Effects of temperature and CO_2 during late incubation on broiler chicken development

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ABSTRACT

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Incubation conditions need to be adjusted to meet embryonic requirements to obtain optimal chick quality and hatchability. Eggshell temperature (EST) can be used as a non-invasive method to determine embryo temperature. A high EST of 38.9°C during the second or third week of incubation negatively affects chicken embryo development and survival compared to a constant EST of 37.8°C during that period. These negative effects of high EST might be due to a dis-balance between metabolic rate and oxygen (O_2) availability. However, effects of lowering EST, which might restore the balance between metabolic rate and O_2 availability, are largely unknown. Besides EST, the carbon dioxide (CO_2) concentration during late incubation also seems to affect embryo development and might even interact with EST. Based on the potential effects of (lower) EST during the last week of incubation and of CO_2 during only the hatching phase, the following three aims are derived: 1, to investigate effects of EST during the last phase of the incubation process, with special attention for EST below the general accepted optimal EST of 37.8°C, 2, to examine from which day of the incubation process onward EST should be changed from 37.8°C, and 3, to investigate whether CO_2 concentrations are interacting with EST during the hatcher phase.

Time until hatch was longer when an EST of 35.6°C was applied during the last week of incubation, followed by 36.7, 37.8, and 38.9°C, which is probably caused by the lower metabolic rate at an EST below 37.8°C. Hatchability of fertile eggs was not affected at low EST, and EST did not affect time between internal pipping (IP) and hatch. An EST of 35.6 and 36.7°C, resulted in a higher yolk-free body mass (YFBM) at hatch compared to 37.8 and 38.9°C, and residual yolk weight was higher at hatch at 38.9°C compared to all other EST treatments. An EST of 35.6°C resulted in higher hepatic glycogen concentration and amount at IP and hatch compared to all other EST treatments. The proposed mechanism involved is that at lower EST, metabolic rate is reduced, which prevents the embryo from O_2 limitation and ensures that fatty acid oxidation from the yolk can be maintained, resulting in energy production to be invested in growth and development. At an EST of 38.9°C, metabolic rate is high, resulting in a relative O_2 shortage for the embryo. Consequently, lipid oxidation is reduced, which forces the embryo to switch to alternative energy sources, such as glycogen. Because glycogen storage is very limited in the egg and embryo, alternative energy sources such as amino acids obtained from muscles might be

used. A clear interaction between EST and start day of treatment was found for relative heart weight. Relative heart weight was higher at an EST of 35.6°C and decreased with increase in EST. The differences among EST became larger when the EST treatment started earlier.

Effects of CO_2 on embryo physiology, embryonic organ development, and chick quality were marginal. EST interacted with CO_2 mainly before IP, but effects were minor at hatch. Interactions between EST and CO_2 were found at an EST of 36.7 and 37.8°C, but remained absent at an EST of 38.9°C, which might indicate that physiological systems are already challenged due to the higher metabolic rate, which limits the capacity to cope with high CO_2 of the embryo.

No effect of start day of treatment was indicated for embryonic organ development and chick quality found at hatch, which suggests that EST affected these parameters only in the last phase of incubation, e.g. from E19 onward. However, first week post-hatch performance was affected by start day of treatment. The beneficial effects of a lower EST of 35.6 and 36.7°C applied during the last week of incubation found at hatch, might contribute to an enhanced development during the first week post-hatch as body weight, carcass weight, and gain to feed ratio were increased.

In conclusion, results of this thesis show that an EST below 37.8°C during late incubation is beneficial for embryo development, organ growth during incubation, and growth performance during the first week post-hatch. In addition, start day of treatment did not affect chick quality and organ growth, except heart weight, at hatch, which implies that effects of EST occur during the hatching phase, e.g. from E19 onward. Although, an effect of start day of treatment was found on first week post-hatch performance, it remains to be investigated whether an EST below 37.8°C leads to improved later life quality characteristics.

CONTENTS

...Nil volentibus arduum…

Chapter 1

General introduction

1.1 INTRODUCTION

Broiler growth and carcass yield has continued to increase during the last 40 years and are likely to increase in the future as genetics will continue to have major contributions to broiler growth (Havenstein et al., 2003a, 2003b). Embryonic growth and development during incubation are of importance as well, as these factors affect chick quality at day of hatch (Lourens et al., 2005, 2007; Molenaar et al., 2010; 2011a) and consequently will affect broiler performance until slaughter age (Hulet et al., 2007; Leksrisompong et al., 2009; Molenaar et al., 2011a). This implies that any factor that affects embryonic growth and development during incubation might have an impact on later broiler performance.

Factors that might play a role on embryonic growth and development are pre-incubation factors, such as egg quality, egg storage, breeder age, and breeder line (Tona et al., 2005), but also factors during incubation, such as temperature and carbon dioxide (CO_2), play a role because these incubation conditions probably affect embryonic growth and development (Lourens et al., 2005; Leksrisompong et al., 2007; Molenaar et al., 2010) and will affect chick quality at hatch.

Incubation conditions need to be adjusted to meet embryonic requirements to obtain optimal chick quality and hatchability (Meijerhof, 2009). One of the important incubation conditions is temperature, as chicken embryonic growth and development are temperature dependent (Ricklefs, 1987; French, 1994; Christensen et al., 1999). To obtain optimal chick quality, embryo temperature, rather than incubator temperature is proven to be important (Lourens et al., 2005; Meijerhof, 2009). Eggshell temperature (EST) can be used as a non-invasive method to determine embryo temperature (Lourens et al., 2005).

Chicken embryos act as poikilotherm and have limited abilities to regulate their own body temperature (Romijn and Lokhorst, 1955). Low incubator temperatures result in low EST and decreases metabolic rate, which slows down growth and development of the embryo. High EST increases metabolic rate and on its turn increases growth and development (Romanoff, 1936; Christensen et al., 1999; Ricklefs, 1987). However, at high EST and consequently high metabolic rate, energy utilization and the conversion of nutrient sources into body development might be hampered by insufficient oxygen (O_2) availability as exchange of O_2 and CO_2 is restricted due to limited eggshell conductance determined by shell and shell membrane porosity, especially during the second part of the incubation process.

Between 2000 and 2015, several studies have applied an EST or incubator temperature treatment during a specific time phase during incubation. From day of incubation (E) 15 until E19, the embryo reaches its growth peak, which leads to a high metabolic rate, and increases heat production. When incubator temperature is not properly

adjusted to maintain a constant optimal EST (French, 1997), insufficient heat removal from the embryo will lead to a higher EST (van den Brand et al., 2015; Lourens et al., 2006). To mimic current practical situations, the majority of the studies focussed on an EST of 38.9°C or higher, starting from the second or third week of incubation and maintained the EST treatment until hatch (Lourens et al., 2005, 2007; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2007, 2009; Molenaar et al., 2010, 2011a, 2011b).

However, effects of EST during only the hatching phase (day 19 of incubation onward) are poorly investigated and in addition, research on an EST below 37.8°C applied during the last week of incubation is lacking. It might be plausible that an EST below 37.8°C applied during the last week of incubation lowers metabolic rate, which may postpone or even prevent embryos from experiencing O_2 shortage during the plateau phase during the second half of incubation, and consequently might prevent the negative effects on embryonic growth, development, and chick quality at hatch. In addition, it remains unknown from which time points during incubation onward certain, particularly lower, EST can be applied.

1.2 TEMPERATURE

Earlier studies have proven that a constant EST of 37.8°C is a more optimal temperature compared to an EST of 38.9°C, to obtain the lowest third week embryonic mortality, the highest hatchability (Lourens et al., 2005), highest chick quality at hatch, expressed in the longest chick length (Lourens et al., 2005; Molenaar et al., 2010) and highest yolk free body mass (YFBM) (Lourens et al., 2005, 2007; Molenaar et al., 2011a).

The application of an EST of 38.9°C starting from the second or third week of incubation resulted in retarded embryonic organ growth and a lower chick quality at hatch compared to a constant EST of 37.8°C. This was expressed by an on average lower YFBM, higher residual yolk weight, and lower relative liver, stomach, and intestines weight at hatch (Lourens et al., 2005, 2007; Joseph et al., 2006; Leksrisompong et al., 2007, 2009; Molenaar et al., 2010, 2011a, 2011b). A consistent negative effect of EST was particularly found on relative heart weight (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011a).

Not only organ weights and chick quality were affected, but also embryo physiology was affected at an EST of 38.9 compared to 37.8°C. Molenaar et al. (2011b) showed that an EST of 38.9°C applied from E7 onward resulted in a lower glycogen amount at E18 compared to an EST of 37.8°C (Molenaar et al., 2011b), which suggests increased glycogen usage or depressed glycogen synthesis, which might be caused by increased glucose oxidation (Molenaar et al., 2013).

Wineland (2000) applied an increased incubator temperature of 38.6°C during the setter phase, followed by a hatcher temperature of 38.2°C during the hatcher phase. He found a lower relative chick weight and lower relative heart weight compared to a lower setter temperature of 37.5°C, followed by a lower hatcher temperature of 36.9°C (Wineland, 2000). However, chick body weight (BW) at hatch consists of YFBM and residual yolk weight of which YFBM might be a better indicator for chick quality than BW (Lourens et al., 2005, 2007; Molenaar et al., 2011a). Nevertheless, the higher relative heart weight found at a lower temperature suggests that a lower temperature might be beneficial for embryonic development. Romanoff (1960) has indicated earlier that a lower incubator temperature (34.5°C) increases the mitotic index of the heart, leading to a higher heart weight at hatch compared to a high incubator temperature (39.5°C), which suggests an improved development.

EST is not only important for chick quality at hatch, but also for post-hatch performance (Lourens et al., 2005; Hulet et al., 2007; Leksrisompong et al., 2009). Decuypere (1984) demonstrated that incubation temperature is important for later life performance as it influences heat production and thyroid status post-hatch, which might affect growth pattern. Leksrisompong et al. (2009) showed that an EST ≥39.5°C applied from E16 onward resulted in a lower feed intake during the first week of life, a lower BW at day 7, and a higher mortality at day 7 (Leksrisompong et al., 2009). BW at day 7 might be a predicting variable for broiler performance, as BW at day 7 is positively correlated to BW at day 35 (Willemsen et al., 2008). In addition, other studies showed that a high EST of 38.9°C applied from the second or third week of incubation affected subsequent broiler results as indicated by a lower BW at day 21 (Hulet et al., 2007) and a higher mortality due to ascites at day 42 (Molenaar et al., 2011a).

1.3 CO_2

Besides temperature, the CO_2 concentration during incubation also seems to affect embryonic development. Several studies suggested some potential effects of different CO_2 concentrations applied during the second or third week of incubation (Taylor et al., 1970; Buys et al., 1998; Everaert et al., 2007, 2008, 2010). The applied CO_2 concentrations ranged between 2,000 and 4,000 ppm in the study of Buys et al. (1998) and between 20,000 and 40,000 ppm in the studies of Everaert et al. (2007, 2008, and 2010).

Everaert et al. (2007) showed that a high CO_2 concentration of 40,000 ppm did not negatively affect embryonic growth, hatchability, and relative growth until 7 days post-hatch. However, they found a 5 h shorter time until internal pipping (IP) and time until hatch at 40,000 ppm compared to a control CO_2 concentration (Everaert et al., 2007).

Broiler and layer embryos exposed to 40,000 ppm CO_2 did show a higher blood pCO_2, higher pH, higher bicarbonate (HCO_3^-), and higher potassium (K^+) concentration (Everaert et al., 2008, 2010). A higher pCO_2 might be caused by the higher O_2 consumption and higher CO_2 production of the embryo as reaction to high CO_2. The higher HCO_3^- concentration could mainly be the result from intracellular exchange of H^+ with K^+, which suggests that embryos can adapt to high CO_2 (Everaert et al., 2008).

Effects of high CO_2 concentrations during incubation described in literature indicated above might not be related to solely CO_2, as in those studies the requested CO_2 concentration was reached by decreasing the ventilation rate. When ventilation rate is decreased, heat transfer from the eggs is decreased as well, which might affect embryo temperature. Therefore, it might be possible that the decreased time until IP found at high CO_2 (Everaert et al., 2007), was caused by an increased EST instead of the higher CO_2 concentration itself. This suggests that effects of CO_2 might be confounded with effects of EST. Studies that control both EST and CO_2 levels were not reported previously.

1.4 AIMS THESIS

Based on the potential effects of (lower) EST during the last week of incubation and of CO_2 during only the hatching phase, as described above, this following three aims are derived for this thesis: 1, to investigate effects of EST during the last phase of the incubation process, with special attention for EST below the general accepted optimal EST of 37.8°C, 2, to examine from which day of the incubation process onward EST should be changed from 37.8°C, and 3, to investigate whether CO_2 concentrations are interacting with EST during the hatcher phase.

1.5 OUTLINE OF THE THESIS

Studies, in which effects of different EST in combination with different CO_2 concentrations during solely the hatching phase are investigated, are lacking. In addition, effects of an EST below 37.8°C during only the hatching phase were never investigated. Therefore, in this thesis, effects of an EST of 36.7, 37.8, and 38.9°C in combination with a CO_2 concentration of 2.000 or 10.000 ppm during only the hatching phase on embryonic organ growth and chick quality were investigated. Chapter 2 shows the results on embryonic organ development and chick quality, and chapter 3 explains effects of EST and CO_2 on physiological parameters, such as hepatic glycogen and blood plasma metabolites.

During the second part of the current thesis, an even lower EST of 35.6°C was applied to investigate additional effects on chick quality and organ development, as the

earlier proposed EST of 36.7°C resulted in improved chick quality and organ development at hatch compared to an EST of 38.9°C. Additionally, it is largely unknown from which time point during incubation onward, a higher or lower EST than 37.8°C will be detrimental or beneficial for embryonic growth, development, and chick quality at hatch. Therefore, chapter 4, 5, and 6 describe effects of an EST of 35.6, 36.7, 37.8, and 38.9°C starting from E15, E17, or E19 onward. During this study, embryonic organ growth and physiology was closely monitored by measuring organ weights and physiological parameters, such as hepatic glycogen and blood plasma metabolites at E15, E17, E19, IP, external pipping (EP), and hatch. Chapter 4 describes effects on embryonic organ growth and chapter 5 describes the effects of the same study on embryo physiology.

Finally, chapter 6 describes the effects of EST started from E15 onward on chick quality at hatch and first week post-hatch growth and performance as BW day 7 might be a predicting variable for later life performance.

In the general discussion, chapter 7, results from chapter 2 until 6 are combined to discuss effects of an EST of 35.6, 36.7, 37.8, and 38.9°C applied from E15, E17, and E19 onward and a CO_2 concentration of 2,000 and 10,000 ppm applied from E19 onward, on chick embryonic organ growth, chick quality, and physiological parameters, such as hepatic glycogen and blood plasma metabolites. In addition, effects of an EST of 35.6, 36.7, 37.8, and 38.9°C on first week post-hatch growth and performance are discussed.

Chapter 2

Temperature and CO_2 during the hatching phase. I. Effects on chick quality and organ development

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2.1 ABSTRACT

The objective of this study was to investigate the effect of eggshell temperature (EST) and carbon dioxide (CO_2) concentration during only the hatching phase on embryonic development and chick quality. Three batches of eggs were incubated at an EST of 37.8°C until d of incubation (E) 19. From E19, embryos were incubated at low (36.7°C), normal (37.8°C), or high (38.9°C) EST and at low (0.2%) or high (1.0%) CO_2 concentration. Organ growth and embryo and chick quality were measured at E19, internal pipping (IP), hatch, and 12 h after hatch.

A few interactions between EST and CO_2 were found at IP, hatch, and 12 h after hatch, but all of these interactions were temporary and in most cases weak. High EST resulted in a lower relative heart weight compared to low (Δ=0.05) and normal EST (Δ=0.06) at IP, compared to low (Δ=0.11) and normal EST (Δ=0.08) at hatch, and compared to low (Δ=0.11) and normal EST (Δ=0.08) at 12 h after hatch. At hatch, high EST resulted in a lower YFBM compared to low EST (Δ=0.65). At 12 h after hatch, high EST resulted in a lower relative liver weight compared to low EST (Δ=0.12). At low EST, higher relative intestines weight was found compared to normal (Δ=0.41) and high EST (Δ=0.37). The effect of CO_2 solely was only found at 12 h after hatch at which a higher relative heart weight (Δ=0.05) and a higher relative lung weight (Δ=0.0542) was found at high CO_2 compared to low CO_2.

High EST during only the hatching phase negatively affected chick development, mainly expressed by the lower relative heart weight at IP, hatch, and 12 h after hatch and lower YFBM at hatch. The resolving effect of CO_2 demonstrates that CO_2 only seem to have a temporary effect during the hatching phase.

Key words: temperature, CO_2, hatching phase, chick quality, organ development

2.2 INTRODUCTION

Growth and development of the avian embryo are temperature dependent (Ricklefs, 1987; French, 1994; Christensen et al., 1999). A constant eggshell temperature (EST) of 37.8°C until d of incubation (E) 19 has proven to be the optimal temperature to obtain the highest chick quality, expressed in the longest chick length (Lourens et al., 2005, 2007) and highest yolk free body mass (YFBM) (Molenaar et al., 2011a) at the day of hatch. Leksrisompong et al. (2007) showed that an increased EST (>39.5°C) from E14 onward retarded organ growth of embryos. Most responsive organs were heart, liver, gizzard, proventriculus, and small intestine. These results indicate that temperature during incubation affects chick quality. However, the mentioned studies are only focusing on temperature contrasts until E19. Joseph et al. (2006) investigated effects of 2 different EST during 3 different periods of incubation (E0 to E10, E11 to E18, and E19 to hatch). However, these incubation periods were always combined. They indicated that high EST (39.5°C) applied from E18 reduced body weight compared to control EST (38.1°C). To our knowledge, no research is available in which a control EST of 37.8°C was applied until E19 and the effect of EST during only the hatching phase (E19 until 12 h after hatch) is studied.

Besides EST, the carbon dioxide (CO_2) concentration during incubation seems to affect body weight of chicks at hatch. A CO_2 concentration of 0.4% compared to 0.2%, from E14 to E19 resulted in a higher body weight at hatch (Buys et al., 1998). This result is in line with the study of Hassanzadeh et al. (2002), who indicated a higher relative embryo weight (ratio embryo weight to egg weight) at external pipping (EP) when embryos were subjected to a CO_2 concentration of 0.4% compared to 0.2% from E15 until E20. However, it is questionable whether BW and relative embryo weight are reliable parameters for chick quality, because residual yolk is included in these parameters and is therefore not comparable with YFBM. The 0.4% CO_2 concentration in both studies was reached by decreasing the ventilation rate. By decreasing the ventilation rate, heat transfer from eggs and chicks decreases (Lourens et al., 2011). Consequently a decrease in heat transfer might increase EST, which results in a potential confounding effect between EST and CO_2 concentration. Therefore, the effect of an increased CO_2 concentration found on BW and relative embryo weight in the studies of Buys et al. (1998) and Hassanzadeh et al. (2002) may be a result of an increased EST.

Little is known about the effect of the interactions between EST and CO_2 concentration during only the hatching phase (E19 until 12 h after hatch) on organ development and chick quality. The aim of this study was therefore to investigate the effect of EST and CO_2 during the hatching phase on embryonic development and subsequent

chick quality. In the accompanying paper, effects of EST and CO_2 during the hatching phase on physiological characteristics of embryos and chicks are described.

2.3 MATERIALS AND METHODS

2.3.1 Experimental Design

The experiment was set up as a 3 x 2 factorial design with three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%). Treatments were applied from E19 until 12 h after hatch. The treatments were divided over three subsequent groups, with two treatments per group. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University.

2.3.2 Egg Storage and Incubation to E19

Before incubation, eggs were stored for 5 d at a storage temperature of 20°C at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands). After storage, 600 first grade eggs (200 eggs per group) of the same Ross 308 broiler breeder flock (41 through 45 weeks of age) were selected on egg weight between 62 and 65 g. For the first 18 days of incubation, the selected eggs were placed per batch in one incubator (HatchTech BV, Veenendaal, the Netherlands) with a capacity of 4,800 eggs. The rest of the incubator was filled with hatching eggs which were not part of the experiment to ensure uniform airflow across eggs.

Eggshell temperatures (EST) were automatically maintained at 37.8°C until E19. The EST was controlled and monitored by 4 eggshell temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK), which were placed halfway the blunted and pointed end of 4 individual fertile eggs. The sensors were attached to the eggshell using heat-conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and tape. Relative humidity was maintained between 50 and 55%, and CO_2 concentration was maintained between 0.25 and 0.35%. Eggs were turned to an angle of 45° and then turned hourly by 90°.

2.3.3 Incubation from E19 Until 12 h After Hatch

At E19, eggs were candled to identify infertile eggs or eggs containing non-viable embryos. All eggs with viable embryos were transported to the experimental facility of Wageningen University (Wageningen, the Netherlands). Treatments were divided over 3 subsequent batches, with two different EST x CO_2 treatments randomly chosen per batch. Eggs and chicks were continuously exposed to light.

At 432 h (E19), 20 embryos per batch were randomly selected to determine baseline measurements on body weight, yolk weight, YFBM, and weights of heart, liver, lungs, stomach, spleen, bursa, and intestines. The remaining eggs per treatment were placed in individually hatching baskets (120 x 135 mm) in 1 of 2 climate respiration chambers (Verstegen et al., 1987).

From 432 h (E19) until hatch, EST was monitored by 4 individual eggshell sensors as described before. Depending on the treatment, EST was set at 36.7°C (low), 37.8°C (normal), or 38.9°C (high). After 3 h (from 432 h until 435 h of incubation), EST treatment levels were reached. The EST was maintained and controlled for an additional 12 h, from 435 h until 447 h of incubation. At 447 h of incubation, temperature of the climate cell was fixed and EST was allowed to increase during the hatching process. The CO_2 concentrations of 0.2% (low) or 1.0% (high) were reached by injecting CO_2 by a continuous flow inside the climate cell. The CO_2 flow was manually adjusted when the 1.0% CO_2 concentration deviated 0.1% absolute from the desired value and when the 0.2% CO_2 concentration deviated 0.05% absolute from the desired value. Relative humidity was maintained between 50 and 55%.

From 441 h of incubation onward, moment of internal pipping (IP), which was determined by candling, external pipping (EP), and hatch were monitored per chick every 3 h during the process of incubation and hatching. The time intervals between IP and EP and between EP and hatch were calculated. From the moment of hatch until 12 h after hatch, chicks remained in individual hatching baskets and food and water was not provided. Temperature remained fixed and CO_2 was controlled until the last chick was sampled 12 h after hatching (525 h after onset of incubation).

2.3.4 Sampling from E19 Until 12 h After Hatch

At 432 h (E19), 20 eggs were randomly assigned to be used for embryo quality and organ measurements at IP. All other eggs were allowed to hatch and chicks were sampled at hatch or at 12 h after hatch. To distribute chicks per EST and CO_2 combination equally across the hatching process, sequential chicks were alternately allocated for measurements at hatch or at 12 h after hatch.

At 525 h of incubation, non-hatched eggs were opened to determine cause of death. Hatch of fertile (HOF) was expressed as percentage of fertile eggs.

2.3.5 Embryo and Chick Quality Determination and Organ Weight Measurements

Quality and organ measurements were performed at four time points: E19, IP, hatch, and 12 h after hatch. For each treatment, approximately 20 chicks were selected for quality and organ measurements at hatch and approximately 20 chicks at 12 h after hatch. At the

moment of sampling, embryos or chicks were sacrificed by decapitation to obtain yolk weight and YFBM. Pipping muscle and liver of each sampled embryo or chick at E19, IP, at hatch, or 12 h after hatch were removed and weighed and immediately frozen in liquid nitrogen. YFBM was frozen and stored at -20ºC for further analysis of organ weights. Weights of heart, lungs, stomach, spleen, bursa, and intestines of all sampled embryos or chicks at E19, IP, hatch or 12 h after hatch, were determined after thawing YFBM at room temperature.

At hatch and 12 h after hatch, navel quality was scored before decapitation. Navel quality was scored as 1 (closed and clean navel), 2 (discolored navel, open navel up to 2 mm, or both), or 3 (black button exceeding 2 mm, open navel exceeding 2 mm, or both).

2.3.6 Statistical Analysis

Data was processed using the statistical software SAS version 9.2 (2009). Distributions of the means and residuals were examined to verify model assumptions. Moment of IP, EP, hatch, and time intervals between IP and EP, between EP and hatch, and between IP and hatch were analyzed using general linear regression (PROC GLM) with EST, CO_2, and their interaction as class variables. Chick weight, residual yolk weight, YFBM, relative weight of heart, liver, lungs, stomach, spleen, bursa, and intestines were analyzed per moment of sampling (IP, hatch, 12 h after hatch) using the same model. Organ weights were expressed as percentages of the YFBM.

Navel condition score and HOF were analyzed using logistic regression analysis (PROC LOGISIC) with EST, CO_2, and their interaction as class variables. For all parameters, embryo or chick was considered as the experimental unit. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Significance was based on $P \leq 0.05$. Data are expressed as least squares means ± SEM.

2.4 RESULTS

2.4.1 Time Intervals During the Hatching Process

An interaction between EST and CO_2 was found for time until IP ($P<0.001$) and time until EP ($P<0.0001$; Table 1). At normal EST, time until IP and time until EP were both 5 h shorter at high CO_2 compared to low CO_2. No effect of CO_2 on time until IP and EP was found at low and high EST. An effect of EST was found on time until hatch ($P<0.001$; Table 1), at which time until hatch was longer at low EST compared to normal (Δ=2 h) and high EST (Δ=4 h).

An interaction between EST and CO_2 was found for the time intervals between start of treatment until IP ($P<0.001$), between IP and EP ($P<0.05$), between EP and hatch

(P<0.003), and between IP and hatch (P<0.0001; Table 1). At normal EST, high CO_2 decreased the time interval between start of treatment until IP with 4.7 h, and extended the time interval between EP and hatch with 3.4 h compared to low CO_2. No effect of CO_2 on the time intervals between start of the treatment until IP and between EP and hatch was found at low and high EST. At low EST, high CO_2 decreased the time intervals between IP and EP (Δ=3.9 h) and between IP and hatch (Δ=3.8 h) compared to low CO_2.

2.4.2 Day 19 of Incubation

On E19 (432 h), baseline values were assessed for chick weight, residual yolk weight, relative weights of liver, pipping muscle, heart, stomach, intestines, spleen, bursa, and lungs. Chick weight and residual yolk weight were 30.80±0.15g and 12.31±0.27g, respectively. Weights of organs were expressed as percentage of YFBM and were 2.17±0.02% for liver, 1.01±0.02% for pipping muscle, 0.48±0.01% for heart, 4.21±0.06% for stomach, 1.62±0.04% for intestines, 0.0299±0.0017% for spleen, 0.0684±0.0031% for bursa, and 0.6063±0.0154% for lungs.

2.4.3 IP

At IP, an interaction between EST and CO_2 was found for chick weight (P<0.0001), residual yolk weight (P< 0.0001), and relative weight of the pipping muscle (P = 0.01), stomach (P<0.0001), intestines (P<0.0001), and bursa (P<0.03; Table 2). At normal EST, chick weight was higher at high CO_2 compared to low CO_2 (Δ=1.01g) and residual yolk weight was higher at high CO_2 compared to low CO_2 (Δ=1.39g). No effect of CO_2 on chick weight and residual yolk weight was found at low and high EST. Relative pipping muscle weight was lower for the low EST, low CO_2 treatment compared to the high EST, low CO_2 treatment (Δ=0.35%), whereas relative pipping muscle weights for other treatments were comparable. At low EST, relative stomach weight was higher at high CO_2 compared to low CO_2 (Δ=0.48%). No effect of CO_2 on relative stomach weight was found at normal and high EST. At normal EST, low CO_2 resulted in higher relative intestines weight compared to high CO_2 (Δ=0.81%) and a higher relative bursa weight compared to high CO_2 (Δ=0.0233%). No effect of CO_2 on relative intestines weight and relative bursa weight was found at low and high EST.

An effect of EST solely was found for relative heart weight (P=0.0003; Table 2). High EST resulted in a lower relative heart weight compared to low EST (Δ=0.05%) and normal EST (Δ=0.06%).

2.4.4 HOF

No interaction was found between EST and CO_2 for HOF (P=0.99). HOF for the applied treatments was on average 98.9%.

2.4.5 Hatch

At hatch, an interaction between EST and CO_2 was found on relative organs weights of intestines (P<0.0001) and bursa (P=0.001; Table 3). At low EST, relative intestines weight was higher at high CO_2 compared to low CO_2 (Δ=0.66%). No effect of CO_2 on relative intestines weight was found at normal and high EST. Relative bursa weight was higher for the low EST, high CO_2 treatment compared to the normal EST, high CO_2 treatment (Δ=0.0286%), whereas relative bursa weights for other treatments were comparable. EST and CO_2 also interacted for relative spleen weight (P=0.02; Table 3). However, after Bonferroni adjustments no significant differences among treatments were found.

The EST affected YFBM (P=0.01) and relative heart weight (P<0.0001; Table 3). Low EST resulted in a higher YFBM compared to high EST (Δ=0.65g). The YFBM at normal EST was not different from low and high EST. High EST resulted in a lower relative heart weight compared to low EST (Δ=0.11%) and normal EST (Δ=0.08%).

2.4.6 Twelve h After Hatch

At 12 h after hatch, an interaction between EST and CO_2 was found for relative stomach weight (P<0.0001; Table 4). At high EST, relative stomach weight was higher at low CO_2 compared to high CO_2 (Δ=0.60%). No effect of CO_2 on relative stomach weight was found at low and normal EST.

The EST affected relative liver weight (P=0.02) and relative intestines weight (P=0.01; Table 4). High EST resulted in lower relative liver weight compared to low EST (Δ=0.12%). Relative liver weight at normal EST was not different from low and high EST. Low EST resulted in a higher relative intestines weight compared to high EST (Δ=0.37%) and normal EST (Δ=0.41%).

For relative heart weight, an EST (P<0.0001) as well as a CO_2 effect (P=0.01) was found (Table 4). High EST resulted in a lower relative heart weight compared to low EST (Δ=0.11%) and normal EST (Δ=0.08%). Low CO_2 resulted in a lower relative heart weight compared to high CO_2 (Δ=0.05%). An effect of CO_2 solely was found for navel quality (P=0.05) and relative lung weight (P=0.05). High CO_2 resulted in a higher navel quality score, representing a worse navel quality, compared to low CO_2 (Δ=0.2) and a higher relative lung weight compared to low CO_2 (Δ=0.0542%).

2.5 DISCUSSION

The aim of the experiment was to determine whether EST and CO_2 applied during only the hatching phase affect embryonic development and subsequent chick quality. Regardless of CO_2, high EST decreased embryo and chick development, expressed by a lower relative heart weight at IP, hatch, and 12 h after hatch, a lower YFBM at hatch, a lower relative liver weight at 12 h after hatch, and lower intestinal weight at normal and high EST at 12 h after hatch.

These results are probably related to the fact that growth and development of the chick embryo are temperature dependent. Embryos are poikilotherm and less able to regulate their own body temperature by increasing or decreasing their heat production (Romijn and Lokhorst, 1955). Therefore, temperature largely determines metabolic rate. High EST (38.9°C) increases metabolic rate and consequently increases embryonic heat production (Lourens et al., 2007; Molenaar et al., 2010). High EST increases glucose oxidation (Molenaar et al., 2013), which can probably be explained by the increased metabolic rate in combination with the low O_2 availability before EP. High EST probably increases energy requirements, which increases the demand for O_2. However, exchange of O_2 and CO_2 is restricted between E15 and E19 due to limited shell and shell membrane porosity, which results in a plateau phase in heat production (Decuypere et al., 1979; Lourens et al., 2007) until EP, when supplementary O_2 becomes available. To meet the demand for energy during the plateau phase, carbohydrate metabolism may increase because less O_2 is necessary for adenosine triphosphate (ATP) production from carbohydrate oxidation than from lipid or protein oxidation (Schreurs et al., 2007).

Toward the end of incubation, predominantly during the hatching process, glucose becomes an indispensable source for energy because muscle activity is high and O_2 availability is low (de Oliveira et al., 2008). Because concentrations of carbohydrate are low in the initial egg, glucose precursors such as; amino acids, glycerol, and lactate are used for gluconeogenesis to build up glycogen stores in heart, liver, intestines, leg and breast muscle, and yolk sac membrane (Garcia et al., 1986). Glycogen is mobilized when the embryo starts the hatching process and is necessary to ensure embryo survival (Freeman, 1969). High EST increases glucose oxidation; therefore, glycogen stores are depleted more rapidly as shown in Molenaar et al. (2011b). Depletion of glycogen stores might affect embryonic development and subsequent chick quality.

In the current study, at IP lower hepatic glycogen concentrations were found at high EST compared to normal and low EST. At hatch, lower hepatic glycogen concentrations were found at high EST compared to low EST (Maatjens et al., 2014b). The lower liver weight at 12 h after hatch found at high EST compared to low EST may be explained by the

depletion of glycogen stores (Maatjens et al., 2014b). This may also hold for the lower relative intestines weight found at high EST compared to low EST at 12 h after hatch, because besides liver and muscles, the intestines are also considered as a glycogen store (Riesenfeld et al., 1982; Garcia et al., 1986).

To compensate for the limited glycogen stores at high EST at IP and hatch, protein might be used as a glycogenic energy source (Hazelwood and Lorenz, 1959) or for immediate ATP production (McArdle et al., 1981). However, when protein is used as an energy source, protein is not used for growth and development, thereby decreasing the efficiency of protein utilization (Molenaar et al., 2010). The study of Molenaar et al. (2013) also showed higher plasma uric acid levels at high EST compared to normal EST which indicates gluconeogenesis from glucogenic amino acids or immediate ATP production from amino acids. Furthermore, lower relative heart weights were found at high EST (Lourens et al., 2007), suggesting that protein sources from e.g. the heart might be used for glycogenic energy supply.

In the studies of Lourens et al. (2007) and Molenaar et al. (2010, 2013), high EST was only applied until E18 or E19. In the current study, high EST was applied during only the hatching phase, and resulted in a lower relative heart weight at IP, hatch, and 12 h after hatch and a lower YFBM at hatch. These results suggest that glucogenic amino acids may be used for energy production instead of development. Already at IP, 38 h after start of the treatment, lower relative heart weights were found at high EST compared to normal and low EST, extending to a larger difference in relative heart weight at high EST compared to normal and low EST at hatch, 63 h after start of the treatment.

The negative effect of high EST on YFBM at hatch was also found in other studies (Lourens et al., 2005, 2007; Molenaar et al., 2010, 2013) and can be explained by the less efficient protein and energy utilization (Molenaar et al., 2010; Lourens et al., 2011) and shorter incubation duration (Molenaar et al., 2010). The YFBM increase between IP and hatch was lower at high EST compared to low EST. These results suggest that efficiency of nutrient utilization before hatch is negatively affected by high EST compared to low EST, but not at high EST compared to normal EST.

Although no main effects of CO_2 were found at IP and hatch, regardless of EST, high CO_2 increased relative heart weight and relative lung weight at 12 h after hatch. Although we only found CO_2 effects at 12 h after hatch, the data need to be interpreted with caution. The potential biological underlying mechanism for the increased relative heart and lung weight at 12 h after hatch may be the following: central chemoreceptors for respiratory control are responsive to CO_2 which affects the respiratory magnitude. The respiratory magnitude increases in response to a higher CO_2 concentration (Harada et al., 1985). In addition, during the energy-demanding hatching process, the metabolic rate is increased

which increases the blood oxygen (O_2) demand. The blood O_2 demand stimulates vasoconstriction in the arterioles of the pulmonary vascular system to activate segments in the lung that are normally not involved in blood O_2 transport (Decuypere et al., 2000). When more O_2 is required, more blood has to be transported through the body to supply main organs of O_2. Subsequently, the heart has to increase the workload to keep up with the request for blood flow. The combination of the aforementioned processes might lead to an increase in relative lung weight subsequently leading to a higher relative heart weight at high CO_2, at 12 h after hatch.

In the current study no other main effects of CO_2 on chick quality were found at IP, hatch, and 12 h after hatch. A few interactions between EST and CO_2 were found at IP, hatch, and 12 h after hatch, but all of these interactions were temporary. However, reasons for these differences remain unclear. In summary, high EST during the hatching phase decreased chick development, mainly expressed by the lower relative heart weight at IP, hatch, and 12 h after hatch and the lower YFBM at hatch. This means that embryo development can be retarded at high EST not only during the first 18 d of incubation as demonstrated earlier, but also during the relatively short hatching phase. Low EST resulted in equal or higher organ weights and chick quality parameters compared to normal EST, therefore low EST did not decrease embryo development and chick quality. Effects of CO_2 during the hatching phase seem to be marginal because EST interacted with CO_2 mainly before IP, but effects were minor at hatch and 12 h after hatch. Therefore, the effect of an increased CO_2 concentration found on relative embryo weight at EP and BW at hatch as described in literature may not be a result of CO_2 itself, but is more likely a result of an increased EST due to a limited ventilation rate.

2.6 ACKNOWLEDGMENTS

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Table 1. Effects of three eggshell temperature (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation through hatch on incubation time until internal pipping (IP), until external pipping (EP), until hatch, and time intervals between start treatment and IP, between IP and EP, between EP and hatch, and between IP and hatch during the hatching process.

	Time IP (h)	Time EP (h)	Time hatch (h)	Time interval Start treatment - IP (h)
EST (°C)				
36.7	472	484	497[a]	39.5
37.8	470	481	495[b]	37.6
38.9	468	480	493[b]	36.1
SEM	0.6	0.7	0.6	0.55
CO_2 (%)				
0.2	470	482	495	38.0
1.0	470	481	495	37.5
SEM	0.5	0.6	0.5	0.45
EST (°C) x CO_2 (%)				
36.7 x 0.2	470[ab]	484[ab]	498	38.2[ab]
36.7 x 1.0	473[a]	483[abc]	497	40.7[a]
37.8 x 0.2	472[a]	484[a]	496	39.9[a]
37.8 x 1.0	467[b]	479[d]	494	35.2[b]
38.9 x 0.2	468[b]	479[cd]	492	35.7[b]
38.9 x 1.0	469[b]	480[bcd]	494	36.5[b]
SEM	0.8	1.0	0.8	0.8
P-value				
EST	<0.001	<0.001	<0.0001	<0.001
CO_2	0.45	0.03	0.82	0.45
EST x CO_2	<0.001	0.02	0.13	<0.001

[a-d]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

Table 1 (continued). Effects of three eggshell temperature (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation through hatch on incubation time until internal pipping (IP), until external pipping (EP), until hatch, and time intervals between start treatment and IP, between IP and EP, between EP and hatch, and between IP and hatch during the hatching process.

	Time interval IP - EP (h)	Time interval EP - hatch (h)	Time interval IP - hatch (h)
EST (°C)			
36.7	11.7	13.6	25.0
37.8	12.7	13.4	25.9
38.9	11.5	13.5	24.9
SEM	0.56	0.39	0.57
CO_2 (%)			
0.2	12.8	12.7	25.3
1.0	11.2	14.2	25.2
SEM	0.46	0.32	0.47
EST (°C) x CO_2 (%)			
36.7 x 0.2	13.7^{a}	13.8^{abc}	26.9^{a}
36.7 x 1.0	9.8^{b}	13.4^{abc}	23.1^{b}
37.8 x 0.2	12.9^{ab}	11.7^{c}	24.6^{ab}
37.8 x 1.0	12.5^{ab}	15.1^{a}	27.3^{a}
38.9 x 0.2	11.7^{ab}	12.7^{bc}	24.4^{ab}
38.9 x 1.0	11.2^{ab}	14.3^{ab}	25.4^{ab}
SEM	0.8	0.6	0.8
P-value			
EST	0.26	0.92	0.39
CO_2	0.02	<0.001	0.92
EST x CO_2	0.05	0.003	<0.001

$^{a-d}$Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

Table 2. Effects of three eggshell temperature (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organ weights of embryos at internal pipping (IP; n=20 per EST x CO_2 treatment).

	Chick weight (g)	Residual yolk (g)	YFBM[1] (g)	Liver (% of YFBM)	Pipping muscle (% of YFBM)
EST (°C)					
36.7	45.88	8.86	37.02	2.08	1.81
37.8	45.72	9.04	36.68	2.07	1.91
38.9	45.94	9.18	36.76	2.03	1.94
SEM	0.17	0.19	0.19	0.02	0.06
CO_2 (%)					
0.2	45.93	8.92	37.01	2.07	1.86
1.0	45.77	9.13	36.64	2.05	1.91
SEM	0.13	0.16	0.16	0.02	0.05
EST (°C) x CO_2 (%)					
36.7 x 0.2	46.19^{ab}	9.15^{ab}	37.04	2.10	1.73^{b}
36.7 x 1.0	45.58^{ab}	8.57^{b}	37.00	2.06	1.89^{ab}
37.8 x 0.2	45.22^{b}	8.35^{b}	36.87	2.10	1.77^{ab}
37.8 x 1.0	46.23^{a}	9.74^{a}	36.49	2.04	2.05^{ab}
38.9 x 0.2	46.38^{a}	9.27^{ab}	37.11	2.01	2.08^{a}
38.9 x 1.0	45.50^{ab}	9.10^{ab}	36.41	2.05	1.80^{ab}
SEM	0.23	0.27	0.27	0.04	0.08
P-value					
EST	0.63	0.51	0.43	0.29	0.24
CO_2	0.41	0.34	0.10	0.52	0.44
EST x CO_2	<0.001	<0.001	0.49	0.29	0.01

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

[1]Yolk free body mass.

Table 2 (continued). Effects of three eggshell temperature (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organ weights of embryos at internal pipping (IP; n=20 per EST x CO_2 treatment).

	Heart (% of YFBM[1])	Stomach (% of YFBM)	Intestines (% of YFBM)	Spleen (% of YFBM)	Bursa (% of YFBM)	Lungs (% of YFBM)
EST (°C)						
36.7	0.52[a]	4.67	2.87	0.0249	0.0768	0.6287
37.8	0.53[a]	4.51	2.78	0.0255	0.0713	0.6170
38.9	0.47[b]	4.64	2.76	0.0271	0.0698	0.5649
SEM	0.01	0.07	0.09	0.0019	0.0038	0.0196
CO_2 (%)						
0.2	0.51	4.62	2.83	0.0247	0.0759	0.5814
1.0	0.50	4.60	2.77	0.0269	0.0694	0.6256
SEM	0.01	0.06	0.07	0.0015	0.0031	0.0161
EST (°C) x CO_2 (%)						
36.7 x 0.2	0.53	4.43[b]	2.62[bc]	0.0231	0.0758[ab]	0.5987
36.7 x 1.0	0.52	4.91[a]	3.12[ab]	0.0268	0.0779[ab]	0.6586
37.8 x 0.2	0.54	4.60[ab]	3.18[a]	0.0262	0.0829[a]	0.6050
37.8 x 1.0	0.51	4.42[b]	2.37[c]	0.0248	0.0596[b]	0.6291
38.9 x 0.2	0.47	4.82[ab]	2.70[abc]	0.0250	0.0691[ab]	0.5406
38.9 x 1.0	0.48	4.47[b]	2.81[abc]	0.0293	0.0706[ab]	0.5892
SEM	0.01	0.10	0.12	0.0026	0.0053	0.0278
P-value						
EST	<0.001	0.21	0.64	0.69	0.40	0.06
CO_2	0.45	0.82	0.51	0.31	0.14	0.06
EST x CO_2	0.21	<0.001	<0.001	0.47	0.03	0.80

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

[1]Yolk free body mass.

Table 3. Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organs weights of chicks at hatch (n=20 per EST x CO_2 treatment).

	Chick weight (g)	Residual yolk (g)	YFBM[1] (g)	Navel score[2]	Liver (% of YFBM)	Pipping muscle (% of YFBM)
EST (°C)						
36.7	46.53	6.25	40.28[a]	1.3	2.42	1.42
37.8	46.30	6.43	39.87[ab]	1.4	2.39	1.46
38.9	45.97	6.35	39.63[b]	1.4	2.32	1.49
SEM	0.17	0.13	0.15	0.1	0.03	0.04
CO_2 (%)						
0.2	46.14	6.29	39.85	1.4	2.40	1.44
1.0	46.39	6.39	40.00	1.4	2.35	1.47
SEM	0.14	0.11	0.12	0.1	0.03	0.03
EST (°C) x CO_2 (%)						
36.7 x 0.2	46.62	6.36	40.26	1.3	2.45	1.45
36.7 x 1.0	46.43	6.14	40.29	1.4	2.38	1.39
37.8 x 0.2	45.94	6.25	39.69	1.5	2.38	1.41
37.8 x 1.0	46.66	6.61	40.05	1.4	2.39	1.51
38.9 x 0.2	45.86	6.27	39.59	1.4	2.36	1.46
38.9 x 1.0	46.08	6.42	39.66	1.5	2.28	1.52
SEM	0.24	0.19	0.21	0.1	0.04	0.05
P-value						
EST	0.07	0.65	0.01	0.61	0.06	0.38
CO_2	0.21	0.54	0.38	0.56	0.21	0.50
EST x CO_2	0.17	0.29	0.71	0.50	0.51	0.28

[a-b]Least squares 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 score of chickens per treatment group scored with 1, 2, or 3, where 1 = closed and clean navel, 2 = discolored navel, open navel up to 2 mm, or both, or 3 = black button exceeding 2 mm, open navel exceeding 2 mm, or both.

Table 3 (continued). Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organs weights of chicks at hatch (n=20 per EST x CO_2 treatment).

	Heart (% of YFBM[1])	Stomach (% of YFBM)	Intestines (% of YFBM)	Spleen (% of YFBM)	Bursa (% of YFBM)	Lungs (% of YFBM)
EST (°C)						
36.7	0.69[a]	5.61	3.97	0.0277	0.0800	0.6666
37.8	0.66[a]	5.53	3.62	0.0254	0.0662	0.6377
38.9	0.58[b]	5.50	3.83	0.0266	0.0789	0.6683
SEM	0.02	0.09	0.10	0.0017	0.0041	0.0228
CO_2 (%)						
0.2	0.65	5.62	3.79	0.0259	0.0743	0.6552
1.0	0.64	5.48	3.82	0.0273	0.0758	0.6598
SEM	0.01	0.07	0.08	0.0014	0.0034	0.0186
EST (°C) x CO_2 (%)						
36.7 x 0.2	0.68	5.57	3.64[b]	0.0231	0.0682[ab]	0.6335
36.7 x 1.0	0.71	5.65	4.30[a]	0.0324	0.0918[a]	0.6997
37.8 x 0.2	0.66	5.61	3.77[ab]	0.0277	0.0693[ab]	0.6432
37.8 x 1.0	0.65	5.46	3.46[b]	0.0230	0.0632[b]	0.6322
38.9 x 0.2	0.60	5.67	3.97[ab]	0.0269	0.0853[ab]	0.6890
38.9 x 1.0	0.56	5.32	3.69[b]	0.0264	0.0725[ab]	0.6475
SEM	0.02	0.12	0.14	0.0025	0.0059	0.0322
P-value						
EST	<0.001	0.64	0.05	0.65	0.04	0.57
CO_2	0.68	0.17	0.84	0.50	0.75	0.87
EST x CO_2	0.31	0.23	<0.001	0.02	0.01	0.23

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Yolk free body mass.

Table 4. Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organs weights of chicks at 12 h after hatch (n=20 per EST x CO_2 treatment).

	Chick weight (g)	Residual yolk (g)	YFBM[1] (g)	Navel score[2]	Liver (% of YFBM)	Pipping muscle (% of YFBM)
EST (°C)						
36.7	44.47	4.84	39.63	1.5	2.82[a]	1.17
37.8	44.70	4.98	39.72	1.6	2.74[ab]	1.23
38.9	44.28	4.95	39.33	1.6	2.70[b]	1.21
SEM	0.17	0.16	0.19	0.1	0.03	0.03
CO_2 (%)						
0.2	44.33	4.82	39.52	1.5[b]	2.77	1.19
1.0	44.64	5.03	39.61	1.7[a]	2.73	1.22
SEM	0.14	0.13	0.15	0.1	0.02	0.03
EST (°C) x CO_2 (%)						
36.7 x 0.2	44.44	4.59	39.85	1.5	2.85	1.19
36.7 x 1.0	44.50	5.08	39.41	1.6	2.79	1.15
37.8 x 0.2	44.32	4.83	39.49	1.4	2.77	1.18
37.8 x 1.0	45.08	5.13	39.96	1.7	2.70	1.28
38.9 x 0.2	44.24	5.03	39.21	1.6	2.70	1.19
38.9 x 1.0	44.33	4.87	39.46	1.7	2.71	1.22
SEM	0.24	0.23	0.26	0.1	0.04	0.04
P-value						
EST	0.22	0.82	0.31	0.71	0.02	0.33
CO_2	0.12	0.28	0.66	0.05	0.23	0.37
EST x CO_2	0.25	0.36	0.21	0.52	0.54	0.31

[a-c]Least squares 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 score of chickens per treatment group scored with 1, 2, or 3, where 1 = closed and clean navel, 2 = discolored navel, open navel up to 2 mm, or both, or 3 = black button exceeding 2 mm, open navel exceeding 2 mm, or both.

Table 4 (continued). Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on chick quality and relative organs weights of chicks at 12 h after hatch (n=20 per EST x CO_2 treatment).

	Heart (% of YFBM[1])	Stomach (% of YFBM)	Intestines (% of YFBM)	Spleen (% of YFBM)	Bursa (% of YFBM)	Lungs (% of YFBM)
EST (°C)						
36.7	0.81[a]	6.17	4.98[a]	0.0259	0.0800	0.7228
37.8	0.78[a]	6.04	4.57[b]	0.0245	0.0712	0.7143
38.9	0.70[b]	6.07	4.61[b]	0.0294	0.0850	0.6698
SEM	0.02	0.08	0.09	0.0022	0.0046	0.0237
CO_2 (%)						
0.2	0.74[b]	6.19	4.71	0.0262	0.0780	0.6752[b]
1.0	0.79[a]	5.99	4.74	0.0270	0.0794	0.7294[a]
SEM	0.01	0.06	0.07	0.0018	0.0038	0.0194
EST (°C) x CO_2 (%)						
36.7 x 0.2	0.77	6.01[abc]	4.83	0.0247	0.0724	0.6988
36.7 x 1.0	0.86	6.32[ab]	5.14	0.0272	0.0877	0.7467
37.8 x 0.2	0.78	6.19[c]	4.68	0.0271	0.0765	0.7063
37.8 x 1.0	0.78	5.89[bc]	4.46	0.0218	0.0657	0.7223
38.9 x 0.2	0.66	6.37[a]	4.62	0.0269	0.0853	0.6206
38.9 x 1.0	0.73	5.77[c]	4.60	0.0320	0.0848	0.7191
SEM	0.02	0.11	0.13	0.0031	0.0065	0.0335
P-value						
EST	<0.001	0.49	0.01	0.26	0.10	0.24
CO_2	0.01	0.03	0.82	0.77	0.80	0.05
EST x CO_2	0.11	<0.001	0.11	0.21	0.14	0.46

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

[1]Yolk free body mass.

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Chapter 3

Temperature and CO_2 during the hatching phase. II. Effects on chicken embryo physiology

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3.1 ABSTRACT

The objective of this study was to investigate the effect of eggshell temperature (EST) and carbon dioxide (CO_2) concentration during only the hatching phase on physiological characteristics of embryos and chicks. Three groups of eggs were incubated at an EST of 37.8°C until d 19 of incubation (E19). From E19, embryos were incubated at a low (36.7°C), normal (37.8°C), or high (38.9°C) EST and at a low (0.2%) or high (1.0%) CO_2 concentration. For E19, internal pipping (IP), hatch, and 12 h after hatch, blood parameters were analysed, and hepatic glycogen was determined.

At IP, hatch, and 12 h after hatch, interactions were found between EST and CO_2, but all these interactions were temporary and in most cases weak. High EST resulted in a lower hepatic glycogen concentration compared to low (Δ=21.1) and normal EST (Δ=14.43) at IP, and a lower hepatic glycogen concentration compared to low EST (Δ=6.24) at hatch. At hatch, high EST resulted in lower hematocrit value (Δ=2.4) and higher potassium (K^+) (Δ=0.5) compared to low EST. At 12 h after hatch, high EST resulted in a higher lactate concentration compared to low (Δ=0.77) and normal EST (Δ=0.65). And high EST resulted in higher potassium compared to low (Δ=0.4) and normal EST (Δ=0.3). An effect of CO_2 solely was only found at IP, at which high CO_2 resulted in a lower pH (Δ=0.03) and a lower hepatic glycogen concentration (Δ=7.27) compared to low CO_2.

High EST during only the hatching phase affected embryo and chick physiology, indicated by the lower hepatic glycogen levels at IP and hatch. High CO_2 affected pH and hepatic glycogen at IP. Effects of CO_2 were only found at low EST, which emphasizes the large effect of EST during the hatching phase.

Key words: temperature, CO_2, hatching phase, chick embryo, physiology

3.2 INTRODUCTION

The hatching phase is characterized by physiological and metabolic processes, which are vital for embryonic survival (Christensen et al., 1999). One of these processes during the hatching phase is the mobilization of hepatic glycogen stores (Freeman, 1969; Pearce, 1971; Garcia et al., 1986, Christensen et al., 2001), because glycogen serves as an energy source during the hatching process (Pearce, 1971). Another process during the hatching phase is the onset of pulmonary respiration which is initiated when the air cell membrane is pierced by the beak of the embryo [internal pipping (IP)]. When the embryo pierces the air cell at IP, more oxygen (O_2) is temporally available and the metabolic rate increases (Vleck et al., 1980). About 24 h later the embryo will start to pip the outer shell membrane and eggshell [external pipping (EP)]. Parallel to this process, the yolk sac begins to enter the body cavity, on d 19 of incubation (E) 19. The yolk is completely drawn into the body on E20 (Christensen, 2009). The combination of the aforementioned processes is necessary to ensure embryo survival and to form a good quality chicken at day of hatch. External factors, such as eggshell temperature (EST) and carbon dioxide (CO_2) concentration, might affect these processes.

Until E19, effects of EST, incubation temperature, and CO_2 have been thoroughly studied. A constant EST of 37.8°C until E19 has proven to be the optimal temperature to gain the highest chick quality (Lourens et al., 2005, 2007; Molenaar et al., 2010, 2011a). Leksrisompong et al. (2007) showed that an increased EST (>39.5°C) from E14 onward retarded organ growth of embryos. Joseph et al. (2006) investigated effects of 2 different EST during 3 different periods of incubation (E0 - E10, E11 - E18, and E19 to hatch). However, these incubation periods were always combined. They indicated that high EST (39.5°C) applied from E18 reduced body weight compared to control EST (38.1°C). In addition, Willemsen et al. (2010) demonstrated that embryo physiology was affected by incubation temperature. Continuous high incubation temperatures (40.6°C) between E16 and E18 affected blood glucose levels, embryonic growth, partial pressure of CO_2 in blood (pCO_2), hepatic glycogen levels, and blood lactate levels at different time points compared to low incubation temperatures (34.6°C). However, in the study of Willemsen et al. (2010), incubation temperature and not EST was used as treatment. Meijerhof and van Beek (1993) demonstrated that incubation temperature is not equal to EST, and that it can be assumed that EST better reflects the metabolic state of an embryo than incubation temperature (Lourens et al., 2005).

Besides temperature, a few studies indicated that the carbon dioxide (CO_2) concentration affects embryonic metabolism during incubation. In the study of Everaert et al. (2008), a CO_2 concentration of 4.0% from E11 until E18 resulted in a higher blood pH,

higher bicarbonate (HCO_3^-) levels from E12 until E16, higher potassium levels, and lower partial pressure of O_2 in blood (pO_2) at E13 compared to normal CO_2 concentrations. The treatment was applied between E11 and E18, but it is possible that effects of CO_2 during the hatching phase may have a more severe effect, because metabolic rate and, subsequently, CO_2 production, are high during the hatching phase. Hassanzadeh et al. (2002) indicated a higher hematocrit level, higher pCO_2 level and lower pO_2 level in blood at EP when embryos were subjected to a CO_2 concentration of 0.4% compared to 0.2% from E15 until E20. However, the CO_2 effect in both studies (Hassanzadeh et al., 2002 and Everaert et al., 2008) might be confounded with EST because the CO_2 concentration of 0.4% (Hassanzadeh et al., 2002) and the CO_2 concentration of 4.0% (Everaert et al., 2008) were both reached by decreasing the ventilation rate. Heat transfer from eggs and chicks is influenced by ventilation and decreases when the ventilation rate decreases. A decrease in heat transfer might increase EST, which might be confounded with CO_2.

The mentioned studies only applied treatments until E18 or from E18 to hatch. However, effects of EST simultaneous with different CO_2 concentrations on embryo physiology during only the hatching phase (E19 until 12 h after hatch) are not studied yet. The aim of this study, therefore, was to investigate the effect of EST and CO_2 during the hatching phase on physiological characteristics of embryos or chicks. In the accompanying paper, effects of EST and CO_2 during the hatching process on embryo and chick development are described.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Design

The experiment was set up as a 3 x 2 experimental design with three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%). Treatments were applied from E19 until 12 h after hatch. The treatments were divided over three subsequent groups, with two treatments per group. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University.

3.3.2 Egg Storage and Incubation to E19

Details about storage and incubation are given in Maatjens et al. (2014a). In short, a total of 600 first grade Ross 308 eggs from the same breeder flock (200 eggs per group, 41-45 wk of age) were stored and incubated until E19 at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands).The eggs were set in one incubator with a capacity of 4,800 eggs (HatchTech BV, Veenendaal, the Netherlands) at an EST of 37.8°C, with at a RH

between 50 and 55%, and a CO_2 concentration between 0.25 and 0.35%. Eggs were turned to an angle of 45° and then turned hourly by 90°.

3.3.3 Incubation From E19 Until 12 h After Hatch

Details about incubation from E19 until 12 h after hatch are given in Maatjens et al. (2014a). In short, at E19 eggs were transported to the experimental facility of Wageningen University. The eggs were incubated in individual hatching baskets (120 x 135 mm) in 1 of 2 climate respiration chambers, which were entered via a lock (Verstegen et al., 1987) at an EST of 36.7, 37.8, or 38.9°C and a CO_2 concentration of 0.2 or 1.0%.

From 432 h (E19) until hatch, EST was monitored and controlled by 4 individual eggshell sensors (as described in Maatjens et al., 2014a). At 447 h of incubation, temperature of the climate cell was fixed and EST was allowed to increase during the hatching process. The CO_2 concentrations of 0.2 or 1.0% were reached by injecting CO_2 by a continuous flow inside the climate cell and were manually adjusted. Relative humidity was maintained between 50 and 55%.

3.3.4 Sampling From E19 Until 12 h After Hatch

From 441 h of incubation onwards, the moment of IP (determined by candling), EP, and hatch were monitored per chick every 3 h during the process of incubation and hatching. The time intervals between IP and EP and between EP and hatch were calculated (Maatjens et al., 2014a).

At 432 h (E19), 20 embryos were randomly assigned to be used for determination of blood parameters and hepatic glycogen measurements at IP. Hatching of the remaining eggs was recorded every 3 h to determine average incubation duration for each treatment group. To distribute chicks per EST and CO_2 combination equally across the hatching process, sequential chicks were alternately allocated for measurements at hatch or at 12 h after hatch.

3.3.5 Blood Parameter Measurements and Hepatic Glycogen Determination

At 432 h (E19), IP, hatch, and 12 h after hatch, blood was extracted from the jugular vein of the embryos or chicks using a 1-mL syringe and 30G needle and collected in heparinized tubes. Thereafter, blood was collected in a heparinized capillary (150 µl) and immediately presented to a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory, Lexington, MA) to determine pH, pCO_2, pO_2, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium (K^+), and calcium (Ca^{2+}). Remaining blood was centrifuged (2,900 x *g*) for 15 minutes. Blood plasma was decanted and stored at -20.0°C for further

analysis. Plasma uric acid was determined with a commercially available enzymatic kit (DiaSys Diagnostic Systems International, Holzheim, Germany).

After blood collection, the embryos or chicks were decapitated to dissect the liver. After determination of liver weight, livers were frozen in liquid nitrogen and stored at -80.0°C for further analysis. Procedures to determine hepatic glycogen were carried out on ice. Approximately 300 mg of liver was homogenized after the addition of the same amount of 7% $HClO_4$ as tissue. The suspension was centrifuged (2,900 x *g*) at 4°C for 15 minutes. The supernatant was decanted, cleaned with 1 mL of petroleum ether, and frozen at -20.0°C for further analysis. 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.

3.3.6 Statistical Analysis

Data was processed using the statistical software SAS version 9.2 (2009). Distributions of the means and residuals were examined to verify model assumptions. Values for pH, pCO_2, pO_2, bicarbonate, glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen were analyzed per moment of sampling (IP, hatch, 12 h after hatch) using general linear regression (PROC GLM) with EST, CO_2, and their interaction as class variables. A log transformation was applied for lactate and plasma uric acid to obtain normally distributed data.

For all parameters, chick was considered as the experimental unit. Least squares means were compared using Bonferroni adjustments for multiple comparisons. Significance was based on $P \leq 0.05$. Data are expressed as least squares means ± SEM.

3.4 RESULTS

3.4.1 Day 19 of Incubation

On E19 (432 h), baseline values were assessed for blood gas parameters, blood (plasma) metabolites, and hepatic glycogen. Values for pH, pCO_2, and pO_2 were 7.4±0.0, 45.7±1.0, and 16.1±0.4 mm of Hg respectively. Values for blood (plasma) metabolites were 29.7±0.5 mmol/L for bicarbonate, 130.0±2.3 mg/dL for blood glucose, 1.54±0.08 mmol/L for lactate, 25.4±0.5% for hematocrit, 3.7±0.1 mmol/L for potassium, and 1.06±0.02 mmol/L for calcium. The value of plasma uric acid was measured at 3.5±0.3 mg/dL, and hepatic glycogen at 46.67±2.00 mg/g wet liver tissue.

3.4.2 Internal Pipping

At IP, an interaction between EST and CO_2 was found for glucose (P=0.01), lactate (P=0.04), and potassium (P=0.01; Table 1). Glucose was lower for the low EST, low CO_2 treatment compared to the normal EST, low CO_2 treatment (Δ=16.8 mg/dL) and high EST, low CO_2 treatment (Δ=16.3 mg/dL). Glucose concentrations for other treatments were comparable. Lactate concentrations were lower for the high EST, high CO_2 treatment compared to the low EST, low CO_2 treatment (Δ=0.49 mmol/L), whereas lactate concentrations for other treatments were comparable. Potassium was lower for the low EST, low CO_2 treatment compared to the normal EST, high CO2 treatment (Δ=0.6 mmol/L) and high EST, low CO_2 treatment (Δ=0.5 mmol/L). An interaction between EST and CO_2 was also found for pO_2 (P=0.03) and hematocrit (P=0.05; Table 1). However, after Bonferroni adjustments no significant differences among treatments were found.

For hepatic glycogen, an effect of EST (P<0.0001) and CO_2 (P=0.02) was found (Table 1). At high EST hepatic glycogen was lower compared to low (Δ=21.43 mg/g) and normal EST (Δ=14.38 mg/g). At high CO_2, hepatic glycogen was also lower compared to low CO_2 (Δ=7.03 mg/g). An effect of CO_2 solely was found for pH (P<0.01; Table 1), at which pH was lower (Δ=0.03) at high CO_2 compared to low CO_2.

3.4.3 Hatch

At hatch, an interaction between EST and CO_2 was found for pH (P=0.05), pCO_2 (P=0.02), and glucose (P=0.02; Table 2). At normal EST, pH was lower at high CO_2 compared to low CO_2 (Δ=0.08), whereas no effect of CO_2 on pH was found at low and high EST. At low EST, the level of pCO_2 was higher at high CO_2 compared to low CO_2 (Δ=6.9 mm Hg). No effect of CO_2 on pCO_2 was found at normal and high EST. Glucose concentration was lower for the normal EST, high CO_2 treatment compared to the high EST, low CO_2 treatment (Δ=19.5 mg/dL). Glucose concentrations for other treatments were comparable. An interaction between EST and CO_2 was also found for lactate concentration (P=0.05; Table 2). However, after Bonferroni adjustments no significant differences among treatments were found.

An effect of EST was found for hematocrit (P=0.01), potassium (P=0.02), and hepatic glycogen (P=0.02; Table 2). High EST resulted in lower hematocrit (Δ=2.4%) and lower hepatic glycogen concentration (Δ=6.28 mg/g) compared to low EST. At normal EST, hematocrit and hepatic glycogen were comparable to low and high EST. High EST resulted in higher potassium levels compared to low EST (Δ=0.5 mmol/L), whereas potassium at normal EST was comparable to low and high EST.

3.4.4 Twelve h After Hatch

At 12 h after hatch, an interaction between EST and CO_2 was found for pCO_2 (P=0.02), bicarbonate (P=0.05), glucose (P<0.001), and calcium (P=0.0002; Table 3). At low EST, pCO_2 was higher at high CO_2 compared to low CO_2 (Δ=8.6 mm Hg), bicarbonate was higher at high CO_2 compared to low CO_2 (Δ=3.4 mmol/L), and calcium was higher at high CO_2 compared to low CO_2 (Δ=0.22 mmol/L). No effect of CO_2 on pCO_2, bicarbonate, and calcium was found at normal and high EST. At low EST, glucose was higher at high CO_2 compared to low CO_2 (Δ=26.9 mg/dL). For glucose, also an effect of CO_2 was found at normal EST at which glucose was higher at low CO_2 compared to high CO_2 (Δ=15.4 mg/dL). No effect of CO_2 on glucose was found at high EST.

An effect of EST was found for lactate (P=0.01) and potassium (P=0.03; Table 3). Lactate was higher at high EST compared to low EST (Δ=0.77 mmol/L) and normal EST (Δ=0.65 mmol/L). Potassium was higher at high EST compared to low EST (Δ=0.4 mmol/L). Potassium at normal EST was not different from low and high EST.

3.5 DISCUSSION

Treatments of EST and CO_2 applied during only the hatching phase affected chick embryo physiology with regard to EST. Results of Maatjens et al. (2014a) showed that high EST affected embryo and chick organ development at IP, hatch, and 12 h after hatch. A few interactions between EST and CO_2 were found at IP, hatch, and 12 h after hatch, but all of these interactions were temporary and, in most cases, weak. An effect of EST was found on time until hatch, at which time until hatch was longer at low EST (497 h) compared to normal (495 h) and high EST (493 h). No interaction was found between EST and CO_2 for hatch of fertile (HOF); HOF for the applied treatments was on average 98.9%. Main effects of high EST were expressed in lower relative heart weights at IP, hatch, and 12 h after hatch, lower yolk free body mass (YFBM) at hatch, and lower relative liver and intestine weight at 12 h after hatch. High EST resulted in a lower relative heart weight (0.47% of YFBM) compared to low (0.52% of YFBM) and normal EST (0.53% of YFBM) at IP, a lower relative heart weight at high EST (0.58% of YFBM) compared to low (0.69% of YFBM) and normal EST (0.66% of YFBM) at hatch, and a lower relative heart weight at high EST (0.70% of YFBM) compared to low (0.81% of YFBM) and normal EST (0.78% of YFBM) at 12 h after hatch. At hatch, high EST resulted in a lower YFBM (39.63g) compared to low EST (40.28g). At 12 h after hatch, high EST resulted in a lower relative liver weight (2.70% of YFBM) compared to low EST (2.82% of YFBM). At low EST, a higher relative intestines weight (4.98% of YFBM) was found compared to normal (4.57% of YFBM) and high EST (4.61% of YFBM). The effect of CO_2 alone was only found at 12

h after hatch. High CO_2 resulted in a higher relative heart weight (0.79% of YFBM) compared to low CO_2 (0.74% of YFBM) and a higher relative lung weight (0.7294% of YFBM) compared to low CO_2 (0.6752% of YFBM). The results in the current paper indicate that high EST results in lower hepatic glycogen concentration at IP and hatch, lower hematocrit at hatch, higher potassium, and higher lactate concentrations at 12 h after hatch.

During the hatching phase, energy is required to maintain physiological processes and to ensure hatching. While metabolic rate of the embryo increases during hatching, the requirement for glucose increases, as glucose is an important source for adenosine triphosphate (ATP) production (de Oliviera et al., 2008). Because the glucose level is low in the initial egg (Romanoff and Romanoff, 1949), hepatic glycogen stores are built up during incubation by gluconeogenesis. Hepatic glycogen is mobilized when the embryo starts the hatching process and is necessary to ensure embryo survival (Freeman, 1969).

In the current study, embryos incubated at high EST had a lower hepatic glycogen concentration at IP compared to embryos incubated at normal and low EST. At hatch, the hepatic glycogen concentration was lower at high EST compared to low EST, whereas at hatch, hepatic glycogen for normal EST was comparable with low and high EST. No difference was found between EST at 12 h after hatch. Overall hepatic glycogen concentration decrease between E19 (when EST treatments started) and 12 h after hatch was similar for all EST treatments. However, hepatic glycogen loss between E19 and IP was substantially higher and between IP and hatch was substantially lower at high EST. This suggests that, at high EST, hepatic glycogen stores are more depleted before IP and that less hepatic glycogen is available during the hatching process. Therefore, it can be assumed that embryos incubated at high EST during the hatching phase used more hepatic glycogen after E19 until hatch compared to normal or low EST.

As a difference of only 2 h in hatching time exists between low and normal EST and between normal and high EST, it can be assumed that energy requirements during the hatching process may be comparable between EST treatments. This suggests that, at high EST, more glycogenic energy is needed for other sources. To compensate for the limited glycogen stores at high EST at IP and hatch, protein might be used as a glycogenic energy source (Hazelwood and Lorenz, 1959) or used for immediate ATP production (McArdle et al., 1981). Protein sources from e.g. the heart may be used for glycogenic energy supply resulting in lower relative heart weights at IP, hatch, and 12 h after hatch (Maatjens et al., 2014a). The effect of EST on hepatic glycogen was earlier described by Molenaar et al. (2011b), who found that high EST decreased hepatic glycogen at hatch when eggs were incubated at normal and high EST from E7 to E19. The treatment duration mentioned was much longer compared to the treatment duration of the current study, which emphasizes the

large effect of high EST during only the hatching phase only. Compared to the study of Molenaar et al. (2011b) at which EST treatments were applied for 12 days, 57.1% of the total amount of hepatic glycogen differences in their study may be clarified by the effect of high EST applied from E19. The effect of high EST in the current study is large because the time interval in which the treatment was applied was much shorter than in the study of Molenaar et al. (2011b). Already at IP, 38 h after start of the treatment, a lower total hepatic glycogen amount was found at high EST compared to normal and low EST. A difference in total hepatic glycogen remained at high EST compared to normal and low EST at hatch, 63 h after start of the treatment. Willemsen et al. (2010) investigated the effect of a short high incubation temperature (40.6°C) treatment between E16 to E18. They also indicated decreased hepatic glycogen levels at E17, E18, IP, and EP compared to the low incubation temperature (34.6°C) treatment. In addition, embryonic stages at which treatments were applied were different between the aforementioned studies; this indicating that, compared to the second week of incubation, physiological processes during the hatching phase might become more responsive to external stimuli as EST.

As indicated previously, high EST decreased hepatic glycogen at IP and hatch. The lower relative heart weight at high EST at IP, hatch, and 12 h after hatch and lower relative intestine weight at high EST at 12 h after hatch (Maatjens et al., 2014a) indicate that protein might be used as an energy source because hepatic glycogen stores might be insufficient for hatching. However, no effect of EST was found on the plasma uric acid concentration. This result is in line with the study of Molenaar et al. (2011b) in which no effect EST on plasma uric acid concentration was found.

High EST increased blood lactate concentration compared to normal and low EST at 12 h after hatch. Increased lactate levels at high EST indicate increased glucose metabolism by the glycolytic pathway and might be caused by the increased metabolic rate at high EST. Muscle activity is high and the O_2 availability is low during the hatching process (de Oliviera et al., 2008). Therefore, the concentration of blood lactate will increase because glucose in the muscles is used for anaerobic glycolysis (Freeman, 1965; Moran, 2007; de Oliviera et al., 2008). Pyruvate is formed during anaerobic glycolysis. The increase in hydrogen ions (H^+) in the blood caused by pyruvate will be buffered by bicarbonate ions (HCO_3^-), thereby preventing blood pH decrease. To preserve the acid-base balance in blood, H^+ in the blood is exchanged with potassium (K^+) from intracellular plasma (Everaert et al., 2008). As a consequence, the level of potassium in blood increased at hatch and 12 h after hatch at high EST in the current study. The increase in buffering capacity of the blood during embryonic development is not only caused by an increase in HCO_3^-, but also due to an increase in hematocrit (Tazawa, 1971; Tazawa and Piiper, 1984). However,

in our study, high EST resulted in a lower hematocrit level at hatch, and was not affected at 12 h after hatch.

Only main effects of CO_2 were found on hepatic glycogen and pH at IP, which were both lower at high CO_2 compared to low CO_2. High EST may have increased the oxidation of carbohydrates expressed by the lower hepatic glycogen concentration in the current study. The higher metabolic rate at high EST causes the embryo to seek anaerobic sources for energy. The respiratory magnitude increases in response to higher CO_2 concentrations (Harada et al., 1985). The increase in respiratory magnitude stimulates activity of muscles, heart, and lung (Maatjens et al., 2014a) to maintain physiological processes, thereby inducing glycogenolysis which results in a lower hepatic glycogen level. Central chemoreceptors for respiratory control react to H^+ in the blood and are therefore responsive to CO_2 which affects the respiratory magnitude (Harada et al., 1985). The increase in H^+ at IP decreases pH at high CO_2.

In summary, high EST in the hatching phase affects embryo and chick physiology clearly demonstrated by the lower hepatic glycogen levels at hatch and the higher lactate level at 12 h after hatch. High CO_2 affects pH and hepatic glycogen at IP. Interactions between EST and CO_2 during the hatching phase were found, but seem to be temporary and were mainly found at low EST. No effect of CO_2 on blood physiology parameters was found at high EST, which might indicate no physiological reactions or adaptive mechanisms to compensate for the effect on CO_2 on the acid-base balance in the blood (Everaert et al., 2008). Production of CO_2 by the embryo or chicks increases during development (Bruggeman et al., 2007). The high metabolic rate and high CO_2 production at high EST compared to low EST by the embryo or chick itself, may be responsible for the little effect of CO_2 at high EST. Results of this study emphasize the negative effect of high EST on chick physiology during the hatching phase.

3.6 ACKNOWLEDGMENTS

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Table 1. Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at internal pipping (IP; n=20 per EST x CO_2 treatment).

	pH	pCO_2 (mm Hg)	pO_2 (mm Hg)	HCO_3^- (mmol/L)	glucose (mg/dL)	lactate[1] (mmol/L)
EST (°C)						
36.7	7.40	44.7	18.7	27.5	136.5	1.82
37.8	7.38	47.5	16.4	27.7	142.2	1.81
38.9	7.41	44.3	19.3	28.2	143.8	1.65
SEM	0.01	1.5	0.9	0.6	2.6	0.08
CO_2 (%)						
0.2	7.41[a]	44.2	17.9	28.1	142.6	1.84
1.0	7.38[b]	46.8	18.4	27.5	139.0	1.68
SEM	0.01	1.2	0.7	0.5	2.2	0.06
EST (°C) x CO_2 (%)						
36.7 x 0.2	7.40	43.7	16.8	26.8	131.6[b]	1.98[a]
36.7 x 1.0	7.40	45.6	20.6	28.1	141.4[ab]	1.67[ab]
37.8 x 0.2	7.40	46.3	16.0	28.4	148.4[a]	1.74[ab]
37.8 x 1.0	7.36	48.7	16.8	27.1	136.0[ab]	1.88[ab]
38.9 x 0.2	7.43	42.6	21.0	29.0	147.9[a]	1.81[ab]
38.9 x 1.0	7.38	46.0	17.7	27.4	139.6[ab]	1.49[b]
SEM	0.01	2.1	1.3	0.9	3.7	0.11
P-value						
EST	0.09	0.24	0.05	0.76	0.15	0.21
CO_2	0.01	0.14	0.68	0.44	0.25	0.07
EST x CO_2	0.16	0.94	0.03	0.26	0.01	0.04

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

Table 1 (continued). Effects of three eggshell temperatures (EST; 36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at internal pipping (IP; n=20 per EST x CO_2 treatment).

	hematocrit (%)	potassium (K^+, mmol/L)	calcium (Ca^{2+}, mmol/L)	uric acid[1] (mg/dL)	hepatic glycogen (mg/g)
EST (°C)					
36.7	22.6	3.9	0.88	4.19	37.77[a]
37.8	25.0	4.2	1.00	4.57	31.10[a]
38.9	24.1	4.2	0.97	4.23	16.67[b]
SEM	0.7	0.1	0.04	0.23	2.49
CO_2 (%)					
0.2	23.9	4.0	0.98	4.26	32.15[a]
1.0	23.9	4.2	0.92	4.40	24.88[b]
SEM	0.6	0.1	0.03	0.19	2.04
EST (°C) x CO_2 (%)					
36.7 x 0.2	23.4	3.8[b]	0.89	4.15	39.99
36.7 x 1.0	21.9	4.0[ab]	0.87	4.24	35.56
37.8 x 0.2	25.7	4.0[ab]	1.08	4.28	37.10
37.8 x 1.0	24.3	4.4[a]	0.93	4.87	25.10
38.9 x 0.2	22.8	4.3[a]	0.98	4.36	19.35
38.9 x 1.0	25.4	4.1[ab]	0.96	4.10	13.99
SEM	1.0	0.1	0.05	0.33	3.52
P-value					
EST	0.07	0.02	0.06	0.42	<0,001
CO_2	0.95	0.15	0.16	0.62	0.02
EST x CO_2	0.05	0.01	0.32	0.41	0.48

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

Table 2. Effects of three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O_2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at hatch (n=20 per EST x CO_2 treatment).

	pH	pCO_2	pO_2	HCO_3^-	glucose	lactate[1]
		(mm Hg)	(mm Hg)	(mmol/L)	(mg/dL)	(mmol/L)
EST (°C)						
36.7	7.46	31.5	27.9	22.5	160.4	2.82
37.8	7.46	30.4	28.0	21.6	156.3	2.61
38.9	7.48	29.6	29.7	21.9	159.9	2.57
SEM	0.01	0.7	1.5	0.6	2.9	0.11
CO_2 (%)						
0.2	7.49	28.7	28.5	21.6	162.7	2.75
1.0	7.45	32.3	28.6	22.4	155.1	2.59
SEM	0.01	0.6	1.2	0.5	2.3	0.09
EST (°C) x CO_2 (%)						
36.7 x 0.2	7.48^{ab}	28.0^{b}	28.7	21.1	156.0^{ab}	2.81
36.7 x 1.0	7.44^{ab}	34.9^{a}	27.2	23.9	164.8^{ab}	2.82
37.8 x 0.2	7.50^{a}	28.9^{b}	29.9	22.1	162.8^{ab}	2.55
37.8 x 1.0	7.42^{b}	32.0^{ab}	26.1	21.0	149.7^{b}	2.68
38.9 x 0.2	7.48^{ab}	29.1^{b}	26.9	21.5	169.2^{a}	2.89
38.9 x 1.0	7.48^{ab}	30.0^{b}	32.4	22.2	150.7^{ab}	2.26
SEM	0.01	1.0	2.1	0.8	4.1	0.16
P-value						
EST	0.43	0.18	0.64	0.49	0.56	0.26
CO_2	0.02	<0.001	0.97	0.25	0.03	0.22
EST x CO_2	0.05	0.02	0.08	0.06	0.02	0.05

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

Table 2 (continued). Effects of three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O_2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at hatch (n=20 per EST x CO_2 treatment).

	hematocrit (%)	potassium (K^+, mmol/L)	calcium (Ca^{2+}, mmol/L)	uric acid[1] (mg/dL)	hepatic glycogen (mg/g)
EST (°C)					
36.7	28.6[a]	3.6[b]	1.00	3.20	17.93[a]
37.8	26.7[ab]	3.8[ab]	1.02	2.90	12.82[ab]
38.9	26.2[b]	4.1[a]	1.02	3.20	11.69[b]
SEM	0.6	0.1	0.03	0.23	1.58
CO_2 (%)					
0.2	27.5	3.8	1.04	3.09	14.90
1.0	26.8	3.8	0.98	3.12	13.39
SEM	0.5	0.1	0.03	0.19	1.39
EST (°C) x CO_2 (%)					
36.7 x 0.2	29.2	3.4	0.99	2.86	17.79
36.7 x 1.0	27.9	3.7	1.01	3.55	18.07
37.8 x 0.2	27.2	4.0	1.08	2.78	13.46
37.8 x 1.0	26.2	3.6	0.95	3.02	12.18
38.9 x 0.2	26.0	4.0	1.06	3.62	13.44
38.9 x 1.0	26.4	4.2	0.98	2.79	9.93
SEM	0.8	0.2	0.05	0.32	2.23
P-value					
EST	0.01	0.02	0.89	0.56	0.02
CO_2	0.37	0.66	0.11	0.91	0.42
EST x CO_2	0.49	0.06	0.25	0.06	0.70

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

Table 3. Effects of three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O_2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at 12 h after hatch (n=20 per EST x CO_2 treatment).

	pH	pCO_2 (mm Hg)	pO_2 (mm Hg)	HCO_3^- (mmol/L)	glucose (mg/dL)	lactate[1] (mmol/L)
EST (°C)						
36.7	7.41	32.6	26.3	20.7	169.3	2.25[b]
37.8	7.43	32.3	30.4	21.1	165.9	2.37[b]
38.9	7.40	32.3	28.4	20.3	172.4	3.02[a]
SEM	0.01	1.1	2.1	0.5	2.7	0.17
CO_2 (%)						
0.2	7.42	31.1	28.6	20.0	167.0	2.58
1.0	7.40	34.4	28.1	21.4	171.4	2.51
SEM	0.01	0.9	1.7	0.4	2.2	0.14
EST (°C) x CO_2 (%)						
36.7 x 0.2	7.44	28.3[b]	26.7	19.0[b]	155.9[b]	2.06
36.7 x 1.0	7.39	36.9[a]	25.9	22.4[a]	182.8[a]	2.43
37.8 x 0.2	7.45	31.2[ab]	31.0	21.2[ab]	173.6[a]	2.43
37.8 x 1.0	7.41	33.4[ab]	29.7	20.9[ab]	158.2[b]	2.31
38.9 x 0.2	7.38	33.7[ab]	28.1	19.9[ab]	171.5[ab]	2.24
38.9 x 1.0	7.41	32.9[ab]	28.7	20.8[ab]	173.4[ab]	2.79
SEM	0.02	1.5	3.0	0.8	3.9	0.24
P-value						
EST	0.41	0.78	0.39	0.63	0.24	0.01
CO_2	0.25	0.01	0.83	0.03	0.17	0.74
EST x CO_2	0.18	0.02	0.95	0.05	<0.001	0.27

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

Table 3 (continued). Effects of three EST (36.7, 37.8, or 38.9°C) and two CO_2 concentrations (0.2 or 1.0%) from d 19 of incubation until 12 h after hatch on pH, partial pressure of CO_2 (pCO_2) and O_2 (pO_2) in blood, bicarbonate (HCO_3^-), glucose, lactate, hematocrit, potassium, calcium, plasma uric acid, and hepatic glycogen at 12 h after hatch (n=20 per EST x CO_2 treatment).

	hematocrit (%)	potassium (K^+, mmol/L)	calcium (Ca^{2+}, mmol/L)	uric acid[1] (mg/dL)	hepatic glycogen (mg/g)
EST (°C)					
36.7	24.3	3.2[b]	0.99	3.17	10.24
37.8	24.1	3.3[ab]	1.02	3.00	9.46
38.9	25.7	3.6[a]	1.06	2.98	9.72
SEM	0.5	0.1	0.03	0.25	1.09
CO_2 (%)					
0.2	25.1	3.4	1.01	3.01	9.40
1.0	24.3	3.4	1.03	3.09	10.21
SEM	0.4	0.1	0.03	0.20	0.90
EST (°C) x CO_2 (%)					
36.7 x 0.2	24.2	3.2	0.88[b]	2.80	11.01
36.7 x 1.0	24.3	3.3	1.10[a]	3.54	9.47
37.8 x 0.2	25.4	3.4	1.10[a]	2.83	8.03
37.8 x 1.0	22.8	3.2	0.94[ab]	3.16	10.89
38.9 x 0.2	25.6	3.5	1.06[ab]	3.39	9.16
38.9 x 1.0	25.8	3.7	1.06[ab]	2.56	10.28
SEM	0.7	0.1	0.04	0.35	1.55
P-value					
EST	0.09	0.03	0.31	0.85	0.86
CO_2	0.23	0.63	0.53	0.79	0.53
EST x CO_2	0.11	0.28	<0.001	0.08	0.32

[a-b]Least squares means followed by different superscripts within a column and factor are significantly different ($P \leq 0.05$).

[1]Transformed from log values back to original least squares means.

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Chapter 4

Temperature during the last week of incubation. I. Effects on hatching pattern and broiler chicken embryonic development

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4.1 ABSTRACT

We investigated effects of an eggshell temperature (EST) of 35.6, 36.7, 37.8, or 38.9°C applied from d of incubation (E) 15, E17, or E19 on hatching pattern and embryonic organ development. A total of 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were incubated at an EST of 37.8°C until E15. From E15, E17, or E19 onward, eggs were incubated at an EST of 35.6, 36.7, 37.8, or 38.9°C. Moment of internal pipping (IP), external pipping (EP), and hatch were determined and organ development was measured at E15, E17, E19, IP, EP, and hatch.

A lower EST extended incubation duration compared to a higher EST. The lower incubation duration was mainly caused by the extended time until IP, whereas time between IP and hatch hardly varied between treatments.

Relative heart weight was affected by EST already from 2 d after start of EST treatment on E15, and effects became more pronounced at longer exposure time to various EST treatments. At hatch, the largest difference in relative heart weight was found between an EST of 35.6 and 38.9°C started at E15 (Δ=64.4%). From E17 onward, EST affected yolk-free body mass (YFBM) and relative stomach weight, where a lower EST resulted in a lower YFBM and relative stomach weight before IP and a higher YFBM and relative stomach weight after IP. From E19 onward, a lower EST resulted in a higher relative liver and spleen weight regardless start time of treatment. Yolk weight and relative intestine weight were not affected by EST before and at E19, but a higher EST resulted in a higher yolk weight and lower relative intestine weight from IP onward.

Based on the higher YFBM and higher relative organ growth found at hatch, we concluded that an EST lower than 37.8°C from E15 onward appears to be beneficial for optimal embryo development.

Key words: temperature, incubation, embryonic organ development

4.2 INTRODUCTION

Incubation conditions need to be adjusted to meet embryonic requirements to obtain optimal chick quality and hatchability (Meijerhof, 2009). Temperature is one of the most important factors during incubation and drives embryonic growth and development (Ricklefs, 1987; French, 1994; Christensen et al., 1999), which impact chick quality and post hatch performance (Lourens et al., 2005). The importance of maintaining the correct embryo temperature during incubation is proven to be more important than incubator temperature settings (Lourens et al., 2005; Meijerhof, 2009). Eggshell temperature can be used as a non-invasive method to determine embryo temperature (Lourens et al., 2005).

Literature has indicated that a constant eggshell temperature (EST) of 37.8°C until d of incubation (E) 19 resulted in the highest hatchability, lowest third week embryonic mortality, and highest chick quality, expressed by a higher yolk free body mass (YFBM) at d of hatch (Lourens et al., 2005, 2007). An EST of 38.9°C from E7 onwards resulted in a lower YFBM at d of hatch compared to an EST of 37.8°C (Lourens et al., 2007, Molenaar et al., 2011). Leksrisompong et al. (2007) showed that an EST of 39.5°C and higher from E14 onwards retarded growth of heart, liver, gizzard, proventriculus, and intestines of embryos. Maatjens et al. (2014a) applied an EST of 36.7, 37.8, and 38.9°C from E19 until hatch. Results indicated that an EST of 38.9°C resulted in a lower relative heart weight already at internal pipping (IP), but also at hatch, and 12 h after hatch. An EST of 36.7°C resulted in a higher YFBM at hatch compared to an EST of 38.9°C, and in a higher liver and intestinal weight at 12 h after hatch compared to an EST of 38.9°C.

All these studies suggested that an EST ≥ 38.9°C should be avoided from E7 onwards or even during only the hatching phase (time from IP till hatch) to prevent negative effects on embryonic development. On the other hand, an EST of 36.7°C during only the hatching phase might have beneficial effects on embryonic development and chick quality.

In the previous study of Maatjens et al. (2014a), an EST of 36.7°C was the lowest investigated EST which was applied from E19. However, it can be questioned whether an even lower EST might be beneficial for embryonic development and additionally, it is largely unknown what the most sensitive moment is to change EST from a constant EST of 37.8°C to a lower or higher EST. Because of this, we aimed to investigate effects of an EST of 35.6, 36.7, 37.8, and 38.9°C starting from E15, E17, and E19 on embryonic organ development and chick quality.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Design

Broiler eggs were incubated at an EST of 37.8°C until E15. From E15, E17, and E19 onwards EST changed to 35.6, 36.7, or 38.9°C or was maintained at 37.8°C. Table 1 shows an overview of the various treatment groups, i.e. changes in EST at E15, E17, or E19. At E15, E17, E19, internal pipping (IP), external pipping (EP), and hatch, embryos or chicks were sampled. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, the Netherlands.

Table 1. Setup of the experiment with 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, 38.9°C) applied from 3 time points (E; E15, E17, or E19) through moment of placement

	Days of incubation			
Treatment	E0 - E15	E15 - E17	E17 - E19	E19 - hatch
1	37.8	35.6	35.6	35.6
2	37.8	36.7	36.7	36.7
3 (control)	37.8	37.8	37.8	37.8
4	37.8	38.9	38.9	38.9
5	37.8	37.8	35.6	35.6
6	37.8	37.8	36.7	36.7
7	37.8	37.8	38.9	38.9
8	37.8	37.8	37.8	35.6
9	37.8	37.8	37.8	36.7
10	37.8	37.8	37.8	38.9

4.3.2 Egg Storage and Incubation up to E15

Before incubation, eggs were stored for 5 d at a storage temperature of 20°C at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands). After storage, 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were selected on an egg weight between 62 and 65 g. For the first 15 d of incubation, the selected eggs were placed in one incubator (HatchTech BV, Veenendaal, the Netherlands) with a capacity of 4,800 eggs. The rest of the incubator was filled with hatching eggs which were not part of the experiment to ensure uniform airflow across eggs.

EST was maintained at 37.8°C until E15. EST was controlled and monitored by 4 EST sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK), which were placed halfway the blunted and pointed end of 4 individual fertile eggs. The sensors were

attached to the eggshell using heat-conducting paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and a small piece of tape. Incubator temperature was adjusted based on the average temperature of the 4 EST sensors. Relative humidity was maintained between 50 and 55%, and CO_2 concentration did not exceed 0.35%. Eggs were placed on setter trays and turned hourly by an angle of 45°.

4.3.3 Incubation from E15 until Hatch

At E14 (332 h), eggs were candled to identify infertile eggs or eggs containing non-viable embryos. All eggs containing viable embryos were transported to the experimental facility of Wageningen University (Wageningen, the Netherlands) for 30 minutes in a climate controlled car.

At E15 (336 h), after arrival at the experimental facility, 3 times 240 eggs were assigned to one out of 4 climate respiration chambers (Heetkamp et al., 2015) in which EST was maintained at 35.6°C (treatment 1), 36.7°C (treatment 2), or 38.9°C (treatment 4). The remaining 2,130 eggs were placed in the Control climate respiration chamber in which EST was maintained at 37.8°C (treatment 3) (Table 1).

At E17 (384 h), 3 times 210 eggs were moved from the Control climate respiration chamber to one of the three climate respiration chambers in which EST was maintained at 35.6°C (treatment 5), 36.7°C (treatment 6), or 38.9°C (treatment 7).

Finally at E19 (432 h), 3 times 180 eggs were moved from the Control climate respiration chamber to one of the 3 climate respiration chambers in which EST was maintained at 35.6°C (treatment 8), 36.7°C (treatment 9), or 38.9°C (treatment 10) (Table 1).

All eggs were placed in individually hatching baskets (120 x 135 mm) and eggs and chicks were continuously exposed to light. From E15 (336 h) until hatch, EST was monitored by the median of 5 individual eggshell sensors per climate respiration chamber as described before. Depending on the treatment, EST was set at 35.6°C, 36.7°C, 37.8°C, or 38.9°C. At E19 (453 h), temperature of the climate respiration chamber was fixed at its current setting and EST was allowed to increase during the hatching process. Relative humidity was maintained between 50 and 55%, and CO_2 concentration did not exceed 0.35%.

From E19 (453 h) of incubation onwards, moment of internal pipping (IP), which was determined by candling all eggs individually, external pipping (EP), and hatch were monitored every 6 h. The time intervals between IP and EP and between EP and hatch were calculated. Chicks remained in individual hatching baskets, without feed and water, until moment of sampling.

4.3.4 Sampling from E15 until Hatch

At E15 (336 h), before dividing the eggs over the treatments, 30 eggs were randomly chosen to determine YFBM, yolk weight, and weights of heart, liver, stomach (gizzard and proventriculus), spleen, bursa, and intestines. At the same moment, at E15, 30 eggs per treatment were randomly chosen to determine YFBM, yolk weight, and weights of heart, liver, stomach (gizzard and proventriculus), spleen, bursa, and intestines at E17, E19, IP, and EP. All other eggs were allowed to hatch and 30 chicks per treatment were sampled at hatch. At E24 (555 h) of incubation, when all chicks had hatched, non-hatched eggs were opened to determine fertility, or cause and moment of death. Hatch of fertile (HOF) was expressed as percentage of fertile eggs, based on hatched chicks from viable eggs after candling at E15.

4.3.5 Organ Weight Measurements

Organ weights were determined at six time points: E15, E17, E19, IP, EP, and hatch. At the moment of sampling, embryos or chicks were sacrificed by decapitation to obtain yolk weight and YFBM. From each embryo or chick, the liver was removed, weighed, and immediately frozen in liquid nitrogen. YFBM was frozen and stored at -20°C for further analysis of organ weights. Weights of heart, stomach (gizzard and proventriculus), spleen, bursa, and intestines of all sampled embryos or chicks sacrificed at E15, E17, E19, IP, EP or hatch, were determined after thawing YFBM at room temperature at a later moment.

4.3.6 Statistical Analysis

Separate analyses were performed for the data collected at E17, E19, IP, EP, and hatch.

At E17, there were four treatments (eggs moved from 37.8°C to another EST, plus a Control treatment of eggs constantly at 37.8°C) in a completely randomised design that was analysed by one-way ANOVA. The F-test was used to test for overall differences between treatments, followed by pairwise comparisons by Fisher's LSD method.

At E19, there were seven treatments, consisting of combinations of EST in 2 time trajectories (E15 to E17 and E17 to E19). By introducing factors for EST (35.6, 36.7, 38.9°C) and for start d of treatment (eggs moved after E15 or E17), the design consists of a 3 x 2 factorial scheme (eggs moved from 37.8°C to another EST after E15 or E17) and an "added" control (eggs constantly at 37.8°C). All data were analysed with a single model in a single analysis. F-tests were performed for interaction and main effects of EST and start d of treatment corresponding to the 3 x 2 factorial scheme. Depending upon the significance of the interaction, pairwise comparisons with Fisher's LSD method were made between the six means of combinations of EST and start d of treatment (P-value interaction ≤ 0.05), or

between the separate 3 means for EST and 2 means for start d of treatment (*P*-value interaction > 0.05). Similarly, depending upon the significance of the interaction, the control treatment was compared with the 6 means of combinations of 3 EST and 2 start d of treatment, or with the separate 3 means for EST and 2 means for start d of the treatment.

At IP, EP, and hatch, there were 10 treatments, consisting of EST in 3 time trajectories (E15 to E17, E17 to E19, and >E19). Again introducing factors for EST and starting day of treatment (eggs moved after E15, E17, or E19), the design consists of a 3 x 3 factorial scheme (eggs moved from 37.8°C to another EST after E15, E17, or E19) and an "added" control (eggs constantly at 37.8°C). Again, F-tests were performed and, depending upon the significance of interaction, appropriate pairwise comparisons by Fisher's LSD method were made, similar to the analysis at E19.

Model assumptions, i.e. normality and equal variance of the error terms in the linear models, were checked by inspection of residual plots. For relative bursa weight, data were log transformed prior to analysis and results are displayed as the inverse log values with accompanying CI. HOF was analysed using logistic regression analysis with main effects and interaction for EST and start d of treatment. All analyses were performed with SAS (Version 9.3, SAS Institute 2010).

4.4 RESULTS

4.4.1 Time Intervals During the Hatching Process

An interaction between eggshell temperature (EST) and start day of treatment was found for time until IP ($P<0.001$), time until EP ($P<0.001$), time until hatch ($P<0.001$), and time interval between start treatment until IP ($P<0.001$; Table 2). Generally, a higher EST decreased time until IP, EP, and hatch.

Averages for time until IP were 485, 473, 469, and 467 h for 35.6, 36.7, 37.8, and 38.9°C respectively. Averages for time until hatch were 508, 495, 491, and 487 h for 35.6, 36.7, 37.8, and 38.9°C respectively.

At 36.7 and 38.9°C, time until IP and time until EP were not affected by start day, and at 38.9°C, time until hatch was not affected by start d. However, at 35.6°C, start d affected time until IP, where time until IP was longer for E15 compared to E17 (Δ=4 h) and E19 (Δ=10 h). At 35.6°C, start d also affected time until EP, at which time until EP was longer for E15 compared to E17 (Δ=3 h) and E19 (Δ=8 h). At 35.6 and 36.7°C, start d affected time until hatch, at which time until hatch was longer for the 35.6°C-E15 treatment compared to 35.6°C-E17 (Δ=4 h) and 35.6°C-E19 (Δ=9 h), and the 36.7°C-E15 treatment compared to 36.7°C-E19 (Δ=2 h).

An effect of EST was found for time interval between IP and EP (P=0.008), time interval between EP and hatch (P<0.001), and for time interval between IP and hatch (P<0.001). However, absolute differences between intervals appear to be relatively small, with the largest difference for time interval between IP and hatch between EST 35.6 and 38.9°C (Δ=2 h). This was caused by a longer time interval between EP and hatch at an EST of 35.6°C compared to 36.7, 37.8, and 38.9°C (Δ=2 h on average).

Table 2. Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on time until internal pipping (IP), until external pipping (EP), and time until hatch

Item	Time IP (h)	Time EP (h)	Time hatch (h)
EST (°C) x start d			
35.6 x E15	490[a]	496[a]	512[a]
35.6 x E17	486[b]	493[b]	508[b]
35.6 x E19	480[c]	488[c]	503[c]
36.7 x E15	473[d]	483[d]	496[d]
36.7 x E17	473[d]	482[d]	496[de]
36.7 x E19	473[d]	481[d]	494[e]
37.8	469[e]	479[e]	491[f]
38.9 x E15	466[e]	473[f]	486[g]
38.9 x E17	467[e]	474[f]	486[g]
38.9 x E19	468[e]	475[f]	488[g]
SEM	0.9	0.7	0.7
P-value			
EST	<0.001	<0.001	<0.001
Start day	<0.001	<0.001	<0.001
EST x start day	<0.001	<0.001	<0.001

[a-g]Least squares means lacking a common superscript within a column and factor differ (P≤0.05).

4.4.2 Day 15 of Incubation (baseline values)

At E15 (336 h), baseline values were assessed for YFBM, yolk weight, and relative weights of heart, liver, stomach, spleen, bursa, and intestine (mean ± SEM). YFBM and yolk weight were 15.51 ± 0.20g and 15.83 ± 0.39g, respectively. Weights of organs were expressed as a percentage of YFBM and were 0.677 ± 0.037% for heart, 1.97 ± 0.04% for liver, 2.52 ± 0.07% for stomach, 0.0330 ± 0.0051% for spleen, 0.0843 ± 0.0085% for bursa, and 0.89 ± 0.13% for intestines.

4.4.3 Yolk Free Body Mass

At E19, YFBM at 35.6°C was lower compared to 36.7 (Δ=0.71g), 37.8 (Δ=0.57g), and 38.9°C (Δ=0.82g) (P=0.008; Table 3). At 36.7°C, 37.8°C, and 38.9°C, YFBM was similar. At hatch, YFBM at 35.6 and 36.7°C was similar, but YFBM was higher compared to 37.8 (Δ=1.31g) and 38.9°C (Δ=0.93g) (P<0.001). At 37.8 and 38.9°C, YFBM was similar.

4.4.4 Yolk Weight

At IP, at 35.6°C, yolk weight was lower compared to 36.7°C (Δ=0.64g), 37.8°C (Δ=1.14g), and 38.9°C (Δ=1.43g). At 36.7°C, yolk weight was lower compared to 38.9°C (Δ=0.79g) (P<0.001; Table 3), but at 37.8°C yolk weight was similar to 36.7 and 38.9°C.

At EP, at 35.6°C, yolk weight was lower compared to 37.8 (Δ=0.69g), and 38.9°C (Δ=0.99g) (P<0.001), but similar to 36.7°C. At 36.7°C, yolk weight was lower compared to 38.9°C (Δ=0.65g) (P<0.001), but yolk weight at 37.8°C was similar to 36.7 and 38.9°C.

At hatch, at 38.9°C, yolk weight was higher compared to 35.6 (Δ=1.25g), 36.7 (Δ=1.35g), and 37.8°C (Δ=1.15g) (P<0.001). At 35.6, 36.7, and 37.8°C, yolk weight was similar.

4.4.5 Relative Heart Weight

At E17, at 35.6°C, relative heart weight was higher compared to 37.8°C (0.706% vs. 0.615% respectively) and 38.9°C (0.706% vs. 0.555% respectively) (P<0.001; Figure 1a). At 36.7°C, relative heart weight was in between 35.6 and 37.8°C. At 38.9°C, relative heart weight was lower compared to all treatments (0.706% vs. 0.555%, 0.667% vs. 0.555%, and 0.615% vs. 0.555% respectively) (P<0.001).

At E19, an interaction effect between EST and start d was found. At 35.6°C and 36.7°C, relative heart weight was higher when treatment was applied from E15 compared to E17 (35.6°C: Δ=0.076%, 36.7°C: Δ=0.053%) (P=0.008; Figure 1a, b). At 37.8°C, relative heart weight was similar to 36.7°C. At 38.9°C, relative heart weight was similar for both starting moments. Overall, compared to an EST of 38.9°C, lower EST treatments showed a consistent higher relative heart weight when treatment duration was longer.

At IP, an interaction between EST and start day was found. At 35.6°C, relative heart weight was higher when treatment was applied from E15 compared to E17 (Δ=0.084%) and to E19 (Δ=0.136%) (P=0.01; Figure 1a, b, c). The relative heart weight of the 35.6°C-E17 treatment was similar to the 35.6°C-E19 treatment. At 36.7 and 38.9°C, no effect of start day was found. At 37.8°C, relative heart weight was comparable to the 36.7°C-E17 and 36.7°C-E19 treatments. At 38.9°C, relative heart weight was lower compared to all other treatments (P=0.01; Figure 1a, b, c).

At EP, an interaction between EST and start d was found. At 35.6°C, relative heart weight was higher when treatment was applied from E15 compared to E19 (Δ=0.110%) and from E17 compared to E19 (Δ=0.126%) (P<0.001; Figure 1a, b, c). Relative heart weight found for the 35.6°C-E15 treatment was similar to the 35.6°C-E17 treatment. At 36.7 and 38.9°C, no effect of start day was found. Relative heart weight was similar for the 35.6°C-E19, 36.7°C-E15, and 36.7°C-E17 treatments. At 37.8°C, relative heart weight was similar to the 36.7°C-E15 and 36.7°C-E19 treatments. At 38.9°C relative heart weight was lower compared to all other treatments (P<0.001; Figure 1a, b, c).

At hatch, an interaction between EST and start d was found. At 35.6°C, relative heart weight was higher when treatment was applied from E15 compared to E19 (Δ=0.081%), and from E17 compared to E19 (Δ=0.057%) (P=0.003; Figure 1a, b, c). No effect of start d was found at 36.7°C, and 37.8°C was different compared to all other treatments. At 38.9°C, relative heart weight was lower when treatment was applied from E15 compared to E19 (Δ=0.089%) (P=0.003; Figure 1a, b, c). Relative heart weight found for the 38.9°C-E17 treatment was similar to the 38.9°C-E15 and 38.9°C-E19 treatments. Overall, at an EST of 35.6 and 36.7°C, at IP, EP, and hatch, an earlier start of treatment resulted in a higher relative heart weight, whereas, at an EST of 38.9°C, the opposite was found.

4.4.6 Relative Liver Weight

At E19, an interaction effect between EST and start d was found. At 35.6°C, relative liver weight was higher when treatment was applied from E15 compared to E17 (Δ=0.18%) (P=0.002; Table 4). At 36.7 and 38.9°C, no effect of onset of treatment was found. At 37.8°C, relative liver weight was comparable to the 35.6°C-E15 treatment, to 36.7°C, and to the 38.9°C-E17 treatment.

At IP, at 35.6°C relative liver weight was higher compared to 36.7 (Δ=0.08%), 37.8 (Δ=0.20%), and 38.9°C (Δ=0.17%) (P<0.001). At 36.7°C, relative liver weight was higher compared to 37.8°C (Δ=0.12%) and 38.9°C (Δ=0.09%) (P<0.001), however, relative liver weight at 37.8°C and 38.9°C were similar.

At EP, at 38.9°C, relative liver weight was lower compared to 35.6°C (Δ=0.16%) and 36.7°C (Δ=0.16%) (P<0.001), but similar to 37.8°C. Relative liver weight at 37.8°C was similar to all other treatments.

At hatch, at 35.6°C, relative liver weight was higher compared to 38.9°C (Δ=0.22%) (P<0.001), but similar to 36.7 and 37.8°C. At 36.7°C, relative liver weight was higher compared to 37.8°C (Δ=0.13%) and 38.9°C (Δ=0.25%) (P<0.001). At 37.8°C, relative liver weight was similar to 38.9°C.

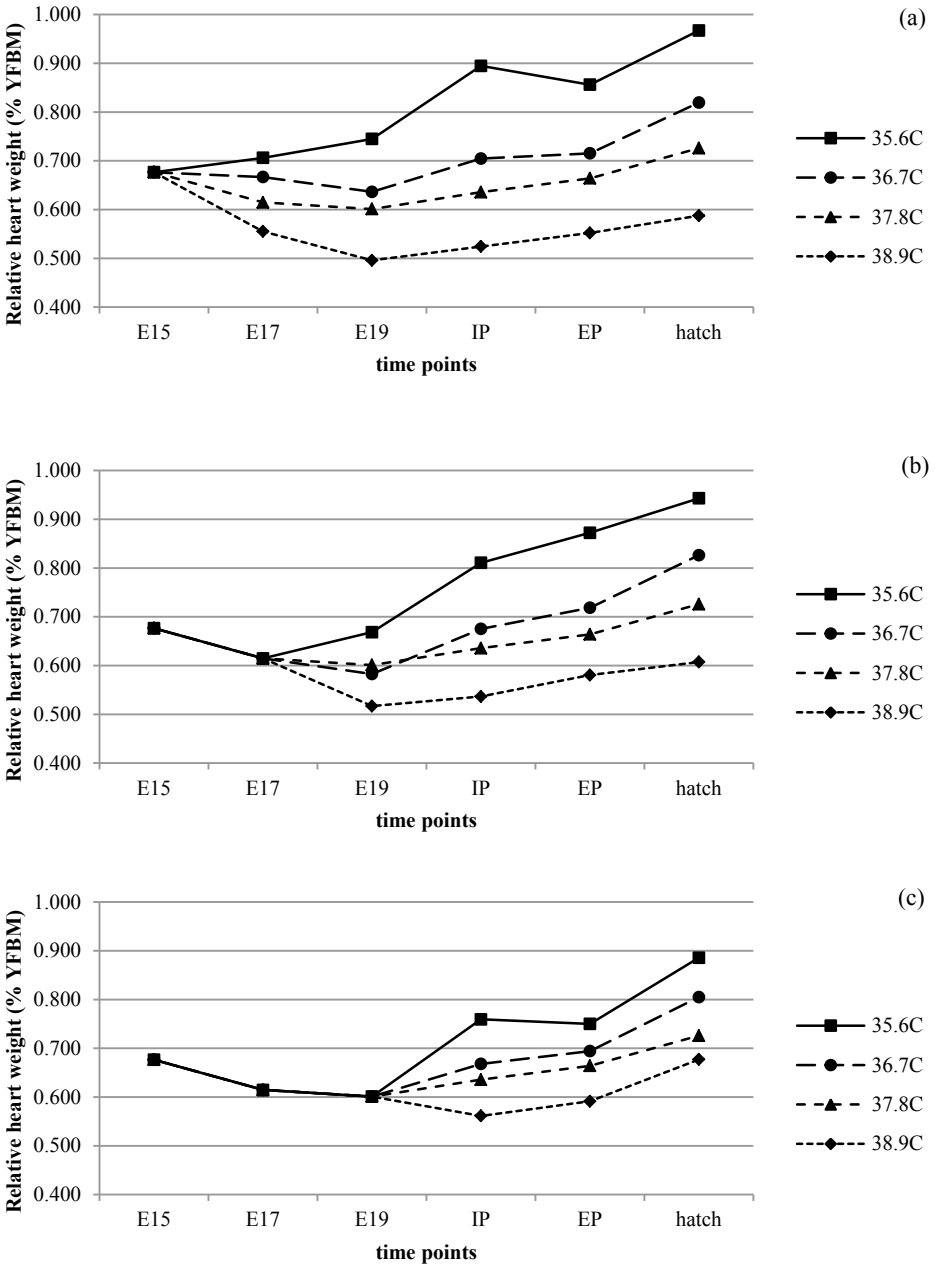

Figure 1. Interactions between eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) and start d of treatment (E; E15 (a), E17 (b), or E19 (c)) through hatch on relative heart weight as percentage of yolk-free body mass (YFBM) at E15, E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment).

4.4.7 Relative Spleen Weight

At E19, at 38.9°C, relative spleen weight was lower compared to 35.6°C (Δ=0.0079%) (*P*=0.02; Table 4). However, at 38.9 and 35.6°C, relative spleen weight was similar to 36.7 and 37.8°C. At IP, at 38.9°C, relative spleen weight was lower compared to 35.6 (Δ=0.0091%), 36.7 (Δ=0.0070%), and 37.8°C (Δ=0.0077%) (*P*=0.003). At 35.6, 36.7, and 37.8°C, relative spleen weight was similar.

At EP, at 35.6°C, relative spleen weight was higher compared to 36.7 (Δ=0.0047%), 37.8 (Δ=0.0072%), and 38.9°C (Δ=0.0062%) (*P*=0.001). At 36.7, 37.8, and 38.9°C, relative spleen weight was similar.

4.4.8 Relative Stomach Weight

At E17, at 37.8°C, relative stomach weight was higher compared to 35.6°C (Δ=0.28%) and 36.7°C (Δ=0.23%) (*P*<0.001; Table 5), but similar to 38.9°C. At 38.9°C, relative stomach weight was similar to all other treatments. At E19, at 38.9°C, relative stomach weight was higher compared to 35.6 (Δ=0.59%), 36.7 (Δ=0.54%), and 37.8°C (Δ=0.39%) (*P*<0.001). At 37.8°C, relative stomach weight was higher compared to 35.6°C (Δ=0.20%) (*P*<0.001). At 36.7°C, relative stomach weight was between 35.6°C and 37.8°C. At hatch, at 35.6°C, relative stomach weight was lower compared to 36.7°C (Δ=0.17%) and 37.8°C (Δ=0.32%) (*P*=0.001), but similar to 38.9°C. At 38.9°C, relative stomach weight was lower compared to 36.7°C (Δ=0.31%) and 37.8°C (Δ=0.46%) (*P*=0.001). At 36.7°C and 37.8°C, relative stomach weight was similar.

At E19, an effect of start d was found. When treatment was applied from E17, relative stomach weight was higher compared to E15 (Δ=0.15%) (*P*=0.02). When no treatment was applied, relative stomach weight was comparable to E15 and E17. At IP, at 35.6°C, relative stomach weight was higher compared to 36.7 (Δ=0.37%), 37.8 (Δ=0.33%), and 38.9°C (Δ=0.47%) (*P*<0.001). At 36.7, 37.8, and 38.9°C, relative stomach weight was similar.

4.4.9 Relative Intestine Weight

At IP, at 35.6°C, relative intestine weight was higher compared to 36.7 (Δ=0.51%), 37.8 (Δ=0.50%), and 38.9°C (Δ=0.67%) (*P*<0.001; Table 5). At 36.7, 37.8, and 38.9°C, relative intestine weight was similar.

At EP, at 35.6°C, relative intestine weight was higher compared to 36.7 (Δ=0.25%), 37.8 (Δ=0.25%), and 38.9°C (Δ=0.52%) (*P*<0.001). At 38.9°C, relative intestine weight was lower compared to 36.7°C (Δ=0.27%) and 37.8°C (Δ=0.27%) (*P*<0.001). At 36.7 and 37.8°C, relative intestine weight was similar.

At hatch, at 38.9°C, relative intestine weight was lower compared to 35.6 (Δ=0.51%), 36.7 (Δ=0.50%), and 37.8°C (Δ=0.36%) (P<0.001). At 35.6, 36.7, and 37.8°C, relative intestine weight was similar.

4.4.10 Relative Bursa Weight

At hatch, at 35.6°C, relative bursa weight was lower compared to 36.7°C (0.0892% vs. 0.1042% respectively) and 37.8°C (0.0892% vs. 0.1059% respectively) (P=0.02), but similar to 38.9°C (0.0892% vs. 0.0924% respectively). At 38.9°C, relative bursa weight was similar to all other treatments.

4.4.11 HOF and Late Dead Embryos

No interaction was found between EST and start d for HOF (P=0.24), and no main effects for EST (P=0.46) and start d (P=0.86). The average HOF for each treatment was: 35.6°C-E15: 98.50%, 35.6°C-E17: 96.3%, 35.6°C-E19: 97.5%, 36.7°C-E15: 96.9%, 36.7°C-E17: 99.1%, 36.7°C-E19: 96.8%, 37.8°C: 99.2%, 38.9°C-E15: 96.3%, 38.9°C-E17: 96.9%, 38.9°C-E19: 96.5%. The HOF was on average 97.4% for all treatments.

The percentage of late dead embryos after E15 for each EST treatment was: 35.6°C: 1.1%, 36.7°C: 1.1%, 37.8°C: 0.5%, 38.9°C: 1.3% (P>0.005).

4.5 DISCUSSION

The aim of the experiment was to investigate whether an EST of 35.6, 36.7, 37.8, or 38.9°C applied from different days (E15, E17, or E19) of incubation onward, affected embryonic development. Generally, results indicate that different organs react differently to applied treatments and duration of applied treatments.

Reasons that organs differ in their response to applied EST treatments during the last wk of incubation might be found in the developmental phases of organs and the sensitivity of specific organs to external factors, e.g. EST. Therefore, E17 and E19 are physiologically not the same moments in development for the different treatments. Another possible explanation might be found in the variation in incubation duration due to applied EST treatments.

4.5.1 Incubation Time

The effect of EST on incubation duration was shown previously by others (Lourens et al., 2007; Molenaar et al., 2010) at which an EST of 38.9°C decreased incubation duration compared to 37.8°C when applied from E7 or E9. The current study showed that even when EST differences were applied from E15 onwards, effects of incubation duration

were considerable. In the current study, the effect of EST on incubation duration was mainly caused by the effect of EST on moment of IP. Time between IP and hatch varied only 2 h between treatments, whereas time until IP varied 24 h between treatments. The reason for the shortened time until IP at a higher EST might be related to the higher metabolic rate (Lourens et al., 2007). The lower metabolic rate at a lower EST resulted in an extended time until IP, which might have contributed to the higher YFBM and higher relative organ growth found at hatch at an EST of 35.6°C and 36.7 compared to an EST of 38.9°C.

4.5.2 Relative Heart Weight

Differences in relative heart weight between the 4 EST treatments increased over time. Effects of an EST of 37.8 compared to 38.9°C, applied during the mid-incubation period (E10 – E18) on relative heart weight was already investigated in other studies which repeatedly show a lower relative heart weight at an EST of 38.9 compared to 37.8°C (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2010, 2013). In the study of Maatjens et al. (2014a), a higher relative heart weight was found at IP and hatch, when an EST of 36.7°C was applied from E19 compared to 38.9°C. Although relative heart weight at an EST of 36.7 and 37.8°C were similar in the study of Maatjens et al. (2014a), present results suggest that relative heart weight was even higher when an EST of 35.6°C is applied.

In addition, current results support the findings that a relative short treatment duration of 39.5 h applied during only the hatching phase, still substantially affects relative heart weights (Maatjens et al., 2014a).

Reasons for the difference in relative heart weight caused by EST and treatment duration might be twofold.

First, Romanoff (1960) showed that the heart is mitotically active up to the first 10 d post hatch. Temperature has shown to affect the number of mitotically active myocytes in the heart. High incubation temperatures of 39.5°C compared to lower temperatures of 34.5 and 36.5°C decrease the mitotic index after E9, which leads to a slower heart weight development and therefore a lower relative heart weight at d of hatch (Romanoff, 1960).

Second, an explanation of the substantial effects of EST during a relative short treatment duration after E19 might be found in the embryonic metabolism between IP and hatch. At high EST metabolic rate and glucose oxidation increase (Molenaar et al., 2013), which depresses the build-up of glycogen stores and results in rapid depletion of glycogen stores between IP and hatch (Maatjens et al., 2014b). To compensate for the limited glycogen stores, glucogenic amino acids might to be deaminated and the carbon skeleton is used as a glycogenic energy source (Hazelwood and Lorenz, 1959) or adenosine

triphosphate (ATP) production (McArdle., 1981) at a high EST of 38.9°C. This results in a lower protein retention efficiency, higher uric acid levels, and a possible lower relative heart weight at hatch (Molenaar et al., 2010, 2013). In addition, when protein sources are used as an energy source, protein is not used for growth and development, which might result in a lower YFBM at hatch at an EST of 38.9°C (Molenaar et al., 2013; Maatjens et al., 2014a).

4.5.3 Relative Liver Weight

At E19, an interaction effect of EST and start d of treatment was found on relative liver weight.

At other stages, an EST effect was found independent of start d of treatment. This implies that liver growth is particularly affected by EST regardless of start d of treatment.

From E19 until hatch, relative liver weights tend to be higher at lower EST compared to higher EST. An explanation for the higher relative liver weight at an EST of 35.6°C might be found in the study of Walter and Seebacher (2007). They indicated that gene expression of PGC-1α in the liver of avian embryos was increased at a relatively low incubation temperature of 35°C during mid-incubation. Their findings suggest that PGC-1α gene expression activates gluconeogenesis. Gluconeogenesis is highly activated during embryogenesis in avian embryos to produce glucose. The carbohydrate concentration is low in the fresh egg and therefore gluconeogenesis is necessary to generate glucose from non-carbohydrate carbon substrates, such as lactate, glycerol, pyruvate, and glucogenic amino acids. Glucose will be transferred to glycogen by glycogenesis and will be stored in liver besides heart, leg and breast muscle, intestines, and yolk sac membrane (Krebs, 1972; Garcia et al., 1986; Christensen et al., 2001). Moreover, as explained before, an EST of 35.6°C extended time until IP, which might have contributed to the higher relative liver weight found at an EST of 35.6°C compared to 37.8 and 38.9°C.

An explanation for the higher relative spleen weight found at a lower EST compared to a higher EST at IP and EP might be the relative growth of organs at different metabolic levels, however at hatch, effects of EST were not significant.

4.5.4 Residual Yolk and Relative Intestine Weight

At IP, EST affected residual yolk and relative intestine weight for the first moment during incubation and contrasts between EST treatments remained until hatch. A higher residual yolk weight and lower intestine weights at hatch at an EST of 38.9 compared to 37.8°C were also found in other studies (Leksrisompong et al., 2007; Maatjens et al., 2014a).

Two reasons might explain the results found. First, at an EST of 35.6°C time intervals between E19 and IP and between EP and hatch were longer compared to 36.7, 37.8, and 38.9°C. These results suggest that more yolk could be used due to the longer time intervals.

Second, at a higher EST, embryos use relatively more glycogen for energy, as explained before, while at a lower EST embryos mainly use lipogenic energy, mostly derived from the yolk, as substrate for energy. If more yolk is used at a lower EST, this may contribute to the explanation that relative intestine weights were higher at a lower EST because yolk is transported to the intestine through the yolk stalk from E19. Once reaching the intestine, anti-peristaltic movements causes the yolk to move towards the gizzard (Esteban et al., 1991a, 1991b).

4.5.5 Yolk Free Body Mass and Relative Stomach Weight

A lower YFBM and relative stomach weight at an EST of 35.6°C compared to all other EST treatments before and at E19 are presumably related to the fact that the embryonic metabolic rate is affected by EST. Embryonic metabolic rate is affected by temperature because embryos act as poikilotherms and have little ability to regulate their own body temperature (Romijn and Lokhorst, 1955). Therefore, they react to environmental temperature at which a low EST decreases metabolic rate, which slows down growth and development of the avian embryo (Romanoff, 1936; Christensen et al., 1999; Ricklefs, 1987). This was indicated by a lower YFBM found at E19 at an EST of 35.6 compared to 36.7, 37.8, and 38.9°C. However, the lower metabolic rate at an EST of 35.6°C increased time until IP already with 12 h compared to 36.7°C, which allows sustained growth, and contributes to a higher YFBM and relative stomach weight at IP at an EST of 35.6 compared to 36.7, 37.8, and 38.9°C.

The lower YFBM found at hatch at a high EST (38.9°C) compared to 37.8°C was found in earlier studies as well (Lourens et al., 2005, 2007; Molenaar et al., 2011). Maatjens et al. (2014a) showed that a lower YFBM at hatch was also found at an EST of 38.9°C compared to 36.7°C. The higher YFBM found at an EST of 35.6°C may be due to the longer time interval between IP and hatch (Δ=2 h) compared to 36.7, 37.8, and 38.9°C. This difference suggests that embryos had more time to develop. However, nutrient efficiency may be higher at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C, resulting in a larger YFBM at hatch at a lower EST. Molenaar et al. (2010) indicated that protein efficiency was 3.2% higher at an EST of 37.8°C compared to an EST of 38.9°C, therefore it might be possible that an even lower EST than 37.8°C will result in higher efficiencies.

4.5.6 Conclusion

Various earlier studies (Lourens et al., 2005, 2007; Molenaar et al., 2011) showed that a constant EST of 37.8°C during incubation resulted in the highest hatchability, lowest third week mortality, and highest chick quality, expressed by a higher YFBM at d of hatch. Moreover, former studies (Lourens et al., 2005, 2007; Molenaar et al., 2011; Maatjens et al., 2014a) and our current study indicated that an EST of 38.9°C resulted in lower relative organ weights and a lower YFBM at hatch.

However, results of the current study indicated that an EST of 35.6 and 36.7°C applied from E15 onward increased chick development also in comparison to 37.8°C. An EST of 35.6 and 36.7°C resulted in the highest YFBM and higher relative organ weights at hatch.

A clear effect of EST and duration of various EST treatments was found particularly on relative heart weight. The development of the heart as a supply organ is of major importance on the embryonic development. Although the effect of EST on the function of the heart is not completely understood, cardiovascular diseases in later life, as ascites, might be reduced when relative heart weight is larger (Molenaar et al., 2011). Therefore, an EST of 35.6°C might be beneficial to increase the relative heart weight and prevent the incidence of ascites during later life.

The higher relative heart weight and higher YFBM at hatch at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C emphasize the importance of EST during the last week of incubation. We conclude that an EST lower than 37.8°C from E15 onward appears to be beneficial for embryo development.

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Table 3. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on yolk-free body mass (YFBM) and residual yolk weight at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (YFBM and residual yolk; n = 30 per treatment)

Item	YFBM (g)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	23.16	31.44[b]	37.95	38.70	40.45[a]
36.7	24.19	32.15[a]	37.42	38.79	40.45[a]
37.8	23.62	32.01[a]	36.96	39.14	39.14[b]
38.9	23.36	32.26[a]	37.21	38.66	39.52[b]
SEM	0.28	0.23	0.27	0.19	0.20
Start d					
C		32.01	36.96	39.14	39.14
E15		31.73	37.41	38.62	40.11
E17		32.17	37.57	38.51	40.44
E19			37.61	39.02	39.87
SEM		0.21	0.27	0.19	0.20
P-value					
EST	0.08	0.008	0.12	0.84	<0.001
Start d		0.07	0.73	0.04	0.09
EST x start d		0.09	0.91	0.25	0.30

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 3 (continued). Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on yolk-free body mass (YFBM) and residual yolk weight at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (YFBM and residual yolk; n = 30 per treatment)

Item	Residual yolk weight (g)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	15.85	12.10	7.93^{c}	7.51^{c}	5.69^{b}
36.7	15.52	12.49	8.57^{b}	7.85^{bc}	5.59^{b}
37.8	16.44	12.10	9.07^{ab}	8.20^{ab}	5.79^{b}
38.9	15.53	12.59	9.36^{a}	8.50^{a}	6.94^{a}
SEM	0.41	0.18	0.27	0.15	0.15
Start d					
C		12.10	9.07	8.20	5.79
E15		12.37	8.44	8.02	6.16
E17		12.42	8.72	8.07	5.89
E19			8.70	7.76	6.17
SEM		0.17	0.26	0.15	0.14
P-value					
EST	0.34	0.09	<0.001	<0.001	<0.001
Start d		0.79	0.66	0.17	0.25
EST x start d		0.09	0.91	0.12	0.21

$^{a\text{-}c}$Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 4. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on relative liver and spleen weight (% of yolk-free body mass) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Relative liver weight (%)				
	E17	E19[1]	IP	EP	H
EST (°C)					
35.6	2.07	2.15	2.19[a]	2.22[a]	2.59[ab]
36.7	2.10	2.00	2.11[b]	2.22[a]	2.62[a]
37.8	2.13	2.04	1.99[c]	2.09[b]	2.49[bc]
38.9	2.11	1.94	2.02[c]	2.06[b]	2.37[c]
SEM	0.04	0.13	0.03	0.03	0.04
Start d					
C		2.04	1.99	2.09	2.49
E15		2.05	2.13	2.19	2.50
E17		2.01	2.11	2.14	2.52
E19			2.08	2.18	2.56
SEM		0.03	0.03	0.03	0.04
P-value					
EST	0.67	<0.001	<0.001	<0.001	<0.001
Start d		0.08	0.63	0.22	0.34
EST x start d		0.002	0.75	0.14	0.52

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

[1]Interactions 35.6°C-E15: 2.24[a], 35.6°C-E17: 2.06[b], 36.7°C-E15: 2.02[b], 36.7°C-E17: 1.98[bd], 37.8°C: 2.04[b], 38.9°C-E15: 1.90[cd], 38.9°C-E17: 1.98[bc].

Table 4 (continued). Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on relative liver and spleen weight (% of yolk-free body mass) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Relative spleen weight (%)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	0.0382	0.0385[a]	0.0366[a]	0.0338[a]	0.0337
36.7	0.0348	0.0336[ab]	0.0345[a]	0.0291[b]	0.0345
37.8	0.0347	0.0352[ab]	0.0352[a]	0.0266[b]	0.0321
38.9	0.0370	0.0306[b]	0.0275[b]	0.0276[b]	0.0307
SEM	0.0026	0.0021	0.0022	0.0015	0.0016
Start d					
C		0.0352	0.0352	0.0266	0.0321
E15		0.0325	0.0312	0.0285	0.0329
E17		0.0359	0.0344	0.0303	0.0336
E19			0.0330	0.0316	0.0324
SEM		0.0020	0.0022	0.0015	0.0016
P-value					
EST	0.74	0.02	0.003	0.001	0.20
Start d		0.09	0.52	0.17	0.67
EST x start d		0.46	0.81	0.22	0.57

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 5. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on relative stomach and intestine weight (% of yolk-free body mass) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Relative stomach weight (%)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	2.92^{b}	3.63^{c}	4.92^{a}	5.03	5.42^{b}
36.7	2.97^{b}	3.68^{bc}	4.55^{b}	4.84	5.59^{a}
37.8	3.20^{a}	3.83^{b}	4.59^{b}	4.99	5.74^{a}
38.9	3.13^{ab}	4.22^{a}	4.45^{b}	4.93	5.28^{b}
SEM	0.07	0.06	0.10	0.07	0.07
Start d					
C		3.83^{ab}	4.59	4.99	5.74
E15		3.77^{b}	4.67	4.92	5.35
E17		3.92^{a}	4.60	4.90	5.46
E19			4.65	4.98	5.47
SEM		0.06	0.10	0.07	0.07
P-value					
EST	<0.001	<0.001	<0.001	0.07	0.001
Start d		0.02	0.81	0.53	0.49
EST x start d		0.24	0.32	0.50	0.14

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 5 (continued). Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on relative stomach and intestine weight (% of yolk-free body mass) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Relative intestine weight (%)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	1.03	1.54	3.12[a]	3.20[a]	3.86[a]
36.7	1.11	1.55	2.61[b]	2.95[b]	3.85[a]
37.8	1.12	1.73	2.62[b]	2.95[b]	3.71[a]
38.9	1.15	1.70	2.45[b]	2.68[c]	3.35[b]
SEM	0.06	0.06	0.11	0.07	0.09
Start d					
C		1.73	2.62	2.95	3.71
E15		1.56	2.74	2.92	3.64
E17		1.64	2.78	2.99	3.81
E19			2.66	2.92	3.61
SEM		0.06	0.11	0.07	0.08
P-value					
EST	0.60	0.06	<0.001	<0.001	<0.001
Start d		0.19	0.50	0.61	0.24
EST x start d		0.95	0.24	0.36	0.22

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

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Appendix 1. Interactions between eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) and start d of treatment (E15, E17, or E19) through hatch on relative heart weight (% of yolk-free body mass) at E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	E19	IP	EP	H
Heart (%)				
EST x start d				
35.6 x E15	0.745^{a}	0.895^{a}	0.856^{a}	0.967^{a}
35.6 x E17	0.669^{b}	0.811^{b}	0.872^{a}	0.943^{a}
35.6 x E19		0.759^{bc}	0.746^{b}	0.886^{b}
36.7 x E15	0.636^{bc}	0.705^{cd}	0.715^{bd}	0.820^{c}
36.7 x E17	0.583^{d}	0.675^{de}	0.719^{bc}	0.826^{c}
36.7 x E19		0.668^{df}	0.694^{cd}	0.805^{c}
37.8	0.601^{cd}	0.636^{ef}	0.664^{d}	0.726^{d}
38.9 x E15	0.496^{e}	0.524^{g}	0.552^{e}	0.588^{f}
38.9 x E17	0.517^{e}	0.537^{g}	0.581^{e}	0.607^{ef}
38.9 x E19		0.561^{g}	0.591^{e}	0.677^{de}
SEM	0.016	0.022	0.019	0.021
P-value				
EST x start d	0.008	0.01	<0.001	0.003

$^{a-g}$Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Chapter 5

Temperature during the last week of incubation. III. Effects on chicken embryo physiology

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5.1 ABSTRACT

We investigated effects of eggshell temperature (EST) of 35.6, 36.7, 37.8, or 38.9°C applied from d of incubation (E) 15, E17, or E19 onward on chicken embryo physiology. A total of 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were incubated at an EST of 37.8°C until E15. From E15, E17, or E19 onward, eggs were incubated at an EST of 35.6, 36.7, 37.8, or 38.9°C. Plasma glucose, uric acid, and lactate concentrations, and hepatic glycogen amount and concentration were measured at E15, E17, E19, internal pipping (IP), external pipping (EP), and hatch.

At E17, an EST of 38.9°C applied from E15 onward resulted in the numerically highest lactate level, followed by an EST of 37.8, 36.7, and 35.6°C. The same pattern was observed at E19, when an EST of 37.8°C showed the highest lactate level, followed by an EST of 36.7 and 35.6°C. These results suggest that with a lower EST, oxygen (O_2) becomes limiting for metabolism at a later stage than with a higher EST. At an EST of 35.6 and 36.7°C, no signs of anaerobic metabolism were observed, as a peak in lactate production remained absent. The higher lactate level combined with a lower hepatic glycogen levels at IP, EP, and hatch found at an EST of 38.9°C suggest a higher embryonic metabolic rate than at an EST of 37.8, 36.7, and 35.6°C. At E17, an EST of 35.6°C applied from E15 onward resulted in a higher plasma glucose and uric acid concentration. From IP onward, an EST of 35.6°C resulted in a higher glycogen amount and concentration compared to all other EST, which might be due to the relative higher O_2 availability due to the lower metabolic rate, which provide time to build up glycogen stores from excessive glucose.
Results of this study stress the negative effects of an EST of 38.9°C from E15 onward and emphasize that an EST of 35.6 and 36.7°C from E15 onward appears to be beneficial for chick embryo physiology.

Key words: temperature, incubation, chicken embryo physiology, hepatic glycogen

5.2 INTRODUCTION

Temperature is one of the most important factors during incubation and affects embryonic growth and development (Ricklefs, 1987; French, 1994; Christensen et al., 1999). Embryo temperature during incubation is proven to reflect embryonic metabolism better than incubator temperature (Lourens et al., 2005; Meijerhof, 2009). As a reflection of embryo temperature, eggshell temperature (EST) can be used as a non-invasive method (Lourens et al., 2005).

Studies have shown that an EST ≥ 38.9°C from d of incubation (E) 14 onward or even during only the hatching phase (E19 till hatch) has negative effects on embryonic development (Leksrisompong et al., 2007; Maatjens et al., 2014a; Maatjens et al., 2016a). An EST of 35.6 or 36.7°C applied during the last week of incubation or an EST of 36.7°C during only the hatching phase has been shown to have beneficial effects on embryonic development and chick quality (Maatjens et al., 2014a and 2016a).

Towards the end of incubation, embryonic energy requirements increase, which increase the demand for oxygen (O_2). However, between E15 and E19, exchange of O_2 and carbon dioxide (CO_2) is restricted due to limited shell and shell membrane porosity, which results in a plateau phase in heat production (Lourens et al., 2007) until IP, when supplementary O_2 from the air cell becomes available. When O_2 becomes limited, yolk lipids cannot be used efficiently for energy production and the embryo will depend more on carbohydrate and protein metabolism, as more O_2 is necessary for lipid oxidation than for carbohydrate and protein oxidation (Moran, 2007; de Oliveira, 2008).

The last week of incubation and the hatching phase are characterized by physiological and metabolic processes, which are essential for embryonic survival and hatching (Christensen et al., 1999). One of these processes involves the synthesis and degradation of glycogen stores (Freeman, 1969; Pearce, 1971; Garcia et al., 1986; Christensen et al., 2001). Effects of EST treatments on hepatic glycogen were described by Molenaar et al. (2011) who found a lower total hepatic glycogen at hatch when an EST of 38.9˚C was applied from E7 onward compared to an EST of 37.8˚C. Maatjens et al. (2014b) showed that when an EST of 38.9˚C was applied during only the hatcher phase, hepatic glycogen concentration was lower at the moment of internal pipping (IP) and hatch compared to an EST of 36.7 or 37.8˚C.

Effects of EST during incubation on plasma metabolites, such as plasma glucose, lactate, and uric acid, are not thoroughly investigated. Molenaar et al. (2013) found a higher plasma lactate at E17.8 and a higher plasma uric acid concentration at E21.6, when an EST of 38.9°C was applied from E10.5 onward compared to an EST of 37.8°C. Maatjens et al. (2014b) only found a higher plasma lactate at 12 h after hatch when an EST of 38.9°C was

applied during the hatcher phase compared to an EST of 37.8 or 36.7°C. Therefore, it can be concluded that a lower EST than 37.8°C applied during the hatching phase appears to be beneficial for embryo physiology.

However, it can be questioned whether a lower EST than 36.7°C might be even more beneficial for embryo physiology and additionally, it is largely unknown from which moment onward these lower EST will affect embryo development, including embryo physiology. Therefore, we aimed to investigate effects of an EST of 35.6, 36.7, 37.8, and 38.9°C starting from E15, E17, or E19 onward on embryo physiology.

5.3 MATERIALS AND METHODS

5.3.1 Experimental Design

The experiment was set up as a 3 x 3 factorial scheme and an "added" control. Eggs were incubated at an EST of 37.8°C until E15. From E15, E17, or E19 onward, EST was changed to 35.6, 36.7, or 38.9°C, or maintained at 37.8°C. Table 1 shows an overview of the different treatment groups, i.e. changes in EST at E15, E17, or E19. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, the Netherlands.

Table 1. Setup of the experiment with 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, 38.9°C) applied from 3 time points (E; E15, E17, or E19) through moment of placement

	Days of incubation			
Treatment	E0 - E15	E15 - E17	E17 - E19	E19 - hatch
1	37.8	35.6	35.6	35.6
2	37.8	36.7	36.7	36.7
3 (control)	37.8	37.8	37.8	37.8
4	37.8	38.9	38.9	38.9
5	37.8	37.8	35.6	35.6
6	37.8	37.8	36.7	36.7
7	37.8	37.8	38.9	38.9
8	37.8	37.8	37.8	35.6
9	37.8	37.8	37.8	36.7
10	37.8	37.8	37.8	38.9

5.3.2 Egg Storage and Incubation up to E15

Before incubation, eggs were stored for 5 d at a storage temperature of 20°C at a commercial hatchery (Lagerwey BV, Lunteren, the Netherlands). In total, 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were selected on egg weight between 62 and 65 g. For the first 15 d of incubation, the selected eggs were placed in one incubator (HatchTech BV, Veenendaal, the Netherlands) with a capacity of 4,800 eggs. The rest of the incubator was filled with hatching eggs which were not part of the experiment to ensure uniform airflow across eggs. Eggshell temperatures (EST) were automatically maintained at 37.8°C until E15. EST was controlled and monitored by 4 eggshell temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK), which were placed halfway the blunted and pointed end of 4 individual fertile eggs. Incubator temperature was adjusted based on the average temperature of the 4 EST sensors. RH was maintained between 50 and 55% and CO_2 concentration did not exceed 0.35%. Eggs were placed on setter trays and turned hourly by an angle of 45°.

5.3.3 Incubation from E15 until Hatch

At E14 (332 h), eggs were candled to identify infertile eggs or eggs containing non-viable embryos. All eggs containing viable embryos were transported to the experimental facility of Wageningen University (Wageningen, the Netherlands) for 30 min in a climate controlled car.

At E15 (336 h), after arrival at the experimental facility, three times 240 eggs were assigned to one out of four climate respiration chambers (Heetkamp et al., 2015) in which EST was maintained at 35.6°C (treatment 1), 36.7°C (treatment 2), or 38.9°C (treatment 4). The remaining 2,130 eggs were placed in the Control climate respiration chamber in which EST was maintained at 37.8°C (treatment 3) (Table 1).

At E17 (384 h), three times 210 eggs were moved from the Control climate respiration chamber to one of the three climate respiration chambers in which EST was maintained at 35.6°C (treatment 5), 36.7°C (treatment 6), or 38.9°C (treatment 7). Finally, at E19 (432 h), three times 180 eggs were moved from the Control climate respiration chamber to one of the three climate respiration chambers in which EST was maintained at 35.6°C (treatment 8), 36.7°C (treatment 9), or 38.9°C (treatment 10) (Table 1).

All eggs were placed in individual hatching baskets (120 x 135 mm) and eggs and chicks were continuously exposed to light. From E15 (336 h) until hatch, EST was monitored by the median of 5 individual eggshell sensors per climate respiration chamber as described before. At E19 (453 h), temperature of each climate respiration chamber was

fixed at its current setting and EST was allowed to increase during the hatching process. RH was maintained between 50 and 55% and CO_2 concentration did not exceed 0.35%.
From E19 (453 h) of incubation onwards, moment of internal pipping (IP), which was determined by candling all eggs individually, external pipping (EP), and hatch, were monitored every 6 h. Eggs were allowed to hatch and chicks remained in individual hatching baskets, without feed and water under continuous light.

5.3.4 Sampling from E15 until Hatch

At E15 (336 h), before dividing the eggs over the treatments, 30 eggs were randomly chosen to be used for determination of embryonic blood parameters and hepatic glycogen determination. At the same moment, at E15, 30 eggs per treatment were randomly assigned to be used for determination of embryonic blood parameters and hepatic glycogen at E17, E19, IP, and EP. All other eggs were allowed to hatch and 30 randomly chosen chicks per treatment, divided over the hatch window, were sampled at hatch for blood parameters and hepatic glycogen. All chicks that were not used for sampling were used for a first week grow-out trial, described in Maatjens et al. (2016b).

5.3.5 Blood Parameter Measurements and Hepatic Glycogen Determination

At E15 (336 h), blood was extracted from the allantoic vein of the chorio allantoic membrane by using a 1-mL syringe and 30-gauge needle and collected in heparinized tubes. At E17 (384 h), E19 (432 h), IP, EP, and hatch, blood was extracted from the jugular vein of the embryos or chicks using a 1-mL syringe and 30-gauge needle and collected in heparinized tubes. Blood was centrifuged (2,900 x *g*) at 4°C for 15 minutes. Blood plasma was decanted and stored at -20.0°C until further analysis. Blood plasma glucose, lactate, and uric acid were determined with commercially available enzymatic kits (blood plasma glucose: Cobas, Roche Diagnostics, the Netherlands; blood plasma lactate and uric acid: DiaSys Diagnostic Systems International, Holzheim, Germany).

After blood collection, the embryos or chicks were decapitated and the liver was dissected. After determination of liver weight, livers were frozen in liquid nitrogen and stored at -80.0°C until further analysis. Procedures to determine hepatic glycogen were carried out on ice. Approximately 300 mg of liver was homogenized after the addition of the same amount in µL of 7% $HClO_4$ as tissue. The suspension was centrifuged (2,900 x *g*) at 4°C for 15 minutes. The supernatant was decanted, cleaned with 1 mL of petroleum ether, and frozen at -20.0°C until further analysis. 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.

For relative weights of heart, liver, spleen, stomach, and intestines at E15, E17, E19, IP, EP, and hatch see Maatjens et al. (2016a).

5.3.6 Statistical Analysis

Separate analyses were performed for the data collected at E17, E19, IP, EP, and hatch. Based on the design, the experiment consists of ten treatment groups, i.e. different EST and changes in EST at E15, E17, or E19 (Table 1).

At E17, there were four treatments (eggs moved from 37.8°C to another EST, plus a Control treatment of eggs constantly at 37.8°C) in a completely randomised design that was analysed by one-way ANOVA. The F-test was used to test for overall differences between treatments, followed by pairwise comparisons by Fisher's LSD method.

At E19, there were seven treatments, consisting of combinations of EST in two time trajectories (E15-E17, E17-E19). By introducing factors for EST (35.6, 36.7, 38.9°C) and for starting day of treatment (eggs moved after E15 or E17), the design consists of a 3 x 2 factorial scheme (eggs moved from 37.8°C to another EST after E15 or E17) and an "added" control (eggs constantly at 37.8°C). All data were analysed with a single model in a single analysis. F-tests were performed for interaction and main effects of EST and starting day of treatment corresponding to the 3 x 2 factorial scheme. Depending upon the significance of the interaction, pairwise comparisons with Fisher's LSD method were made between the six means of combinations of EST and start day of treatment (P-value interaction ≤ 0.05), or between the separate three means for EST and two means for start day of treatment (P-value interaction > 0.05). Similarly, depending upon the significance of the interaction, the control treatment was compared with the six means of combinations of three EST and two start days of treatment, or with the separate three means for EST and two means for starting time of the treatment.

At IP, EP, and hatch, there were 10 treatments, consisting of EST in three time trajectories (E15-E17, E17-E19, >E19). Introducing factors for EST and starting day of treatment (eggs moved after E15, E17, or E19), the design consists of a 3 x 3 factorial scheme (eggs moved from 37.8°C to another EST after E15, E17, or E19) and an "added" control (eggs constantly at 37.8°C). Again, F-tests were performed and, depending upon the significance of interaction, appropriate pairwise comparisons by Fisher's LSD method were made, similar to the analysis at E19.

Model assumptions, i.e. normality and equal variance of the error terms in the linear models, were checked by inspection of residual plots. For the analyses of uric acid concentration, a log transformation was applied at E17, E19, IP, and hatch to obtain normally distributed data. For the analyses of total glycogen and glycogen concentration at E17, E19, and IP, normality of the residuals could not be attained. Preliminary analyses

indicated that a Poisson distribution fit the data better. Therefore, total glycogen and glycogen concentration were analysed using PROC GLIMMIX, using a Poisson distribution with a log link to model the linear regression analysis. Models included the same variables as described above. Results are displayed as the inverted natural logarithm least squares means and the corresponding confidence interval (CI). All analyses were performed with SAS (Version 9.3, SAS Institute 2010).

5.4 RESULTS

5.4.1 Day 15 of Incubation (baseline values)

At E15 (336 h), baseline values were assessed for total hepatic glycogen, hepatic glycogen concentration, plasma glucose, uric acid, and lactate (mean ± SEM). Total hepatic glycogen and hepatic glycogen concentration were 2.24 ± 0.34 mg and 7.23 ± 1.05 mg/g respectively. Values for plasma metabolites were 115.65 ± 4.63 mg/mL for glucose, 3.85 ± 0.35 mg/mL for uric acid, and 1.18 ± 0.09 mmol/L for lactate.

5.4.2 Plasma Glucose

No interactions between EST and start day of treatment were found for plasma glucose. At E17, glucose was higher at an EST of 35.6 and 38.9˚C compared to 36.7, with 37.8˚C intermediate (P=0.02; Table 2). At IP, glucose was higher at an EST of 36.7˚C compared to 35.6 and 37.8, with 38.9˚C intermediate (P=0.008). At EP, glucose was higher at an EST of 36.7 and 37.8˚C compared to 35.6 and 38.9˚C (P=0.02). At E19 and hatch, no effect of EST or start day of treatment was found.

5.4.3 Plasma Uric Acid

No interactions between EST and start day of treatment were found for plasma uric acid. At E17, uric acid was higher at an EST of 35.6 and 38.9˚C compared to 36.7, with 37.8˚C intermediate (P=0.02; Table 3). At E19, uric acid was higher at an EST of 35.6˚C compared to 36.7 and 37.8˚C. Uric acid at an EST of 38.9˚C was comparable to 35.6 and 36.7, but higher compared to 37.8˚C (P=0.03). At EP, uric acid was higher at an EST of 35.6 compared to 38.9˚C, with 36.7 and 37.8˚C intermediate (P=0.008). At hatch, uric acid was higher at an EST of 35.6˚C compared to all other EST treatments (P<0.001). At IP, no effect of EST or start day of treatment was found.

5.4.4 Plasma Lactate

At IP, EP, and hatch, no effect of EST or start day of treatment was found.

At E19, an interaction between EST and start day of treatment was found for lactate (P=0.01; Table 4). Lactate was similar at an EST of 35.6 and 36.7˚C for all start days of treatment. At an EST of 38.9˚C, lactate was higher at E17 compared to E15. Lactate at an EST of 37.8°C was similar to the 38.9˚C-E17 treatment, but higher compared to all other treatments.

5.4.5 Hepatic Glycogen

No interactions between EST and start day of treatment were found for total hepatic glycogen and hepatic glycogen concentration. At IP, total hepatic glycogen amount differed between all EST treatments with the highest amount at an EST of 35.6°C, followed by 36.7, 37.8, and 38.9°C (P<0.001; Table 5). At EP, total hepatic glycogen amount was higher at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C (P=0.05). At hatch, total hepatic glycogen amount was higher at an EST of 35.6°C compared to all other EST treatments (P<0.001). At E19, no effect of EST or start day of treatment was found on total hepatic glycogen.

At E19, the hepatic glycogen concentration was higher at an EST of 36.7°C, compared to 35.6 and 38.9 with 37.8°C intermediate (P =0.05; Table 6). At IP, hepatic glycogen concentration was different between all EST treatments with the highest amount at an EST of 35.6˚C, followed by 36.7, 37.8, and 38.9˚C (P<0.001). At EP, hepatic glycogen concentration were both higher at an EST of 35.6 and 36.7˚C compared to 37.8 and 38.9˚C (P=0.05). At hatch, hepatic glycogen concentration was higher at an EST of 35.6˚C compared to all other EST treatments (P<0.001).

An effect of start day of treatment was found at hatch, at which hepatic glycogen concentration was higher when treatment was applied from E19 compared to E15 (P=0.05) where the Control treatment and E17 were intermediate.

5.5 DISCUSSION

The aim of the experiment was to investigate whether an EST of 35.6, 36.7, 37.8, or 38.9°C applied from different days (E15, E17, or E19) of incubation onward, affected embryonic physiology.

Maatjens et al. (2016a) showed that an EST of 35.6 and 36.7°C from E15 onward appears to be beneficial for embryo development, expressed by a higher yolk-free body mass (YFBM) and higher relative organ weights at hatch. In the current study, EST affected

blood plasma glucose, uric acid, and lactate concentration. In addition, total hepatic glycogen and hepatic glycogen concentration were affected by EST as well.

An increase in EST from 37.8 to 38.9°C from E15 onward might have resulted in an immediate increase in metabolic rate which on its turn affects embryonic heat production (Janke et al., 2002) and embryonic growth and development (Romanoff, 1936; Ricklefs, 1987; Christensen et al., 1999). However, there is a limitation in the relationship between EST and metabolic rate, because metabolic rate drives the request for O_2. The exchange of O_2 and CO_2 between E15 and IP becomes limited due to limited shell and shell membrane porosity, which results in a plateau phase in heat production (Romijn and Lokhorst, 1956; Lourens et al., 2007). Therefore, at high EST and consequently high metabolic rate, energy utilization and the conversion of egg nutrient sources into body development may be hampered by insufficient O_2 availability, especially during the second part of the incubation process.

At E17, an EST of 38.9°C probably increased anaerobic metabolism, which resulted in the numerically highest lactate level, followed by an EST of 37.8 (Δ=0.25), 36.7 (Δ=0.47), and 35.6°C (Δ=0.70 mmol/L). The same pattern was observed at E19, where an EST of 37.8°C resulted in a higher lactate level than at an EST of 36.7 and 35.6°C, which suggests that O_2 became limited at a later stage compared to an EST of 38.9°C. At an EST of 35.6 and 36.7°C, no signs of anaerobic metabolism were observed, as a peak in lactate production remained absent. The peak in lactate probably indicates that glycolysis stopped due to a lack of O_2. The drop in lactate from E17 to E19 at an EST of 38.9°C, and from E19 to IP at an EST of 37.8°C, suggest hepatic gluconeogenesis, which converts lactate into glucose by the Cori-cycle (Sato et al., 2006). From IP onward, O_2 availability was restored due to O_2 availability from the air cell to sustain plasma glucose levels, and embryonic growth and development. The suggested higher metabolic rate at an EST of 38.9°C compared to all other EST was supported by the decrease in hepatic glycogen amount from E19 to IP and the lower glycogen amount at IP at an EST of 38.9°C, followed by 37.8, 36.7, and 35.6°C, which indicates that glucogenic energy was needed to support embryonic growth and that glucose was not used for hepatic glycogen synthesis. Earlier research indicated that at a high EST of 38.9°C, glucose oxidation was increased (Molenaar et al., 2013) and that glycogen synthesis was depressed (Maatjens et al., 2014b), compared to an EST of 37.8°C, which necessitates the use of blood glucose for immediate energy instead of glycogen synthesis.

At EP, when O_2 becomes largely available, an EST of 38.9°C resulted in a lower plasma glucose concentration compared to 36.7 and 37.8°C. In combination with the higher glycogen amount and glycogen concentration at EP compared to IP, this suggests that plasma glucose between IP and EP at an EST of 38.9°C might be mainly used for building

up hepatic glycogen reserves for the energy demanding hatching phase. However, at an EST of 38.9°C, hepatic glycogen levels remain considerably lower at IP, EP, and hatch compared to an EST of 35.6 and 36.7°C.

A decrease in EST from 37.8 to 35.6°C from E15 onward probably decelerated embryonic metabolic rate because chick embryos act as poilkilotherm and have limited abilities to regulate their own body temperature (Romijn and Lokhorst, 1955). However, embryos become more responsive to external stimuli, such as temperature (Lourens et al., 2006) between E14 and E18 (Nichelmann and Tzschentke, 2003). The decrease in EST might have caused a decreased blood flow in the chorioallantoic membrane (CAM) (Tzschentke, 2007), reflecting in a higher uric acid and glucose concentration found at E17 at an EST of 35.6°C. This might be due to the fact that the metabolic rate is low at an EST of 35.6°C, which sustained sufficient O_2 supply by the CAM, which ensures that embryos can utilize yolk fat as their main energy source (Moran, 2007). During that process, triacylglycrol is mobilised from yolk fat to free fatty acids. Fatty acids will release Acetyl-CoA, which can be oxidized to produce ATP (McArdle., 1981). Glycerol can generate glucose by gluconeogenesis (Hazelwood and Lorenz, 1959). This major metabolic pathway which is called beta-oxidation is active in the liver in order to maintain homeostasis (de Oliveira et al., 2008). In addition, Walter and Seebacher (2007) have indicated that gene expression of PGC-1α in the liver of avian embryos was increased at a relatively low incubation temperature of 35°C during mid-incubation, which might activate gluconeogenenis during embryogenesis to produce glucose. Both reasons appear to be confirmed by the numerically higher relative liver weight found at an EST of 35.6°C at E19 compared to all other EST treatments (Maatjens et al., 2016a), which suggests a higher liver activity.

At IP, an EST of 35.6°C resulted in a higher glycogen amount and concentration compared to all other EST, which was not accompanied by a higher plasma glucose level at IP. It might be possible that the 24 h longer time frame between E15 and IP at an EST of 35.6°C (Maatjens et al., 2016a), provided time to build up glycogen stores from excessive glucose to an abundant level, which in addition could be used as energy for embryonic growth and development. This is supported by the lower relative yolk weight and expressed by the numerically higher YFBM at IP at an EST of 35.6°C (Maatjens et al., 2016a). An even lower residual yolk weight at EP compared to IP at an EST of 35.6°C, in combination with the higher relative heart, spleen, stomach, and intestine weight (Maatjens et al., 2016a), suggests that the higher uric acid level found at EP originate from amino acid metabolism from the yolk and oral amnion consumption (Moran, 2007). The higher residual yolk weight at IP and EP at an EST of 38.9°C compared to 35.6 and 36.7°C, indicates lower yolk consumption and suggests that the higher uric acid levels found in

other studies possibly originate from another source than yolk, as for example from degradation of muscle protein as suggested by Molenaar et al. (2013). It can be suggested that depending on the EST which the embryos experience, variation in uric acid level originates from different sources.

From EP until hatch, an EST of 35.6°C resulted in a decreased hepatic glycogen amount due to glycolysis (Moran, 2007), but at hatch, the hepatic glycogen amount remained higher compared to all other EST. In combination with the higher YFBM found at an EST of 35.6 and 36.7°C compared to an EST of 37.8 and 38.9°C (Maatjens et al., 2016a), this might suggest that nutrient efficiency may be higher at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C. Molenaar et al. (2010) indicated that protein efficiency was 3.2% higher at an EST of 37.8°C compared to an EST of 38.9°C, which support our current findings. Therefore it appears that an even lower EST than 37.8°C will result in higher efficiencies for protein deposition. For the embryo, hepatic gluconeogenesis is crucial, because glucose is the major source of energy during the time from IP until the hatch, particularly during the energy demanding hatching process. Therefore, the suggested improved physiological status, expressed by the higher hepatic glycogen amount, might ease the hatching process and might contribute to an improved chick quality at hatch.

In summary, an EST of 38.9°C applied from E15 onward affected embryo metabolic rate and consequently chick embryo physiology, which was demonstrated by the early lactate peak, though numerically, already at E17 and a lower glycogen amount and concentration at E19 to hatch. An EST of 35.6°C applied from E15 onward, immediately influenced plasma glucose and uric acid concentration at E17. The continue lower lactate concentration from E15 onward and the large increase in hepatic glycogen amount and concentration from E19 to hatch, suggest that an EST of 35.6°C results in a lower metabolic rate and consequently it appears that O_2 is less limited during the last week of incubation. Results of this study stress the negative effects of an EST of 38.9°C from E15 onward and emphasize that an EST of 35.6 and 36.7°C from E15 onward might be beneficial for chick embryo physiology.

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Table 2. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on plasma glucose concentration (mg/100mL) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Plasma glucose (mg/100mL)				
	E17	E19	IP	EP	H
EST (°C)					
35.6	133.84[a]	152.97	165.83[b]	167.53[b]	225.99
36.7	100.82[b]	148.11	188.50[a]	177.25[a]	225.32
37.8	117.28[ab]	139.67	163.35[b]	182.80[a]	230.48
38.9	126.43[a]	153.38	176.06[ab]	163.86[b]	218.45
SEM	7.16	3.53	6.16	4.13	3.49
Start day					
C		139.67	163.35	182.80	230.48
E15		149.78	171.10	167.82	223.72
E17		153.19	183.30	170.67	228.29
E19			176.00	170.14	217.76
SEM		3.37	6.16	4.13	3.45
P-value					
EST	0.02	0.40	0.008	0.02	0.19
Start day		0.40	0.11	0.82	0.08
EST x start day		0.07	0.06	0.96	0.45

[a-b]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 3. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on plasma uric acid concentration (mg/100mL) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Plasma uric acid (mg/100mL)								
	E17	CI[1] E17	E19	CI[1] E19	IP	CI[1] IP	EP	H	CI[1] H
EST (°C)									
35.6	3.56[a]	2.97 - 4.15	2.65[a]	2.32 - 2.72	4.23	3.46 - 4.44	3.86[a]	4.51[a]	3.96 - 4.63
36.7	2.43[b]	1.95 - 2.83	2.28[bc]	2.00 - 2.36	4.24	3.62 - 4.40	3.48[ab]	3.78[b]	3.14 - 3.65
37.8	3.00[ab]	2.38 - 3.39	2.06[c]	1.72 - 2.22	3.82	2.93 - 4.11	3.34[ab]	3.44[b]	2.75 - 3.64
38.9	3.13[a]	2.57 - 3.57	2.40[ab]	2.12 - 2.52	4.03	3.35 - 4.17	2.96[b]	3.66[b]	3.17 - 3.84
SEM							0.23		
Start day									
C			2.06	1.72 - 2.22	3.82	2.93 - 4.11	3.34	3.44	2.75 - 3.64
E15			2.46	2.19 - 2.51	4.34	3.59 - 4.53	3.72	3.95	3.34 - 3.95
E17			2.43	2.16 - 2.47	3.83	3.29 - 4.04	3.12	3.82	3.30 - 3.90
E19					4.31	3.56 - 4.45	3.46	4.18	3.57 - 4.20
SEM							0.23		
P-value									
EST	0.02		0.03		0.68		0.008	<0.001	
Start day			0.89		0.33		0.11	0.65	
EST x start day			0.096		0.93		0.86	0.22	

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

[1]Confidence Interval.

Table 4. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on plasma lactate concentration (mmol/L) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Plasma lactate (mmol/L)				
	E17	E19[1]	IP	EP	H
EST (°C)					
35.6	1.39	1.72	1.90	2.12	2.53
36.7	1.64	1.78	2.08	2.23	2.44
37.8	1.86	2.24	1.82	2.09	2.42
38.9	2.09	1.85	1.91	2.13	2.55
SEM	0.22	0.09	0.11	0.13	0.10
Start day					
C		2.24	1.82	2.09	2.42
E15		1.69	2.04	2.28	2.52
E17		1.88	1.93	2.12	2.51
E19			1.92	2.08	2.49
SEM		0.09	0.11	0.13	0.10
P-value					
EST	0.15	0.46	0.18	0.76	0.53
Start day		0.03	0.76	0.43	0.89
EST x start day		0.01	0.07	0.83	0.23

[1]Interactions between EST and start day: 35.6°C-E15: 1.58^{cd}, 35.6°C-E17: 1.86^{bc}, 36.7°C-E15: 1.84^{bd}, 36.7°C-E17: 1.68^{cd}, 37.8°C: 2.24^{a}, 38.9°C-E15: 1.61^{cd}, 38.9°C-E17: 2.11^{ab}.

[a-d]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 5. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on total hepatic glycogen (mg) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Total hepatic glycogen (mg)							
	E17	CI[1] E17	E19	CI[1] E19	IP	CI[1] IP	EP	H
EST (°C)								
35.6	3.22	1.04 - 9.99	9.71	7.25 - 13.00	21.99[a]	18.29 - 26.45	24.90[a]	17.08[a]
36.7	1.00	0.13 - 7.64	13.70	10.81 - 17.36	17.98[b]	15.15 - 21.33	23.31[a]	10.15[b]
37.8	5.01	2.14 - 11.72	10.43	7.16 - 15.18	13.46[c]	9.56 - 18.95	12.88[b]	12.07[b]
38.9	1.37	0.24 - 7.79	9.11	6.79 - 12.23	5.56[d]	3.97 - 7.79	14.16[b]	11.56[b]
SEM							3.79	1.20
Start day								
C			10.43	7.16 - 15.18	13.46	9.56 - 18.95	12.88	12.07
E15			9.47	7.44 - 12.05	12.15	9.59 - 15.38	18.32	10.91
E17			12.01	9.77 - 14.74	13.89	11.39 - 16.94	18.26	13.77
E19					13.03	10.45 - 16.25	25.80	14.10
SEM							3.79	1.19
P-value								
EST	0.33		0.08		<0.001		0.05	<0.001
Start day			0.18		0.30		0.22	0.06
EST x start day			0.54		0.53		0.76	0.96

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

[1]Confidence Interval.

Table 6. Effects of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on hepatic glycogen concentration (mg/g) at E17, E19, internal pipping (IP), external pipping (EP), and hatch (H) (n = 30 per treatment)

Item	Hepatic glycogen (mg/g)							
	E17	CI[1] E17	E19	CI[1] E19	IP	CI[1] IP	EP	H
EST (°C)								
35.6	7.57	2.48 - 23.12	13.82[b]	10.28 - 18.57	26.13[a]	21.66 - 31.53	27.53[a]	16.01[a]
36.7	2.07	0.24 - 17.50	20.94[a]	16.61 - 26.39	22.35[b]	18.85 - 26.50	27.91[a]	9.67[b]
37.8	10.92	4.58 - 26.05	15.95[ab]	11.05 - 23.02	18.02[c]	12.95 - 25.03	15.08[b]	12.46[b]
38.9	3.08	0.54 - 17.72	14.09[b]	10.58 - 18.74	7.19[d]	5.17 - 9.98	16.67[b]	12.31[b]
SEM							4.10	1.11
Start day								
C			15.95	11.05 - 23.02	18.02	12.98 - 25.03	15.08	12.46[ab]
E15			14.03	11.04 - 17.82	15.13	11.96 - 19.13	21.34	10.75[b]
E17			18.19	14.85 - 22.27	17.31	14.21 - 21.08	20.98	13.30[ab]
E19					16.02	12.85 - 19.98	29.79	13.94[a]
SEM							4.10	1.10
P-value								
EST	0.35		0.05		<0.001		0.05	<0.001
Start day			0.14		0.28		0.18	0.05
EST x start day			0.70		0.53		0.83	0.89

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

[1]Confidence Interval.

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Chapter 6

Temperature during the last week of incubation. II. Effects on first week broiler development and performance

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6.1 ABSTRACT

Little is known about applying various eggshell temperatures (EST) during the last week of incubation. In particular, the effect of an EST below 37.8°C during the last week of incubation is poorly investigated. Therefore, we investigated effects of EST of 35.6, 36.7, 37.8, or 38.9°C applied from d of incubation (E) 15, E17, or E19 on first week broiler development and performance. A total of 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were incubated at an EST of 37.8°C until E15. From E15, E17, or E19 onward, eggs were incubated at an EST of 35.6, 36.7, 37.8, or 38.9°C. Chick quality was determined at placement in the broiler house and organ development was measured at d7. BW was determined at placement, d4, and d7. Feed intake (FI) was measured at d4 and d7 and G:F was calculated between placement and d4, and between d4 and d7.

Chick quality at placement was higher at an EST of 35.6°C compared to all other EST treatments, expressed by a longer chick length and highest prevalence of closed navels. BW d7 was higher at an EST of 36.7°C compared to all other EST treatments, which was not caused by a higher FI during the first week. A higher G:F between d0 and d7 was found at an EST of 36.7°C compared to 35.6 and 38.9°C. At d7, a higher relative heart weight was found at an EST of 35.6 compared to 38.9°C.

This study indicates that an EST of 38.9°C applied from E15 onward negatively affected chick quality, organ development, and G:F until d7 compared to 37.8°C. Moreover, an EST of 36.7°C had a clear positive effect on chick quality, organ development, G:F, and growth performance until d7. An EST of 35.6°C resulted in equal or higher chick quality and organ weights compared to 36.7°C, but this was not reflected in performance parameters.

Key words: temperature, incubation, organ development, broiler performance

6.2 INTRODUCTION

Incubation temperature is an important factor affecting embryonic growth, development (French, 1994; Christensen et al., 1999), and post-hatch performance (Lourens et al., 2005, Leksrisompong et al., 2009). Earlier studies (Lourens et al., 2005, 2007; Molenaar et al., 2010, 2011) showed that a constant eggshell temperature (EST) of 37.8°C during incubation resulted in the highest hatchability, lowest third week mortality, and highest chick quality, expressed by a higher yolk-free body mass (YFBM) at hatch. An EST of ≥38.9°C applied from the second or third week of incubation or only during the hatching phase resulted in a lower YFBM and impaired organ development at hatch (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011; Maatjens et al., 2014).

Optimizing EST to support embryonic development is important to generate good day old chick quality and to ensure growth and performance during later life (Hulet et al., 2007). Already in 1984, Decuypere demonstrated that incubation temperature is important for later life performance (Decuypere, 1984). However, effects of EST, instead of incubation temperature, are not investigated thoroughly. A few studies investigated the effect of EST applied from the second or third week of incubation on subsequent broiler performance (Hulet et al., 2007; Leksrisompong et al., 2009; Molenaar et al., 2011). Molenaar et al. (2011) found that an EST of 38.9°C applied from d of incubation (E) 7 onward resulted in a higher mortality due to ascites at d42 post-hatch compared to an EST of 37.8°C. Other studies showed that an EST ≥39.5°C applied from E16 onward resulted in a lower feed intake (FI) during the first week of life, a lower BW at d7, a higher mortality at d7 (Leksrisompong et al., 2009), and a lower BW at d21 (Hulet et al., 2007) compared to an EST of 37.8°C. These studies show that EST can have lasting effects on subsequent broiler performance. Maatjens et al. (2014) indicated that an EST of 38.9°C during the relative short hatching phase (from E19 onward) already had substantial negative effects on embryonic development as well, expressed by a lower relative heart weight at hatch compared to 37.8°C and a lower YFBM at hatch compared to 36.7°C. On the other hand, they demonstrated that an EST <37.8°C in the hatching phase had positive effects on embryonic development.

According to the results of the study of Maatjens et al. (2014), the aim of the current study was to investigate the effect of EST lower or higher than 37.8°C (35.6, 36.7, and 38.9°C) from different starting points already earlier than E19 (E15 and E17) on chick quality and first week broiler development and performance.

6.3 MATERIALS AND METHODS

6.3.1 Experimental Design

Eggs were incubated at an EST of 37.8°C until E15. From E15, E17, or E19 onward, EST was changed to 35.6, 36.7, or 38.9, or maintained at 37.8°C. Table 1 shows an overview of the different treatment groups, i.e. changes in EST at E15, E17, or E19. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University, the Netherlands.

Table 1. Experimental setup of the treatments with 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, 38.9°C) applied from 3 time points (E; E15, E17, or E19) through moment of placement.

	Days of incubation			
Treatment	E0 - E15	E15 - E17	E17 - E19	E19 - hatch
1	37.8	35.6	35.6	35.6
2	37.8	36.7	36.7	36.7
3 (control)	37.8	37.8	37.8	37.8
4	37.8	38.9	38.9	38.9
5	37.8	37.8	35.6	35.6
6	37.8	37.8	36.7	36.7
7	37.8	37.8	38.9	38.9
8	37.8	37.8	37.8	35.6
9	37.8	37.8	37.8	36.7
10	37.8	37.8	37.8	38.9

6.3.2 Incubation

In total, 2,850 first grade eggs of a 43 wk old Ross 308 broiler breeder flock were selected on egg weight between 62 and 65 g. For the first 15 d of incubation, the selected eggs were placed in one incubator (HatchTech BV, Veenendaal, the Netherlands) with a capacity of 4,800 eggs. The rest of the incubator was filled with hatching eggs which were not part of the experiment to ensure uniform airflow across eggs. EST was automatically maintained at 37.8°C until E15. EST was controlled and monitored by 4 eggshell temperature sensors (NTC Thermistors: type DC 95; Thermometrics, Somerset, UK), which were placed halfway the blunted and pointed end of 4 individual fertile eggs. Incubator temperature was adjusted based on the average temperature of the 4 EST sensors. RH was maintained between 50% and 55% and CO_2 concentration did not exceed 0.35%. Eggs were placed on setter trays and turned hourly by an angle of 45°. At E14 (332 h), all eggs

containing viable embryos were transported to the experimental facility of Wageningen University (Wageningen, the Netherlands) for 30 minutes in a climate controlled car.

At E15 (336 h), after arrival at the experimental facility, three groups of 240 eggs were assigned to one out of four climate respiration chambers (Heetkamp et al., 2015) in which EST was maintained at 35.6°C (treatment 1), 36.7°C (treatment 2), or 38.9°C (treatment 4). The remaining 2,130 eggs were placed in the Control climate respiration chamber in which EST was maintained at 37.8°C (treatment 3) (Table 1).

At E17 (384 h), three groups of 210 eggs were moved from the Control climate respiration chamber to one of the three climate respiration chambers in which EST was maintained at 35.6°C (treatment 5), 36.7°C (treatment 6), or 38.9°C (treatment 7).

Finally, at E19 (432 h), three groups of 180 eggs were moved from the Control climate respiration chamber to one of the three climate respiration chambers in which EST was maintained at 35.6°C (treatment 8), 36.7°C (treatment 9), or 38.9°C (treatment 10) (Table 1).

All eggs were placed in individual hatching baskets (120 x 135 mm) and eggs and chicks were continuously exposed to light. From E15 (336 h) until hatch, EST was monitored by the median of 5 individual eggshell sensors per climate respiration chamber as described before. At E19 (453 h), temperature of the climate respiration chamber was fixed at its current setting and EST was allowed to increase during the hatching process. RH was maintained between 50% and 55% and CO_2 concentration did not exceed 0.35%.

From E19 (453 h) of incubation onwards, moment of internal pipping (IP), which was determined by candling, external pipping (EP), and hatch were monitored every 6 h. Eggs were allowed to hatch and chicks remained in individual hatching baskets, without feed and water under continuous light, until moment of placement in the grow out facility.

6.3.3 Hatch and First Week Grow-Out

Directly after the last chick had hatched for each treatment, chick quality was determined and chicks were moved to the grow-out facility, which was located in the same building. The moment was defined by a lack of IP or EP in unhatched eggs. As a result, placement times varied between treatments. At moment of determination, chicks were classified as first or second grade chicks. A chick was classified as first grade when it was clean and without deformities or lesions (Tona et al., 2004), other chicks were classified as second grade chicks. Only first grade chicks were placed in the grow-out facility. Chick quality of all individual first grade chicks was determined by measuring chick weight, chick length, and navel quality for all individual chicks. Chick length was measured from the tip of the beak to the tip of the middle toe, excluding the nail (Hill, 2001). Navel quality 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).

A total of 90 chicks per treatment was wing tagged and placed in the grow-out facility with 6 replicate floor pens per treatment and 15 chicks per pen. Each pen (1.10 x 0.90m) was prepared with wood shavings, a bell drinker, and feed trough. For the first day, an egg flat filled with starter feed was used as supplemental feeder. Feed and water were provided ad libitum and chicks were reared under continuous light. Chicks were fed a crumbled starter diet (2,846 kcal of ME/kg, 219 g/kg CP). Room temperature decreased from 35°C at moment of placement to 30°C at d7.

6.3.4 Data Collection

At d4 (96 h post-placement) and d7 (168 h post-placement), body weight was recorded for all chicks per treatment, and FI per pen was determined. Mortality was recorded daily and dead chicks were weighed. G:F between d0 and d4, d4 and d7, and between d0 and d7 was calculated based on weight gain and FI per pen.

At 7 d (168 h post-placement), 10 chicks per pen were sacrificed by decapitation. The liver was removed, weighed, and immediately frozen in liquid nitrogen. Carcasses were frozen and stored at -20°C for further analysis of organ weights. Weights of heart, stomach, spleen, bursa, and intestines of all sampled chicks were determined after thawing carcasses at room temperature at a later moment. The empty weight of the duodenum (duodenal loop excluding pancreas), jejunum (end duodenum to Meckels' diverticulum), ileum (Meckels' diverticulum to ileal-cecal junction), and ceca were measured. Intestines were first emptied by gentle squeezing.

6.3.5 Statistical Analysis

Separate analyses were performed for the data collected at placement, 4 d, and 7 d of age. Based on the design, the experiment consists of ten treatment groups, i.e. different EST and changes in EST at E15, E17, or E19 (Table 1).

At hatch, there were 10 treatments, consisting of EST in three time trajectories (E15-E17, E17-E19, >E19). Introducing factors for EST and starting day of treatment (eggs moved after E15, E17, or E19), the design consists of a 3 x 3 factorial scheme (eggs moved from 37.8°C to another EST after E15, E17, or E19) and an "added" control (eggs constantly at 37.8°C). F-tests were performed for interaction and main effects of EST and starting day of treatment corresponding to the 3 x 3 factorial scheme. Depending upon the significance of the interaction, pairwise comparisons with Fisher's LSD method were made between the combinations of EST and start day of treatment (P-value interaction ≤ 0.05), or between the separate three means for EST and three means for start day of treatment (P-

value interaction > 0.05). Similarly, depending upon the significance of the interaction, the control treatment was compared with the nine means of combinations of three EST and three start days of treatment, or with the separate three means for EST and three means for starting time of the treatment.

Model assumptions, i.e. normality and equal variance of the error terms in the linear models, were checked by inspection of residual plots. Navel quality score and mortality were analysed as binary variables using logistic regression analysis with main effects and the interaction between EST and starting day of treatment on the logit scale. For navel quality score the required number of observations of each navel score (1, 2, or 3) for each treatment was not attained, therefore navel scores were grouped to score 1 (score 1; closed and clean navel area) and score 2 (score 2 and 3; black button, string, or open navel area). Parameters were estimated by maximum likelihood estimation. Wald tests were used to test for interaction and main effects on the logit scale. Estimated means on the logit scale were back transformed to obtain estimated prevalence for score 2. Table 2 presents the prevalence of navel score 1, 2, and 3. Data for chick quality at placement and data for organ weights at 7 d of age were analysed using chick as experimental unit. At 4 and 7 d of age, data for BW, weight gain, FI, and G:F were analysed using pen as experimental unit. All analyses were performed with SAS (Version 9.3, SAS Institute 2010).

6.4 RESULTS

6.4.1 Second Grade Chicks, Navel Quality Score, and Chick Length at Placement

No interactions between EST and start day of treatment were found for percentage of second grade chicks, navel quality score, and chick length.

No main effects for EST (P=1.00) and start day of treatment (P=1.00) were found for percentage of second grade chicks. The percentage of second grade chicks for an EST of 35.6, 36.7, 37.8, or 38.9°C was 0.0, 0.0, 0.0, and 1.1%, respectively.

A higher prevalence of navel quality score 2 was found at an EST of 38.9°C compared to all other EST treatments (P<0.001; Table 2), which were similar. Furthermore, a higher prevalence of navel quality score 2 was found when treatment was applied from E15 compared E17 and E19. Navel score for the Control treatment was similar to E15 and E17 (P<0.001). Chick length differed between all EST treatments, with the longest chick length for an EST of 36.7°C, followed by 35.6, 37.8, and 38.9°C (P<0.001; Table 3).

Table 2. Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through moment of placement on prevalence (percentage) navel score 1, 2, and 3 and total navel score 2 and 3 at placement (n=90 per treatment).

Item	Score 1 (%)[1]	Score 2 (%)[1]	Score 3 (%)[1]	Score 2 and 3 (%)
EST (°C)				
35.6	65.8	29.4	4.9	33.9[b]
36.7	60.5	34.4	5.1	38.9[b]
37.8	62.8	31.9	5.3	37.2[b]
38.9	29.2	68.8	2.0	70.9[a]
Total (%)[2]	53.6	42.3	4.2	46.5
Start day				
C	62.8	31.86	5.31	37.2[ac]
E15	46.0	48.16	5.83	56.0[a]
E17	52.4	45.77	1.88	47.8[bc]
E19	59.6	36.03	4.38	40.7[b]
Total (%)[2]	53.6	42.3	4.2	46.5
P-value				
EST	<0.001			
Start day	<0.001			
EST x start day	0.08			

[a-c] Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

[1] Score 1, 2, and 3 show original prevalence.

[2] Total percentages are based on original prevalence.

6.4.2 Body Weight and Weight Gain at 4 and 7 d of Age

No interactions between EST and start day of treatment were found for BW d0 at placement, d4, and d7, weight gain between d0 and d4, d4 and d7, and between d0 and d7.

At placement, BW d0 differed between all EST treatments, with the highest BW d0 for an EST of 38.9°C, followed by 37.8, 36.7, and 35.6°C ($P<0.001$; Table 3). BW at d4 was higher at an EST of 37.8 and 38.9°C compared to 35.6 and 36.7°C ($P=0.005$; Table 3), which were similar. BW at d7 was higher at an EST of 36.7°C compared to all other EST ($P<0.001$). BW at d7 was higher at an EST of 38.9°C compared to 35.6°C, whereas BW at d7 at an EST of 37.8°C was intermediate. Weight gain between d4 and d7 and between d0 and d7 were higher at an EST of 36.7°C compared to all other EST ($P \leq 0.001$), which were similar.

At placement, the highest BW d0 was found for the Control treatment compared to all other start days of treatment (P=0.01; Table 3). BW d0 for E15 and E17 were both higher compared to E19. BW d4 for the Control treatment was higher compared to E15 and E17 (P=0.006; Table 3), but similar to E19. BW d4 for E19 was intermediate the Control treatment and E17, but higher compared to E15 (P=0.006). BW d7 for E17 and E19 were higher compared to E15 (P=0.004) with the Control treatment intermediate. Weight gain between d0 and d4 for E15 was lower compared to all other start days of treatment (P=0.001), which were similar. Weight gain between d0 and d7 for E17 and E19 was higher compared to E15 and the Control treatment (P=0.003), which were similar.

6.4.3 Feed Intake and Gain to Feed Ratio at 4 and 7 d of Age

No interaction between EST and start day was found for FI between d0 to d4, d4 to d7, d0 to d7 and for G:F between d0 to d4, d4 to d7, and d0 to d7.

FI between d0 and d4 at an EST of 35.6°C was higher compared to all other EST treatments (P<0.001; Table 4). FI between d0 and d4 at an EST of 36.7 and 37.8°C were both higher compared to 38.9°C (P<0.001). FI between d4 and d7 at an EST of 38.9°C was higher compared to all other EST treatments (P<0.001). FI between d4 and d7 at an EST of 36.7 and 37.8°C was higher compared to 35.6°C (P<0.001). G:F between d0 to d4 at an EST of 37.8 and 38.9°C were higher compared to 35.6 and 36.7°C (P<0.001). G:F between d0 to d4 at an EST of 36.7 was higher compared to 35.6°C (P<0.001). G:F between d4 and d7 at an EST of 35.6 and 36.7°C were higher compared to 37.8 and 38.9°C (P<0.001). G:F between d0 to d7 at an EST of 36.7°C was higher compared to 35.6 and 38.9°C (P=0.003), whereas 37.8°C was intermediate.

FI between d0 and d4 was higher for E19 compared to E15 and the Control treatment (P=0.01), whereas E17 was intermediate. FI between d0 and d7 was higher for E19 compared to E15 (P=0.04) where the Control treatment and E17 were intermediate.

6.4.4 Body Development and Organ Growth at 7 d of Age

An interaction for EST and start day was found for relative liver (P<0.001), ileum (P=0.01), and caeca weight (P=0.05; Table 5). Relative liver weight was similar at an EST of 35.6 and 38.9°C for all start days of treatment. At an EST of 36.7°C, relative liver weight at E15 and E17 was higher compared to E19. The largest difference in relative liver weight was found between 36.7°C-E17 and 36.7°C-E19 (Δ=0.74%).Relative ileum weight was similar at an EST of 35.6°C for all start days of treatment. At an EST of 36.7°C, relative ileum weight at E19 was higher compared to E17, whereas E15 was intermediate. At an EST of 38.9°C, relative ileum weight at E15 was lower compared to E17 and E19, which were similar. Relative caeca weight was similar at an EST of 35.6 and 38.9°C for all start

days of treatment. At an EST of 36.7°C, relative caeca weight at E19 was higher compared to E15 and E17, which were similar.

At an EST of 36.7°C, carcass weight at d7 was higher compared to all other EST treatments (P=0.004; Table 5), which were similar. At an EST of 35.6 and 36.7°C, relative heart weight was higher compared to 38.9°C (P=0.005), whereas 37.8°C was intermediate. At an EST of 37.8 and 38.9°C, relative stomach weight was higher compared to 35.6 and 36.7°C (P<0.001), which were similar. At an EST of 35.6°C, relative bursa weight was lower compared to 36.7 and 38.9°C (P=0.002), whereas 37.8°C was intermediate. At an EST of 35.6°C, relative duodenum and jejunum weight was higher compared to all other EST treatments (both P<0.001). At an EST of 36.7°C, relative duodenum and jejunum weight was higher compared to 37.8 and 38.9°C (both P<0.001), which were similar.

6.4.5 Mortality at 7 d of Age

No interaction was found between EST and start day for mortality at d7 (P=1.00) and no main effects were found for EST (P=1.00) and start day (P=1.00). Mortality at d7 for 35.6, 36.7, 37.8, and 38.9°C was 1.1, 0.0, 0.0, and 2.5%, respectively.

6.5 DISCUSSION

The aim of the experiment was to investigate whether an EST of 35.6, 36.7, 37.8, or 38.9°C applied from E15, E17, or E19 of incubation affected chick quality at placement in the grow out facility and first week broiler development and performance.

6.5.1 Chick Quality at Placement

High chick quality can be quantified by a high YFBM (Lourens et al., 2005; Maatjens et al., 2014), long chick length, and good navel quality (Hill, 2001) and may be related to high chick performance potential (Tona et al., 2005). Although investigations to correlate day-old-chick BW and performance are ambiguous, chick BW at 7 to 10 d of age has been shown to be related to BW at slaughter age (Tona et al., 2003, 2004).

In the current study, BW d0 at placement showed a gradual decrease from a high EST towards a lower EST. In the study of Hulet et al. (2007) also a lower chick weight was found at an EST of 37.5°C compared to 38.6, and 39.7°C. However, opposite results were found in other studies (Lourens et al., 2005; Leksrisompong et al., 2007; Molenaar et al., 2011) at which a lower chick weight was found at a higher EST. It is questionable whether chick weight at hatch is a reliable parameter for chick quality, because residual yolk is included in this parameter. YFBM might be a better indicator to determine chick quality at day of hatch because residual yolk is not included (Molenaar et al., 2011). Earlier studies

showed that YFBM at hatch was higher at an EST of 37.8°C compared to 38.9°C, when EST was increased from 37.8 to 38.9°C at E7 or E9 (Lourens et al., 2005, 2007; Molenaar et al., 2011, 2013), and during only the hatching phase (from E19 onward) (Maatjens et al., 2014). The current study showed a lower YFBM at hatch when an EST of 37.8 or 38.9°C was applied from E15 onward compared to 35.6 and 36.7°C, and a higher residual yolk weight at hatch when an EST of 38.9°C was applied from E15 onward compared to 35.6, 36.7, and 37.8°C (Maatjens et al., 2016). Therefore, the higher BW d0 found at a higher EST in the current study is probably due to a larger amount of residual yolk.

At placement, chick length was longer at a lower EST compared to a higher EST, which might reflect a better chick quality as proposed by Hill (2001). A first reason for the increased chick length in the current study might be found in the up to 21 h longer incubation time at an EST of 35.6°C compared to all other EST treatments (Maatjens et al., 2016), which suggests that embryos had more time to develop. A second reason might be that protein efficiency may be higher at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C, resulting in a higher YFBM at hatch (Maatjens et al., 2016). Molenaar et al. (2010) indicated that protein efficiency was 3.2% higher at an EST of 37.8°C compared to 38.9°C. Possibly when protein is used as an energy source, protein is not used for growth and development, thereby decreasing the efficiency of protein utilization (Molenaar et al., 2010) and affecting development of YFBM and consequently, chick length.

At placement, the prevalence of navel quality score 1 (good quality) was lower at an EST of 38.9°C compared to 35.6, 36.7, and 37.8°C. Navel quality is highly related to the amount of retracted yolk (Tona et al., 2005). Earlier results of the current study showed a higher residual yolk weight at hatch at an EST of 38.9°C compared to 35.6, 36.7, and 37.8°C (Maatjens et al., 2016). Therefore the lower prevalence of navel score 1 might be explained by a higher amount of residual yolk

6.5.2 First Week Broiler Performance

At 7 d of age a higher BW was found at an EST of 36.7°C compared to 35.6, 37.8, and 38.9°C. Earlier studies indicated a higher BW at d7 at an EST of 37.8 compared to 38.9°C, suggesting that an EST lower than 38.9°C might be beneficial for growth during the first week (Leksrisompong et al., 2009; Molenaar et al., 2011). The higher BW at d7 might be related to the longer chick length at hatch at an EST of 36.7°C compared to all other EST treatments. This corresponds with the study of Willemsen et al. (2008) who found a significant correlation between chick length and BW at d7, which remained significant until d35 (Willemsen et al., 2008). Leksrisompong et al. (2009) found that a higher BW at d7 was caused by a higher FI between d0 and d7, but their result was not in line with our study as we found no difference in FI between d0 and d7 between EST

treatments. Although FI was not different, weight gain between d0 and d7 was higher at an EST of 36.7°C compared to all other EST. This was particularly due to a higher weight gain between d4 and d7 at an EST of 36.7°C compared to all other EST. Willemsen et al. (2008) found a significant correlation between relative growth during the first week and chick length at hatch. Therefore, it might be possible that the longer chick length at hatch found at an EST of 36.7°C in our study is related to the higher weight gain found between d0 and d7.

Total FI between d0 and d7 did not show significant differences between EST but FI from d0 to d4 showed a gradual decrease from an EST of 35.6 to 38.9°C. Reason for the lowest FI between d0 and d4 at an EST of 38.9°C might be the nutrient availability of the residual yolk. After hatch, residual yolk provides energy in the chick and precedes the initiation of growth (Chamblee et al., 1992). Yolk size decreases to less than 1 g at 96 h after hatching (Noy et al., 1996). The current study showed a higher residual yolk weight at hatch when an EST of 38.9°C was applied from E15 onward compared to 35.6, 36.7, and 37.8°C (Maatjens et al., 2016). In the current study, we found no effect of EST or start day of treatment on the amount of residual yolk at d7, indicating that the amount of yolk uptake during the first 7 d was higher at an EST of 38.9°C compared to lower EST. This in turn suggests that chicks incubated at a low EST depend more on exogenous feed than endogenous feed, compared to chicks that were incubated at a higher EST, which have more endogenous feed available and consequently depend to a lesser extend on exogenous feed to obtain equal weight gain between d0 and 4.

Although FI between d0 and d7 was not different between EST treatments, a higher weight gain was found between d0 and d7 at an EST of 36.7°C compared to all other EST treatments, resulting in a higher G:F between d0 and d7 at an EST of 36.7°C compared to 35.6 and 38.9°C. This higher G:F might be due to better intestinal development. Earlier results of the same study showed that at an EST of 35.6, 36.7, or 37.8°C applied from E15 onward resulted in a higher relative intestine weight at hatch compared to 38.9°C (Maatjens et al., 2016).

The higher relative intestinal weight at an EST <38.9°C might be caused by the uptake of yolk by the yolk stalk between E19 and E20 and the transport of the yolk toward the intestine and gizzard (Esteban et al., 1991a, 1991b). Residual yolk uptake and early access to feed stimulate small intestine development (Noy and Sklan, 1998, 1999; Uni et al., 1998), as it changes the villus volume which continues after hatch (Noy and Sklan, 1997). From hatch until d4, the duodenum undergoes a major increase in villus volume, which contributes to feed digestion. From hatch until d10, the jejunum increases in villus volume and plays a major role in feed absorption (Noy and Sklan, 1997). However, in the current study, the highest relative duodenum and jejunum weight at d7 was found at an EST

of 35.6°C compared to all other EST treatments, and not at an EST of 36.7°C. It can be speculated that relative intestine weight is not a reliable parameter to determine intestinal development in relation to G:F.

Although effects of start day of treatment are not significant for BW d0, BW d4, and weight gain between d0 and d4, effects of EST seemed to be more pronounced when applied from E15 onward compared to E17 or E19. Therefore, it cannot be completely ruled out that longer treatment duration might emphasize the EST effect.

Development of the heart was affected by EST, at which a higher relative heart weight at d7 was found at an EST of 35.6 and 36.7°C compared to 38.9°C. The heart is mitotically active until d10 after hatching (Romanoff, 1960), but heart growth rate peaks at approximately 5 to 6 d after hatching and continues growth similar to body growth (Christensen, 2009). This suggests that the higher relative heart weight found at d7 at an EST of 35.6 and 36.7°C will remain until slaughter age in the case that body weight would increase equally for all different EST. The development of the heart as a supply organ is of major importance on the quality of the day old chick and later life performance as mortality due to ascites was decreased when an EST of 37.8 was applied from E7 compared to 38.9°C (Molenaar et al., 2011). Data of the current study suggest that EST below 37.8°C from E15 onward might be beneficial for heart development and might show positive effects during later life performance.

To conclude, results of the current study indicate that an EST applied from E15 onward affected chick quality, first week organ development, and performance. An EST of 38.9°C negatively affected chick quality, organ development, and G:F compared to 37.8°C. Moreover, an EST of 36.7°C had a clear positive effect on chick quality, organ development, G:F, and growth performance. At an EST of 35.6°C equal or higher chick quality and organ weights were found compared to 36.7°C, but this was not reflected in performance parameters.

An EST of 36.7°C during the last week of incubation might be beneficial for chick quality and growth performance during the first week of life. However, it remains to be investigated whether these results lead to carry over effects and improved slaughter characteristics.

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Table 3. Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through moment of placement on chick length and chick body weight at placement (n = 90 per treatment), body weight at d4 and d7, weight gain between d0 to d4, d4 to d7, and d0 to d7 (n = 6 per treatment)

Item	Chick length (cm)	BW d0 (g)	BW d4 (g)	BW d7 (g)
EST (°C)				
35.6	20.5^{b}	41.5^{d}	107.3^{b}	186.9^{c}
36.7	20.6^{a}	43.0^{c}	108.5^{b}	196.9^{a}
37.8	20.3^{c}	44.2^{b}	112.5^{a}	190.1^{bc}
38.9	20.1^{d}	45.1^{a}	110.3^{a}	192.9^{b}
SEM	0.04	0.12	0.81	1.50
Start day				
C	20.4	44.2^{a}	112.5^{a}	190.1^{ab}
E15	20.4	43.3^{b}	107.0^{c}	188.9^{b}
E17	20.4	43.2^{b}	109.1^{b}	194.0^{a}
E19	20.4	42.9^{c}	109.8^{ab}	193.9^{a}
SEM	0.04	0.12	0.81	1.50
EST x Start day				
35.6 x E15	20.5	41.4	105.5	183.5
35.6 x E17	20.6	41.7	107.9	187.8
35.6 x E19	20.5	41.4	108.4	189.4
36.7 x E15	20.7	43.1	108.3	194.1
36.7 x E17	20.7	43.0	109.0	199.5
36.7 x E19	20.6	42.8	108.0	197.1
37.8	20.4	44.2	112.5	190.1
38.9 x E15	20.1	45.5	107.2	189.0
38.9 x E17	20.1	45.1	110.2	194.8
38.9 x E19	20.2	44.6	113.5	194.9
SEM	0.05	0.18	1.15	2.14
P-value				
EST	<0.001	<0.001	0.005	<0.001
Start day	0.91	0.01	0.006	0.004
EST x start day	0.12	0.06	0.08	0.89

[a-d]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 3 (continued). Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through moment of placement on chick length and chick body weight at placement (n = 90 per treatment), body weight at d4 and d7, weight gain between d0 to d4, d4 to d7, and d0 to d7 (n = 6 per treatment)

Item	Weight gain d0-d4 (g)	Weight gain d4-d7 (g)	Weight gain d0-d7 (g)
EST (°C)			
35.6	65.8	81.7[b]	145.7[b]
36.7	65.5	89.2[a]	154.2[a]
37.8	68.2	79.3[b]	145.9[b]
38.9	65.2	84.0[b]	147.8[b]
SEM	0.81	1.53	1.53
Start day			
C	68.2[a]	79.3	145.9[b]
E15	63.7[b]	83.0	145.7[b]
E17	65.8[a]	86.1	150.8[a]
E19	67.1[a]	85.8	151.2[a]
SEM	0.81	1.53	1.53
EST x Start day			
35.6 x E15	64.0	77.4	142.0
35.6 x E17	66.4	83.2	146.2
35.6 x E19	67.1	84.5	148.8
36.7 x E15	65.0	87.8	151.5
36.7 x E17	66.1	90.5	156.6
36.7 x E19	65.2	89.2	154.4
37.8	68.2	79.3	145.9
38.9 x E15	61.8	83.8	143.6
38.9 x E17	65.0	84.6	149.6
38.9 x E19	68.9	83.5	150.3
SEM	1.15	2.17	2.18
P-value			
EST	0.81	0.001	<0.001
Start day	0.001	0.16	0.003
EST x start day	0.06	0.51	0.81

[a-d]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 4. Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on feed intake per chick between d0 to d4, d4 to d7, and d0 to d7, and G:F between d0 to d4, d4 to d7, and d0 to d7 (n = 6 per treatment)

Item	Feed intake/chick d0-d4 (g)	Feed intake/chick d4-d7 (g)	Feed intake/chick d0-d7 (g)
EST (°C)			
35.6	75.7[a]	89.6[c]	165.3
36.7	66.7[b]	98.7[b]	165.3
37.8	63.2[b]	96.7[b]	159.9
38.9	58.3[c]	107.0[a]	166.6
SEM	1.36	3.19	3.48
Start day			
C	63.2[b]	96.7	159.9[ab]
E15	64.3[b]	95.8	159.8[b]
E17	67.2[ab]	99.5	167.3[ab]
E19	69.2[a]	100.4	170.2[a]
SEM	1.36	3.19	3.48
EST x start day			
35.6 x E15	72.3	85.7	157.3
35.6 x E17	77.3	91.4	168.7
35.6 x E19	77.5	91.7	170.0
36.7 x E15	65.7	97.7	163.4
36.7 x E17	67.2	98.9	166.1
36.7 x E19	67.1	99.4	166.5
37.8	63.2	96.7	159.9
38.9 x E15	54.9	104.0	158.8
38.9 x E17	57.2	108.2	167.0
38.9 x E19	63.0	110.2	174.0
SEM	1.91	4.67	5.02
P-value			
EST	<0.001	<0.001	0.94
Start day	0.01	0.44	0.04
EST x start day	0.34	0.98	0.75

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 4 (continued). Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through hatch on feed intake per chick between d0 to d4, d4 to d7, and d0 to d7, and G:F between d0 to d4, d4 to d7, and d0 to d7 (n = 6 per treatment)

Item	G:F d0-d4	G:F d4-d7	G:F d0-d7
EST (°C)			
35.6	0.846[c]	0.869[a]	0.858[b]
36.7	0.988[b]	0.896[a]	0.932[a]
37.8	1.061[a]	0.796[b]	0.901[ab]
38.9	1.081[a]	0.772[b]	0.879[b]
SEM	0.02	0.02	0.02
Start day			
C	1.061	0.796	0.901
E15	0.980	0.854	0.898
E17	0.975	0.855	0.896
E19	0.960	0.828	0.875
SEM	0.02	0.02	0.02
EST x start day			
35.6 x E15	0.860	0.898	0.880
35.6 x E17	0.838	0.853	0.844
35.6 x E19	0.842	0.857	0.085
36.7 x E15	0.997	0.856	0.912
36.7 x E17	1.006	0.919	0.854
36.7 x E19	0.960	0.911	0.930
37.8	1.061	0.796	0.901
38.9 x E15	1.083	0.809	0.901
38.9 x E17	1.082	0.792	0.890
38.9 x E19	1.079	0.716	0.846
SEM	0.03	0.03	0.03
P-value			
EST	<0.001	<0.001	0.003
Start day	0.68	0.51	0.48
EST x start day	0.91	0.16	0.47

[a-c]Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

Table 5. Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through moment of placement on carcass weight at d7 and relative organ weights, calculated as percentage of carcass weight at d7 (n = 60 per treatment)

Item	Carcass weight (g)	Heart (% carcass)	Liver (% carcass)	Stomach (% carcass)	Spleen (% carcass)
EST (°C)					
35.6	169.7[b]	0.944[a]	4.82	3.86[b]	0.0836
36.7	174.3[a]	0.942[a]	4.82	3.80[b]	0.0843
37.8	166.0[b]	0.921[ab]	4.77	4.00[a]	0.0838
38.9	168.7[b]	0.908[b]	5.07	3.98[a]	0.0790
SEM	1.5	0.01	0.07	0.04	0.0033
Start day					
C	166.0	0.921	4.77	4.00	0.0838
E15	169.0	0.937	4.93	3.90	0.0789
E17	171.9	0.930	5.06	3.86	0.0859
E19	171.9	0.926	4.72	3.88	0.0821
SEM	1.5	0.01	0.06	0.04	0.0033
EST x start day					
35.6 x E15	167.7	0.963	4.74[bc]	3.90	0.0813
35.6 x E17	169.7	0.928	4.94[ac]	3.85	0.0913
35.6 x E19	171.7	0.942	4.75[c]	3.81	0.0780
36.7 x E15	173.9	0.966	4.94[ac]	3.82	0.0836
36.7 x E17	175.1	0.939	5.12[a]	3.76	0.0874
36.7 x E19	173.8	0.922	4.38[d]	3.81	0.0821
37.8	166.0	0.921	4.77[c]	4.00	0.0838
38.9 x E15	165.2	0.890	5.04[ab]	3.97	0.0723
38.9 x E17	170.9	0.919	5.08[a]	3.97	0.0789
38.9 x E19	169.9	0.917	5.06[ab]	4.00	0.0856
SEM	2.1	0.02	0.10	0.06	0.0048
P-value					
EST	0.004	0.005	<0.001	<0.001	0.33
Start day	0.15	0.55	<0.001	0.74	0.23
EST x start day	0.67	0.08	<0.001	0.88	0.22

[a-d]Least squares means lacking a common superscript within a column and factor differ (P≤0.05).

Table 5 (continued). Effect of 4 eggshell temperatures (EST; 35.6, 36.7, 37.8, or 38.9°C) applied from 3 starting points (E15, E17, or E19) through moment of placement on carcass weight at d7 and relative organ weights, calculated as percentage of carcass weight at d7 (n = 60 per treatment)

Item	Bursa (% carcass)	Duodenum (% carcass)	Jejunum (% carcass)	Ileum (% carcass)	Caeca (% carcass)
EST (°C)					
35.6	0.1621^{b}	1.17^{a}	1.90^{a}	1.23	0.54
36.7	0.1756^{a}	1.09^{b}	1.82^{b}	1.25	0.51
37.8	0.1737^{ab}	0.99^{c}	1.70^{c}	1.33	0.53
38.9	0.1769^{a}	0.96^{c}	1.69^{c}	1.25	0.51
SEM	0.0039	0.02	0.02	0.02	0.01
Start day					
C	0.1737	0.99	1.70	1.33	0.53
E15	0.1665	1.05	1.81	1.22	0.52
E17	0.1708	1.08	1.79	1.24	0.51
E19	0.1774	1.09	1.81	1.27	0.53
SEM	0.0039	0.02	0.02	0.02	0.01
EST x start day					
35.6 x E15	0.1547	1.18	1.89	1.22^{cd}	0.52^{ad}
35.6 x E17	0.1614	1.18	1.90	1.26^{bcd}	0.54^{ab}
35.6 x E19	0.1702	1.17	1.90	1.22^{cd}	0.55^{a}
36.7 x E15	0.1773	1.07	1.85	1.25^{bcd}	0.50^{cd}
36.7 x E17	0.1765	1.06	1.75	1.21^{cd}	0.49^{d}
36.7 x E19	0.1737	1.13	1.87	1.29^{ab}	0.54^{ab}
37.8	0.1737	0.99	1.70	1.33^{a}	0.53^{ac}
38.9 x E15	0.1687	0.92	1.70	1.19^{d}	0.53^{ac}
38.9 x E17	0.1743	1.00	1.71	1.27^{ac}	0.50^{cd}
38.9 x E19	0.1877	0.97	1.66	1.30^{ab}	0.51^{bcd}
SEM	0.0057	0.03	0.03	0.02	0.01
P-value					
EST	0.002	<0.001	<0.001	0.44	0.01
Start day	0.09	0.30	0.55	0.06	0.09
EST x start day	0.33	0.16	0.08	0.01	0.05

$^{a-d}$Least squares means lacking a common superscript within a column and factor differ ($P \leq 0.05$).

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6

Chapter 7

General discussion

7.1 INTRODUCTION

During incubation, temperature drives chicken embryonic metabolism, development, and growth (Ricklefs, 1987; French, 1994; Christensen et al., 1999). To optimize embryonic development, incubator temperature should be set to control and maintain the correct embryo temperature during incubation (Lourens et al., 2005; Meijerhof, 2009). Eggshell temperature (EST) measurements can be used as a non-invasive method to determine embryo temperature (Lourens et al., 2005). Earlier studies indicated that a constant EST of 37.8°C throughout incubation has been proven to be a more optimal temperature compared to an EST of 38.9°C, to obtain the lowest third week embryo mortality, the highest hatchability (Lourens et al., 2005), the highest chick quality, expressed in the longest chick length (Lourens et al., 2005; Molenaar et al., 2010), and the highest yolk free body mass (YFBM) (Lourens et al., 2005, 2007; Molenaar et al., 2011a) at hatch.

Chicken embryos act as poikilotherm and have limited abilities to regulate their own body temperature (Romijn and Lokhorst, 1955). Low incubator temperatures result in low EST, which decreases metabolic rate and slows down growth and development of the embryo. High EST increases metabolic rate and on its turn increases growth and development (Romanoff, 1936; Ricklefs, 1987; Christensen et al., 1999). However, at high EST and consequently high metabolic rate, energy utilization and the conversion of nutrient sources into body development might be hampered by insufficient oxygen (O_2) availability, as exchange of O_2 and carbon dioxide (CO_2) is restricted due to limited shell and shell membrane porosity (eggshell conductance), especially during the second part of the incubation process (Figure 1).

The freshly laid egg consists besides minerals and water of 0.9% of carbohydrates, 12.1% protein, and 10.5% fat (Romanoff, 1960). Part of these energy sources will be deposited in the embryo and part will be lost as heat. Heat production can be determined by indirect measurements, such as the rate of O_2 consumption and CO_2 production. O_2 consumption and CO_2 production during the oxidation of metabolic fuels (carbohydrates, protein, fat) varies depending on the energy source used, resulting in different respiratory quotients. For carbohydrate, protein, and fat the respiratory quotient is 1.0, 0.809, and 0.707 CO_2/O_2, respectively. The average amount of O_2 consumed for metabolizing carbohydrates, protein, and fat is 0.829, 0.966, and 2.016 L/g, respectively (Bender, 2002). This means that more O_2 is required for the oxidation of fat compared to the oxidation of carbohydrates.

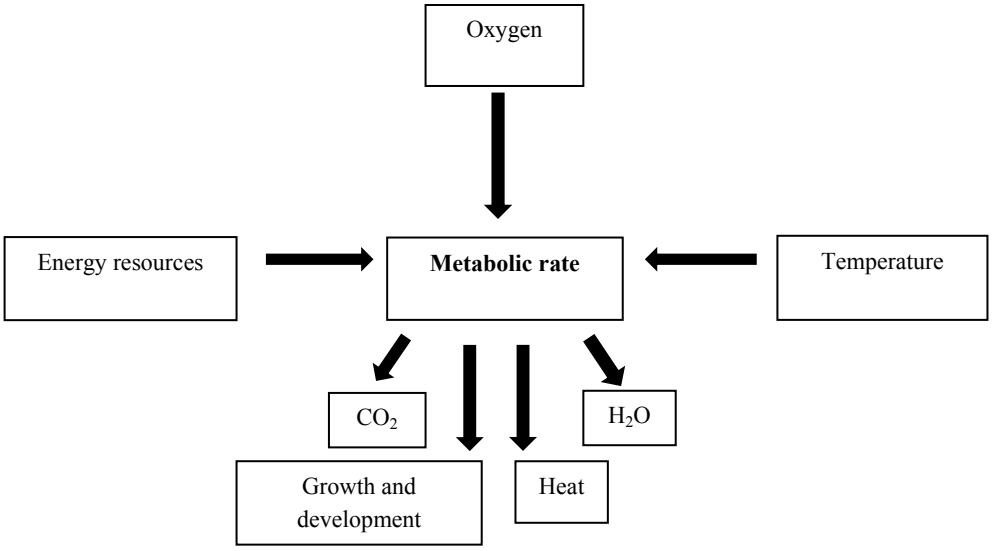


Figure 1. Schematic overview of the drivers for embryonic metabolic rate and the output of embryonic metabolism.

During the first week of incubation, the chorio allantoic membrane (CAM) cannot provide sufficient O_2 for fatty acid oxidation, and therefore carbohydrates are the main energy source for the growing embryo (Moran, 2007). From day of incubation (E) 9 onward, yolk fatty acids become the dominant energy source as adequate O_2 can be supplied by the CAM (Moran, 2007). However, from halfway during incubation onward, between E15 and internal pipping (IP), exchange of O_2 and CO_2 is restricted due to limited eggshell conductance determined by shell and shell membrane porosity, which results in a plateau phase in heat production (Figure 2; Lourens et al., 2007) until IP. This period of limited gas exchange, finishes at IP when supplementary O_2 becomes available. Consequently, in a situation of limited O_2 availability, oxidation of fat becomes more difficult than the oxidation of carbohydrates, particularly when embryos have a high metabolic rate (e.g. when exposed to high EST). In these circumstances, the embryo will use glycogen stores for body reserves and in case of limited availability of these glycogen stores it needs to increase gluconeogenesis. Lowering EST will lower the metabolic rate and ensures that O_2 remains sufficiently available. Consequently, yolk lipids can be used efficiently for energy production, which implies that embryos become less dependent on gluconeogenesis for growth and development (Moran, 2007; de Oliveira et al., 2008).

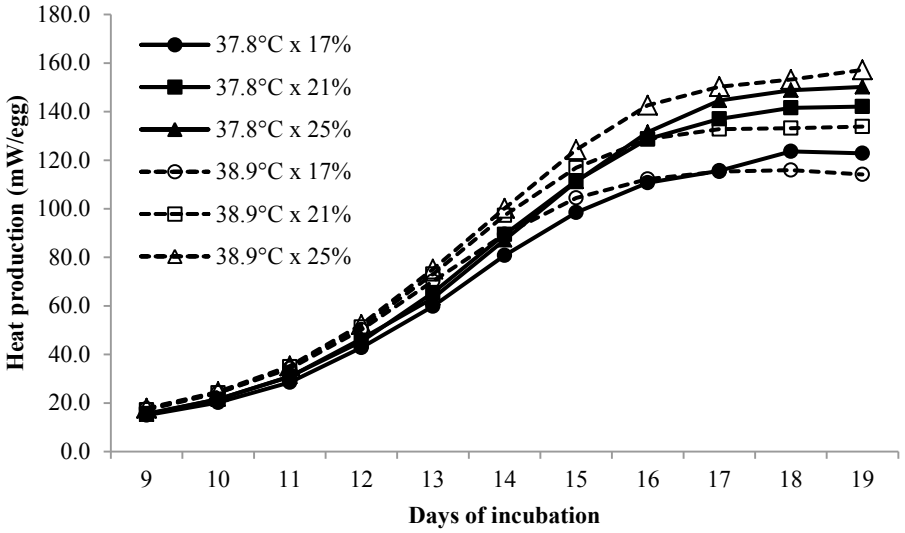


Figure 2. Heat production (mW/egg) in broiler eggs (60-65g) incubated at 2 eggshell temperature (EST) (37.8 and 38.9°C) and 3 O_2 concentrations (17, 21, and 25%) from day of incubation (E) 9 onward (Lourens et al., 2007).

Towards the end of incubation, glucose becomes a very important energy source to survive the energy demanding hatching process, because O_2 availability is low and muscle activity is high due to movements to initiates IP (de Oliveira et al., 2008). The initial carbohydrate level in the egg at the start of incubation is low, therefore during incubation glycogen stores need to build up from glucose precursors, such as glucogenic amino acids, glycerol, and lactate by gluconeogenesis and glycogenesis (Garcia et al., 1986). Glycerol originating from fat is the main glucogenic energy source before the O_2 limiting phase. Molenaar et al. (2013) showed that an EST of 38.9°C compared to 37.8°C from E10.5 onward increased both metabolic rate and glucose oxidation, but depressed hepatic glycogen storage. To compensate for the limited glycogen stores towards the end of incubation, protein might be used as an alternative glycogenic energy source (Hazelwood and Lorenz, 1959) or immediate adenosine triphosphate (ATP) production (McArdle et al., 1981). However, when protein is used as an alternative energy source, protein is not used for embryonic growth and development, resulting in decreased protein deposition efficiency as indicated by Molenaar et al. (2010).

It is generally known that a high EST of 38.9°C in the second or third week of incubation results in higher third week embryonic mortality, lower hatchability (Lourens et

al., 2005), and lower chick quality, expressed as a shorter chick length (Lourens et al., 2005; Molenaar et al., 2010) and lower YFBM (Lourens et al., 2005, 2007; Molenaar et al., 2011a) at hatch (Appendix 1).

It can be hypothesized that the negative effects of a high EST on hatchability and chick quality are mainly caused by O_2 limitation. To counteract the effects of low O_2 at high metabolic rate, O_2 levels during incubation can be increased. At a high EST of 38.9°C, the metabolic rate is higher compared to an EST of 37.8°C. Increasing O_2 from 21to 25% at an EST of 38.9°C results in higher embryonic development and a heat production, which indicates a higher metabolic rate (Figure 2). This is supported by the findings of Lourens et al. (2007) and Molenaar et al. (2010), who found a longer chick length and higher YFBM at an O_2 concentration of 25% compared to 21% (Lourens et al., 2007), and a lower third week embryo mortality, higher hatch of fertile, and higher YFBM at hatch at an EST of 38.9°C with an O_2 concentration of 25% compared to 17% (Molenaar et al., 2010). An alternative for increasing O_2 levels to restore the imbalance between metabolic rate and O_2 availability, may be to lower the metabolic rate during the last phase of incubation by lowering the EST. Lowering the EST may postpone or even prevent the embryos from experiencing O_2 shortage during a plateau phase halfway during incubation. Effects of an EST below 37.8°C during the incubation phase when O_2 availability becomes limited, is so far poorly investigated and it was also unknown from which time points during incubation onward a certain lower EST should be applied.

Besides temperature, CO_2 might play a role as well during the O_2 limiting stage of incubation. Temperature drives the request for O_2 as it influences the metabolic rate. A higher O_2 consumption however leads to a higher CO_2 production, which increases the partial pressure of CO_2 in blood (pCO_2) (Everaert et al., 2008). High levels of CO_2 might interact with EST, as both increase the request for O_2. Several studies investigated the effect of CO_2 levels during incubation. However, the effects found might not be related to solely CO_2 as in those studies an increased level of CO_2 was reached by a decreased ventilation rate (Everaert et al., 2007, 2008, 2010; Buys et al., 1998). A decreased ventilation rate, decreases the heat transfer from the eggs, which might affect EST. Effects found in these studies may therefore, at least partly, be related to EST, and not to CO_2 itself. Studies that control both EST and CO_2 levels were not reported previously.

Based on the potential effects of (lower) EST during the last week of incubation and of CO_2 during only the hatching phase, as described above, the following three aims are derived for this thesis: 1, to investigate effects of EST during the last phase of the incubation process, with special attention for EST below the general accepted optimal EST of 37.8°C, 2, to examine from which day of the incubation process onward EST should be

changed from 37.8°C, and 3, to investigate whether CO_2 concentrations are interacting with EST during the hatcher phase.

7.2 TEMPERATURE

Several studies from 2000 until 2015 have applied an EST or incubator temperature treatment during a specific time period during incubation (Table 1, 2). In these studies, eggs were incubated at a constant EST of 37.8°C or a set incubator temperature of 37.5°C, until start of the treatment. From E15 until E19, the embryo reaches its growth peak as it doubles its own YFBM during these 5 days (chapter 4). This high growth rate leads to a high metabolic rate, which increases heat production. When incubator temperature is not properly adjusted to maintain a constant optimal EST (French, 1997), insufficient heat removal from the embryo will lead to a higher EST (van den Brand et al., 2015; Lourens et al., 2006). To mimic current practical situations, the majority of the studies focussed on an EST of 38.9°C or higher, starting from the second or third week of incubation and maintained the EST treatment until hatch (Table 1; Lourens et al., 2005, 2007; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2007, 2009; Molenaar et al., 2010, 2011a, 2011b). Wineland (2000) investigated effects of a high incubator temperature of 38.6°C until transfer, without measuring EST, followed by an incubator temperature of 38.3°C until hatch.

Although several studies focussed on an EST of 38.9°C or higher during the last phase of incubation, or an incubator temperature above 37.5°C, effects of an EST below 37.8°C or incubator temperature below 37.5°C were poorly investigated. Therefore, in the current thesis, effects of both higher and lower EST than the assumed optimal EST of 37.8°C were investigated (Appendix 2). Effects of an EST of 35.6, 36.7, 37.8, and 38.9°C were investigated to cover the generally accepted higher EST, but also to examine the impact of an EST below 37.8°C, which might be beneficial for embryo development as the hypotheses is that a lower EST will not or later during incubation result into O_2 limitation during the second part of the incubation process.

7.2.1 Effects of EST during the second part of incubation on hatching time and embryo development

Chapter 2 and 4 indicate that time until hatch was longer at an EST of 35.6°C, followed by 36.7, 37.8, and 38.9°C (Figure 3a). The effect of EST on total incubation duration was mainly caused by the effect on time until IP (Figure 3a). At an EST of 35.6°C, time until IP extended to 485 h compared to 469 h at an EST of 37.8°C. An EST treatment did not substantially affect time between IP and hatch, as this time period varied only 2 h

between EST treatments, whereas time until IP varied 24 h between treatments (chapter 2 and 4). A possible explanation for the extended time until IP might be the lower metabolic rate at an EST below 37.8°C, which sustained O_2 availability. Therefore, fat oxidation can be prolonged and glycogenic energy requirements would be less, which seem to have resulted in a more sustained embryonic growth, expressed in increments in YFBM between E19 and IP, of 6.5, 5.3, 4.9, and 4.9g, at an EST of 35.6, 36.7, 37.8, and 38.9°C, respectively. This increment contributed to a numerically higher YFBM already found at IP at an EST of 35.6 compared to 36.7, 37.8, and 38.9°C. The higher YFBM at IP at lower EST resulted in a higher YFBM at hatch at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C (Figure 3c). The average hatch of fertile (HOF) percentages showed no significant difference between all EST treatments, indicating that the longer incubation time at lower EST did not affect embryonic survival (Figure 3b). A high EST of 38.9°C resulted in the shortest time interval between start treatment and IP and consequently between start treatment and hatch compared to all other EST treatments.

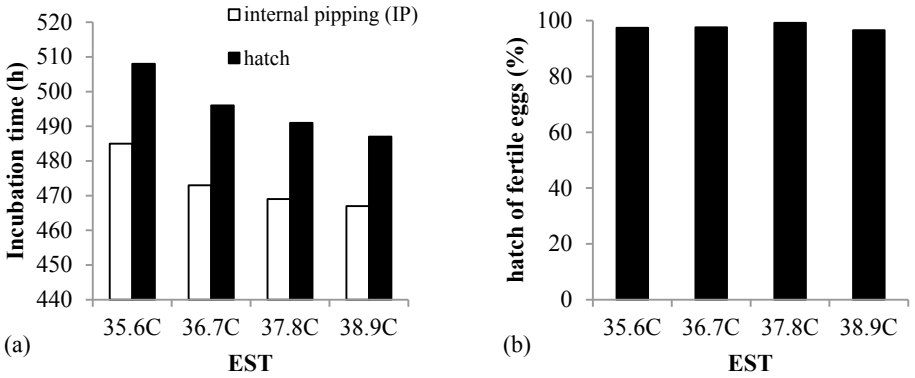


Figure 3. Average time until internal pipping (IP) and time until hatch (a), and hatch of fertile (HOF, b) at an eggshell temperature (EST) of 35.6, 36.7, 37.8, and 38.9°C (based on chapter 4).

Opposite to current results, Willemsen et al. (2010) found an extended time until IP and time until hatch at an EST of 41.1°C compared to an EST of 38.3°C. They applied an even higher EST of 41.1°C from E16 to E18, which might have been too high to keep up with the O_2 request for the increased metabolic rate. Due to the insufficient O_2 supply, metabolic processes were probably restricted, which lead to a higher embryonic mortality during and after treatment of an EST of 41.1°C compared to an EST of 35.5 (Δ=13.0%) and 38.3°C (Δ=14.8%) (Willemsen et al., 2010). The higher embryonic mortality at an EST of 41.1°C indicates that there seems to be an upper limit to incubator temperature settings.

Chapter 2 and 4 show that an EST below 37.8°C might contribute to improved body development. Earlier studies indicated that an EST of 37.8°C resulted in a higher body weight at hatch compared to a higher EST between 39.5 and 41.1°C (Leksrisompong et al., 2007, 2009; Willemsen et al., 2010). However, body weight is a combination of residual yolk weight and YFBM of which the latter might be a better indicator for chick quality (Lourens et al., 2005, 2007; Molenaar et al., 2011a). A higher YFBM was found at an EST of 35.6 and 36.7°C (both 40.5g) compared to 37.8 (Δ=1.4g) and 38.9°C (Δ=1.0g) (Figure 3c). At a lower EST, metabolic rate is reduced, which prevents O_2 limitation and subsequently ensures that fat from the yolk can be used efficiently for a prolonged amount of time, and that the energy produced is invested in growth and development, which contributes to a higher YFBM.

The higher YFBM at a lower EST may suggest that protein from yolk and albumen is used for growth and development and that these sources are not used as an alternative energy source to compensate for possible depleted glycogen stores. Glucose precursors are necessary for gluconeogenesis and glycogenesis to build up glycogen stores (Garcia et al., 1986), which serve as an energy source for the hatching process. Molenaar et al. (2013) showed that an EST of 38.9°C increased glucose oxidation compared to an EST of 37.8°C, which might result in lower glycogen stores as demonstrated in chapter 3 and 5. The lack of glycogenic energy stores may lead to protein catabolism, especially during the energy demanding hatching phase. Chapter 3 and 5 indicate that hepatic glycogen stores are increasing more between E19 and IP at an EST of 35.6 (Δ=12.3mg) compared to an EST of 36.7 (Δ=4.3mg), and 37.8°C (Δ=3.0mg) (Figure 3e). At an EST of 38.9°C, hepatic glycogen levels are already decreasing between E19 and IP (Δ= -3.6%). In addition, hepatic glycogen levels remain higher until hatch at an EST of 35.6°C compared to all other EST treatments. This result suggests that at low EST glycogen stores are not limited and therefore it might be possibly that it is not necessary to use protein as an alternative glycogenic energy source (Hazelwood and Lorenz, 1959) or immediate ATP production (McArdle et al., 1981).

At hatch, residual yolk was lower at an EST of 35.6, 36.7, and 37.8°C compared to 38.9°C (on average Δ=1.3g) (Figure 3d). The lower metabolic rate at a lower EST during the last phase of the incubation process sustained O_2 availability, which possibly prolonged the period of fat oxidation from the yolk, and resulted in more yolk uptake during incubation.

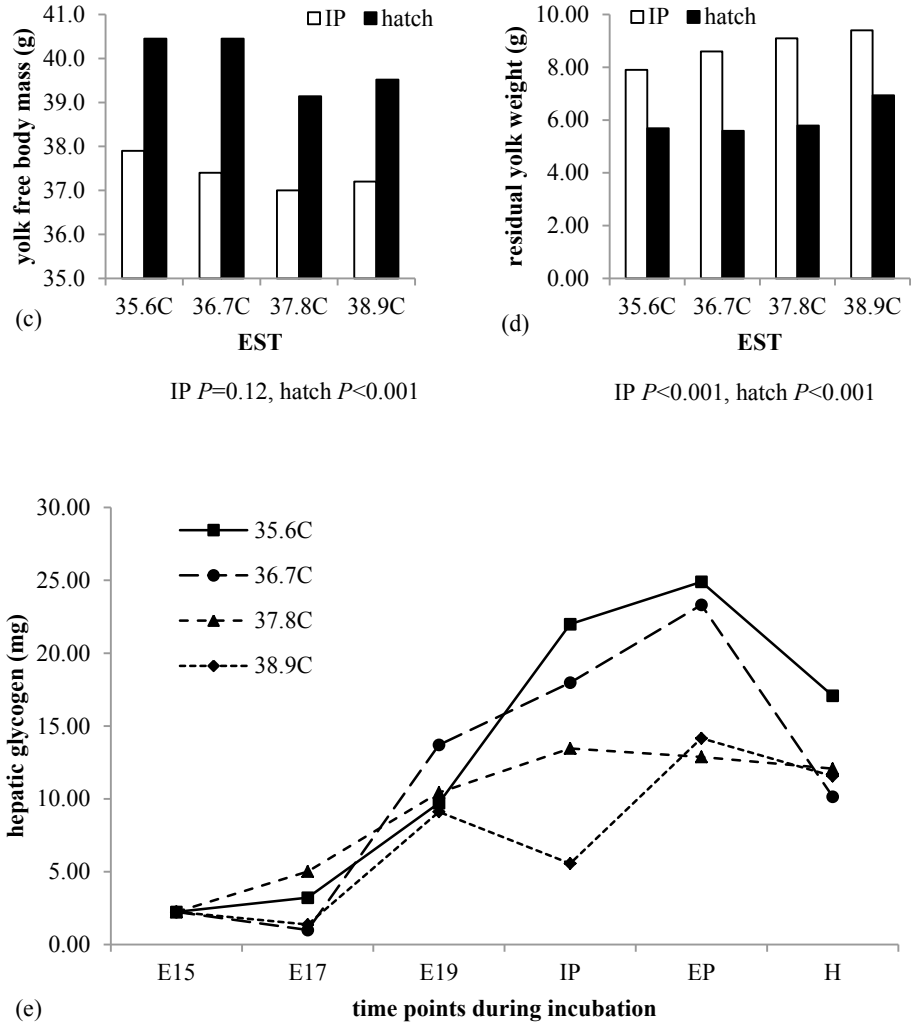


Figure 3. Average yolk free body mass (YFBM) at internal pipping (IP) and hatch (c), residual yolk weight at internal pipping (IP) and hatch (d), hepatic glycogen amount (e) at an eggshell temperature (EST) of 35.6, 36.7, 37.8, and 38.9°C (based on chapter 4).

7.2.2 Effects of EST during the second part of incubation on embryo organ growth

An interaction between EST and start day of treatment was found for relative heart weight, at which the largest difference was found when treatments were applied from E15 onward between an EST of 35.6 and 38.9°C (Δ=0.38%). This interaction will be pointed out further when discussing 'start day of treatment' (see further). In general, the highest relative heart weight was found at an EST of 35.6°C, followed by an EST of 36.7, 37.8, and

38.9°C (Figure 4a). Earlier studies repeatedly have shown than an EST of 38.9°C resulted in lower relative heart weights at hatch compared to an EST of 37.8°C (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011b). One of the reasons might be that temperature affects the number of mitotically active myocytes in the heart. The mitotic index is decreased when an incubation temperature of 39.5°C is applied compared to 34.5 and 36.5°C. The lower mitotic index might lead to a slower heart development, which results in a lower relative heart weight at hatch (Romanoff, 1960).

Another reason might be found in the higher metabolic rate at high EST. At high EST, glucose oxidation increases (Molenaar et al., 2013), which depresses the build-up of glycogen stores and results in a rapid depletion of glycogen stores between IP and hatch (chapter 3 and 5). To compensate for possible limitation of glycogen stores, protein catabolism will ensure that amino acids can be used as a glycogenic energy source (Hazelwood and Lorenz, 1959) or immediate ATP production (McArdle, 1981). The lower protein retention efficiency, higher uric acid levels, and a lower relative heart weight (Molenaar et al., 2010, 2013) might indicate that protein catabolism occurred at a high EST of 38.9 compared to 37.8°C.

The lower metabolic rate at an EST of 35.6°C might contribute to the higher relative liver weight found (Figure 4b). Walter and Seebacher (2007) indicated that gene expression of PGC-1α in the liver of avian embryos was increased at a relatively low incubation temperature of 35°C. Their findings suggest that PGC-1α gene expression activates gluconeogenesis, which might contribute to the higher relative liver weight found at hatch.

The higher relative intestines weight at a low EST is probably also related to the lower metabolic rate based on two reasons (Figure 4d). First, at a lower EST of 35.6°C, time until IP was on average longer compared to 36.7 (Δ=12 h), 37.8 (Δ=16 h), and 38.9°C (Δ=18 h). During the last 3 days of incubation, the weight of the intestine, as proportion of embryo weight, increased from 1% at E17 to 3.5% at hatch (Uni et al., 2003), which suggests that the largest part of intestinal growth occurred during the last part of incubation. The prolonged time until IP might have contributed to the increased intestinal weight at an EST of 35.6°C compared to all other EST treatments.

Second, at lower EST, embryos mainly use lipogenic energy, mostly derived from the yolk. Yolk is transported to the intestine through the yolk stalk from E19 onward which might be supported by the lower yolk weight found at IP at an EST of 35.6 compared to 36.7 (Δ=0.64g), 37.8 (Δ=1.14g), and 38.9°C (Δ=1.43g) (chapter 4). The lower yolk weight suggests higher yolk uptake, which might contribute to the higher relative intestine weight (Esteban et al., 1991a, 1991b). Once reaching the intestine, anti-peristaltic movements causes the yolk to move towards the gizzard (Esteban et al., 1991a, 1991b), which might contribute to a higher relative stomach weight. However, the amount of yolk uptake was

not related to the higher relative stomach weights found (Figure 4c). Therefore, the ambiguous results in relative stomach weight found at various EST could not be clarified.

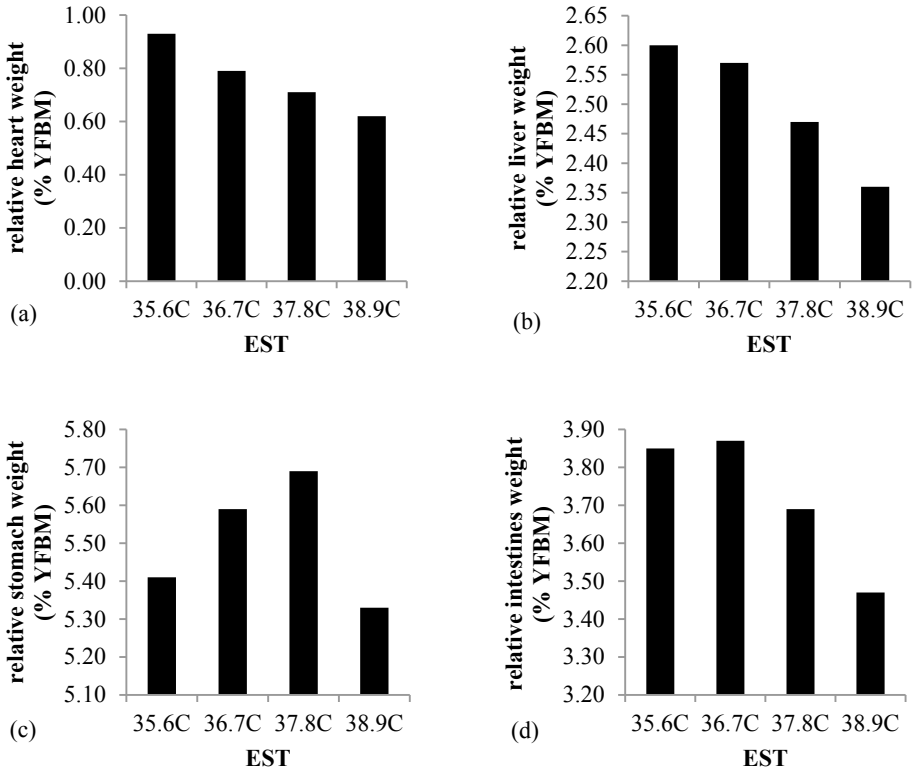


Figure 4. Average relative heart weight (a), relative liver weight (b), relative stomach weight (c), and relative intestines weight (d) at an eggshell temperature (EST) of 35.6, 36.7, 37.8, and 38.9°C (based on chapter 4).

Our studies confirm that the application of an EST above 37.8°C negatively affected embryonic organ growth and chick quality at hatch compared to a constant EST of 37.8°C. This was expressed by on average lower YFBM, higher residual yolk weight, and lower relative heart, liver, stomach, and intestines weight at hatch. In addition, incubation duration was shorter (Figure 3, 4; chapter 2, 4, and Appendix 1). An EST of 35.6 or 36.7°C, positively affected embryonic organ growth and chick quality at hatch, as an EST of 35.6 and 36.7°C resulted in higher YFBM, lower residual yolk weight, higher relative heart, liver, and intestines weight at hatch compared to a constant EST of 37.8°C (Figure 3, 4; chapter 2, 4, and Appendix 2).

Besides the effect of EST on chick development at hatch, later broiler performance during the grow-out phase is of high interest. Decuypere (1984) demonstrated that incubation temperature is important for later life performance as an incubation temperature of 33.8°C applied from E17 onward, resulted in a lower body mass at 3 days post-hatch, but an accelerated growth from the second week post-hatch onward. Leksrisompong et al. (2009) showed that an EST ≥39.5°C applied from E16 onward resulted in a lower feed intake (FI) during the first week of life, a lower body weight (BW) at day 7, and a higher mortality at day 7. BW at day 7 might be a predicting variable for broiler performance, as BW at day 7 is positively correlated with BW at day 35 (Willemsen et al., 2008). In addition, other studies showed that a high EST of 38.9°C applied from the second or third week of incubation affected subsequent broiler results, as indicated by a lower BW at day 21 (Hulet et al., 2007) and a higher mortality due to ascites at day 42 (Molenaar et al., 2011a).

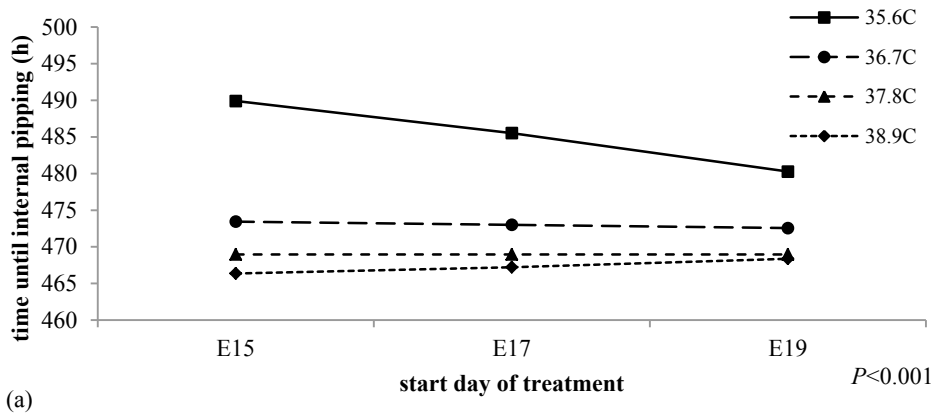
Chapter 6 shows that EST of 35.6°C resulted in a longer chick length at day of placement compared to 36.7 (Δ=0.1cm), 37.8 (Δ=0.2cm), and 38.9°C (Δ=0.4cm), a higher relative duodenum weight at day 7 compared to 36.7 (Δ=0.08g), 37.8 (Δ=0.18g), 38.9°C (Δ=0.21g), and higher relative jejunum weight at day 7 compared to 36.7 (Δ=0.08g), 37.8 (Δ=0.20g), and 38.9°C (Δ=0.21g). An EST of 36.7°C resulted in a higher BW at day 7 compared to 35.6 (Δ=9.7g), 37.8 (Δ=6.8g), and 38.9°C (Δ=4.3g), a higher weight gain between day 0 and day 7 compared to 35.6 (Δ=8.5g), 37.8 (Δ=8.3g), and 38.9°C (Δ=6.4g), a higher gain to feed ratio between day 0 and day 7 compared to 35.6 (Δ=0.074) and 38.9°C (Δ=0.053), a higher carcass weight at day 7 compared to 35.6 (Δ=4.6g), 37.8 (Δ=8.3g), and 38.9°C (Δ=5.6g) (chapter 4). This suggests that beneficial effects of a lower EST of 35.6 and 36.7°C applied during the last week of incubation found at hatch, might contribute to an enhanced development during the first week post-hatch. However, effects of EST on later performance remain to be studied.

7.3 START DAY OF TREATMENT

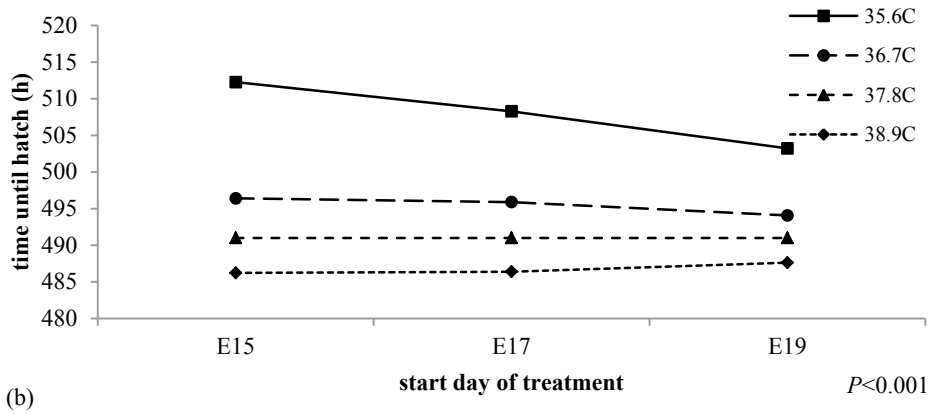
Results as described above show that EST during the last phase of incubation affect embryonic development, however it remained a question from which day onward different EST setting should be applied. Earlier studies (Table 1; Lourens et al., 2005, 2007; Joseph et al., 2006; Hulet et al., 2007; Leksrisompong et al., 2007, 2009; Molenaar et al., 2010, 2011a, 2011b) have in common that treatments were all started during the setter phase, between E7 and E19. None of these studies applied an EST treatment during only the last week of incubation or the hatcher phase. The first part of the current thesis focusses on effects of an EST treatment of 36.7, 37.8, and 38.9°C during only the hatcher phase, from

E19 onward (chapter 2 and 3), whereas the second part focusses on an EST treatment from E15, E17, or E19 onward (chapter 4, 5, and 6).

Two interactions between EST and start day of treatment were found on time until IP and time until hatch (chapter 4). Time between IP and hatch changed only marginally and therefore both figures show the same pattern (Figure 5a, b). At an EST of 35.6°C, time until IP was longer when treatment was started at E15, compared to E17 (Δ=+4h) and E19 (Δ=+10h). In addition, the effect of start day of treatment was larger at an EST of 35.6°C compared to 36.7 and 38.9°C, which was probably caused by the low metabolic rate. This implies that decreasing or increasing EST compared to 37.8°C from E15 onward will affect time until IP and consequently time until hatch.



(a)



(b)

Figure 5. Time until internal pipping (IP, a) and time until hatch (b) at an eggshell temperature (EST) of 35.6, 36.7, 37.8, and 38.9°C and start day of treatment E15, E17, and E19 (based on chapter 4).

No effect of start day of treatment was found on YFBM, residual yolk weight, and relative organ weights at hatch, which include liver, stomach, spleen, and intestines weight (chapter 2, 4). This means that increasing EST from 37.8 to 38.9°C from E15 onward will result in the same detrimental effects regardless of start day of treatment, and that decreasing EST from 37.8 to 35.6 or 36.7°C results in the same positive effects on chick development or organ growth regardless the start day of treatment (chapter 4). The most obvious reason for absent effects of start day of treatment is that EST affects these parameters only in the last phase of incubation, e.g. from E19 onward.

Results in chapter 4 describe the increase of relative organ weights. Relative liver and spleen weight were both affected by EST from E19 onward. Relative stomach weight, and YFBM, were both affected by EST from E17 onwards. Relative intestine weight, and residual yolk weight, were both affected from IP onwards. These results imply that organs respond differently to the applied EST treatments and treatment duration, which might be because of the various developmental phases of organs and sensitivity of organs to EST during the last week of incubation.

For practical implications, lowering EST from the start of the hatcher phase (E19 till hatch), will provide enough time to obtain beneficial effects at hatch, but also higher EST applied from that moment onward will result in the same negative effects on chick development and organ growth at hatch, than changing EST from an earlier day of incubation onward. Taking into account the later hatching moment of chicks exposed to a lower EST than 37.8C, it appears most beneficial to lower the EST only during the hatching phase (from E19 onward).

As mentioned earlier, an interaction between EST and start day of treatment was found on relative heart weight (chapter 4). Various studies have shown that an EST of 38.9°C applied from the second or third week of incubation onward resulted in a lower relative heart weight at hatch compared to 37.8°C (Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011a). Chapter 2 shows that even a relative short EST treatment duration up to 38 h, which means starting from E19 onward, resulted in a lower relative heart weight at 38.9°C compared to 36.7 and 37.8°C (chapter 2). A clear interaction between EST and start d of treatment was described in chapter 4, at which the difference in relative heart weight increased over time, resulting in an absolute difference of 0.39% at hatch between an EST of 35.6 and 38.9°C applied from E15 onward. When treatments were applied from E17 and E19 onward, absolute differences in relative heart weight were 0.34% and 0.21% respectively, between an EST of 35.6 and 38.9°C (Figure 6).

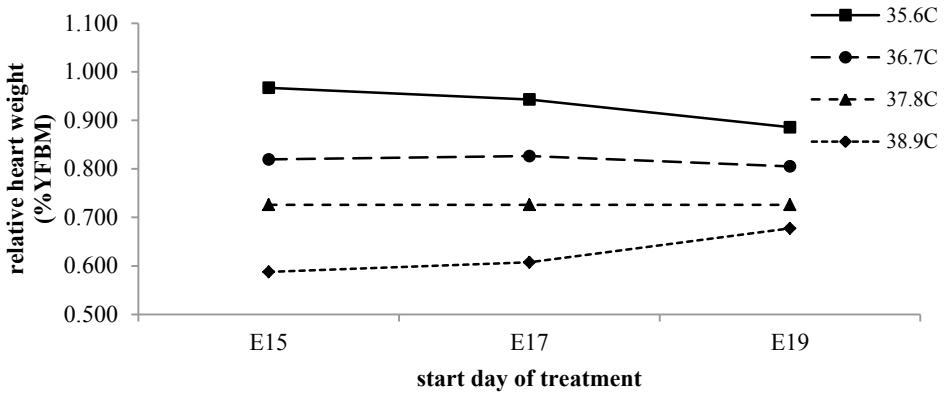


Figure 6. Relative heart weight (% of YFBM) at an eggshell temperature (EST) of 35.6, 36.7, 37.8, and 38.9°C and start day of treatment E15, E17, and E19 (based on chapter 4).

This result showed that EST determines relative heart weight at hatch, with the largest effect found at longer treatment duration. The heart remains mitotically active until day 10 after hatching (Romanoff, 1960), and heart growth rate peaks at approximately 5 to 6 days after hatching and continues growth similar to body growth (Christensen, 2009). This may suggest that an improved heart development found at hatch, which remained until day 7 (chapter 6; 35.6°C: 0.944%, 36.7°C: 0.942%, 37.8°C: 0.921%, and 38.9°C: 0.908% of YFBM), would maintain until slaughter age. The heart, as being a supply organ in the cardiovascular system might be suggested to be of major importance for the quality of the day old chick and for later life performance.

Earlier research has indicated that an EST of 38.9°C applied from E7 onward resulted a higher mortality due to ascites at 42 days of age compared to an EST of 37.8°C (Molenaar et al., 2011a), which might be due to the lower relative heart weight at hatch. A possible decreased heart development might lead to insufficient pulmonary respiratory capacity, because the heart cannot keep up with the request for O_2. The increased O_2 requirement, due to a high growth rate, leads to an increased number of red blood cells. The increase in viscosity of the blood, leads to an increased cardiac output, pulmonary hypertension, and right ventricle hypertrophy, which are predisposing factors for ascites (Decuypere et al., 2000).

The large difference in relative heart weight caused by EST and treatment duration might be the results of two different causes which are probably based on the difference in metabolic rate. The high metabolic rate at an EST of 38.9°C during the last week of incubation leads to a decrease in glucose oxidation, which negatively affects glycogen

synthesis and might even lead to protein catabolism instead of anabolism during the hatching phase. The higher uric acid levels in combination with the lower protein retention efficiency, and lower relative heart weight found in the study of Molenaar et al. (2010, 2013) suggest that protein sources from the heart might be used as an energy source at a high EST of 38.9°C. The low metabolic rate at an EST of 35.6°C during the last week of incubation sustained O_2 availability. Therefore, fat oxidation can be prolonged and glycogenic energy requirements would be less, which may imply that amino acid metabolism from the yolk and oral amnion consumption is increased (Moran, 2007). This may prevent degradation of muscle protein, which may have occurred at a high EST as suggested by Molenaar et al. (2013). Although catabolism may not be prevented towards hatching, lowering EST to 35.6 or 36.7°C will still lead to a higher relative heart weight at hatch compared to an EST of 38.9°C. At 7 days post-hatch, relative heart weight remains larger at an EST of 35.6°C, followed by 36.7, 37.8, and 38.9°C however the effect of start day of treatment remained absent.

Data of the current study suggest that an EST below 37.8°C applied from E15 onward might be beneficial for heart development, compared to lowering EST from E17 or E19 onward. However, the question remains whether this suggested improved heart development might contribute to an even more improved performance and minimization of the occurrence of ascites during later life. Relative heart weight at hatch is still higher at an EST of 35.6°C when treatment was started at E19 compared to all other treatment groups, regardless start day of EST treatment.

Starting an EST from E15, E17, or E19 might affect later life performance regardless of EST (Figure 7a, b; chapter 6). Regardless of the relative change in EST, weight gain during the first 7 days post-hatch was higher when EST changed from E19 onward compared to E15 (Δ=5.5g) and comparable to E17 (Δ=0.4g) (Figure 7a). Consequently, BW at day 7 was higher when EST changed from E19 onward compared to E15 (Δ=5.0g) and comparable to E17 (Δ=0.1g) (Figure 7a). This higher weight gain might be explained by the higher FI during the first week post-hatch when EST changed from E19 onward compared to E15 (Δ=10.4g) or E17 (Δ=2.9g) (Figure 7b).

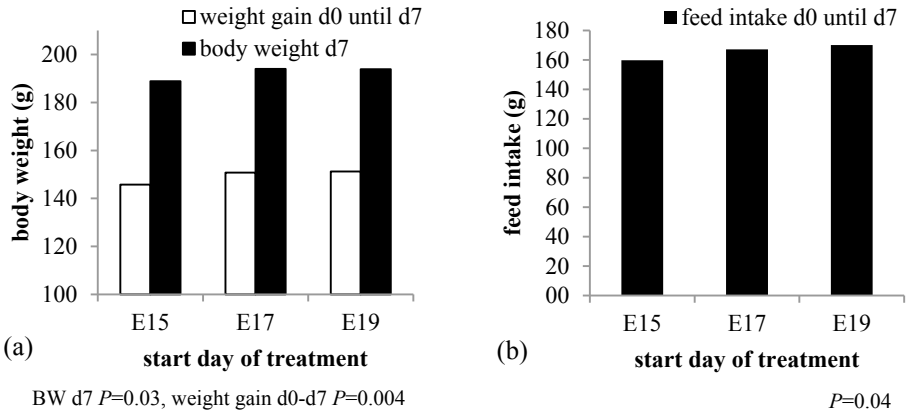


Figure 7. Weight gain between day 0 to day 7, body weight day 7 (a), and feed intake (FI) between day 0 and day 7 (b) at start day of treatment E15, E17, and E19 (based on chapter 6).

Results on chick development and organ weight at hatch, weight gain in the first week post hatch, and BW at day 7, suggest that changing EST from the hatching phase onward and not earlier, might be beneficial as changing EST from E15 or E17, results in equal chick development and relative organ weights at hatch, but lower weight gain during the first 7 days post-hatch and BW at day 7. Reasons for the start day of treatment effect on 7 days post-hatch weight gain and BW at day 7 remain unclear.

7.4 CO_2

Several studies suggest potential effects of different CO_2 concentrations applied during the second or third week of incubation on embryonic development, hatching parameters, changes in acid-base balance, physiological responses of broiler and layer embryos, and post-hatch growth of broiler chicks (Everaert et al., 2007, 2008, 2010; Buys et al., 1998). The applied CO_2 concentrations ranged between 20,000 and 40,000 ppm in the studies of Everaert et al. (2007, 2008, and 2010) and between 2,000 and 4,000 ppm in the study of Buys et al. (1998). Very high levels of CO_2 seem to result in embryonic mortality. Taylor et al. (1970) investigated hatch percentage for layer embryos at high CO_2 levels, increasing from 6,000 to 186,000 ppm from E17 onward, which resulted in 0.00% hatchability at 186,000 ppm.

Everaert et al. (2007) showed that a high CO_2 level did not negatively affect embryonic growth, hatchability, and relative growth until 7 days post-hatch. However, broiler and layer embryos exposed to high CO_2 levels showed a consistent higher blood pH, higher blood pCO_2, higher bicarbonate (HCO_3^-), and higher potassium (K^+) concentration

(Everaert et al., 2008, 2010). The higher HCO_3^- concentration, which could mainly be the result from intracellular exchange of H^+ with K^+, suggests that embryos are able to adapt to high CO_2 (Everaert et al., 2008).

Chapter 2 shows an interaction between EST and CO_2 for time until IP and time until external pipping (EP). At an EST of 37.8°C, a 5 h shorter time until IP and time until EP was found at 10,000 compared to 2,000 ppm. In addition, a 3.4 h longer time interval between EP and hatch was found at 10,000 compared to 2,000 ppm. Everaert et al. (2007) also showed a 5 h shorter time until IP and time until hatch at 40,000 ppm compared to a control CO_2 level. Both results suggest that increasing CO_2 levels might affect time interval until IP and consequently time until hatch.

Chapter 2 and 3 describe effects of 10,000 ppm compared to 2,000 ppm applied during only the hatching phase, while EST was controlled from E19 onward. In this study, the increased CO_2 level was reached by injecting CO_2 in a climate respiration chamber. A CO_2 effect was only found at 12h after hatch, at which a high CO_2 of 10,000 ppm resulted in a higher relative heart and lung weight compared to 2,000 ppm. It might be possible that a higher CO_2 level decreased the blood O_2 affinity in the tissue capillaries. In lung capillaries, the outflow of CO_2 to alveolies increases blood O_2 affinity and improves haemoglobin O_2 binding (Bohr et al., 1904). The adapted blood O_2 affinity may in turn activate segments of the lung that are normally not involved in blood O_2 transport (Decuypere et al., 2000). It might also be possible that central chemoreceptors for respiratory control react to H^+ in the blood and are therefore responsive to CO_2, which affects the respiratory magnitude (Harada et al., 1985). The heart need to increase its workload to transport blood through the body to supply organs of O_2, which might contribute to a higher relative heart and lung weight (chapter 3).

Interactions between EST and CO_2 were found at IP on chick and residual yolk weight, relative pipping muscle, stomach, intestines, and bursa weight, but were in all cases weak. At hatch, interactions between EST and CO_2 were only found on relative intestines and bursa weight, at which higher relative intestine and bursa weight was found at an EST of 36.7°C in combination with 10,000 ppm. At 12 h after hatch only an interaction between EST and CO_2 was found on relative stomach weight (chapter 2). Reasons for these differences remain unclear.

In chapter 3, at hatch, interactions between EST and CO_2 were found on blood pH and pCO_2, at which a higher blood pH was found at 2,000 ppm compared to 10,000 ppm at an EST of 37.8°C. A higher blood pCO_2 was found at 10,000 ppm compared to 2,000 ppm at an EST of 36.7°C, which might be caused by a suggested increased metabolism. However, metabolic rate is thought to be lower at an EST of 36.7°C compared to 37.8 and 38.9°C. Therefore, the higher blood pCO_2 at an EST of 36.7°C in combination with 10,000

ppm compared to 2,000 ppm might be caused by adaptive mechanisms or physiological reactions to compensate for the effect of CO_2 in the surrounding air, such as a decreased blood O_2 affinity in the tissue capillaries (Bohr et al., 1904) or by an increased respiratory magnitude as response to high CO_2 (Harada et al., 1985).

At 12h after hatch, interactions between EST and CO_2 were found on blood pCO_2, HCO_3^-, glucose, and calcium. Following the results at hatch, a higher blood pCO_2 was found at 10,000 ppm compared to 2,000 ppm at an EST of 36.7°C. The suggested decreased blood O_2 affinity in the tissue capillaries or increased respiratory magnitude might have induced gluconeogenesis, resulting in a higher blood glucose level. High CO_2 also resulted in a higher blood HCO_3^- concentration, which could mainly be the result from intracellular exchange of H^+ with K^+ to stabilize blood pH which indicates that embryos can adapt to high CO_2 as suggested by Everaert et al. (2008). The reason for the higher calcium concentration at a high CO_2 remains unknown, but it cannot be ruled out completely that increased resorption of eggshell minerals ($CaCO_3$), thereby releasing calcium and HCO_3^-, might have occurred (Everaert et al., 2008).

The presence of a CO_2 effect at hatch at an EST of 36.7°C (blood pCO_2) and 12 h after hatch (blood pCO_2, HCO_3^-, glucose, and calcium) might indicate that the embryos due to the lower metabolic rate are able to show adaptive mechanisms or physiological reactions to compensate for the effect of CO_2 on the acid-base balance in the blood (Everaert et al., 2008).

At IP, hatch, and 12 h after hatch, no effect of CO_2 was found at an EST of 38.9°C. At a high EST, physiological systems are possibly already challenged due to the higher metabolic rate of the embryos which limits the adaptive mechanisms or physiological responses of the embryo to deal with additional high CO_2 levels in the surrounding air. Therefore, results found in the studies of Everaert et al. (2007, 2008, 2010) and Buys et al. (1998), which suggest the tolerance of the embryo to high CO_2 level, might be the result of limited adaptive mechanisms or physiological responses due to the high metabolic rate at a possible high EST, as EST was not controlled.

7.5 LATER PERFORMANCE

Incubation temperature is an important factor which affects embryonic growth and development (French, 1994; Christensen et al., 1999), but also post-hatch growth and performance (Decuypere, 1984; Lourens et al., 2005; Hulet, 2007; Leksrisompong et al., 2009). Results of the current thesis suggest that an EST below 37.8°C might contribute to a higher first week broiler growth. Although FI between day 0 and day 7 did not differ between EST treatments, FI between day 0 and day 4 was higher at an EST of 35.6°C

compared to 36.7 (Δ=9.1g), 37.8 (Δ=7.1g), and 38.9°C (Δ=17.4g). However FI between day 4 and day 7 was higher at an EST of 38.9°C compared to 35.6 (Δ=17.4g), 36.7 (Δ=8.3g), and 37.8°C (Δ=10.3g) (chapter 6). After hatch, residual yolk provides energy in the chick and precedes the initiation of growth (Chamblee et al., 1992). Yolk size decreases to less than 1g at 96 h after hatching (Noy et al., 1996). As indicated in chapter 4, residual yolk weight was lower at hatch at an EST of 35.6, 36.7, and 37.8°C compared to 38.9°C. At day 7 post-hatch, residual yolk weight was not different between EST treatments, which means that residual yolk uptake during the first week post-hatch was higher at an EST of 38.9°C compared to 35.6°C (Δ=1.25g), 36.7°C (Δ=1.35g), and 37.8°C (Δ=1.15g). Therefore, the result in chapter 6 suggests that chicks incubated at a lower EST depend more on exogenous feed directly after hatch, compared to chicks that were incubated at a higher EST, which depend more on endogenous feed to obtain an equal weight gain between day 0 and day 4. Another explanation might be that yolk uptake during incubation and first week post-hatch might contribute to an improved intestinal development as suggested by Noy and Sklan (1997, 1998, and 1999) and Uni et al. (1998). This might be supported by a higher relative intestinal weight found at a lower EST compared to a higher EST at hatch (chapter 4), 12 h after hatch (chapter 3), but also at day 7 post-hatch (chapter 6). The higher gain to feed ratio at day 7 at an EST of 36.7 compared to 35.6°C (Δ=0.074) and 38.9°C (Δ=0.053), might suggest an improved intestinal development (chapter 2, 4, and 5). However, the reason for the difference in gain to feed ratio between an EST of 35.6 and 36.7°C remains unclear because a higher intestinal weight was found at an EST of 35.6°C compared to all other EST, suggesting the most optimal intestinal development. Therefore, it can be discussed whether intestinal weight is a valuable parameter to determine intestinal development.

After hatching, the chick gradually transforms from acting as poikilotherm into full blown homeotherm, which means the chick is able to regulate its own body temperature within limits. Full blown homeothermy occurs around 10 days after hatching, when thermoregulatory mechanisms have matured and body temperature has stabilized (Whittow and Tazawa, 1991; Nichelmann and Tzschentke, 2002). Optimal brooding conditions are well known to be critical for later life performance. Leksrisompong et al. (2009) showed that when chicks were incubated from E15 onward at an EST >39.4°C, cool brooding conditions of 28.5°C resulted in lower FI between day 0 and day 2 and between day 0 and day 7. Provision of hot (36.1°C) or cool (28.5°C) brooding conditions after a high EST treatment did not affect BW at day 7 (Leksrisompong et al., 2009). The effect of EST and brooding temperature on rectal temperature was investigated by Lourens et al. (2005). They showed that rectal temperature was numerically lower at day 7 when embryos were incubated at 38.9°C during the last week of incubation and placed in cold brooding

conditions in which temperature gradually decreased from 30 to 25°C from day 0 to day 7. Based on the theory of Whittow and Tazawa (1991), the thermoregulatory capacity might be used to classify day old chicks to their maturity. This might indicate that chicks incubated at a high EST have a less mature thermoregulation system than chicks incubated at a lower EST. This suggests that the capacity to maintain the correct body temperature is lower for chicks incubated at a higher EST than for chicks incubated at a lower EST.

Although effects of EST and brooding conditions on body temperature are poorly investigated, it might be possible that an EST of 35.6 or 36.7°C applied from E15 onward stimulates maturity of the thermoregulation system, which might contribute to stabilizing body temperature and in turn contributes to an improved performance.

7.6 CONCLUSIONS

Based on the findings of this thesis, the following conclusions can be drawn.

1) An EST of 38.9°C during the last week of incubation should be avoided, as it has repeatedly been show to result in lower chick quality and lower relative organ weights at hatch (chapter 2, 4). The generally accepted optimal EST of 37.8°C throughout incubation can be questioned as a lower EST of 35.6 or 36.7°C applied during the last week of incubation resulted in beneficial effects on broiler embryonic development. A beneficial effect of an EST of 36.7°C remained for the first week post hatch, expressed by higher carcass weight, higher relative organ weights, higher weight gain, higher BW, and higher gain to feed ration between day 0 and day 7, compared to 38.9°C (chapter 6).

2) No effect of start day of treatment was found on YFBM, residual yolk weight, and relative organ weights at hatch (chapter 4), which suggests that changing EST from 37.8°C to 35.6, 36.7, or 38.9°C from E15 onward results in the same negative or positive effects regardless of start day of treatment on chick development or organ growth (chapter 4). The most obvious reason for the lack of effects of start day of treatment, is that EST affects these parameters particularly during the last phase of incubation, e.g. from E19 onward.

3) A CO_2 concentration of 10,000 ppm compared to 2,000 ppm from E19 onward affects embryo physiology at an EST of 36.7, 37.8, as well as at an EST of 38.9°C. At an EST of 38.9°C, embryos seem to lack physiological reactions or adaptive mechanisms to compensate for a high CO_2 level, probably due to the high metabolic rate. Effects of high CO_2 on chick quality at hatch remain absent.

4) An effect of start day of treatment was found on first week post-hatch performance, but it remains to be investigated whether an EST below 37.8°C lead to improved later life quality characteristics.

Table 1. Time periods of EST treatments >37.8°C

Treatment based on EST		Days of incubation																				
reference	EST (°C)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	H
Wineland et al., 2000[1]	38.6-38.3																						
Molenaar et al., 2010	38.9																						
Molenaar et al., 2011b	38.9																						
Molenaar et al., 2011a	38.9																						
Lourens et al., 2005	38.9																						
Lourens et al., 2007	38.9																						
Joseph et al., 2006	38.0-39.4																						
Leksrisompong et al., 2007	39.9-40.3																						
Leksrisompong et al., 2009	39.4-40.7																						
Maatjens et al., 2016	38.9																						
Hulet et al., 2007	38.6-39.7																						
Maatjens et al., 2016	38.9																						
Maatjens et al., 2014a	38.9																						
Maatjens et al., 2016	38.9																						

[1]use of incubator temperature: setter 38.6°C-hatcher 38.3°C.

Table 2. Time periods of EST treatments <37.8°C

Treatment based on EST		Days of incubation																				
reference	EST (°C)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	H
Wineland et al., 2000[1]	37.5-36.9																						
Maatjens et al., 2016	36.7																						
Maatjens et al., 2016	35.6																						
Maatjens et al., 2016	36.7																						
Maatjens et al., 2016	35.6																						
Maatjens et al., 2014a	36.7																						
Maatjens et al., 2016	36.7																						
Maatjens et al., 2016	35.6																						

[1]use of incubator temperature: setter 38.6°C-hatcher 38.3°C.

Appendix 1. Difference between an EST >37.8°C compared to ±37.8°C on hatch time, yolk free body mass (YFBM), residual yolk weight, and relative heart weight (% YFBM) at hatch

Treatment based on EST							
Reference	EST (°C)	Time	Breed, PS age (wk)	Hatch time (h)	YFBM (g)	Residual yolk (g)	Heart (% YFBM)
Wineland et al., 2000[1]	38.6-38.3	E0 onward	52 wk				-0.251
Molenaar et al., 2010	38.9	E7 onward	Hybro, 45-51 wk	-8	-1.60	1.20	
Molenaar et al., 2011a	38.9	E7 onward	Ross x Cobb 500, 33 wk				
Molenaar et al., 2011b	38.9	E7 onward	Hybro, 45-51 wk	-8	-1.20		-0.12
Lourens et al., 2005 (exp. 1)	38.9	E7 onward	Hybro G, 28 wk		-0.40	0.00	
Lourens et al., 2005 (exp. 2)	38.9	E7 onward	Hybro G, 60 wk		-0.30	-0.10	
Lourens et al., 2007	38.9	E9 onward	Hybro G^{+}	-0.6d	-1.60	0.00	-0.20
Joseph et al., 2006	38.0-39.4	E10 onward	Ross 308, 31 wk		-2.50	0.20	
Leksrisompong et al., 2007 (exp. 2)	40.0	E14 onward	Ross 308, 65 wk		-2.69	-0.41	-0.14
Leksrisompong et al., 2007 (exp. 3)	40.3	E14 onward	Ross 308, 69 wk		-5.21	2.91	-0.25
Leksrisompong et al., 2007 (exp. 4)	39.9	E14 onward	Ross 308, 48 wk		-1.64	0.54	-0.14
Leksrisompong et al., 2009 (exp. 1)	39.4	E15 onward	Ross 308, 48 wk				
Leksrisompong et al., 2009 (exp. 2)	40.7	E14 onward	Ross 308, 52 wk				
Maatjens et al., 2016	38.9	E15 onward	Ross 308, 43 wk	-5	0.02	1.41	-0.14
Hulet et al., 2007	38.6-39.7	E16 onward	Cobb, 29 and 57 wk				
Maatjens et al., 2016	38.9	E17 onward	Ross 308, 43 wk	-5	0.61	0.96	-0.12
Maatjens et al., 2014a	38.9	E19 onward	Ross 308, 41-45 wk	-2	-0.24	-0.08	-0.08
Maatjens et al., 2016	38.9	E19 onward	Ross 308, 43 wk	-3	0.49	1.07	-0.05

[1]reference temperature: setter 37.5°C-hatcher 36.9°C.

Appendix 2. Difference between an EST <37.8°C compared to ±37.8°C on hatch time, yolk free body mass (YFBM), residual yolk weight, and relative heart weight (% of YFBM) at hatch

Treatment based on EST							
Reference	EST (°C)	Time	Breed, PS age (wk)	Hatch time (h)	YFBM (g)	Residual yolk (g)	Heart (% YFBM)
Wineland et al., 2000[1]	37.5-36.9	E0 onward	52 wk				0.251
Maatjens et al., 2016	35.6	E15 onward	Ross 308, 43 wk	21	1.52	-0.27	0.24
Maatjens et al., 2016	36.7	E15 onward	Ross 308, 43 wk	5	1.22	0.04	0.09
Maatjens et al., 2016	35.6	E17 onward	Ross 308, 43 wk	17	1.70	-0.04	0.21
Maatjens et al., 2016	36.7	E17 onward	Ross 308, 43 wk	5	1.55	-0.55	0.10
Maatjens et al., 2014a	36.7	E19 onward	Ross 308, 41-45 wk	2	0.41	-0.18	0.03
Maatjens et al., 2016	35.6	E19 onward	Ross 308, 43 wk	12	0.70	0.06	0.16
Maatjens et al., 2016	36.7	E19 onward	Ross 308, 43 wk	3	1.15	-0.04	0.08

[1]Reference temperature: setter 38.6°C-hatcher 38.3°C.

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Summary

SUMMARY

During incubation, temperature drives chicken embryonic metabolism, development, and growth. Incubation conditions need to be adjusted to meet embryonic requirements to obtain optimal chick quality and hatchability. To obtain optimal chick quality, embryo temperature, rather than incubator temperature, needs to be controlled. Eggshell temperature (EST) can be used as a non-invasive method to determine embryo temperature.

Several studies from 2000 until 2015 have applied an EST or incubator temperature treatment during a specific time period during incubation. In these studies, eggs were incubated at a constant EST of 37.8°C or a set incubator temperature of 37.5°C, until start of the treatment. To mimic current practical situations, the majority of earlier research focussed on an EST of 38.9°C or higher, starting from the second or third week of incubation and maintained the EST treatment until hatch. A high EST of 38.9°C in the second or third week of incubation resulted in higher third week embryonic mortality, lower hatchability, and lower chick quality at hatch, expressed as a shorter chick length and lower yolk free body mass (YFBM) compared to a constant EST of 37.8°C throughout incubation. These negative effects of a high EST in the second part of incubation might be explained by, first, the high metabolic rate of the embryos due to the high incubation temperature and, second, the limited availability of oxygen (O_2), due to relatively low eggshell conductance. Because of these two factors, it appears that at a high EST the balance between metabolic rate and O_2 availability is disturbed, which may result in higher embryonic mortality and impaired embryonic development. Lowering EST might restore the balance between metabolic rate and O_2 availability and may postpone or even prevent the embryos from experiencing O_2 shortage during a plateau phase halfway during incubation.

Besides temperature, the carbon dioxide (CO_2) concentration during incubation also might affect embryonic development. However, effects of high CO_2 concentrations during incubation described in literature might not be related to solely CO_2 because the requested CO_2 concentration was reached by decreasing the ventilation rate. When ventilation rate is decreased, heat transfer from the eggs is decreased as well, which might affect embryo temperature.

Based on the potential effects of (lower) EST during the last week of incubation and of CO_2 during only the hatching phase, as described above, the following three aims are derived for this thesis: 1, to investigate effects of EST during the last phase of the incubation process, with special attention for EST below the general accepted optimal EST of 37.8°C, 2, to examine from which day of the incubation process onward EST should be

changed from 37.8°C, and 3, to investigate whether CO_2 concentrations are interacting with EST during the hatcher phase.

Chapter 2 and 3 are based on the first experiment in which 600 Ross 308 eggs were individually monitored from E19 until hatch. During that experiment, effects of an EST of 36.7, 37.8, and 38.9°C in combination with a CO_2 concentration of 2,000 or 10,000 ppm were investigated.

It was concluded that time until hatch was longer at an EST of 36.7°C compared to 37.8 and 38.9°C, which might be caused by the lower metabolic rate of embryos exposed to an EST of 36.7°C. Although time until hatch was prolonged at an EST of 36.7°C, hatch of fertile (HOF) was not affected (chapter 2).

A high EST of 38.9°C resulted in a lower relative heart weight at internal pipping (IP), hatch, and 12h after hatch. This implies that a higher EST of 38.9°C compared to an EST of 36.7 or 37.8°C, even applied for a relative short treatment duration up to 38 h, starting from day of incubation (E) 19 onward, resulted in a lower relative heart weight (chapter 2). For chick quality at hatch, effects of EST were limited, as only a lower yolk free body mass (YFBM) was found at an EST of 38.9°C compared to 36.7°C (chapter 2).

Effects of CO_2 on chick organ development and chick quality at hatch were marginal. Effects of CO_2 were mainly found before IP on chick weight, residual yolk weight, relative pipping muscle, stomach, intestines, and bursa weight, but effects mostly disappeared. At hatch, only an effect of CO_2 on relative stomach weight was found and at 12h after hatch on relative intestines and bursa weight (chapter 2).

A high EST of 38.9°C affected chick physiology, which was demonstrated by the lower hepatic glycogen levels at hatch and lower lactate levels at 12h after hatch compared to an EST of 37.8°C. This was probably caused by the increased oxidation of carbohydrates due to the higher metabolic rate. A high CO_2 level of 10,000 ppm resulted in lower blood pH and hepatic glycogen at IP compared to a low CO_2 level of 2,000 ppm. Interactions between EST and CO_2 were found for several physiological variables at an EST of 36.7 and 37.8°C, but remained absent at an EST of 38.9°C (chapter 3). At a low EST of 36.7°C, metabolic rate was lower compared to a high EST of 38.9°C, which suggests that embryos were able to show adaptive mechanisms or physiological reactions on the acid-base balance in the blood to compensate for the effect of CO_2. However, no effect of CO_2 was found at a high EST of 38.9°C, which might indicate that physiological systems are already challenged due to the higher metabolic rate, and consequently, embryos cannot show their adaptive mechanisms anymore.

Chapter 4, 5, and 6 are based on the second experiment in which 2,870 Ross 308 eggs were individually monitored from E15 until hatch. During that experiment, effects of

an EST of 35.6, 36.7, 37.8, and 38.9°C starting from E15, E17, or E19 onward were investigated. In addition, 900 chicks were placed in a broiler grow-out facility to investigate effects on first week post-hatch development and growth performance.

It was concluded that time until hatch was longer at an EST of 35.6°C, followed by 36.7, 37.8, and 38.9°C. The effect of EST on total incubation duration was mainly caused by the effect of EST on time until IP. EST treatment did not substantially affect time between IP and hatch, as this time period varied only 2 h, whereas time until IP varied 24 h between EST treatments (chapter 4). A possible explanation for the extended time until IP might be the lower metabolic rate at an EST below 37.8°C. Following the results of chapter 2, hatchability of fertile eggs was not affected by the extended incubation duration at an EST of 35.6°C (chapter 4).

At a lower EST, metabolic rate is reduced which reduces O_2 limitation and subsequently ensures that fatty acid oxidation can be maintained for a longer period. Consequently, energy produced can be used for growth and development, resulting in a higher YFBM at an EST of 35.6 and 36.7°C compared to 37.8 and 38.9°C. The higher YFBM at a lower EST may suggest that protein from yolk and albumen is used for growth and development which was supported by the lower residual yolk weight at an EST of 35.6, 36.7, and 37.8°C compared to 38.9°C. This suggests that protein sources from yolk and albumen are not used as an alternative energy source to compensate for the limited glycogen stores at an EST of 38.9°C (chapter 5). In addition, yolk uptake by the yolk stalk from E19 onward towards the intestines might contribute to higher relative intestinal weight found at an EST of 35.6, 36.7, and 37.8°C compared to 38.9°C and might even contribute to improved intestinal development.

A clear interaction between EST and start day of treatment was found for relative heart weight (chapter 4). Differences in relative heart weight between EST treatments increased over time, resulting in an absolute difference of 0.39% at hatch between an EST of 35.6 and 38.9°C applied from E15 onward. At 7 days post hatch, relative heart weight remains larger at an EST of 35.6°C followed by 36.7, 37.8, and 38.9°C, but the effect of start day of treatment was absent. The question remains whether this higher relative heart weight at low EST might contribute to an improved performance and minimization of the occurrence of ascites during later life.

Chapter 6 shows that an EST of 36.7°C resulted in a higher body weight (BW) and higher carcass weight at day 7 compared to all other EST, and a higher weight gain and gain to feed ratio between day 0 and day 7 compared to 35.6 and 38.9°C. This suggests that beneficial effects of a lower EST of 36.7°C applied during the last week of incubation found at hatch, might contribute to an enhanced development during the first week post-

hatch. However, effects of EST on later performance until slaughter age remain to be studied.

For the results found at hatch, no effect of start day of treatment was indicated. The most obvious reason for absent effects of start day of treatment is that EST affects the determined parameters only in the last phase of incubation, e.g. from E19 onward. Taking into account the later hatching moment of chicks exposed to a lower EST than 37.8°C, it appears most beneficial to lower the EST only during the hatching phase (from E19 onward).

Starting an EST treatment from E15, E17, or E19 affects first week post-hatch performance (chapter 6). Weight gain during the first 7 days post-hatch and BW at day 7 were higher when EST changed from E19 onward compared to a change in EST from E15 onward.

CONCLUSIONS

Results of this thesis show that an EST below 37.8°C is beneficial for embryo development and growth performance during the first week post-hatch. In addition, start day of treatment hardly affects chick quality and organ weights at hatch, which implies that effects of EST mostly occur during the hatching phase, e.g. from E19 onward. It remains to be investigated whether an EST below 37.8°C leads to improved later life performance.

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Liefs, Conny

Curriculum Vitae

CURRICULUM VITAE

Conny Cornelia Maria Maatjens was born on January 18, 1979 in Oudenbosch (Noord-Brabant) where she also spent her childhood. In 1998 she graduated from Thomas More College, after which she started the study Animal Husbandry and Health Care at Hogeschool Delft. In 2002 she graduated and started her study at Wageningen University, where she obtained her MSc degree in Animal Sciences on Animal Breeding and Genetics and Adaptation Physiology. She investigated the effect of inbreeding depression on milk production and fertility traits, in cooperation with CRV holding B.V., and the use of rumination characteristics as predictor for metabolic disorders, in cooperation with Lely Industries B.V., both in Holstein dairy cattle. After her graduation in 2004, she started to work for Hybro B.V. as an operational geneticist, where she kept the breeding program up to date by being in close contact with the pure line breeding farm. In 2007, she started to work at the Research and Development department at HatchTech B.V., where she was involved in developing, improving, and implementing the current and new incubation techniques and products related to incubation. From 2009 to 2011, she was responsible for the technical implementation of the HatchBrood system. A system where chicks spend their first 4 days of life, the early brooding period, before they are transported to the farm. In January 2011 she started her PhD project at Wageningen University in collaboration with HatchTech B.V. in Veenendaal, in which she investigated the effect of temperature and CO_2 during the last week of incubation on embryonic development, physiology, chick quality, and first week broiler performance. The results of this project are written in this thesis. After finishing her PhD project, Conny will start working as Manager Research and Development at Wimex Agrarprodukte Import und Export GmbH, Regenstauf, Germany.

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TRAINING AND SUPERVISION PLAN OF GRADUATE SCHOOL WIAS

Description	Year
Education and Training	
The Basic Package (3.0 ECTS)	
WIAS Introduction Course	2010
Course on philosophy of science and/or ethics	2012
Scientific Exposure	
International conferences (6.0 ECTS)	
5th International Poultry Conference, Taba, Egypt	2009
IFRG meeting, Ede, the Netherlands	2011
IFRG meeting and PDP workshop, Pisa, Italy	2012
FACTA congress, Campinas, SP, Brazil	2013
XIVth European Poultry conference, Stavanger, Norway	2014
IFRG meeting and PDP workshop, Berlin, Germany	2015
Seminars and workshops (3.3 ECTS)	
The embryonic life of chickens: factors that influence development Wageningen, the Netherlands	2010
WIAS Science Day, Wageningen, the Netherlands	2011
WIAS Science Day, Wageningen, the Netherlands	2012
WIAS Science Day, Wageningen, the Netherlands	2013
38th Animal Nutrition and Research Forum, Roeselare, Belgium	2013
WIAS Science Day, Wageningen, the Netherlands	2014
WIAS Science Day, Wageningen, the Netherlands	2015
The 5th Workshop on Fundamental Physiology and Perinatal Development in Poultry (PDP), Ede, the Netherlands	2011
Presentations (8.0 ECTS)	
Incubation, chick quality, and later performance, 5th IPC, Taba, Egypt	2009
Effect of body temperature and relative humidity in the hatcher on body weight loss and chick length of the broiler chick, IFRG, Ede, the Netherlands, poster presentation	2011

Effects of temperature and CO_2 during the last 4 days of incubation on chick quality,
IFRG, Pisa, Italy 2012

Effect of diet composition and partical size on the performance of chicks hatched from small eggs,
38th Animal Nutrition and Research forum, Roeselare, Belgium 2013

Incubation process from egg to chick, hatching, and aftercare,
FACTA congress, Campinas, SP, Brazil 2013

Effects of temperature and CO_2 during the last 3 days of incubation on chick quality,
WIAS Science Day, Wageningen, the Netherlands 2014

Effects of temperature and CO_2 during the hatching phase on embryo physiology,
XIVth European Poultry conference, Stavanger, Norway 2014

Effect of temperature during the last week of incubation on embryonic organ development and chick quality,
IFRG and PDP, Berlin, Germany 2015

In-Depth Studies

Disciplinary and interdisciplinary courses (2.9 ECTS)

Epigenesis and epigenetics - Physiological consequences of perinatal programming,
Wageningen, the Netherlands 2008

Orientation on Mathematical Modelling in Biology,
Wageningen, the Netherlands 2011

Incubation biology and management,
Wageningen, the Netherlands 2014

Advanced statistics courses (3.0 ECTS)

Statistics for the Life Sciences, Wageningen, the Netherlands 2012

Design of Experiments, Wageningen, the Netherlands 2012

PhD students' discussion groups (0.7 ECTS)

Animal Welfare Discussion group 2011

Chicken Discussion group 2015

Statutory Courses (4.0 ECTS)	
Use of Laboratory Animals	2008
Professional Skills Support Courses (4.8 ECTS)	
Culture and change programme,	
Den Dolder, the Netherlands	2007
Course Techniques for Scientific Writing,	
Wageningen, the Netherlands	2012
Course Techniques for Writing and Presenting a Scientific paper,	
Wageningen, the Netherlands	2012
Project and Time Management,	
Wageningen, the Netherlands	2012
Research Skills Training (6.0 ECTS)	
Preparing own PhD research proposal	2011
Didactic Skills Training	
Lecturing (0.2 ECTS)	
Presenting Adaptation Physiology college	2012
Supervising theses (4.0 ECTS)	
Supervising 1 MSc thesis	2012
Supervising 2 BSc thesis	2015
Tutorship (3.7 ECTS)	
Supervising Adaptation Physiology project group	2012
Supervising Adaptation Physiology project group	2013
Supervising Adaptation Physiology project group	2014
Supervising Adaptation Physiology project group	2015
Education and Training Total	**50 ECTS[1]**

[1]1 ECTS credit equals a study load of approximately 28 hours

Colophon

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Providing superior chick quality

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