High- and low-temperature manipulation during late incubation: Effects on embryonic development, the hatching process, and metabolism in broilers

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ABSTRACT Temperatures continuously higher and lower than the standard incubation temperature by 3°C from embryonic d 16 until embryonic d 18.5 result in differential effects on embryonic development, the hatching process, and embryonic metabolism. Embryos in the high-temperature group were forced into a state of malnutrition by the temperature treatment, as reflected by reduced embryo growth and yolk consumption, resulting in a significantly lower chick weight at hatch. In addition, altered air cell and blood gases as well as a retarded hatching process further indicated reduced growth of embryos exposed to higher incubation temperatures during the latter part of incubation. In addition, hatchability was significantly reduced by the high-temperature treatment due to higher embryonic mortality during the treatment period and the hatching process. Levels of blood glucose, lactate, liver glycogen, plasma triglycerides, and nonesterified fatty acids indicated an altered carbohydrate and lipid metabolism for the high-temperature group. Although the hatching process of embryos exposed to lower incubation temperatures was also significantly retarded, their embryonic development and growth were strikingly similar to those of the control group.

Key words: broiler, incubation temperature manipulation, embryonic development, hatching process

INTRODUCTION

Incubation temperature is one of the most important single physical factors determining or influencing chicken embryo development and hatchability (Decuypere and Michels, 1992). Temperature influences both the time needed for development to hatching and the percentage of hatch (Romanoff, 1960). Generally, high temperatures are known to accelerate embryonic development, leading to a shorter duration of incubation (Kaplan et al., 1978), whereas low temperatures result in the opposite effect (Black and Burggren, 2004). In addition, if the incubation temperature is too low or too high, embryonic mortality will be increased, and therefore hatchability will be decreased (Decuypere et al., 1979; Suarez et al., 1996).

According to Nichelmann and Tzschentke (2002), the development of the thermoregulatory system of precocial birds is characterized by a prenatal phase in the development of the thermoregulatory system, which is characterized by endothermic reactions with a low efficiency. However, these reactions ultimately have an influence on the efficiency of the thermoregulatory system (Nichelmann and Tzschentke, 2002; Moraes et al., 2003). Therefore, they may support adaptivity to postnatal environmental conditions and play a role in epigenetic temperature adaptation processes (Nichelmann and Tzschentke, 2002). Interestingly, near the end of incubation, embryonic reactions to changed environmental conditions change from being uncoordinated, nonadaptive reactions (aspecific reactions) to coordinated, adaptive reactions (specific reactions; Tzschentke et al., 2004). Hence, it is hypothesized that these characteristic reaction patterns at the end of incubation could be used as a physiological tool to help characterize this critical period in the development of the thermoregulatory system (Tzschentke and Plagemann, 2006). In addition, the end phases in the ontogeny of the hy-
pothalamus-pituitary-thyroid axis and hypothalamus-pituitary-adrenal axis, which are important in metabolism, the thermoregulatory response, and the stress response, as well as being major factors in the hatching process (Decuypere et al., 1991), are situated in this same period near the end of incubation.

Incubation temperature manipulation is currently being studied as a tool to induce epigenetic temperature adaptation to increase thermostolerance posthatch (Tzschentke and Plagemann, 2006; Tzschentke, 2007). The key element in inducing epigenetic temperature adaptation is finding the right time frame, frequency, duration, and amplitude of temperature manipulation. Many studies have focused on intermittent temperature manipulations during late incubation, ranging from 3 to 12 h/d (Moraes et al., 2003; Yalçın and Siegel, 2003; Collin et al., 2005, 2007; Yalçın et al., 2005, 2008a; Piestun et al., 2008, 2009). In addition, most studies have investigated only temperatures that are higher than standard incubation temperatures. A recent study by Shinder et al. (2009) reported preliminary results of repetitive acute cold exposure of chick embryos late in incubation on hatchability, hatching weight, and acquisition of cold resistance postnatally.

Therefore, this study aimed to investigate the effect of thermal manipulations applied continuously during late incubation on embryonic development and the hatching process because these affect the quality of day-old broiler chicks. Embryonic growth, yolk consumption, partial pressure of CO₂ (pCO₂), and partial pressure of O₂ (pO₂) in both the air cell and blood were analyzed to characterize embryonic development. Broiler chicks were followed individually to map the hatching process. Plasma thyroid hormone concentrations, both triiodothyronine (T₃) and thyroxine (T₄), are reference measurements for evaluating the level of metabolism of the embryos (McNabb, 2000). Furthermore, an idea of the stress level created by the temperature treatment can be found through the level of corticosterone (Piestun et al., 2009). In addition, these hormones play a major role in the hatching process (Decuypere et al., 1991). Because synthesis and degradation of glycogen are vital for embryonic survival during the last phase of incubation (Christensen et al., 2001), glycogen levels are determined in the liver, the most metabolically active tissue of the embryo. In addition, different metabolite levels, such as plasma uric acid levels, blood glucose and lactate levels, and plasma triglyceride (TG) and nonesterified fatty acid (NEFA) levels were determined to give an indication of the effect of temperature manipulation on protein, carbohydrate, and lipid metabolism during the last days of incubation and the transition to posthatch life.

**Experimental Design**

Two identical experiments, separated in time, with 1,200 eggs from a different flock of the same strain (Cobb-500) and age (38 wk old), were conducted. Broiler breeder eggs (Belgabroed, Merksplas, Belgium) were individually numbered and weighed before being evenly divided among 3 forced-draft incubators with a maximum capacity of 600 eggs (Pas Reform, Zeddam, the Netherlands), which were adjusted with a focus controller from Petersime NV (Zulte, Belgium). All eggs were stored for 3 to 4 d before incubation. The incubators were set at a standard dry bulb temperature of 37.6°C and a wet bulb temperature of 30°C. At the beginning of embryonic day (ED) 16, 3 experimental groups (low, control, high; 400 eggs/group) were created by redistributing the eggs so that each group contained a mixture of eggs from the 3 incubators. All 400 eggs/treatment were placed on 3 trays (133 to 134 eggs/tray) in the incubator. Eggs were turned more than 90° every hour during the whole incubation period, until ED 18.5.

After redistributing the eggs at the beginning of ED 16, the incubator temperature (dry bulb temperature) was set at 34.6°C (low), 37.6°C (control), or 40.6°C (high) and remained at this temperature continuously until ED 18.5. The wet bulb temperature was kept constant at 30°C. At ED 18.5, the eggs were transferred from the turning trays to hatching baskets, and all the eggs were incubated at the standard temperature of 37.6°C until the end of incubation. Average egg weights at setting were 63.45 ± 0.20 g (experiment 1) and 63.98 ± 0.19 g (experiment 2) for the low-temperature group, 63.30 ± 0.20 g (experiment 1) and 64.10 ± 0.18 g (experiment 2) for the control group, and 63.64 ± 0.20 g (experiment 1) and 64.10 ± 0.18 g (experiment 2) for the high-temperature group.

**Sampling**

At ED 16, ED 17, and ED 18, internal pipping (IP), external pipping (EP; for experiment 2 only), and hatch, samples of 12 eggs or chicks/group were taken. To limit tray variation, 4 eggs were taken randomly from each of the 3 trays at ED 16, ED 17, and ED 18. At ED 16, samples were taken before the thermal manipulation treatment for analysis of incubator variation. At IP, samples were taken 2 h after the occurrence of IP. At EP (for experiment 2 only) and hatch, samples were taken immediately after the occurrence of EP or the emergence of the chick from the egg.

**Eggshell Temperature and Egg Weight Loss.** Eggshell temperature, as an indicator of embryo temperature (French, 1997; Lourens et al., 2005; Joseph et al., 2006; Hulet et al., 2007), was measured at the equator of the egg immediately after opening the incubator by using a digital thermometer based on infrared technology (IRT 4520, Thermoscan, Braun, Germany). Eggshell temperature measurements were performed on sampling days ED 16, ED 17, and ED 18. On each
sampling day except hatch, eggs used for sampling were weighed before sampling to calculate egg weight loss:

\[
\text{egg weight loss} \% = \left( \frac{\text{egg weight at setting} - \text{egg weight}}{\text{egg weight at setting}} \right) \times 100.
\]

**Gases in the Air Cell.** At ED 16, ED 17, ED 18, and IP, the pCO₂ and pO₂ in the air cell were analyzed using a gas analyzer (Synthesis 10 model, Instrumentation Laboratory, Lexington, KY) by making a hole in the air cell, after candling, with an 18-gauge needle. The needle of the gas analyzer was immediately introduced in the air cell for air aspiration and measurement.

**Gases and Metabolites in the Blood.** On all sampling days, blood was drawn from the vena jugularis of the embryo or chick with a 1-mL needle and 27-gauge syringe and was collected in heparinized tubes. Blood was collected in a heparinized capillary tube (150 μL) and introduced into a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory) for the determination of pCO₂, pO₂, glucose, and lactate, except on ED 16 in experiment 1. The remaining blood was centrifuged for 10 min at 3,024 × g at 4°C. The plasma was collected and stored at −20°C for later analyses of T₃, T₄, corticosterone, TG, and uric acid levels.

**Embryo and Yolk Weights.** After careful separation of the yolk and embryo, both were weighed. Embryos were weighed after excessive fluid was dried off with an absorbent paper. Relative embryo and yolk weights were calculated using the following formulas:

\[
\text{relative embryo weight} \% = \left( \frac{\text{yolk-free embryo weight}}{\text{egg weight at setting}} \right) \times 100,
\]

and

\[
\text{relative yolk weight} \% = \left( \frac{\text{yolk weight}}{\text{egg weight at setting}} \right) \times 100.
\]

A sample of the liver was snap-frozen in N₂ and stored at −80°C until later glycogen determination.

**Water Content of the Embryo and Yolk.** Both the embryo and yolk (experiment 1) were dried at 105°C until a stable end weight was recorded, to determine the water content (%) of the embryo and yolk:

\[
\text{water content of embryo/yolk} \% = \left( \frac{\text{wet embryo/yolk weight} - \text{dry embryo/yolk weight}}{\text{wet embryo/yolk weight}} \right) \times 100.
\]

In experiment 2, the process of drying the yolk was done to preserve the yolk for further analysis of lipid content and was carried out by lyophilization for approximately 72 h (until a stable end weight was obtained). Thereafter, the yolk was crushed in a food processor and stored at −20°C for later water content determination. The final water content of the yolk was calculated by adding the water content lost in the lyophilization process and the water content lost by overnight drying of the lyophilized yolk at 105°C.

**Hatching Events**

After transfer to the hatching baskets, eggs were checked individually every 2 h between 452 and 509 h of incubation for the occurrence of IP, EP, and chick emergence (hatch). These data were then used to calculate the average IP, EP, and hatching times as well as the time interval between IP and EP, the time interval between EP and hatch, and the time interval between IP and hatch. After each collection of hatched chicks, the chicks were weighed.

**Embryonic Mortality**

Eggs that did not hatch after 509 h of incubation were opened and checked for incubation stage at death. Death before treatment was defined as dead before ED 16, whereas death during treatment was defined as dead during the temperature treatment period from ED 16 until ED 18.5. Embryos that did not survive the hatching process or embryos that were alive but that did not emerge out of the egg before 509 h were defined as dead after treatment. These data were used to calculate hatchability and mortality (before, during, and after treatment) relative to the number of fertile eggs:

\[
\text{hatchability} \% = \left( \frac{\text{number of hatched chicks}}{\text{number of fertile eggs}} \right) \times 100,
\]

and

\[
\text{mortality} \% = \left( \frac{\text{number of dead embryos}}{\text{number of fertile eggs}} \right) \times 100.
\]

**Hormones and Metabolites**

Plasma T₃ and T₄ concentrations were measured by RIA as described by Darras et al. (1996). The antisera for T₃ and T₄ were purchased from Byk-Belga (Brussels, Belgium). Intraassay CV were 4.5 and 5.4% for T₃ and T₄, respectively. Plasma corticosterone levels were measured by using a commercially available double-antibody RIA (IDS Ltd., Boldon, UK). The intraassay CV for corticosterone was 3.9%.

Plasma TG (VetTest 9820377) and plasma uric acid (VetTest 9820378) concentrations were determined using a VetTest 8008 analyzer (Idexx Laboratories Inc., Westbrook, ME). The apparatus is based on dry chemical technology and colorimetric reaction. Sample analysis is carried out on selective testing discs (Idexx Laboratories Inc.) by means of a laser reading the bar codes. For the quantitative determination of NEFA
in plasma, an enzymatic color test was used according to the manufacturer’s recommendations (NEFA C, ACS-ACOD method, WAKO Chemicals GmbH, Neuss, Germany; Rogiers, 1978; Aufenanger and Kattermann, 1995; Krebs et al., 2000). Because of the lack of plasma on ED 16, plasma TG, uric acid, and NEFA levels could not be determined on this day.

### Glycogen Determination

For determination of the glycogen concentrations in liver, a method based on that of Dreiling et al. (1987) was used. First, tissue was homogenized in the same amount (μL) of 7% HClO₄ as the amount (mg) of tissue taken. Homogenates were centrifuged at 4°C at 14,000 × g until clear supernatants were obtained. The supernatants were washed with 1 mL of petroleum ether and stored at −20°C. An iodine color reagent [0.39 mL of an iodine solution (0.104 g of I₂, 1.04 g of KI₂ in 4 mL of Milli-Q water; Millipore, Billerica, MA) + 30 mL of 10% CaCl₂] was added to standards or tissue extracts in a microtiter plate. After mixing and a waiting period of 10 min, the absorbance was measured at 450 nm (Victor 1420 Multilabel Counter, PerkinElmer, Wellesley, MA). Tissue glycogen concentration could then be calculated using the standard curve of rabbit liver glycogen (Sigma, Bornem, Belgium).

### Lipid Content Analysis of the Yolk

For determination of the lipid content in yolk, a method adapted from that of Folch et al. (1957) was used. Approximately 0.5 g of lyophilized yolk was homogenized for 30 s in 4.5 mL of CH₃OH (containing 100 mg/L of hydroxytoluene). An additional 9 mL of CHCl₃ was added and the mixture was homogenized for another 60 s. After 20 min of centrifugation at 2,000 × g, the extract was decanted. Twelve milliliters of CHCl₃:CH₃OH (2:1; vol/vol) was added to the precipitate and the mixture was homogenized for 60 s. The second extract was added to the first after centrifugation (20 min, 2,000 × g). Thereafter, 6.2 mL of 0.88% KCl was added to the total extract and the mixture was shaken vigorously. After 10 min of centrifugation (10 min, 2,000 × g), the top aqueous layer was removed. The bottom layer, consisting of a chloroform:methanol mixture and lipid components, was filtered and evaporated to dryness at 40°C in a water bath with N₂ until a constant weight was reached. This amount of lipid was expressed as a percentage of dry yolk weight.

### Statistical Analysis

The data were processed with the statistical software package SAS version 9.2 (SAS Institute Inc., Cary, NC). To evaluate differences between experiments, data were analyzed for the effect of experiment (experiment 1 or 2), incubation treatment (low, control, or high), embryonic age, and their interactions with a general linear regression model. Because no significant second-degree interactions were found between experiment, incubation treatment, and embryonic age and no significant first-degree interactions were found between experiment and incubation treatment or experiment and age, the explanatory variable experiment was discarded from the model. Although, for all parameters, no significant interactions with the classification variable experiment were found and significant effects of experiment were found only for pCO₂ in blood, pO₂ in the air cell and blood, and blood glucose, the results of the 2 incubation experiments were analyzed and are presented separately to illustrate repeatability between the incubation experiments. In addition, to quantify interexperimental variability, the CV for every parameter (per group, per ED) was calculated.

Therefore, data were reanalyzed per experiment for the effect of embryonic age, incubation treatment (low, control, or high), and their interaction. When a significant incubation treatment or interaction effect was found, the means were compared by Tukey’s test, per ED. The period investigated, from ED 16 until hatch, was divided into 3 periods, namely, ED 16 (before the temperature treatment), ED 17 and 18 (during the temperature treatment), and IP until hatch (after the temperature treatment), which were analyzed separately. At ED 16, incubator and group effects were analyzed separately, and when no significant differences were found for either factor, the results were pooled to give 1 average value as a pretreatment starting point. Because the response variables describing the hatching process were found to be not normally distributed (Kolmogorov-Smirnov test), the effects of incubation treatment (low, control, or high) on time of IP, EP, and hatch and their time intervals were analyzed using a nonparametric test (Kruskal-Wallis test). Hatchability and embryonic mortality were analyzed using a logistic regression model. A 5% degree of significance was used. All data are shown as mean ± SEM.

### RESULTS

Because no incubator effect or group effect was found on ED 16 for any parameter in either experiment, an average pooled value across incubation treatments (n = 36) at ED 16 was calculated and is shown in all figures. The average egg weight at setting was not significantly different between treatment groups for the 2 experiments.

### Eggshell Temperature and Egg Weight Loss

During the treatment period, ED 17 to 18 for both experiments, the eggshell temperature of the low-temperature group (35.5 ± 0.1°C) was significantly lower (P < 0.0001) than that of the control temperature group (38.3 ± 0.1°C), which was significantly lower (P < 0.0001) than that of the high-temperature group (41.1 ± 0.1°C). No significant differences in egg weight...
loss between incubation treatments were found in the 2 experiments on any sampling day.

Embryo Growth and Yolk Consumption

In experiment 1 (during and after treatment, \( P < 0.0001 \); Figure 1a), the high-temperature group had a lower relative yolk-free embryo or chick weight at ED 18, IP, and hatch compared with both the low-temperature and control groups, which were similar. In experiment 2 (during treatment, \( P = 0.0002 \); after treatment, \( P < 0.0001 \); Figure 1b), at ED 17, the high-temperature group had a significantly lower relative yolk-free embryo (chick) weight compared with the low-temperature group, whereas the control group did not differ from the 2 experimental groups. Thereafter, with the exception of EP, the relative yolk-free embryo or chick weight of the high-temperature group was significantly lower compared with that of the control and low-temperature groups. In both experiments, no significant difference in relative yolk-free embryo or chick weight was found between the low-temperature and control groups.

The high-temperature group exhibited higher relative yolk weights at ED 18 and IP in the first experiment (during treatment, \( P = 0.0159 \); after treatment, \( P < 0.0001 \); Figure 2a) and at IP and EP in the second experiment (during treatment, \( P = 0.8420 \); after treatment, \( P < 0.0001 \); Figure 2b) compared with the low-temperature and control groups. For both experiments, at hatch, the relative yolk weight of the high-temperature group was significantly higher compared with that of only the low-temperature group.

The water content of yolk-free embryos (data not shown) was significantly higher only for the high-temperature group in comparison with other groups at IP in experiment 1 (during treatment, \( P = 0.5850 \); after treatment, \( P = 0.0051 \)) and at EP in experiment 2 (during treatment, \( P = 0.7130 \); after treatment, \( P = 0.0005 \)). No differences in water content of the yolk (data not shown) were found between incubation treatments in the first experiment. However, in the second experiment (during treatment, \( P = 0.0270 \); after treatment, \( P = 0.0015 \)), the high-temperature group had a significantly lower water content in the yolk than did the control group on ED 18. At IP, the yolk of the high-temperature group had a significantly higher water content than those of the 2 other groups, whereas at hatch, this difference remained only in comparison with the control group.

Gases in the Air Cell

At ED 17, the pCO\(_2\) level in the air cell (Figure 3a and 3b) of the high-temperature group was significantly lower than that of the control group in the first experiment (during treatment, \( P = 0.0026 \)) and was significantly higher than that of the low-temperature group in the second experiment (during treatment, \( P = 0.0435 \)). At ED 18, the high-temperature group exhibited a significantly lower pCO\(_2\) level compared with the control treatment in both experiments and compared with the low-temperature group in the second experiment. At IP, the pCO\(_2\) level of the high-temperature group remained lower than that of the other 2 experimental groups, numerically so in the first experiment (\( P = 0.1246 \)) and significantly in the second experiment (\( P = 0.0004 \)). No difference in pCO\(_2\) level in the air cell was found between the low-temperature and control groups.

In the first experiment (during treatment, \( P < 0.0001 \)), at ED 17, the pO\(_2\) in the air cell (Figure 3a and 3b) of both experimental groups was significantly higher compared with that of the control group, whereas at ED 18, it remained significantly higher only in the high-temperature group compared with the control group. At IP, the pO\(_2\) levels of the temperature-manipulated groups were significantly higher than that of the
control group, numerically so in the first experiment ($P = 0.0884$) and significantly in the second experiment ($P = 0.0003$).

**Gases in the Blood**

In experiment 1 (during treatment, $P = 0.0009$; after treatment, $P = 0.0174$) and experiment 2 (during treatment, $P = 0.0013$; after treatment, $P = 0.0514$), at ED 18, the pCO$_2$ in the blood (Figure 4a and 4b) was significantly lower for the high-temperature group compared with the low-temperature and control groups. At IP, the high-temperature group had significantly lower blood pCO$_2$ compared with the control group in the first experiment and compared with the low-temperature group in the second experiment.

The blood pO$_2$ (Figure 4a and 4b) was significantly higher for the high-temperature group compared with the control group on ED 18 in the first experiment (during treatment, $P = 0.0425$; after treatment, $P = 0.2465$). In the second experiment (during treatment, $P = 0.0013$; after treatment, $P < 0.0009$) only at hatch, significantly increased blood pO$_2$ was found for both temperature-manipulated groups compared with the control group.

**Hatching Events**

The hatching process was clearly affected by the temperature treatment in both experiments, as shown in Table 1. In both experiments, the average incubation times to occurrence of IP ($P < 0.0001$), EP ($P < 0.0001$), and hatch ($P < 0.0001$) were significantly delayed for the high- and low-temperature groups compared with the control group.
pared with the control group. In addition, the time to IP, EP, and hatch (in experiment 1 only) was delayed more for the high-temperature group than for the low-temperature group.

Although, in the first experiment, the duration of the entire hatching process (IP to hatch) was not significantly different between temperature groups, the duration of the different stadia (IP to EP and EP to hatch) was. The time interval between IP and EP was significantly \( P < 0.0001 \) shorter for the low-temperature group than for the high-temperature and control groups. Consequently, the time interval between EP and hatch was significantly \( P < 0.0001 \) longer for the low-temperature group than for the other groups. In the second experiment, the duration of the entire hatching process (IP to hatch) was significantly \( P < 0.0001 \) longer (by 2 h) for the low-temperature group than for the high-temperature and control groups. The time interval between IP and EP was the shortest \( P < 0.0001 \) and, logically, the EP to hatch interval was the longest \( P < 0.0001 \) for the low-temperature group compared with the other groups.

**Hatchability and Embryonic Mortality**

Table 1 summarizes hatchability and embryonic mortality for both experiments as a function of the number of fertile eggs. Hatchability was significantly lower for the high-temperature group than for the low-temperature and control groups in both experiments \( P < 0.0001 \). In the first experiment, the low-temperature group also had significantly lower hatchability than the control group. The significantly higher embryonic mortality during the treatment period (first experiment, \( P = 0.0135 \); second experiment, \( P = 0.0059 \)) and during the hatching process (both experiments, \( P < 0.0001 \)) was mostly responsible for the significantly lower hatching percentage in the high-temperature group in both experiments.

**Chick Weight at Hatch**

Absolute chick weight at hatch (Table 1) was significantly reduced in the high-temperature group compared with the low-temperature and control groups in the first experiment \( P = 0.0119 \) and compared with the control group in the second experiment \( P = 0.0219 \). Moreover, the relative chick weight at hatch (Table 1) was significantly lower for the high-temperature group than for the other 2 groups in both experiments \( P < 0.0001 \).

**Plasma Hormone Levels**

In both experiments, plasma \( T_3 \) concentrations (Figure 5a and 5b) were low until d 18 of incubation, but were followed by a steep increase from IP to reach maximum levels at hatch. Plasma \( T_4 \) concentrations (Figure 6a and 6b) increased gradually from 3 to 4 ng/mL at ED 16 to 8 to 11 ng/mL at hatch. In both experiments, at ED 17 and ED 18, the high-temperature group had significantly reduced plasma \( T_3 \) (during treatment: first experiment, \( P < 0.0001 \); second experiment, \( P = 0.0001 \)) and \( T_4 \) (during treatment: first experiment, \( P < 0.0001 \); second experiment, \( P < 0.0001 \)) concentrations compared with the low-temperature and control groups. In experiment 1 (after treatment: \( T_3, P = 0.0014 \)), but not in experiment 2, this difference was still significant at hatch. In the second experiment (after treatment, \( P = 0.0081 \)), at EP, the plasma \( T_3 \) concentration of the control group was significantly higher compared with that of the low-temperature group. At ED 18, the low-temperature group had a significantly lower level of plasma corticosterone compared with the high-temperature group in the first experiment (during treatment, \( P = 0.0096 \); data not shown) and compared
with the control and high-temperature groups in the second experiment (during treatment, \( P = 0.0139 \); data not shown). In the second experiment (after treatment, \( P < 0.0001 \)), at hatch, the high-temperature group had a significantly higher corticosterone concentration compared with the low-temperature group, which in turn had a higher concentration compared with the control group.

### Glycogen Concentration

In both experiments, the glycogen concentration of the liver was determined (first experiment: during treatment, \( P < 0.0001 \); after treatment, \( P = 0.0112 \); second experiment: during treatment, \( P = 0.0270 \); after treatment, \( P = 0.0015 \); Figure 7a and 7b). At ED 18, peak levels of liver glycogen were reached in all temperature groups, and this was followed by a decline to minimum values at hatch. In both experiments, liver glycogen was significantly lower in the high-temperature group compared with the low-temperature and control groups from ED 17 until EP. The low-temperature group had higher liver glycogen reserves compared with the control group, and were significant at ED 17 and ED 18 in the first experiment and at ED 17 in the second. From ED 18 to IP, the liver glycogen level of the low-temperature group decreased sharply, resulting in equal (experiment 1) or even significantly lower (experiment 2) levels at IP. Thereafter, the low-temperature group had levels comparable with those of the control group.

### Metabolite Levels and Lipid Content of the Residual Yolk

Figure 7a and 7b show the blood glucose levels for the first (during treatment, \( P = 0.0011 \); after treatment, \( P = 0.0369 \)) and second experiment (during treatment, \( P < 0.0001 \); after treatment, \( P < 0.0001 \)), respectively. During the temperature treatment in both experiments, blood glucose levels were lowest for the low-temperature group and highest for the high-temperature group (significant at ED 18 for the first experiment and at ED 17 and ED 18 for the second experiment). At IP, however, this order was reversed and the lowest values were found for the high-temperature group (significant for the first experiment) and the highest values were found for the low-temperature group (significant for the second experiment), and these remained higher at hatch for the low-temperature group (for the second experiment only).

Increasing levels of blood lactate for the first experiment (during treatment, \( P = 0.0241 \); after treatment, \( P < 0.0001 \)) and the second experiment (during treatment, \( P = 0.005 \); after treatment, \( P < 0.0001 \)) are shown in Figure 8a and 8b. At ED 17, the blood lactate concentration was significantly higher at ED 17 for the high-temperature group and highest for the high-temperature group (significant at ED 18 for the first experiment and at ED 17 and ED 18 for the second experiment). At hatch, the high-temperature group exhibited a significantly lower blood lactate level than the low-temperature and control groups.

**Table 1. Time of internal pipping (IP), external pipping (EP), and hatch (h) and their intervals (h), chick weight at hatch (g), and relative chick weight at hatch (%), hatching percentage, and embryonic mortality (%) for experiments 1 and 2**

<table>
<thead>
<tr>
<th>Item</th>
<th>Low</th>
<th>Control</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP (h)</td>
<td>472 ± 1b</td>
<td>464 ± 0c</td>
<td>474 ± 1a</td>
</tr>
<tr>
<td>EP (h)</td>
<td>480 ± 1b</td>
<td>474 ± 0c</td>
<td>485 ± 1a</td>
</tr>
<tr>
<td>Hatch (h)</td>
<td>496 ± 1b</td>
<td>487 ± 0c</td>
<td>498 ± 0a</td>
</tr>
<tr>
<td>IP to EP (h)</td>
<td>8 ± 0b</td>
<td>10 ± 0a</td>
<td>11 ± 0a</td>
</tr>
<tr>
<td>EP to hatch (h)</td>
<td>16 ± 0b</td>
<td>13 ± 0b</td>
<td>13 ± 0b</td>
</tr>
<tr>
<td>IP to hatch (h)</td>
<td>24 ± 0</td>
<td>23 ± 0</td>
<td>24 ± 0</td>
</tr>
<tr>
<td>Chick weight (g)</td>
<td>47.59 ± 0.28a</td>
<td>47.81 ± 0.26a</td>
<td>46.63 ± 0.26b</td>
</tr>
<tr>
<td>Relative chick weight (%)</td>
<td>75.50 ± 0.18a</td>
<td>75.66 ± 0.21a</td>
<td>74.38 ± 0.16b</td>
</tr>
<tr>
<td>Hatching (%)</td>
<td>85.8b</td>
<td>93.1a</td>
<td>74.20</td>
</tr>
<tr>
<td>Mortality before treatment (%)</td>
<td>8.6</td>
<td>3.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Mortality during treatment (%)</td>
<td>2.1b</td>
<td>1.3b</td>
<td>6.0b</td>
</tr>
<tr>
<td>Mortality after treatment (%)</td>
<td>3.5b</td>
<td>2.5b</td>
<td>13.8b</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP (h)</td>
<td>474 ± 0b</td>
<td>465 ± 0c</td>
<td>477 ± 1a</td>
</tr>
<tr>
<td>EP (h)</td>
<td>484 ± 0b</td>
<td>476 ± 0c</td>
<td>490 ± 1a</td>
</tr>
<tr>
<td>Hatch (h)</td>
<td>498 ± 0a</td>
<td>486 ± 0b</td>
<td>499 ± 0a</td>
</tr>
<tr>
<td>IP to EP (h)</td>
<td>10 ± 0b</td>
<td>11 ± 0b</td>
<td>12 ± 0a</td>
</tr>
<tr>
<td>EP to hatch (h)</td>
<td>14 ± 0a</td>
<td>11 ± 0b</td>
<td>10 ± 0b</td>
</tr>
<tr>
<td>IP to hatch (h)</td>
<td>24 ± 0a</td>
<td>22 ± 0b</td>
<td>22 ± 0b</td>
</tr>
<tr>
<td>Chick weight (g)</td>
<td>48.28 ± 0.25ab</td>
<td>48.32 ± 0.22a</td>
<td>47.40 ± 0.26b</td>
</tr>
<tr>
<td>Relative chick weight (%)</td>
<td>75.43 ± 0.19a</td>
<td>75.33 ± 0.12a</td>
<td>74.51 ± 0.17b</td>
</tr>
<tr>
<td>Hatching (%)</td>
<td>87.5a</td>
<td>88.9a</td>
<td>77.3b</td>
</tr>
<tr>
<td>Mortality before treatment (%)</td>
<td>8.9</td>
<td>7.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Mortality during treatment (%)</td>
<td>2.2b</td>
<td>0.7b</td>
<td>5.8b</td>
</tr>
<tr>
<td>Mortality after treatment (%)</td>
<td>1.4b</td>
<td>1.1b</td>
<td>9.6b</td>
</tr>
</tbody>
</table>

\( a-c \)Different superscript letters indicate a significant difference between treatment groups (\( P < 0.05 \)).

1 Incubation temperature was decreased (low: 34.6°C) or increased (high: 40.6°C) from embryonic day 16 to embryonic day 18.5. The control group was incubated at 37.6°C throughout incubation. Data are shown as mean ± SEM.
control groups in the first experiment and than the low-temperature group in the second experiment. Plasma TG (during treatment, \(P < 0.0001\); after treatment, \(P = 0.0002\)) and NEFA (during treatment, \(P = 0.0003\); after treatment, \(P = 0.0050\)) in experiment 2 are shown in Figure 9a and 9b, respectively. The high-temperature group had significantly reduced plasma TG and NEFA levels at ED 17, ED 18, and hatch compared with the control group. At hatch, plasma TG and NEFA levels of the low-temperature group were significantly lower than those of the control group, resulting in equal levels compared with the high-temperature group. For plasma uric acid levels, a significant age effect was found, but not a significant effect of temperature treatment (data not shown). No significant differences between treatment groups were found in the decreasing lipid content of the residual yolk at any time point (data not shown).

**DISCUSSION**

The present study clearly demonstrates differential effects of continuous higher and lower temperatures, by 3°C, compared with the standard incubation temperature during late incubation (ED 16 to ED 18.5) on embryonic development, the hatching process, thyroid hormone levels, glycogen reserves, and metabolite levels. Because 2 identical experiments were performed with similar starting material, it also gives an idea of the interexperimental variability for incubation experiments, because the average variability between the present 2 incubations was only 1.83%, with a minimum value of 0.02% (pO2 air cell, IP, low-temperature group) and a maximum of 9.8% (pO2 blood, hatch, control group).

**Effect of the High-Temperature Treatment**

Relative embryo and yolk weights, as well as gases in the air cell and blood, all point to a slower embryonic development of the embryos subjected to a continuous 3°C higher incubation temperature from ED 16 to ED 18.5. In both experiments, the high-temperature group had a significantly lower relative yolk-free embryo weight than the control group, together with even higher water content in the embryo at IP and EP. Pies-tun et al. (2009) also found significantly reduced embryonic growth when subjecting embryos to continuous thermal (39.5°C) manipulation from ED 7 to ED 16. Embryos subjected to the high-temperature treatment consumed less yolk, as reflected by the higher remaining yolk weight, which can be only partially explained by the higher water content in the yolk. The slower development of the high-temperature group was further supported by the higher pO2 and lower pCO2 in the air cell and blood, which all indicate a significantly lowered metabolism of the high-temperature group. Moreover, the reduced plasma thyroid hormone levels, as indicators of metabolism level (McNabb, 2000), confirm the alterations in metabolism. Indeed, measurements of plasma metabolites and liver glycogen clearly showed a downward shift in carbohydrate and lipid metabolism. The nonsignificantly affected level of plasma uric acid suggests no effect of the high-temperature treatment on amino acid metabolism. The lower yolk consumption can account for the observed lower plasma TG and NEFA levels of the high-temperature group during and after temperature manipulation. In addition, the equal lipid content (g of lipid/g of dry yolk) present in the residual yolk suggests that the reduced plasma TG and NEFA levels were not caused by reduced absorption of lipids from the yolk by the embryos of the high-temperature group. However, it is still possible that the fatty acid profiles of the residual yolk are different between temperature manipulation treatments because this has been related to selective absorption of fatty acids from the yolk by the embryo (Yalçın et al., 2008b). Because of the lack of O2 in the last stages of incubation, the chick embryo cannot use yolk lipids efficiently for energy and
becomes dependent on gluconeogenesis (Moran, 2007; de Oliveira, 2009). The increased anaerobic production of lactate, which is converted to glucose by hepatic gluconeogenesis, could explain the elevated blood glucose levels found in the high-temperature group. Further measurement of lactate dehydrogenase could confirm this hypothesis. Embryos in the high-temperature group were unable to accumulate a sufficient amount of glycogen in the liver, as indicated by the significantly lower liver glycogen concentration and higher blood glucose concentration during the treatment period, which are needed as energy sources during the last days of incubation (Christensen et al., 2001), and therefore were possibly too weakened by the incubation treatment and energy-demanding hatching process. This is reflected in the significantly lower hatching percentage because of the higher embryonic mortality during the incubation treatment and the higher mortality during the hatching process. The significant reductions in lipid and carbohydrate metabolism would also explain the enormous delay in the hatching process caused by the continuous 3°C higher incubation temperature. In general, temperature manipulations during the first week of incubation will shorten the total duration of incubation according to Van’t Hoff’s rule regarding biochemical reactions (Tzschentke, 2007). However, in the last week of incubation, the Van’t Hoff principle will be overruled by the physiological processes of the embryo, resulting in an increased duration of incubation, depending on the duration, amplitude, and period of incubation temperature manipulation (Moraes et al., 2003; Yahav et al., 2004a; Babacanoğlu et al., 2006; Yalçin et al., 2006, 2008a; Lourens et al., 2007; Piestun et al., 2008, 2009). Depending on the period, and mainly the daily dura-

Figure 6. Plasma thyroxine (T₄) concentration (ng/mL) for experiment 1 (a) and experiment 2 (b). Incubation temperature was decreased (low: 34.6°C) or increased (high: 40.6°C) from ED 16 to ED 18.5. The control group was incubated at 37.6°C throughout incubation. Different letters (a, b) indicate a significant difference between treatment groups at that time point (P < 0.05); n = 12. Data are expressed as mean ± SEM. ED = embryonic day; IP = internal pipping; EP = external pipping.

Figure 7. Liver glycogen concentration (μg of glycogen/mg of wet liver tissue) and blood glucose concentration (mg/dL) for experiment 1 (a) and experiment 2 (b). Incubation temperature was decreased (low: 34.6°C) or increased (high: 40.6°C) from ED 16 to ED 18.5. The control group was incubated at 37.6°C throughout incubation. Different small letters (a–c) indicate a significant difference between treatment groups for liver glycogen levels at that time point (P < 0.05). Different capital letters (A, B) indicate a significant difference between treatment groups for blood glucose levels at that time point (P < 0.05); n = 12. Data are mean ± SEM. ED = embryonic day; IP = internal pipping; EP = external pipping.
tion of temperature manipulation, hatchability can be decreased (Joseph et al., 2006; Collin et al., 2007; Piestun et al., 2008), increased (Yahav et al., 2004a; Collin et al., 2007), or not influenced (Yahav et al., 2004a,b; Collin et al., 2005; Joseph et al., 2006; Piestun et al., 2008; Yalçin et al., 2008a). The deleterious effect of the high-temperature treatment is further reflected in the lower absolute and relative chick weights at hatch, which is in agreement with other continuous higher temperature manipulations (Lourens et al., 2005, 2007; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2007; Piestun et al., 2008). Interestingly, temperature manipulations performed in shocks for 3 to 12 h/d have no effect (Moraes et al., 2003; Yalçin and Siegel, 2003; Yahav et al., 2004a,b; Collin et al., 2005, 2007; Babacanoğlu et al., 2006; Yalçin et al., 2006; Piestun et al., 2008) or even slightly increase the chick weight at hatch (Yalçin et al., 2008a). This makes comparison with the results of other authors difficult because many factors, such as duration, frequency, amplitude, and period of temperature manipulation, all influence the embryonic development and hatching process differently. Moreover, other interfering factors, such as breeder flock age and storage conditions, may be different between experiments and authors may further blur comparisons in the literature. It would seem from these results that the higher incubation temperature manipulation alters the developmental or growth trajectory, also known as developmental plasticity (Schew and Ricklefs, 1998).

**Effect of the Low-Temperature Treatment**

Although many studies have focused on higher temperature manipulation during incubation, few studies have investigated lower incubator temperatures. Our results clearly indicate that a continuously decreased incubator temperature from ED 16 to ED 18.5 affects embryo development to a lesser extent than an opposite temperature increase. Indeed, no differences in relative yolk-free embryo or chick weight, hatchling weight, yolk consumption, water content of both parameters, plasma thyroid hormones, and TG and NEFA levels were observed compared with those of the control group. Only air cell and blood pO2 might suggest a somewhat altered development of embryos exposed to lower incubation temperatures. No effect of the continuous low-temperature treatment was found on hatchability. Shinder et al. (2009) also found no effect on hatchability when exposing chick embryos to a repetitive acute cold treatment on ED 18 and ED 19. However, they did find a greater to significantly greater hatchling weight for cold-treated chicks. The main effect of a decreased incubation temperature was observed on the hatching process, namely, a delay of 9 to 12 h. In addition, the time needed to complete the different stadia of the hatching process was significantly altered by the low incubation treatment. Plausible explanations for this retardation in hatching process could be suggested from liver glycogen reserves and blood glucose levels. It appears that the low treatment group accumulated higher liver glycogen reserves during the treatment period, as reflected by the significantly higher liver glycogen and numerically lower blood glucose levels on ED 17 and ED 18. Thereafter, when reaching the stage of IP, the liver glycogen level of the low-temperature group declined substantially in both experiments, resulting in a significantly lower liver glycogen level and a catching up of the blood glucose level. This greater breakdown of liver glycogen might suggest that the embryos used many of their liver glycogen reserves to adapt to the newly set standard incubation temperature at ED 18.5, which could result in a delayed hatching process. In addition, the lower level of plasma T3 at the stage of IP suggests a delay in development.
of EP (Decuypere et al., 1991; Tona et al., 2007), the lower levels of plasma TG and NEFA at hatch, and the higher level of plasma corticosterone at hatch (De Smit et al., 2006; Everaert et al., 2008), all in experiment 2, could be linked to this retardation of the hatching process in the low-temperature group, as substantiated by a significant increase between EP and hatch in both experiments.

For both temperature treatments, the eggshell temperature followed the incubation temperature. This can be explained by the poikilothermic reaction nature of the chick embryo (Nichelmann and Tzschentke, 2002). In addition, the decreased temperature of embryos exposed to the low incubation temperature indicates that the embryo is not able to increase its metabolic rate because of limitations in gas exchange through the eggshell membranes and eggshell.

**Conclusion**

In conclusion, a continuous 3°C increase in the incubation temperature between ED 16 and ED 18.5 resulted in reduced embryo growth as well as a delay in the hatching process and increased embryonic mortality, which is linked to reduced thyroid hormone levels; decreased liver glycogen reserves, resulting in a decrease in the availability of blood glucose during the hatching process; hyperglycemia, or both in the days before hatch; and decreased plasma TG and NEFA levels. Although the hatching process of embryos exposed to lower incubation temperatures was also significantly retarded, they showed embryonic development and growth strikingly similar to those of the control temperature group, confirming that broiler embryos are more sensitive to high incubation temperatures than to low incubation temperatures (French, 1997).

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