PHYSIOLOGY, ENDOCRINOLOGY, AND REPRODUCTION

Effects of Eggshell Temperature and Oxygen Concentration on Embryo Growth and Metabolism During Incubation

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ABSTRACT Embryo development and heat production (HP) were studied in eggs of similar size (60 to 65 g) that were incubated at normal (37.8°C) or high (38.9°C) eggshell temperature (EST) and exposed to low (17%), normal (21%), or high (25%) O_2 concentration from d 9 through 19. High EST initially increased HP, but gradually O_2 became more important for HP than EST. Finally, HP was highest for the combination of high EST with high O_2 and lowest for the combination of high EST with low O_2. High EST decreased hatch time, BW, yolk free BW, and relative heart weight. The EST had no effect on residual yolk weight, chick length, or relative liver weight. Increased O_2 increased yolk free BW and chick length and decreased residual yolk weight at hatch. No interactions between EST and O_2 were observed with regard to embryo development and hatchling characteristics. If embryo development is reflected by HP, it can be concluded that high EST primarily increased embryonic development until the second week of incubation. During the third week of incubation, O_2 had a greater effect in determining embryo development than EST.

Key words: eggshell temperature, oxygen concentration, embryo development, heat production

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INTRODUCTION

Temperature determines the metabolic rate of yolk and albumen utilization and hence embryo development during incubation (Romanoff, 1972; Deeming and Ferguson, 1991). Temperature may have a divergent effect during the course of incubation because a constant higher temperature initially accelerates embryonic growth and utilization of nutrients and energy from yolk and albumen, but decreases embryonic growth later in development (Romanoff, 1972). Rahn et al. (1974) suggested that later in incubation, metabolic processes are limited by the insufficient exchange of oxygen, which would explain the plateau phase for gas exchange and heat production (**HP**). In the plateau phase, the maximum rate of oxygen diffusion into the egg is determined by the oxygen conductance and the partial pressure gradient of the gas between the ambient air and the inner side of the shell (Romijn and Roos, 1938; Romijn, 1950; Visschedijk, 1968; Visschedijk et al., 1985). Oxygen conductance is specific for breed (Janke et al., 2004; O'Dea et al., 2004), age, or egg size (Visschedijk, 1980). However, the partial pressure gradient for oxygen during incubation depends on metabolic rate and O_2 concentration of the ambient air. Because oxygen consumption and HP increase rapidly from d 9 to 10 of incubation onward (Lourens et al., 2006a), the demand for oxygen increases accordingly and gas exchange reaches the plateau phase some days later. During this plateau phase, large eggs incubated at the same eggshell temperature (**EST**) of 37.8°C produce more heat than small eggs (Lourens et al., 2006a). The EST would have increased more in large eggs than in small eggs when incubated at the same machine temperature (**MT**). Long-term high EST during the plateau phase is detrimental for embryo development (Lourens et al., 2005). However, even during the plateau phase, HP can be further increased by moderate, short-term increments of EST when eggs are incubated at normal O_2 concentration (Lourens et al., 2006b). It is unknown how HP and embryo development are affected by long-term high EST in eggs exposed to different O_2 concentrations. Therefore, an experiment was conducted where eggs of 1 breed and of similar size were incubated at normal (37.8°C) or high (38.9°C) EST and exposed to low (17%), normal (21%), or high (25%) O_2 concentration from d 9 through 19. It was hypothesized that high EST is not detrimental for embryonic development and metabolic rate when O_2 is sufficiently available.

MATERIALS AND METHODS

Experimental Design

In a 2×3 experimental design with EST and O_2 concentration as factors, HP, hatch time (**HT**), and chick develop-

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ment parameters were recorded. Each combination of EST × O_2 was repeated twice.

Hatching Eggs and Incubation

In 6 trials, a total of 2,040 graded hatching eggs from Hybro G$^+$ grandparent flocks were used. Eggs weighing between 60.0 and 65.0 g were selected at the Torsius Breeder Hatchery in Putten, the Netherlands, and were stored between 3 and 7 d at 16 to 18°C. Eggs were transported to the incubation and hatching facilities of Wageningen University, the Netherlands, and incubated in a HT-combi incubator with a maximum setting capacity of 4,800 eggs (Hatchtech B.V., Veenendaal, the Netherlands). Each trial had 240 eggs equally divided across 4 incubator trays and turned every hour. On d 8, eggs were candled, clear eggs were removed, and between 160 and 200 eggs containing living embryos were transferred and equally divided across 2 identical small open circuit climate respiration chamber (**CRC**; Lourens et al., 2006a,b). In the CRC, eggs were turned every half hour. At d 8 and 19, individual eggs were weighed and weight loss (**WL**) was determined to explain possible variation in yolk free BW (**YFB**) and residual yolk weight (**RY**) at hatch due to temperature treatment affecting water vapor conductance and to identify possible outliers due to, for example, eggshell cracks. Oxygen and carbon dioxide concentrations were measured every 9 min in both chambers and in fresh air. Carbon dioxide concentration was measured with a non-dispersive infrared CO_2 analyzer (type Uras 3G, Hartmann & Braun, Frankfurt, Germany). Oxygen concentration was measured with a paramagnetic oxygen analyzer (type ADC7000, Analytical Development Co. Ltd., Hertfordshire, UK). The exact air volumes were measured with a Schlumberger G1.6 dry gas meter. The HP was calculated from oxygen consumption and carbon dioxide production according to Romijn and Lokhorst (1961) and adjusted for fertility and embryo mortality based on the description of Lourens et al. (2006a).

Eggshell Temperature and Oxygen Concentration

Thermistors were attached to the eggshells of 5 individual, fertile eggs at the start of each trial in each CRC as described by Lourens et al. (2005). The EST was measured every 30 s and MT was automatically adjusted every 5 min when the median EST drifted away from the EST set point. The MT was adjusted using the median EST of 5 eggs per CRC to avoid the low EST of eggs containing dead embryos from affecting the decision for the direction of the next MT step. The EST in both CRC remained constant at 37.8°C (normal) or 38.9°C (high) in each trial. The O_2 of the air entering the CRC was adjusted by using a mixture of air with pure nitrogen (17% O_2) or of air with pure oxygen (25% O_2). Using a constant ventilation airflow, O_2 of the air entering the CRC was set at about 17% (low = 17.2% ± 0.19 SD), about 21% (normal = 21.0% ± 0.03 SD), or about 25% (high = 25.1% ± 0.21 SD). Because ventilation of each CRC was not changed during the 11 d measurements; consequently, O_2 concentration of the outgoing air decreased from d 8 to 19 in the low O_2 treatment from 17.1 to 16.5%; in the normal O_2 treatment from 20.9 to 20.2%, and in the high O_2 treatment from 24.8 to 24.2%. Air pressure inside the chambers exceeded barometric pressure by +100 Pa.

Embryo Mortality, Hatch Time, and Hatchling Measurements

At d 8 and 19 of incubation and at the day of hatch, clear eggs and dead in shell were opened to determine true fertility and the timing of embryonic mortality by visual appraisal as described by Lourens et al. (2006a). The HP calculations were corrected for the number of embryos that were alive at any day of incubation. At 19 d of incubation, per EST × O_2 treatment and per repetition, 60 randomly chosen eggs containing living embryos were transferred to identical hatching boxes. All hatching boxes were placed in 1 large CRC that allowed entrance of personnel. From 19 d onward, only EST was set as factor at 37.8 or 38.9°C, and O_2 concentration was no longer controlled. The EST was measured from 8 individual eggs, and MT was adjusted as described above. At 19.5 d of incubation, the thermistors were removed, MT remained constant, and so EST was allowed to increase. Individual HT per chick was recorded using a video camera and recorder. At 21.5 d of incubation, all hatched chicks were killed with a mixture of CO_2 and O_2. All chicks were weighed and individual chick length (**CL**) was measured by stretching the chick along a ruler and taking the length between the top of the beak and the tip of the middle toe of the right feet (Hill, 2001). Next, RY was removed from all chicks and weighed, and from 15 chicks chosen at random per EST × O_2 repetition heart and liver were removed and weighed to determine heart weight (**HW**) and liver weight (**LW**).

Statistical Analyses

The nonlinear, sigmoid curves for HP and MT were analyzed in Genstat 6.1 (2002) with a REML-procedure according to the following model for repeated measurements: $Y_{ijk} = \mu + EST_i + O2_j + D_k + \text{interactions} + \varepsilon_{ijk}$, where Y_{ijk} is HP or MT, μ is the overall mean, EST_i is eggshell temperature (i = normal, high), $O2_j$ is oxygen concentration (j = low, normal, high), D_k is day number (k = 9 to 19), and ε_{ijk} is the residual error term. The CRC was used as experimental factor. The HP and MT were calculated per day of incubation, so day of incubation was the repeated factor.

Chick characteristics were analyzed with the GLM procedure of Genstat software (Genstat 6.1, 2002), where the average of 1 group of eggs in 1 CRC was used as the experimental unit. The model was $Y_{ij} = \mu + EST_i + O2_j + (EST \times O2)_{ij} + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the overall mean, EST_i is eggshell temperature (i =

Table 1. Characteristics of hatchlings incubated at 2 different eggshell temperature (EST) profiles (37.8 or 38.9°C) and 3 different O_2 concentrations (17, 21, and 25%) from d 9 through 19[1]

Item	CL (cm)	BW (g)	YFB (g)	RY (g)	HW (%YFB)	LW (%YFB)	HT (d)
EST, °C							
37.8	19.7	41.5[a]	37.4[a]	4.0	1.1[a]	4.4	20.5[a]
38.9	19.8	39.8[b]	35.8[b]	4.0	0.9[b]	4.3	19.9[b]
O_2, %							
17	19.0[c]	40.6	35.3[c]	5.3[a]	1.0	4.2	20.2
21	19.9[b]	40.6	36.9[b]	3.7[b]	1.0	4.4	20.2
25	20.4[a]	40.7	37.7[a]	3.0[c]	1.0	4.4	20.2
EST, °C × O_2, %							
37.8 × 17	19.0	41.5	36.3	5.2	1.1	4.3	20.4
37.8 × 21	19.8	41.2	37.6	3.6	1.0	4.5	20.5
37.8 × 25	20.1	41.7	38.5	3.2	1.1	4.5	20.5
38.9 × 17	18.9	39.7	34.3	5.4	1.0	4.2	19.9
38.9 × 21	20.0	39.9	36.2	3.8	0.9	4.3	20.0
38.9 × 25	20.6	39.7	36.9	2.9	0.9	4.3	19.9
Overall SEM	0.08	0.20	0.15	0.06	0.01	0.05	0.04
Source of variation							
EST	NS	*	*	NS	*	NS	**
O_2	**	NS	*	**	NS	NS	NS
EST × O_2	NS	NS	NS	NS	NS	NS	NS

[a–c]Refer to significant differences ($P < 0.05$) between treatments.

[1]CL = chick length; YFB = yolk free BW; HW = heart weight; LW = liver weight; and HT = hatch time.

*$P < 0.01$; **$P < 0.001$.

normal, high), $O2_j$ is oxygen concentration (j = low, normal, high), and ε_{ij} is the residual error term.

RESULTS

Embryonic Development

No interactions between EST and O_2 concentration were observed with regard to embryo development and hatchling characteristics (Table 1). High EST compared with normal EST reduced HT and decreased BW, YFB, and relative HW. The EST had no significant effect on CL, RY, or relative LW. High O_2 concentration decreased RY and increased YFB and CL at hatch. The O_2 concentration had no effect on HT, relative HW, or relative LW.

Heat Production and Machine Temperature

The effects of EST and O_2 concentration on HP are summarized in Figure 1. From d 9 through 15, high EST significantly increased HP compared with normal EST. From d 14 to 17, in both EST treatments, HP was lowest in the low O_2 treatment compared with the normal or high O_2 treatments. From d 16 onward, HP in the normal O_2 treatment was still significantly higher compared with the low O_2 treatment, but also significantly lower compared with the high O_2 treatment. At d 18 and 19 of incubation, an interaction between EST and O_2 occurred. At low and normal O_2, HP was lower in the high EST treatment. At high O_2, HP remained high in the high EST treatment. A consequence of differences in HP between treatments was that MT needed to be adapted to maintain EST constant at 37.8 or 38.9°C. At d 18 of incubation, eggs incubated at 37.8°C EST and at 17, 21, or 25% O_2 concentration required a MT of 37.2, 37.0, and 36.7°C (differences of 0.6, 0.8, and 1.1°C, respectively). Likewise, eggs incubated at 38.9°C EST and at 17, 21, or 25% O_2 concentration required a MT of 38.0, 37.9, and 37.9°C (differences of 0.9, 1.0, and 1.0°C, respectively).

DISCUSSION

Embryo Development and Hatch Time

It was hypothesized that high EST is not detrimental for embryonic development and metabolic rate when O_2 is sufficiently available, but no interactions were observed for EST × O_2 concerning embryo development. In the present study it was clearly shown that high EST decreased embryo development in terms of BW, YFB, and HW of hatchlings that were taken out of the hatching baskets at 21.5 d of incubation. The EST, however, had no effect on RY, comparable with findings of Lourens et al. (2005). Differences in RY between breeds may be more related to genetics and eggshell conductance; Christensen et al. (1999) showed that growth selected embryos did not respond to increased oxygen by increasing fat metabolism, whereas embryos from their randombred controls did.

High EST decreased HT, and regression analysis showed that within each EST group, HT had no effect on YFB ($P > 0.05$). Within each EST group, late hatching chicks had larger RY, and as a consequence, also BW increased with HT. Also, Hager and Beane (1983), Reis et al. (1997), Suarez et al. (1997), and Joseph and Moran (2005) concluded that heavier BW and RY at hatch were associated with late emergence. Nutrient utilization from the RY in the hatcher period is poorly understood. Newly hatched chicks may absorb the nutrients left in the RY for growth and maintenance of the YFB (Noy et al., 1996;

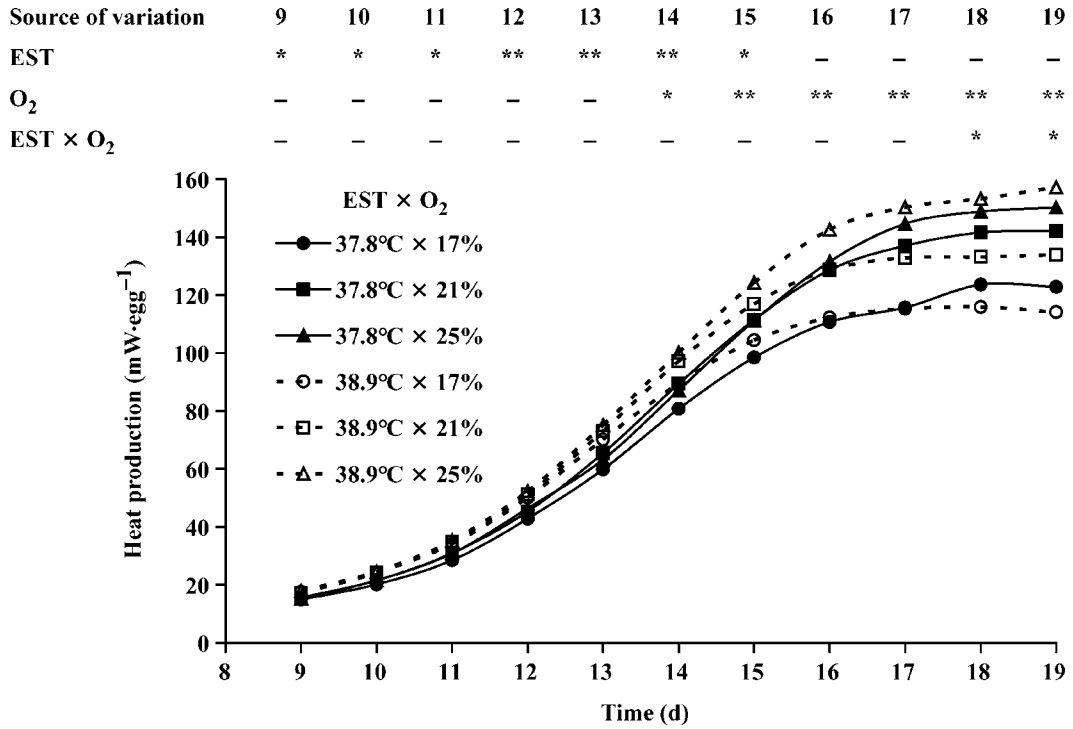

Figure 1. Heat production (mW·egg^{-1}) in eggs (60 to 65 g) incubated at 2 eggshell temperature (EST) settings (37.8 and 38.9°C) and 3 O_2 settings (17, 21, and 25%) between d 9 through 19. *$P < 0.05$; ** = $P < 0.01$.

Noy and Sklan, 1998, 2002). At the same time, dehydration occurs with extended holding time in the hatcher baskets (Hager and Beane, 1983; Reis et al., 1997). The HT decreased as flock age increased (Smith and Bohren, 1975; Shanawany, 1984; Yannakopoulos, 1988; Christensen et al., 2001; Joseph and Moran, 2005). This may be related to higher EST due to the effect of increased HP with increased egg weight (Lourens et al., 2006a). However, when egg weight is held constant, HT will still decrease with increasing flock age (Fanguy et al., 1980; Hager and Beane, 1983; Burke, 1992, Christensen et al., 2000). Older hens produce thin-shelled eggs (Roland, 1976; Britton, 1977; Peebles and Brake, 1987). Thin eggshells not only increase the rate of water loss (Tullett and Board, 1977), but also the eggshell oxygen conductance (Christensen et al., 1996) compared with thick eggshells. Oxygen conductance was observed to be inversely related to the incubation period (Rahn et al., 1974). Contrary to previous research, oxygen conductance in the present study was artificially increased, and EST and not O_2 determined HT. Different from all other experiments is that in the present experiment EST was maintained constant at 37.8 or at 38.9°C, and EST was not allowed to increase with increased O_2. So the decrease in HT by increased oxygen conductance as described in Rahn et al. (1974) may be explained more by increased EST than by oxygen conductance.

The partial pressure of oxygen can decrease by decreasing concentration, or by increasing altitude and hence by decreasing barometric air pressure. Because incubating embryos respire totally by diffusion, when barometric air pressures decrease, the diffusion rate and respiration decrease. Reduced diffusion rates at the same barometric pressure have been shown to affect organ maturation and BW (Bagley and Christensen, 1989). In the present study, air pressure in the CRC exceeded outside barometric air pressure by +100 pa to avoid leakage, which relates to height difference of 9 to 10 m. Barometric pressure was the same in all treatments, and so barometric pressure did not likely affect results between treatments.

At sea level, oxygen makes up 20.9% of the atmosphere, and the equivalent percentage of oxygen drops approximately 1% for every 500 m rise in altitude (Julian, 2000). Lokhorst and Romijn (1965) observed decreased hatchability when eggs were incubated at low air pressures. Heat production was highest (151 mW·egg^{-1}) at sea level. Above a simulated altitude of 3,000 m (<15% O_2), HP decreased to below 80 mW·egg^{-1} and embryos did not survive. At high altitude of 2,000 m, embryos hatched earlier than those incubated at sea level (Hassanzadeh et al., 2004). The difference in partial pressures of 4% in the study by Hassanzadeh et al. (2004) was the same as in the present study, where it was concluded that HT was not affected by O_2 at all. In the study by Hassanzadeh et al. (2004), eggs were incubated at a constant MT of 37.8°C at both altitudes. Because it was observed that eggs incubated at high altitude experience more difficulties to exchange heat (Meijerhof, 2002), these eggs are likely to be incubated at higher EST, which may have decreased HT as a result. Next to EST, incubation weight loss was also not measured in the study by Hassanzadeh et al. (2004), which could have explained the lower BW of chicks hatched at high altitude. Increased weight loss may also have reduced YFB of chicks incubated at high EST in

the present study because high EST increases the partial water vapor pressure between egg and air (Meijerhof and Van Beek, 1993). Weight loss between setting and transfer at 19 d of eggs that hatched was 11.5% in eggs incubated at 37.8°C EST and 12.4% in eggs incubated at 38.9%. Regression analysis showed that BW and YFB decreased with WL at a significant higher rate in chicks that hatched after exposure to high EST compared with normal EST ($P < 0.05$) and that RY was not affected at all by WL or EST. There were no significant correlations between WL and HT for BW, YFB, or RY ($P > 0.05$).

Heat Production

Until d 15, eggs incubated at high EST produced more heat than eggs incubated at normal EST. Independently from EST, O_2 determined HP at d 16 to 17. Between d 18 to 19 of incubation, HP was decreased by a combination of high EST with low or normal O_2. Van Golde et al. (1998) showed that already in the middle of the incubation period O_2 availability can be a limiting factor for growth, well before metabolism exceeds the oxygen diffusion capacity of the eggshell. Similar to the present study, McCutcheon et al. (1982), Stock et al. (1983), Stock and Metcalfe (1984), and Asson-Batres et al. (1989) also observed increased embryonic development at increased oxygen availability only late in incubation. Rahn et al. (1979) suggested that for normal embryo development, the total amount of oxygen consumed per gram of fresh egg weight averages about 100 mL. In the present study, O_2 concentration and not EST affected total oxygen consumption per gram of fresh egg weight. Embryos incubated at O_2 of 17% consumed less than 100 $mL \cdot g^{-1}$; eggs incubated at O_2 of 21 or 25% consumed more than 100 $mL \cdot g^{-1}$. High EST initially increased oxygen consumption, and gradually O_2 became more important. Therefore, we believe that the level of oxygen consumption during the plateau phase is of more importance for embryonic growth, development, and maturation than the total amount of oxygen consumed. Accordingly, embryo development in eggs incubated at high O_2 could not be increased by increased EST. It can be speculated that the total oxygen consumption in eggs incubated at low O_2 can be increased by decreasing EST, which would slow down development and decrease HP, but also increase HT, which would allow the embryos more time to develop and mature.

Normal O_2 limited HP even in eggs incubated at normal EST, which was confirmed by the study of Visschedijk (1980), who showed that at increased O_2 above normal O_2, a small but significant increase in carbon dioxide production was found. Lokhorst and Romijn (1965) incubated eggs at 38.0°C MT and at different O_2. Highest HP (143 $mW \cdot egg^{-1}$) was found for eggs incubated at the highest O_2 of 20.9%. At 17% O_2, HP dropped to 107 $mW \cdot egg^{-1}$, comparable with results of the present experiment. When eggs were incubated at low O_2 after setting, O_2 reached lethal levels below 15%. Bjønnes et al. (1987) exposed embryos between 18 to 21 d of incubation to reduced (6 to 7%) oxygen concentration. The effect of hypoxia on oxygen consumption was most pronounced in the 18- to 19-d-old embryos and less in older embryos after internal and external pipping when relatively more uptake occurs via the lungs and not through the chorioallantois membrane by diffusion (Rahn et al., 1979).

From this study it was concluded that high EST initially increased HP, but gradually O_2 became more important for HP than EST. Finally, HP was highest for the combination of high EST with high O_2 and lowest for the combination of high EST with low O_2. If embryo development is reflected by the course of HP, it can be concluded that high EST increased embryonic development only until the second week of incubation. During the third week of incubation it was O_2 that determined embryo development more than EST. The hypothesis that high EST is not detrimental for embryonic development and metabolic rate when O_2 is sufficiently available was rejected.

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