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Interaction between eggshell temperature and carbon dioxide concentration after day 8 of incubation on broiler chicken embryo development

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ABSTRACT

Carbon dioxide (**CO_2**) is considered to be an important factor during incubation of eggs. Effects attributed to higher CO_2 concentrations during experiment might be due to confounding effects of other environmental conditions, such as incubation temperature. To disentangle effects of eggshell temperature (**EST**) and CO_2 concentration, an experiment was conducted. A total of 630 Cobb 500 hatching eggs from 37 to 45 wk commercial breeder flocks were collected and incubated according to treatments. The experiment was setup as a complete randomized 2 × 3 factorial design, resulting in 6 treatments. From day 8 of incubation onward, broiler eggs were exposed to one of two EST (37.8 or 38.9 °C) and one of three CO_2 concentrations (0.1, 0.4 or 0.8%). Eggs were incubated in climate-respiration chambers and metabolic heat production was determined continuously. At day 18 of incubation and at 6 h after hatching, embryo and chicken quality were determined by evaluation of organ weights, navel condition, blood metabolites and hepatic glycogen. Hatching time and chicken length at 6 h after hatching showed an interaction between EST and CO_2 concentration (both $P = 0.001$). Furthermore, no effect of CO_2 concentration was found on embryo development or chicken quality. Metabolic heat production between day 8 and 18 of incubation was not affected by either EST or CO_2. At day 18 of incubation, an EST of 38.9 °C resulted in a higher egg weight loss, longer embryos, higher yolk free body mass (**YFBM**) and lower heart weight than an EST of 37.8 °C (all $P < 0.008$). At 6 h after hatching, an EST of 38.9 °C resulted in a higher residual yolk weight and lower YFBM, liver weight and heart weight than an EST of 37.8 °C (all $P < 0.003$). Lactate, uric acid and hepatic glycogen were not affected by EST at either day 18 of incubation or at hatch. Glucose was not affected by EST at day 18 of incubation, but at hatch, it was higher at an EST of 37.8 °C than at an EST of 38.9 °C ($P = 0.02$). It can be concluded that effects of CO_2 concentration (at concentrations ≤0.8%) on embryonic development and chicken quality appear to be limited when EST is maintained at a constant level. Moreover, a higher EST from day 8 of incubation onward appears to negatively affect chicken quality at hatch.

Implications

Effects of carbon dioxide concentrations ≤0.8% after day 8 of incubation, without potential confounding effects of changes in eggshell temperature, appear to be marginal. However, a high incubation temperature during this period does have a significant negative effect on hatchling quality. These findings imply that: (1) in commercial hatcheries, one should focus on eggshell temperature rather than CO_2 concentration, as long as the latter remains below 0.8% and (2) in studies related to CO_2 concentrations, eggshell temperature and possibly relative humidity and oxygen concentration should be maintained at a constant level to prevent confounding factors, possibly affecting the results.

Introduction

It has been suggested that carbon dioxide (**CO_2**) is an important factor for embryo development and chicken quality during artificial incubation (Tona et al., 2007; Willemsen et al., 2008).

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CO_2 concentrations seem to be especially important in early (first week) and late (last week) incubation. CO_2 concentrations >1% during the first 10 days of incubation have been shown to stimulate embryonic development, induce earlier hatching moment and increase hatchability (De Smit et al., 2006; Tona et al., 2007; Willemsen et al., 2008). These findings might be due to the stimulatory effect of CO_2 on vascularization of the chorio-allantoic membrane (Verhoelst et al., 2011; Fernandes et al., 2017) and thereby facilitating a higher oxygen uptake capacity. CO_2 concentrations >1% in the last week of incubation have been found to result in an earlier start of the hatching process (Buys et al., 1998; Tona et al., 2013 and 2015) and to increase the resistance against ascites in later life (Buys et al., 1998; Everaert et al., 2010, Everaert et al., 2012). However, no effect on blood glucose and lactate was found (Tong et al., 2015).

Studies about CO_2 concentrations vary considerably in experimental design. Firstly, higher CO_2 concentrations can be obtained in two different ways, namely by addition of CO_2 in the incubator (Bruggeman et al., 2007; Maatjens et al., 2014; Burggren et al., 2015) or by reduction of the ventilation rate in the incubator (Buys et al., 1998; Tong et al., 2015; Özlü et al., 2019). Secondly, the used CO_2 concentrations that are considered as high vary considerably among studies, ranging from 0.4 to 0.8% (Buys et al., 1998; Özlü et al., 2019) to 4 to 5% (Mueller et al., 2013) to 6 to 8% (Taylor and Kreutziger, 1966 and 1969; Taylor et al., 1971) to even 10% or higher (Barott, 1937; Burggren et al., 2015).

CO_2 addition to the incubator or reduced ventilation rate to increase the CO_2 concentration in the incubator can result in side effects, such as reduced O_2 concentration or increased incubation temperature or relative humidity in the incubator (Maatjens et al., 2014; Boleli et al., 2016). However, these potential confounding effects of CO_2 increase in the incubator are hardly investigated and are thus largely unknown. Because incubation temperature is the most important factor affecting embryonic development and hatchling quality (Wilson, 1991), in the current study, the interaction between CO_2 concentration and incubation temperature will be investigated. Therefore, the aim of this study was to investigate effects of CO_2 concentration after day 8 of incubation in interaction with eggshell temperature on embryonic development and hatchling quality. Other potential confounding factors of higher CO_2 concentration were ruled out by maintaining O_2 concentration and relative humidity constant.

Material and methods

Experimental design

The experiment was setup as a 2 × 3 factorial design with eggshell temperature (**EST**) and CO_2 concentration as main factors. The experiment was executed in six consecutive batches. Two EST (37.8 or 38.9 °C) and 3 CO_2 concentrations (0.1, 0.4 or 0.8%) were applied from day 8 of incubation onward. Within each batch, both EST were applied, but CO_2 concentration varied among batches. Each EST × CO_2 combination was tested twice. CO_2 concentrations were based on the following findings: 0.1% CO_2 is a common concentration in a multistage incubator, 0.8% CO_2 can be obtained in a single stage incubator at the end of incubation and 0.4% CO_2 is intermediate. During incubation, heat production was determined continuously and embryo and hatchling quality were determined.

Eggs and incubation

In total, 630 Cobb 500 eggs (105 eggs per batch) were obtained from commercial broiler breeder farms. First grade hatching eggs were selected with an egg weight between 62.0 and 65.0 g from broiler breeder flocks varying in age between 37 and 45 weeks.

From the start of incubation till day 8 of incubation (**E8**), all eggs within a batch were incubated in one incubator (HatchTech, Veenendaal, the Netherlands) with a maximum capacity of 1 408 eggs, at an EST of 37.8 °C. EST was controlled and monitored by four sensors (NTC Thermistors, type DC 95, Thermometrics, Somerset, UK), which were attached to the equator of four individual eggs, using heat conduction paste (Dow Corning 340 Heat Sink Compound, Dow Corning GmbH, Wiesbaden, Germany) and a small piece of tape (2 × 2 cm). EST was controlled by the median temperature of the four sensors. Relative humidity was maintained between 50 and 60% throughout incubation.

At E8, 90 fertile eggs were selected per batch. These eggs were randomly divided over two small climate-respiration chambers (**CRCs**; Lourens et al., 2006), which were used as incubator. For further details about the use of CRC as incubator, see Heetkamp et al. (2015) and Van den Brand et al. (2015). Eggs in one CRC were subjected to an EST of 37.8 °C from E8 till hatching, whereas eggs in the other CRC were subjected to an EST of 38.9 °C. EST was controlled and monitored by sensors (Pt-100, Sensor Data BV, Rijswijk, the Netherlands) attached to the equator of four individual eggs, as described above. Based on the median of the four sensors, CRC setpoint temperature was adjusted automatically when necessary to maintain the set EST.

Within each batch, one CO_2 concentration setpoint was used for both CRCs. CO_2 concentration in the CRC was organized as follows. First, pure CO_2 (>99.95%) was injected in a buffer (400 L), using a Mass Flow Controller (Bronkhorst, Veenendaal, the Netherlands, model F-201CV-500-RBD-33-Z, adjustment range 0 to 200 mL/min) at a rate taking internal production into account to obtain a CO_2 concentration of 0.1, 0.4 or 0.8% on average inside both CRCs, depending on setpoint per batch. In this buffer, air was continuously mixed. From this buffer, air samples were collected each 9 min and analyzed on O_2 and CO_2 concentration (Heetkamp et al., 2015). From this buffer, air was injected into both CRCs via a gas volume meter (Schlumberger/Itron G1.6, Liberty Lake, USA). Air samples of both CRCs were also collected each 9 min and analyzed on O_2 and CO_2 concentrations. Based on these concentrations, the injection volume from the buffer was automatically adjusted to maintain the CO_2 setpoint.

Measurements

Individual egg weight (n = 630) was determined before incubation (E0), at E8 and at E18 of incubation. Egg weight loss was calculated for the period between E0 and E18 and expressed as a percentage of egg weight at E0. At E8, E14 and E18, eggs were candled and infertile eggs and eggs containing a dead embryo were removed. Removed eggs were opened to determine fertility or moment of embryonic death, using the method described by Lourens et al. (2006).

At E18, 15 eggs per batch per CRC were randomly selected for blood sampling and embryonic development measurements. Eggs were opened at the blunt end and blood was collected from the jugular vein, using a 30-Gauge needle and a syringe of 1 mL that was flushed with heparin before collection. Blood was put in Eppendorfs and centrifuged for 3 min at 1 000g and plasma was stored at −20 °C until analyses. Weight and length of the embryo (Hill, 2001) were determined, where after the embryo was decapitated. The liver, heart and residual yolk were removed and weighed. The liver was immediately frozen in liquid nitrogen and then stored at −80 °C until further analysis of hepatic glycogen.

From 467 h of incubation onward, the number of hatchlings emerged from the eggshell was checked every 6 h. When chickens were hatched, they were removed from the CRC 6 h later for

measurements (n = 13–15 per batch per treatment). Weight and length (Hill, 2001) of the chicken were determined and navel condition was scored as described by Molenaar et al. (2010a), using a scale of 0 (closed and clean navel area), 1 (navel containing a black button up to 2 mm or a black string), or 2 (navel containing a black button exceeding 2 mm or an open navel area). Thereafter, the chicken was killed by cervical dislocation, followed by decapitation. Blood was collected in a 4-mL blood tube containing 10 mg of sodium fluoride and 8 mg of potassium oxalate (BD Vacutainer, Franklin Lakes, NJ). An extra droplet of 10% heparin was added and mixed into the tube before sampling to prevent coagulation. Blood was centrifuged at 1 500*g* for 10 min at room temperature and plasma was stored at −20 °C till further analyses. The liver, heart and residual yolk were weighed and the liver was immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Yolk free body mass (**YFBM**) was calculated as BW minus residual yolk weight. At the end of incubation, all unhatched eggs were opened to determine the moment of embryonic death, using the method described by Lourens et al. (2006).

Heat production was determined from E8 onward, using indirect calorimetry (Heetkamp et al., 2015; Van den Brand et al., 2015). Oxygen consumption and carbon dioxide production were determined at 9-min intervals and heat production was calculated per day, using the formula described by Romijn and Lokhorst (1961). Heat production was corrected for embryonic mortality and expressed as mW/egg.

Blood and glycogen analyses

Hepatic glycogen levels at E18 and 6 h after hatch were analyzed, using the procedure described by Molenaar et al. (2010a). All procedures during the analysis were executed on ice. Plasma glucose, lactate and uric acid concentrations were determined with commercially available enzymatic photometric kits (DiaSys Diagnostic Systems International, Holzheim, Germany).

Statistical analyses

All data were analyzed, using SAS (version 9.4, Cary, NC, USA). Data were checked for normality of variance for both means and residuals, using the Shapiro Wilk test. All continuous data showed a normal distribution. Continuous data were analyzed with the Mixed procedure, using the model: $Y_{ijk} = \mu + EST_i + CO_{2j} + EST \times CO_{2ij} + e_{ijk}$ [model 1], where Y = the dependent variable, μ = the overall mean, EST = effect of eggshell temperature (i = 37.8 or 38.9 °C), CO_2 = effect of CO_2 concentration (j = 0.1, 0.4 or 0.8%), EST × CO_2 =-interaction between eggshell temperature and CO_2 concentration, e = residual error. CO_2 nested within batch was used as a random effect. To test relationships between hatching time at one side and chicken length and YFBM at the other side, hatching time was added as a covariable to model 1. Metabolic heat production was analyzed per day (E8 to E18) with the Mixed procedure, using model 1. Navel score was analyzed with the Glimmix procedure, using model 1.

Except for the analyses of metabolic heat production, the egg, embryo or chicken was used as experimental unit. For metabolic heat production, the CRC was used as the experimental unit. Data are expressed as LSmeans ± SEM and multiple comparisons between treatments were corrected for Bonferroni. Differences were considered significant at $P \leq 0.05$.

Results

No interaction between EST and CO2 was found on egg weight at days 0, 8 and 18, egg weight loss and embryo characteristics at E18 (all $P > 0.05$; data not shown). Egg weight at day 0, 8 and 18 of incubation was not affected by EST or CO_2 (Table 1). Egg weight loss between E0 and E18 was higher at an EST of 38.9 °C than at an EST of 37.8 °C (Δ = 0.7%; P = 0.005). At E18, no effect of CO_2 was found on embryo characteristics. At E18, a high EST of 38.9 °C from E8 onward resulted in longer chickens (Δ = 0.2 cm; $P < 0.001$), higher YFBM (Δ = 0.57 g; P = 0.008) and lower heart weight (Δ = 0.14% of YFBM; $P < 0.001$) than an EST of 37.8 °C.

An interaction was found between EST and CO_2 concentration for hatching time (P = 0.001; Table 2). At an EST of 37.8 °C, no effect of CO_2 concentration was found on hatching time (492, 493 and 492 h for 0.1, 0.4 and 0.8% CO_2, respectively), but at an EST of 38.9 °C, chickens incubated at a CO_2 concentration of 0.1 and 0.8% hatched earlier than chickens incubated at 0.4% (480, 491 and 484 h for 0.1, 0.4 and 0.8% CO_2, respectively). The latter group of chickens hatched at the same incubation duration than the chickens incubated at an EST of 37.8 °C. An interaction between EST and CO_2 concentration was also found for chicken length at 6 h after hatching (P = 0.001). At an EST of 37.8 °C, no effect of CO_2 concentration was found on chicken length (19.5, 19.5 and 19.3 cm for 0.1, 0.4 and 0.8% CO_2, respectively), but at an EST of 38.9 °C, a CO_2 concentration of 0.1% resulted in shorter chickens than a CO_2 concentration of 0.4 and 0.8% (19.1, 19.8 and 19.7 cm for 0.1, 0.4 and 03.8% CO_2, respectively). No other interactions between EST and CO_2 (data not shown), nor main effects of CO_2 concentration were found on chicken characteristics at 6 h after hatching. A high EST of 38.9 °C from E8 onward resulted in a larger RY (Δ = 0.67 g; $P < 0.001$), lower YFBM (Δ = 0.70 g; $P < 0.001$), lower relative liver weight (Δ = 0.11% of YFBM; P = 0.003) and lower relative heart weight (Δ = 0.17% of YFBM; $P < 0.001$) than an EST of 37.8 °C. Chicken weight and navel condition at 6 h after hatching were not affected by EST or CO_2 concentration.

Plasma glucose, lactate and uric acid concentrations at day 18 of incubation and plasma lactate and uric acid concentrations at 6 h after hatching were not affected by the interaction between EST and CO_2 concentration from E8 onward (data not shown) nor by EST or CO_2 concentration (Table 3). Plasma glucose concentration at 6 h after hatching was higher at an EST of 37.8 °C than at an EST of 38.9 °C (Δ = 0.26 mmol/L; P = 0.02) and was also higher at a CO_2 concentration of 0.8% than at a CO_2 concentration of 0.1% (Δ = 1.00 mmol/L; P = 0.02), with a CO_2 concentration of 0.4% in between. Hepatic glycogen at both sampling moments was not affected by EST or CO_2 concentration. Heat production between E8 and E18 was not affected by the interaction between EST and CO_2 ($P > 0.37$), EST ($P > 0.27$) or CO_2 ($P > 0.32$) during any of the incubation days (data not shown).

Discussion

The aim of the current experiment was to investigate whether EST and CO_2 concentration from E8 onward are interacting in their effect on embryonic and hatchling characteristics. To our knowledge, this is the first study that has investigated the interaction between EST and CO_2 concentrations, without potential confounding effects of relative humidity, ventilation rate or oxygen concentration. For this study, a maximum CO_2 concentration of 0.8% was applied, because that level can be obtained during the last phase of artificial incubation.

Interaction effects between EST and CO_2 were only found for hatching moment and chicken length at 6 h after hatching. As consistently found in other studies, eggs incubated at an EST of 38.9 °C hatched on average earlier than eggs incubated at an EST of 37.8 °C from E8 onward (Lourens et al., 2007; Molenaar et al., 2010b). However, this effect was only found with a CO_2 concentration of 0.1 and 0.8%, but not with a CO_2 concentration of 0.4%. The latter

Table 1
Effects of eggshell temperature (EST) and CO_2 concentration from day 8 of incubation (E8) onward on egg weight (loss; E0 = day of set) and broiler chicken embryo characteristics at day 18 of incubation (E18; Least Square means ± SEM).

	EST, °C			CO_2, %				*P*-values	
Item[1]	37.8	38.9	SEM	0.1	0.4	0.8	SEM	EST	CO_2
Egg wt E0, g	63.5	63.6	0.1	63.6	63.5	63.7	0.1	0.47	0.55
Egg wt E8, g	60.9	61	0.1	61.1	61	60.9	0.1	0.48	0.49
Egg wt E18, g	57.4	57.1	0.1	57.4	57.4	57	0.2	0.14	0.3
Egg wt loss, E0-E18, %	9.6[b]	10.3[a]	0.2	9.7	9.6	10.6	0.3	0.005	0.18
Embryo length, cm	18.3[b]	18.5[a]	0.1	18.3	18.4	18.4	0.1	<0.001	0.76
YFBM, g	33.65[b]	34.22[a]	0.41	34.66	33.76	33.39	0.68	0.008	0.49
RY wt, g	11.77	11.36	0.19	11.38	11.83	11.48	0.27	0.09	0.54
Liver wt, % YFBM	1.96	1.95	0.02	1.89	2	1.97	0.03	0.92	0.12
Heart wt, % YFBM	0.69[a]	0.55[b]	0.01	0.61	0.61	0.64	0.02	<0.001	0.54

[1] wt = weight, YFBM = yolk free body mass, RY = residual yolk.
[a,b] Least Square means within a row and factor lacking a common superscript differ ($P < 0.05$).

Table 2
Effects of eggshell temperature (EST) and CO_2 concentration from day 8 of incubation onward on hatching time and broiler chicken characteristics at 6 h after hatching (Least Square means ± SEM).

	EST, °C			CO_2, %				*P*-values	
Item[1]	37.8	38.9	SEM	0.1	0.4	0.8	SEM	EST	CO_2
Hatching time, h	492	485	1	486	492	488	1	<0.001	0.6
Chicken length, cm	19.4	19.5	0.1	19.3	19.7	19.5	0.1	0.4	0.18
Chicken wt, g	46.54	46.48	0.13	46.43	46.36	46.74	0.17	0.75	0.38
RY, g	6.51[b]	7.18[a]	0.2	7.05	6.72	6.76	0.31	<0.001	0.85
YFBM, g	40.02[a]	39.32[b]	0.17	39.38	39.65	39.99	0.24	<0.001	0.35
Liver wt, % YFBM	2.43[a]	2.32[b]	0.03	2.35	2.4	2.38	0.04	0.003	0.51
Heart wt, % YFBM	0.85[a]	0.68[b]	0.01	0.75	0.79	0.76	0.01	<0.001	0.23
Navel condition[2]	0.71	0.76	–	0.68	0.59	0.93	–	0.56	0.34

[1] wt = weight, YFBM = yolk free body mass, RY = residual yolk.
[2] Scored as: 0 (closed and clean navel area), 1 (navel containing a black button up to 2 mm or a black string) or 2 (navel containing a black button exceeding 2 mm or an open navel area).
[a,b] Least Square means within a row and factor lacking a common superscript differ ($P < 0.05$).

Table 3
Effects of eggshell temperature (EST) and CO_2 concentration from day 8 of incubation onward on plasma glucose, lactate, and uric acid and on liver glycogen of broiler chickens at day 18 of incubation and 6 h after hatching (Least Square means ± SEM).

	EST, °C			CO_2, %				*P*-values	
Item	37.8	38.9	SEM	0.1	0.4	0.8	SEM	EST	CO_2
Day 18 of incubation									
Glucose, mmol/L	8.42	8.42	0.13	8.5	8.17	8.6	0.19	0.47	0.77
Lactate, mmol/L	2.14	2.31	0.14	2	2.27	2.4	0.23	0.12	0.5
Uric acid, μmol/L	190.5	209.8	21.2	231	199.9	169.5	34.8	0.17	0.54
Liver glycogen, mmol/L	20.61	19.12	1.6	17.99	19.95	21.67	2.47	0.32	0.63
Six hours after hatching									
Glucose, mmol/L	11.26[a]	11.00[b]	0.08	10.71[b]	10.97[ab]	11.71[a]	0.11	0.02	0.02
Lactate, mmol/L	3.18	3.11	0.22	2.58	3.44	3.4	0.36	0.62	0.31
Uric acid, μmol/L	165.1	175.7	10	169.1	180.3	161.8	15.3	0.26	0.72
Liver glycogen, mmol/L	12.95	12.66	0.97	13.59	12.36	12.46	1.56	0.7	0.84

[a,b] Least Square means within a row and factor lacking a common superscript differ ($P < 0.05$).

CO_2 concentration was also used in the experiments of Lourens et al. (2007) and Molenaar et al. (2010b), but in these studies, hatching time was advanced with higher EST. The reason for this discrepancy is unclear, but the current finding that a CO_2 concentration of approximately 0.4% resulted in a delayed hatching time of chickens than a lower or higher CO_2 concentration was supported by a study of Wilgus and Sadler (1954). They found that at an oxygen concentration of 20.5% and a CO_2 concentration of 0.44%, the percentage of chickens hatched at 21 days were lower (70%) than when a CO_2 concentration was used of 0.25, 0.89 or 1.20% (90–95%). Buys et al. (1998) found no effect or a stimulating effect of a higher CO_2 concentration (0.2 vs 0.4% from E14 to E19) on hatching moment, depending on the genetic make-up of the broiler line they used. However, in these studies, only one incubation temperature was used and/or a higher CO_2 concentration was obtained by reducing the ventilation rate, which may have affected EST. Based on the interaction between EST and CO_2 concentration for both hatching moment and chicken length, it appears that at higher EST from E8 onward, chickens become more sensitive for differences in CO_2 concentration than at a control EST of 37.8 °C.

The interaction between EST and CO_2 concentration regarding the chicken length was quite strong. Again, no effect of CO_2 concentration was found at an EST of 37.8 °C, but at an EST of 38.9 °C and CO_2 concentration of 0.4 or 0.8% appeared to increase chicken length. These findings in chicken length appear to be (partly) related to the YFBM, in which numerically the same effects of EST and CO_2 concentrations were seen. Both effects might be related to the differences in hatching time, because both chicken

length (β = 0.03 cm/h; P < 0.001) and YFBM (β = 0.04 g/h; P = 0.003) showed a positive relationship with hatching time.

For none of the other embryo or chicken quality variables at E18 or at 6 h after hatching, an effect of CO_2 concentration was found. This might suggest that: (1) the used CO_2 concentrations were too low to affect embryonic and chicken development or (2) CO_2 concentration from E8 onward does not affect embryonic and chicken development at all when other factors, such as EST, relative humidity or O_2, are ruled out by keeping them at a constant and comparable level between treatments.

Regarding the first suggestion, most studies about CO_2 concentrations after day 8 of incubation indeed used considerable higher concentrations of CO_2, even up to 20% (Taylor and Kreutziger, 1966; Mueller et al., 2013; Burggren et al., 2015) and in these studies, effects on hatchability, hatching time and/or chicken quality were found. However, Buys et al. (1998) found quite strong stimulating effects of a small increase in CO_2 concentration on chicken characteristics during the late embryonic phase and at hatch. They increased the CO_2 concentration from 0.2% to 0.4% between E14 and E19 by reducing the ventilation rate. Tong et al. (2015) also increased CO_2 concentration to 1.0% from E18 till hatching by reducing the ventilation rate. They found some effects on hatching moment, but no effects on chicken quality. These studies may be confounded by changes in environmental conditions, such as EST, RH and O_2 concentration by reducing the ventilation rate (see also second suggestion below).

The second suggestion that CO_2 concentrations up to 0.8% do not have an effect on embryonic and chicken characteristics at all would suggest that obtained results found in literature might be due to confounding effects of CO_2 with other factors, such as incubation temperature, relative humidity, oxygen concentration or ventilation rate. Based on the present results, it can be suggested that effects of an increased EST on embryonic and chicken development are much stronger than the effects of increased CO_2 concentration to a concentration of 0.8% or 1.0%, as used in the current study and by Tong et al. (2015), respectively.

In the current study, effects of a higher EST after day 8 of incubation on embryonic and chicken development are in accordance with earlier studies (Lourens et al., 2005, Molenaar et al., 2010b; Maatjens et al., 2014). An EST of 38.9 °C stimulated embryonic development at E18, by increasing the YFBM and chicken length compared to an EST of 37.8 °C. This is comparable with Nangsuay et al. (2016), who found stimulating effects of high EST on embryo development till E16. However, at hatching opposite results were found, where an EST of 38.9 °C resulted in a lower YFBM and/or higher RY than an EST of 37.8 °C (Lourens et al., 2005; Molenaar et al., 2010b, Nangsuay et al., 2016). This might be related to the disbalance between metabolic rate and the oxygen availability in the last phase of incubation with a high EST, due to limited eggshell conductance (Nangsuay et al., 2017). Consequently, less lipids may be metabolized for energy production and the embryo starts to utilize more glycogen as alternative energy source and finally protein as alternative nutrient source for gluconeogenesis (Nangsuay et al., 2017). However, in the current study, this was not expressed in a lower liver glycogen level or in a tendency toward a higher uric acid level at hatching, as earlier seen (Molenaar et al., 2011).

Conclusions

It can be concluded that effects of CO_2 (at concentrations $\leq$0.8%) from day 8 of incubation onward on embryonic development and chicken quality appear to be limited when oxygen concentration, EST and relative humidity are kept constant. Moreover, high EST from day 8 of incubation onward appeared to negatively affect embryonic development and chicken quality at hatch.

Ethics approval

The experimental protocols were approved by the Animal Use and Care Committee of Wageningen University, the Netherlands (approval number 2016.W-0087).

Data and model availability statement

None of the data were deposited in an official repository. Data are available upon request.

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Author contributions

Conceptualization: **HvdB, RMeij, BK**; Methodology: **HvdB, MJWH, IvdA, MO**; Software: **MJWH**; Validation: **MJWH, HvdB**; Formal analysis: **HvdB, RMol**; Investigation: **HvdB, MJWH, IvdA, MO**; Resources: **HvdB, IvdA**; Data curation: **MO**; Writing Original Draft: **HvdB**; Writing Review and Editing: all authors; Visualization: **HvdB**; Supervision: **HvdB**; Project administration: **HvdB, IvdA**; Funding acquisition: non-applicable.

Declaration of interest

None.

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