter 4. In the normal EST, body weights at 6 weeks of age were 156 g (~5%) lower in chickens grown in cold compared with regular temperature. However, the growth in the fifth week of chickens incubated at a normal EST did not differ between the cold (702 ± 26 g) and regular (671 ± 13 g) growout temperature ($P > 0.05$; Chapter 4). This result indicates that 10°C does not negatively affect growth after the fifth week of age or is within the thermal neutral zone of broiler chickens. Lubritz and McPherson (1994) also found that negative effects of cold growout temperatures (~15 to 18°C) on body weight diminished after 5 weeks of age. Results in Chapter 4 and the study by Lubritz and McPherson (1994) suggest that the growout temperature of 20°C is relatively high at the end of the growout period. Chickens incubated at high EST compared with normal EST may be better able to cope with these relatively high temperatures (Yahav et al., 2004; Piestun et al., 2008b; Yalçin et al., 2008b, 2010). This coping mechanism may be further expressed in the smaller weight difference between the high and normal EST reared at a normal growout temperature at week 6 of age (43.8 g) compared with week 5 of age (59.8 g) (Chapter 4).

In addition to the lower body weight in later life in broiler chickens after high EST compared with normal EST incubation, the efficiency of feed utilization was also negatively affected after high

EST compared with normal EST incubation (Chapter 5). Feed conversion ratios (FCR) adjusted to a 2 kg body weight were 0.04 higher in chickens incubated at high (38.9°C) EST compared with normal (37.8°C) EST from day 7 of incubation onward in Chapter 5. This finding is not consistent with results of Hulet et al. (2007), who found that chickens incubated at an EST of 38.6°C compared with 37.5 or 39.7°C from day 17 until 21 of incubation had the lowest FCR adjusted to a 2 kg body weight at 44 days of age. The discrepancy between the two studies in FCR is unclear but may be related to the duration of the EST and the effect on body and gastro-intestinal tract development. Body weights at hatch were not negatively affected by the high EST of 38.6 or 39.7°C in the study of Hulet et al. (2007), whereas body weights were reduced by 3.4 g at hatch after high EST (38.9°C) incubation in Chapter 5. Because body weights were not largely affected in the study of Hulet et al. (2007) due to high EST, relative weights of the gastro-intestinal tract may also not be greatly impaired. Feed conversion ratios may only be increased in later life when gastro-intestinal development or maturation (Wineland et al., 2006) is impaired at hatch after high EST incubation.

Although high EST impaired body weights and FCR in Chapter 5, relative breast meat yield increased by 1.0% at high EST. Joseph et al. (2006) and Hulet et al. (2007) found no indications for a high relative breast meat yield in their studies using high EST incubation, but the high EST were applied for a shorter period than that used in Chapter 5. However, Collin et al. (2007) and Piestun et al. (2009b) showed that short periods of high temperatures during incubation can increase muscle growth in the growout period. The positive effect of high EST incubation on muscle growth during later life is probably related to increases in muscle cell proliferation and cell differentiation during incubation (Piestun et al., 2009b).

Conclusions

From the current thesis, it can be concluded that high EST or low O_2 availability from the first week of incubation onward negatively affect the survival and development of chickens from the perinatal period until the age of slaughter. These negative effects can be partly explained by changes in nutrient utilization and metabolite levels.

High EST increased the glucose oxidation in broiler embryos during the second half of incubation. This was expressed in lower hepatic glycogen at day 18 of incubation after high EST incubation, which may increase embryo mortality at the end of incubation.

Body development of broiler embryos was reduced at hatch after high EST incubation because of a lower efficiency in protein utilization for growth. This lower efficiency was possibly due to the use of amino acids as a glucogenic energy source to compensate for the limited hepatic glycogen stores.

In broiler embryos, body development was proportional to the O_2 availability during incubation. In addition, differences in O_2 concentration during incubation seem to affect the development of adaptive mechanisms, and these mechanisms might possible influence nutrient utilization and body development.

Finally, effects of EST were long-lasting in broiler chickens expressed by a lower body weight and a higher incidence of ascites, which is probably the result of the reduced heart and lung development at hatch.

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Summary

Several studies have shown that suboptimal incubation conditions, such as a high eggshell temperature (EST) or low O_2 availability, can negatively affect the survival and development of chicken embryos. However, the physiological mechanisms that may explain these effects are largely unknown. Furthermore, the negative effects of suboptimal incubation conditions may have long-lasting effects on survival and development of chickens throughout their production cycle, which has not been intensively investigated. Therefore, the first aim of this thesis was to investigate effects of EST and O_2 availability during incubation on the survival, development, physiology, and nutrient utilization of chicken embryos. The second aim was to investigate the long-lasting effects of suboptimal EST on the survival and subsequent performance of broiler chickens.

In the first experiment, effects of a high (38.9°C) EST or a normal (37.8°C) EST combined with a low (17%), normal (21%), or high (25%) O_2 concentration from day 7 until 19 of incubation were investigated in broiler embryos (Chapter 1, 2). After day 19 of incubation, the EST difference was maintained, but the O_2 concentration was 21% for all treatments. A high EST, compared with a normal EST, reduced the yolk-free body mass (YFBM = body weight – residual yolk) after emergence from the eggshell, possibly as a result of the reduced incubation duration, the lower weight of supply organs (i.e., heart and lung), or the lower efficiency of protein utilization for growth. The amino acids that were not used for development in the high EST may be used as an energy source to compensate for the limited hepatic glycogen stores that were found at day 18 of incubation in the high EST. Hepatic glycogen is used as an energy source during the energy-demanding hatching process that starts around day 19 of incubation. Although hepatic glycogen was lower at day 18 of incubation in the high EST compared with the normal EST, no differences in hepatic glycogen were found between the EST treatments at 12 and 48 hours after emergence from the eggshell. This result may indicate that less hepatic glycogen was used at the end of incubation in the high EST treatment and that glucogenic amino acids were used as an additional glucogenic energy source. The deamination of amino acids was further indicated by a tendency for a higher plasma uric acid concentration in the high EST treatment compared with the normal EST treatment.

The results concerning the effect of O_2 concentration showed that YFBM increased with an increase in O_2 concentration at day 18 of incubation and that organ weights were proportional to the YFBM. The differences in YFBM at 12 hours after emergence from the eggshell were larger between the low and normal O_2 than they were between the normal and high O_2, and this difference might be due to the lower efficiency in nutrient utilization for growth at the low O_2 concentration. The effects of O_2 that were found at 12 hours after emergence from the eggshell were less pronounced at 48 hours after emergence from the eggshell. When O_2 was shifted to 21% for all treatments at day 19 of incubation, embryos incubated at a low O_2 seemed to use nutrients more efficiently than those incubated at a normal or high O_2. This result may suggest that embryos develop adaptive mechanisms that still continue in the posthatch period.

An additional negative effect on survival occurred when embryos were exposed to both a high EST and low O_2 concentration. Possible explanations for the lower survival include the reduced body development, the higher incidence of malpositions, or the lower nutrient availability for hatching (e.g., hepatic glycogen).

The results of the first experiment showed that hepatic glycogen stores were negatively affected by high EST incubation, and this suggests that glucose metabolism is affected by a high EST. Therefore, the effect of a high EST on glucose metabolism was investigated using [U-^{13}C] glucose and measuring ^{13}C enrichments in the intermediate pools and the end-products of glucose metabolism in the second experiment (Chapter 3). Embryos were incubated at a high (38.9°C) or normal (37.8°C) EST from day 10.5 of incubation onward and were injected with a single bolus of [U-^{13}C]glucose in the chorio-allantoic fluid at day 17.5 of incubation. Compared with the normal EST, the high EST showed a higher ^{13}C enrichment in expired CO_2 from day 17.6 until 17.8 of incubation, a tendency for a higher recovery of ^{13}C enrichment in the expired CO_2, a higher ^{13}C enrichment in plasma lactate at day 17.8 of incubation, and a lower ^{13}C enrichment in hepatic glycogen at day 18.8 of incubation. These results showed that glucose oxidation from the initial [U-^{13}C]glucose was larger in the high EST compared with the normal EST, and this may explain the lower hepatic glycogen concentrations that were found at day 18.2 and 18.8 of incubation in the high EST treatment. The results indicated again that amino acids were used as a glucogenic energy source at a high EST. Hepatic glycogen use seemed to be smaller during the hatching process and plasma uric acid concentrations were 43% higher at day 21.6 of incubation in the high EST. The use of glucogenic amino acids for energy instead of body development may explain the decrease in body development that was observed specifically after hatch in the high EST compared with the normal EST.

The long-lasting effects of suboptimal incubation conditions in broiler chickens on subsequent performance and mortality have not been intensively described in literature. Broiler embryos were therefore incubated at a high (38.9°C) EST or normal (37.8°C) EST from day 7 of incubation onward and were raised at a normal or cold growout temperature; the latter was used to induce ascites (Chapter 4). Body weights were lower at hatch and continued to be lower during the growout period in the high EST compared with the normal EST. During the growout period, total mortality was 4.1% higher and mortality associated with ascites was 3.8% higher in the high EST compared with the normal EST treatment. Mortality associated with ascites may be related to the 26% lower heart weights at hatch in the high EST compared with the normal EST treatment.

Because studies on the effect of incubation conditions on layer embryos are limited, the effect of a high (38.9°C) EST compared with a normal (37.8°C) EST and an increased O_2 availability, by puncturing a hole in the air cell, was investigated in layer embryos in the last week of incubation (Chapter 5). Embryo mortality was not affected by the EST or hole treatment. Yolk-free body mass was lower and residual yolk weight was higher in the high compared with the normal EST at 12 hours after emergence. Yolk-free body mass did not differ between hatchlings with or without a hole in the air cell during the last week of incubation, but residual yolk weight was slightly lower in hatchlings that had a hole in their air cell during the last week of incubation. Hepatic glycogen was lower in the high EST compared with the normal EST at 12 hours after emergence from the eggshell, and this effect may be related to the shorter hatching process that was found at the high EST. Hepatic glycogen levels were lower in hatchings with a hole in the air cell during the last week of incubation compared with the hatchlings without a hole in the air cell, and this may be related

to the longer period between external pipping and hatching in the hatchlings that received a hole in their air cell. A high EST negatively affected hatchling development and seemed to change the carbohydrate metabolism in layer embryos. The effect of a hole in the air cell was limited.

From the current thesis, it can be concluded that high EST or low O_2 availability from the first week of incubation onward negatively affect the survival and development of chickens from the perinatal period until the age of slaughter. These negative effects can be partially explained by changes in nutrient utilization and metabolite levels. High EST increased the glucose oxidation in broiler embryos during the second half of incubation. This was expressed in lower hepatic glycogen at day 18 of incubation after high EST incubation, which may increase embryo mortality at the end of incubation. Body development of broiler embryos was reduced at hatch after high EST incubation because of a lower efficiency in protein utilization for growth. This lower efficiency was possibly due to the use of glucogenic amino acids as a glucogenic energy source to compensate for the limited hepatic glycogen stores. In broiler embryos, body development was proportional to the O_2 availability during incubation. Differences in O_2 concentration during incubation seems to affect the development of adaptive mechanisms, and these mechanisms might possible influence nutrient utilization and body development. Finally, effects of high EST were long-lasting in broiler chickens expressed by a lower body weight and a higher incidence of ascites, which is probably the result of the reduced heart and lung development at hatch.

Samenvatting

Verschillende studies hebben laten zien dat suboptimale condities tijdens het broedproces, zoals een hoge eischaaltemperatuur of een lage zuurstofbeschikbaarheid, een negatief effect heeft op de overleving en de ontwikkeling van het kippenembryo. De fysiologische processen die deze negatieve effecten mogelijk kunnen verklaren zijn grotendeels onbekend. De negatieve effecten van suboptimale broedcondities hebben wellicht niet alleen op de korte termijn maar tevens op de lange termijn gevolgen voor de overleving en de ontwikkeling van het kuiken. Het eerste doel van dit proefschrift was om te onderzoeken wat de effecten van eischaaltemperatuur en zuurstofbeschikbaarheid zijn op de overleving, ontwikkeling, fysiologie en het nutriëntengebruik van het kippenembryo. Het tweede doel van dit proefschrift was om te onderzoeken wat de lange termijn effecten zijn van suboptimale eischaaltemperaturen op de overleving en de groei van vleeskuikens.

In het eerste experiment is gekeken naar het effect van een hoge (38.9°C) of een normale (37.8°C) eischaaltemperatuur gecombineerd met een lage (17%), normale (21%) of hoge (25%) zuurstofconcentratie van dag 7 tot 19 van het broedproces op de ontwikkeling van vleeskuikenembryo's (Hoofdstuk 1, 2). Vanaf dag 19 van het broedproces werd het verschil in eischaaltemperatuur gehandhaafd, maar werd het zuurstofgehalte voor alle embryo's op 21% gezet. Een hoge vergeleken met een normale eischaaltemperatuur gaf een lager dooiervrij lichaamsgewicht (= lichaamsgewicht - dooierrest) bij uitkomst. Mogelijke oorzaken hiervoor zijn de kortere tijd die het broedproces in beslag nam, het lagere gewicht van organen die het lichaam van bloed of zuurstof voorzagen, zoals het hart en de longen, of de lagere efficiëntie waarmee eiwitten gebruikt werden voor de groei van het embryo. De eiwitten die niet voor de ontwikkeling van het embryo werden gebruikt, werden wellicht als energiebron gebruikt, omdat leverglycogeen mogelijk limiterend werd op dag 18 van het broedproces. Leverglycogeen wordt gebruikt als energiebron tijdens het energieverslindende uitkomstproces dat omstreeks dag 19 van het broedproces begint. Alhoewel een lager leverglycogeengehalte werd gevonden op dag 18 van het broedproces in de hoge vergeleken met de normale eischaaltemperatuur, was er geen verschil in leverglycogeen tussen de eischaaltemperatuur behandelingen op 12 en 48 uur na uitkomst. Dit resultaat suggereert dat er minder leverglycogeen werd gebruikt aan het einde van het broedproces in de hoge vergeleken met de normale eischaaltemperatuur en dat wellicht glucogene aminozuren als energiebron werden gebruikt. De trend voor een hoger plasma urinezuurconcentratie in de hoge vergeleken met de normale eischaaltemperatuur suggereert ook dat aminozuren werden afgebroken.

Een hogere zuurstofconcentratie tijdens het broedproces zorgde op dag 18 van het broedproces voor een hoger dooiervrij lichaamsgewicht. De organen uitgedrukt als percentage van het dooiervrij lichaamsgewicht waren niet verschillend tussen de zuurstofconcentraties. Het verschil in dooiervrij lichaamsgewicht op 12 uur na uitkomst was groter tussen de lage en normale zuurstofconcentratie dan tussen de normale en hoge zuurstofconcentratie. Dit verschil kan wellicht veroorzaakt zijn door de lagere efficiëntie waarmee nutriënten gebruikt werden voor de groei van het embryo in de lage zuurstofconcentratie. De effecten tussen de zuurstofconcentraties waren minder groot op 48 uur na uitkomst vergeleken met 12 uur na uitkomst. De embryo's die bij een laag zuurstofgehalte uitgebroed werden, konden waarschijnlijk hun nutriënten efficiënter gebruiken

toen het zuurstofgehalte naar 21% werd gebracht op dag 19 van het broedproces dan de embryo's die bij een normaal of hoog zuurstofgehalte zaten. Dit resultaat suggereert dat embryo's adaptatie mechanimsen kunnen ontwikkelen die mogelijk doorwerken tot in de periode na het uitkomen als de kuikens bloot worden gesteld aan andere omstandigheden.

De hoogste embryonale sterfte trad op als de embryo's werden blootgesteld aan een hoge eischaaltemperatuur gecombineerd met een lage zuurstofconcentratie tijdens het broeden. Mogelijke verklaringen hiervoor zijn een slechte lichaamsontwikkeling, malposities van het embryo of een lage beschikbaarheid van essentiële nutriënten voor het uitkomstproces, zoals leverglycogeen.

De resultaten uit het eerste experiment lieten zien dat leverglycogeen afnam bij een hoge eischaaltemperatuur en dit suggereert dat het glucosemetabolisme wordt beïnvloed door de eischaaltemperatuur. Dit is de reden waarom we in het tweede experiment hebben gekeken naar het effect van een hoge eischaaltemperatuur op het glucosemetabolisme van vleeskuikenembryo's. In het experiment werd gebruik gemaakt van [U-^{13}C]glucose en werden ^{13}C verrijkingen in de tussen- en eindproducten van glucosemetabolisme gemeten (Hoofdstuk 3). Embryo's werden gebroed op een hoge (38.9°C) of normale (37.8°C) eischaaltemperatuur vanaf dag 10.5 van het broedproces en werden eenmalig geïnjecteerd met een bolus van [U-^{13}C]glucose in het chorio-allantois op dag 17.5 van het broedproces. De hoge vergeleken met de normale eischaaltemperatuur had een hogere ^{13}C verrijking in uitgeademd CO_2 van dag 17.6 tot 17.8 van het broedproces, een trend voor een hogere totale hoeveelheid ^{13}C verrijking in uitgeademd CO_2 van de initiële dosis [U-^{13}C]glucose, een hogere ^{13}C verrijking in plasma lactaat op dag 17.8 van het broedproces en een lagere ^{13}C verrijking in leverglycogeen op dag 18.8 van het broedproces. Al deze resultaten suggereren dat glucose-oxidatie van de initiële hoeveelheid [U-^{13}C]glucose hoger was in de hoge vergeleken met de normale eischaaltemperatuur en dat verklaart mogelijk de lagere leverglycogeen concentratie die gevonden werd op dag 18.2 en 18.8 van het broedproces bij de hoge eischaaltemperatuur. Verder werden er wederom indicaties gevonden dat aminozuren werden gebruikt als een glucogene energiebron bij een hoge eischaaltemperatuur. Bij de hoge vergeleken met de normale eischaaltemperatuur leek het gebruik van leverglycogeen lager tijdens het uitkomen en de concentratie urinezuur in het plasma was 43% hoger op dag 21.6 van het broedproces. Het gebruik van glucogene aminozuren voor energie in plaats van lichaamsontwikkeling kan mogelijk de lagere lichaamsgewichten die voornamelijk werden gevonden na de uitkomst van de vleeskuikens verklaren in de hoge ten opzichte van de normale eischaaltemperatuur.

De effecten van suboptimale broedcondities op groei en sterfte in het latere leven van vleeskuikens is niet uitvoerig beschreven in de literatuur. Vleeskuikenembryo's werden daarom gebroed bij een hoge (38.9°C) of normale (37.8°C) eischaaltemperatuur vanaf dag 7 van het broedproces (Hoofdstuk 4). De kuikens groeiden daarna op bij een normaal of koud temperatuursschema, waarbij het laatste gebruikt werd om ascites op te wekken. Lichaamsgewichten van de vleeskuikens die gebroed waren bij een hoge vergeleken met een normale eischaaltemperatuur waren lager bij uitkomst en bleven lager gedurende de zes-weekse groeiperiode. De totale sterfte was 4.1% hoger en de sterfte gerelateerd aan ascites was 3.8% hoger in de hoge vergeleken met de normale

eischaaltemperatuur. De sterfte gerelateerd aan ascites werd mogelijk veroorzaakt door de reductie in hartgewichten (26%) die bij uitkomst werden gevonden in de hoge ten opzichte van de normale eischaaltemperatuur.

Er is weinig bekend over het effect van suboptimale broedcondities bij leghenembryo's. Daarom werd het effect van een hoge (38.9°C) eischaaltemperatuur vergeleken met een normale (37.8°C) eischaaltemperatuur bij leghenembryo's (Hoofdstuk 5). Tevens werd de zuurstofbeschikbaarheid verhoogd bij de helft van de eieren door middel van een gaatje in de luchtkamer. Deze twee behandelingen werden toegepast in de laatste week van het broedproces. Embryonale sterfte werd niet beïnvloed door de hoge eischaaltemperatuur of een gaatje in de luchtkamer. In de hoge vergeleken met de normale eischaaltemperatuur op 12 uur na uitkomst was het dooiervrij lichaamsgewicht lager en het gewicht van de dooierrest hoger. Het dooiervrij lichaamsgewicht was niet verschillend tussen kuikens die een gaatje of geen gaatje hadden in de luchtkamer tijdens het broeden. De hoeveelheid leverglycogeen was lager in de hoge dan in de normale eischaaltemperatuur op 12 uur na uitkomst en dit effect kan mogelijk verklaard worden door de kortere tijd die het uitkomen in beslag nam in de hoge eischaaltemperatuur. De hoeveelheid leverglycogeen was lager in kuikens die een gat in de luchtkamer hadden dan kuikens die geen gat in de luchtkamer hadden tijdens het broedproces. Dit kan wellicht verklaard worden door de langere periode tussen extern aanpikken en uitkomen in de kuikens die een gaatje in de luchtkamer hadden tijdens het broedproces. Een hoge eischaaltemperatuur verminderde de ontwikkeling bij uitkomst en leek het glucosemetabolisme te veranderen in de leghenembryo's. Het effect van een gaatje in de luchtkamer was minimaal.

Aan de hand van de resultaten van dit proefschrift kan worden geconcludeerd dat een hoge eischaaltemperatuur of een lage zuurstofbeschikbaarheid vanaf de eerste week van het broedproces een negatief effect heeft op de overleving en de ontwikkeling van kippenembryo's. Dit heeft effect van de perinatale periode tot aan de slachtleeftijd. Deze negatieve effecten kunnen gedeeltelijk worden verklaard door veranderingen in het gebruik van nutriënten en concentraties van verschillende bloedmetabolieten zoals glucose en urinezuur. Hoge eischaaltemperaturen verhoogden de glucose-oxidatie in vleeskuikens tijdens de tweede helft van het broedproces en dit veroorzaakte waarschijnlijk een lager leverglycogeengehalte op dag 18 van het broedproces. De ontwikkeling van de vleeskuikens was minder bij uitkomst na het broeden op een hoge eischaaltemperatuur door een lagere efficiëntie van het gebruik van eiwitten voor groei. Deze lagere eiwitefficiëntie was mogelijk een gevolg van het gebruik van aminozuren voor glucogene energie om zo de limiterende leverglycogeengehaltes te compenseren die optraden bij hoge eischaaltemperaturen. De ontwikkeling van vleeskuikenembryo's was grotendeels evenredig met de zuurstofbeschikbaarheid tijdens het broeden. Er leken echter adaptatie mechanismen op te treden door de verschillende zuurstofconcentraties tijdens het broeden en deze mechanismen beïnvloeden mogelijk het nutriëntengebruik en de ontwikkeling van het kuiken. Tenslotte gaf een hoge eischaaltemperatuur een lager lichaamsgewicht en een hogere sterfte als gevolg van ascites tijdens de zes-weekse groeiperiode van vleeskuikens. Het verhoogde aantal ascites gevallen werd mogelijk veroorzaakt door de reductie in hart- en longontwikkeling na het broeden op een hoge eischaaltemperatuur.

Curriculum Vitae

Curriculum Vitae (English)

Rosanne (Roos) Molenaar was born on December 21 1979 in Delft and was raised in Wateringen. In 1998, she graduated from high school 'Christelijk Lyceum Delft'. In the same year, she started with her BSc Animal Husbandry at Hogeschool Delft with the specialization Animal Health. During her study, she graduated for the study veterinarian assistent and she did an internship at the Adaptation Physiology Group at Wageningen University in Wageningen. She did her major thesis at the same department and investigated the location and migration of embryos in the early gestation of the Meishan pig.

Rosanne Molenaar graduated in 2002 and started with the MSc Animal Sciences at Wageningen University in Wageningen. Her major specialization was in Animal Health and Welfare and her second specialization was in Animal Nutrition. For the specialization Animal Health and Welfare, she investigated the effect of egg size on embryo development, heat production, and chick quality. For her second specialization Animal Nutrition, she investigated the efficiency, distribution, and faecal excretion of a copper oxide wire particle bolus in red deer at the Institute of Veterinary, Animals and Biomedical Sciences at Massey University in Palmerston North, New Zealand.

Rosanne Molenaar graduated for her MSc in 2005 and started as a junior researcher at HatchTech Incubation Technology B.V. in Veenendaal. In October 2005, she started as a PhD student at the Adaptation Physiology Group of Wageningen University. The results of her study are described in the current thesis. During her PhD project, she continued her activities at the research group at HatchTech Incubation Technology B.V. After graduation, she will continue her activities as a senior researcher at the research group of HatchTech Incubation Technology B.V. in Veenendaal.

Curriculum Vitae (Nederlands)

Rosanne (Roos) Molenaar werd geboren op 21 december 1979 te Delft en groeide op in Wateringen. In 1998 behaalde zij haar VWO diploma aan het Christelijk Lyceum Delft. In september van het jaar 1998 begon ze aan de studie Dier- en Veehouderij met de specialisatie Diergezondheidszorg aan de Hogeschool Delft te Delft. Tijdens deze studie heeft ze het diploma voor MBO Dierenartsassistent Paraveterinair behaald en heeft ze stage gelopen bij de leerstoelgroep Adaptatiefysiologie van Wageningen University te Wageningen. Tijdens haar afstudeervak bij dezelfde leerstoelgroep heeft ze gekeken naar de locatie en migratie van embryo's tijdens de vroege dracht bij het Meishan varken.

In 2002 is Rosanne Molenaar afgestudeerd en begonnen met de opleiding Dierwetenschappen met als hoofdspecialisatie Diergezondheid en Welzijn en als tweede specialisatie Diervoeding aan de Wageningen University te Wageningen. Voor haar hoofdspecialisatie deed ze onderzoek naar het effect van eigrootte op embryonale ontwikkeling, warmteproductie en kuikenkwaliteit. Voor haar tweede specialisatie Diervoeding deed ze onderzoek naar de efficiëntie, distributie en fecale excretie van koper oxide draadjes toegediend als een bolus bij herten. Dit onderzoek vond plaats aan het Institute of Veterinary, Animals and Biomedical Sciences aan de Massey Universiteit in Palmerston North in Nieuw Zeeland.

In 2005 is ze afgestudeerd en begonnen als junior onderzoeker op de onderzoeksafdeling van HatchTech Incubation Technology B.V. in Veenendaal. In oktober van dat jaar begon ze als promovenda bij de leerstoelgroep Adaptatiefysiologie van de Wageningen University te Wageningen aan het onderzoek dat is beschreven in dit proefschrift. Tevens bleef ze werkzaam bij HatchTech Incubation Technology B.V. Na het afronden van het promotie onderzoek blijft Rosanne Molenaar werkzaam op de onderzoeksafdeling van HatchTech Incubation Technology B.V.

Publications

Refereed Scientific Journals

Soede, N. M., M. J. W. van Sleuwen, R. Molenaar, F. W. Rietveld, W. P. G. Schouten, W. Hazeleger, and B. Kemp. 2006. Influence of repeated regrouping on reproduction in gilts. Anim. Reprod. Sci. 96:133-145.

Lourens, A., R. Molenaar, H. van den Brand, M. J. W. Heetkamp, R. Meijerhof, and B. Kemp. 2006. Effect of egg size on heat production and the transition of energy from egg to hatchling. Poult. Sci. 85:770-776.

Castillo-Alcala, F., P. R. Wilson, R. Molenaar, and N. Lopez-Villalobos. 2007. Efficacy, distribution and faecal excretion of copper oxide wire particles in a novel bolus in red deer (*Cervus elaphus*). N. Z. Vet. J. 55:81-86.

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Molenaar, R., S. de Vries, I. van den Anker, R. Meijerhof, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and a hole in the air cell on the perinatal development and physiology of layer hatchling. Poult. Sci. 89:1716-1723.

Molenaar, R., R. Meijerhof, I. van den Anker, M. J. W. Heetkamp, J. J. G. C. van den Borne, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. Poult. Sci. 89:2010-2021.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, and H. van den Brand. 2010. Meeting embryonic requirements of broilers throughout incubation: A review. Rev. Bras. Cienc. Avic. 12:137-148.

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Molenaar, R., I. A. M. Reijrink, R. Meijerhof, and H. van den Brand. 2007. Correlation between chick length and chick weight at hatch and slaughter weight and breast yield in broilers. Book of abstracts of the 29th Poultry Science Symposium Biology of Breeding Poultry. 23-25 July, Edinburgh, Scotland. pp. 40.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, and H. van den Brand. 2007. Relationship between chick length and chick weight at hatch and slaughter weight and breast meat yield in broilers. Book of Abstracts of the 3th Workshop on Fundamental Physiology and Perinatal Development in Poultry, 5-8 October, Berlin, Germany. pp. 29.

Molenaar, R., I. A. M. Reijrink, R. Meijerhof, J. K. W. M. Sparla, and P. J. A. Wijtten. 2008. Effect of protein and energy level in feed on posthatch chick performance. Book of Abstracts of International Poultry Scientific Forum, 21-22 January, Atlanta, Georgia, USA. pp. 12.

Molenaar, R., L. der Kinderen, R. Meijerhof, and H. van den Brand. 2008. The effect of post-hatch feed on chick development. Book of Abstracts of the 23rd World's Poultry Congress, 30 June-4 July, Brisbane, Queensland, Australia. pp. 324.

Molenaar, R., R. Meijerhof, B. Kemp, and H. van den Brand. 2009. The effect of eggshell temperature on malpositions, protein energy and heart weight in broiler embryos. Book of Abstracts of the 4th Workshop on Fundamental Physiology and Perinatal Development in Poultry, 10-12 September, Bratislava, Slovakia. pp. 31.

Molenaar, R., R. Meijerhof, B. Kemp, and H. van den Brand. 2009. High eggshell temperatures: A matter of life and death importance? Book of Abstracts of the Incubation and Fertility Research Group, 7-8 July, Norwich, UK. pp. 25.

Molenaar, R., J. J. G. C. van den Borne, E. Hazejager, R. Meijerhof, B. Kemp, and H. van den Brand. 2010. Glucose oxidation in chicken embryos incubated at a normal or high eggshell temperature - Preliminary results of a tracer study. Proceedings of the Incubation and Fertility Research Group, 23 August, Tours, France.

Molenaar, R., S. de Vries, I. van den Anker, R. Meijerhof, B. Kemp, and H. van den Brand. 2010. Effect of eggshell temperature and drilling a hole in the air cell on survival and development in layer hatchlings. Book of Abstracts of the XIIIth European Poultry Conference, 23-27 august 2010, Tours, France. pp. 496.

Other publications

Molenaar, R. The impact of high eggshell temperatures during incubation. 2010. International Hatchery Practice 24, number 4, pp. 17-19.

Training and supervision plan

The basic package (3.0 ECTS)

WIAS Introduction Course	2006
WIAS Course on Philosophy of Science and Ethics	2007

International conferences (7.2 ECTS)

XIIth European Poultry Conference, Verona, Italy	2006
Poultry Science Association 95th Annual meeting, Edmonton, Canada	2006
Poultry Science Symposium Biology of Breeding Poultry Congress, Edinburgh, UK	2007
International Poultry Scientific Forum, Atlanta, USA	2008
XXIIIth World's Poultry Congress, Brisbane, Australia	2008
IFRG meeting, Norfolk, UK	2009
IFRG meeting, Tours, France	2010
XIIIth European Poultry Conference, Tours, France	2010

Seminars and workshops (4.2 ECTS)

The 2nd combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Berlin, Germany	2005
WIAS Science Day, Wageningen, the Netherlands	2006
The 3rd combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Berlin, Germany	2007
WIAS Science Day, Wageningen, the Netherlands	2008
Studiedag Agrivaknet, GGL en WPSA, Wijchen, the Netherlands	2008
The 4th combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2009
WIAS Science Day, Wageningen, the Netherlands	2009
Studiedag Agrivaknet, GGL en WPSA, Veldhoven, the Netherlands	2009
WIAS Science Day, Wageningen, the Netherlands	2010
ANR Forum 2010, Lelystad, the Netherlands	2010
Incubation 2010, Utrecht, the Netherlands	2010

Presentations (10.0 ECTS)

Poster at Poultry Science Symposium Biology of Breeding Poultry Congress, Edinburgh, UK	2007
Oral presentation at The 3rd combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Berlin, Germany	2007
Oral presentation at International Poultry Scientific Forum, Atlanta, USA	2008
Oral presentation at XXIIIth World's Poultry Congress, Brisbane, Australia	2008

Oral presentation at IFRG meeting, Norfolk, UK	2009
Oral presentation at WIAS Science day, Wageningen, the Netherlands	2010
Oral presentation at ANR Forum 2010, Lelystad, the Netherlands	2010
Oral presentation at The 4th combined Workshop of Fundamental Physiology of the European Working Group of Physiology and Perinatal Development in Poultry, Bratislava, Slovakia	2010
Oral presentation at IFRG meeting, Tours, France	2010
Poster presentation at XIIIth European Poultry Conference, Tours, France	2010

In-depth studies (6.4 ECTS)

Incubation Training, Penn State University, State College, USA	2005
Summercourse Glycosciences, Wageningen	2006
Design of Animal Experiments	2007
Poultry Discussion Group	2007-2008
Epigenesis and Epigenetics	2008
Statistics for the Life Science	2008

Professional Skills Support Courses (5.7 ECTS)

PhD Competence Assessment	2005
Project and Time Management	2006
Theatervaardigheden en Presenteren	2006
Working with EndNote	2006
Techniques for Presenting and Writing a Scientific Paper	2006
Supervising MSc thesis work	2006
Scientific Writing	2009
NWO Talents day	2010

Research Skills Training (3.0 ECTS)

Preparing own PhD research proposal	2006

Didactic Skills Training (9.5 ECTS)

Supervising 4 MSc students	2007-2009
Lecture Adaptation Physiology I, Wageningen, the Netherlands	2009
Lecture Penn State University, State College, USA	2009
Eight-week course 'Module Veldwerk' at high school Pantarijn, Wageningen, the Netherlands	2009

Management Skills Training (2.0 ECTS)

Committee of WIAS Science Day	2008-2009

Education and Training total	***51.0 ECTS***

Colophon

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